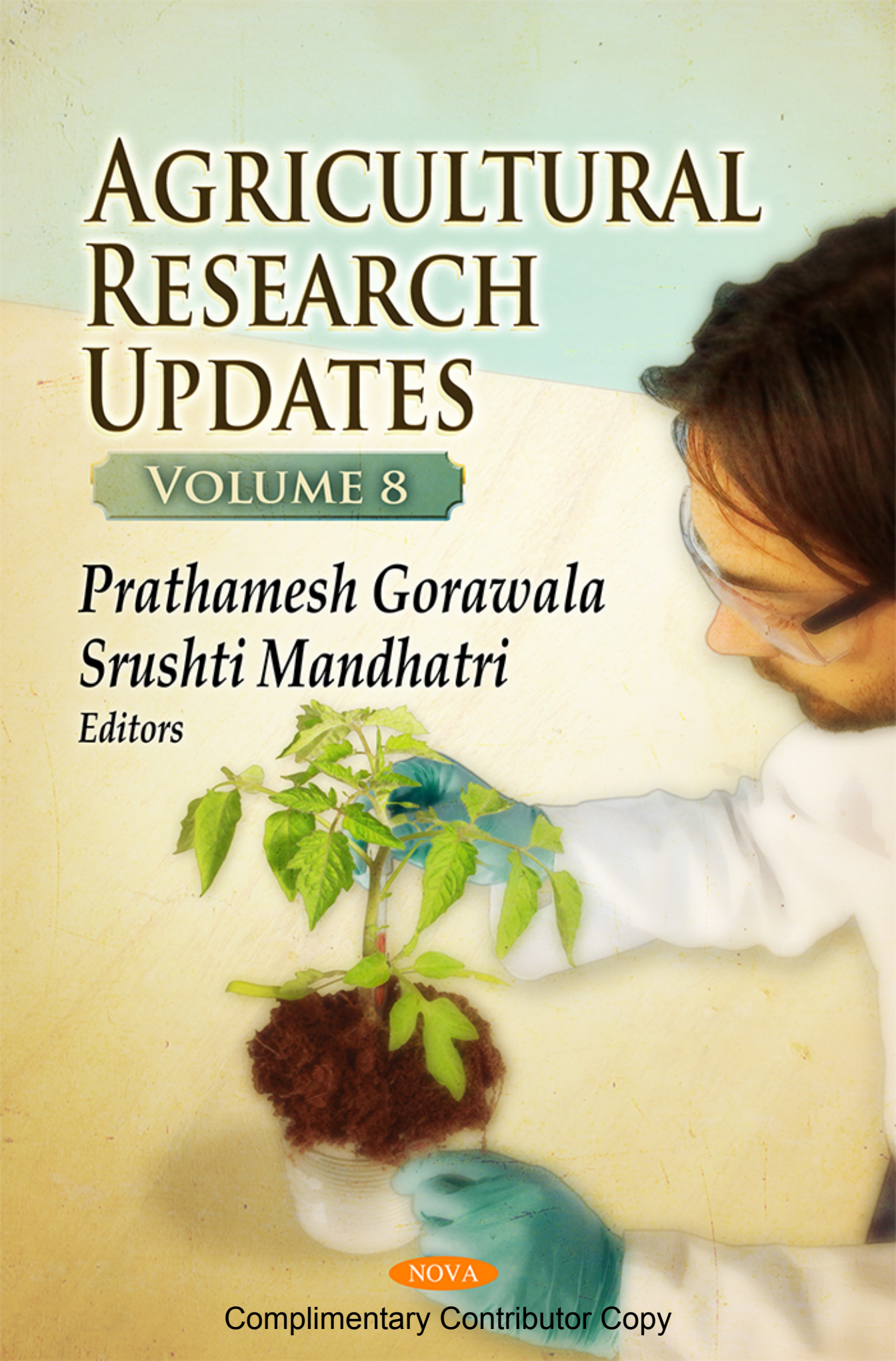
Agricultural Research Updates

Volume 8

Prathamesh Gorawala
Srushti Mandhatri
Editors



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VOLUME 8

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PREFACE

This compilation examines agricultural research from across the globe and covers a broad spectrum of related topics. In this book, the authors discuss research including cheese whey as a source of active peptides; technological and chemical characterization of PDO cheeses of Italy; biotechnological conversion of whey into high-value products; apple pomace; trends in precision agriculture for sugarcane biorefineries competitiveness; and fruit-based functional beverages.

Chapter 1 – Whey, a co-product in cheese manufacture, has been traditionally reused by its high content in soluble proteins. Complete, delactosed or low salts content powder whey are commonly used as food ingredients. Whey protein concentrates (WPC) or isolates (WPI) obtained by membrane technology (MT) with different proteins content are in fact one of the most important products in whey processing industries. These products are present in many human foods.

In recent years a number of bioactive peptides present in cheese whey have been described giving this co-product a new extra pharmaceutical and medical interest.

Bioactive peptides have been defined as specific proteins fragments that have a positive impact on body functions and can positively affect human health. These biopeptides are inactive within protein sequence and can be released in a proteolysis mainly made by specific enzymes. Main limitations to biopeptides production at the industrial scale are the development of appropriate technologies to fractionate and purify the peptides in economical and competitive processes. From the point of view of human health, real activity of most of them must be still demonstrated and clinical tests must be performed in the next years.

In this chapter a review of possible biopeptides obtained from single whey proteins (mainly alpha-lactalbumin, beta-lactoglobulin and caseinomacropeptide - CMP) as well as from raw whey and WPC is made. The different biological and physiological activities of main peptides described in the literature are also included.

Enzymatic hydrolysis conditions, peptides fractionation by membrane technology, peptides analysis and animal and human trials to demonstrate the biological effects of these products are also reviewed.

Chapter 2 – Whey is an important surplus product of food industry. Based on the point of view, huge quantities of whey produced worldwide can represent an environmental problem with its disposal, or, due to the fact that whey is rich in fermentable nutrients, it can be considered as an attractive substrate for biotechnological production of various industrially interesting products. Therefore, this chapter is intended to summarize possible up-stream

processing methods, general principles and fermentation strategies for the microbial productions using whey as a substrate. Further, among huge amount of potential products, this chapter focuses on the production of high value substances and materials which find applications especially in the fields of health care, medicine and pharmacy. At first, many biologically active substances such as vitamins, carotenoids, antibiotics etc. can be produced from whey employing various microorganisms and cultivation strategies. Moreover, also number of biopolymers which can be used in the form of drug carriers, scaffolds, sutures, adhesives *etc.* can be produced by using whey as a cheap complex substrate. Apart from poly (lactic acid), these materials include polyhydroxyalkanoates - bacterial polyesters and also several polysaccharides such as xanthan, alginate, hyaluronic acid, gellan, pullulan, dextran or chitosan. In addition, the aim of this review is also to provide basic economical consideration of fermentation processes. In conclusion, utilization of whey as a substrate for microbial productions of high value products could result in a very promising process meeting both economic and ecological requirements.

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Chapter 4 – The solid residue of apple juice extraction is the apple pomace. This processing waste represents up to 30% of the original fruit and consists of a complex mixture of peel, core, seed, calyx, stem, and soft tissue. It has high water content and is mainly composed of insoluble carbohydrates such as cellulose, hemicellulose, and lignin. Simple sugars, such as glucose, fructose, and sucrose, as well as small amounts of minerals and vitamins can also be found. This residual material is a poor animal feed supplement because of its extremely low protein content and high amount of sugar, and its production in large amounts has been reported world-wide. The direct disposal of agro-industrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabolites, with a higher commercial value. The application of agro-industrial by-products offers a wide range of alternative substrates, thus helping solve pollution problems related to their disposal. Attempts have been made to use apple pomace to generate several value-added products, such as pectin, polyphenols, edible fibers, enzymes,

single cell protein, pigments, aroma compounds, alcohols, organic acids, polysaccharides, biohydrogen, antioxidant substances and mushrooms among others. This chapter reviews recent developments regarding process and products, as well as research works that employed apple pomace as a substrate for different industrial and biotechnological applications.

Chapter 5 – The world sugarcane agroindustry participates with 70% of the sucrose production and integrates agricultural activities of growing, harvesting and transportation of sugarcane with production in sugar mills. However, it has challenges related to low productivity and socio-economic aspects that are a risk to food security and the agro industrial conversion to biorefineries. Regarding sugarcane productivity, Remote sensing (RS) is a vital component of a wide variety of applications across disciplines as a decision support system that combines multi-source information and could be very useful for agricultural applications and has been used to detect or assess a variety of agricultural variables: crop discrimination, and inventory, growth stage, health, nutrient requirement, spatial variations in productivity, biomass estimation and area estimation and soil physical and chemical properties sampling with complex biophysical environments by satellite imagery. It can provide farm managers data that can allow them to make quick decisions concerning their operations at various spatial scales. However, remote sensing data has not been used to its fullest potential for management of sugarcane crops largely because these data are not readily available to researchers and farmers, to understanding of effects of sensor characteristics such as spatial, spectral and radiometric resolution and scene characteristics such as acquisition date etc. and as well as integration of conventional field survey information with remote sensing data. Potentially, one of the most powerful tools, processes and techniques in precision agriculture (PA) is the use of remote sensing, Geographic Information System (GIS) and Global Positioning System (GPS), which has the ability to rapidly provide a description of canopy crops, mapping spatial variability, quality and Modeling to predict the yields for reduction of costs of production and competitiveness. The objective of this paper is to review the status and development of the documented applications of the role of remote sensing technology to optimize sugarcane production and improve productivity and diversification projects. The authors discuss advantages, limitations, strategies and future perspective with special emphasis on farm sugarcane, contribution of vegetation components to reflectance signals, as well as mechanistic relationships between biochemical processes and spectral indexes, which are compared and summarized in detail.

Chapter 6 – Fruit juice has been considered as one of the staple beverages from many decades. Fruit juice and juice-based beverages have increasingly been promoted on a health platform and has been consumed worldwide due to the natural abundance of carbohydrate, essential vitamins, minerals and other nutrients in them.Fruit juices also possess several reported health benefits, such as aid in digestion, high antioxidant properties, anti-inflammatory properties, and combating several diseases. A functional beverage is a non-alcoholic drink product that is formulated with ingredients, such as raw fruits, herbs, vitamins, minerals, amino acids and other bioactive compounds that provide specific health benefits. In the recent years, functional beverages have become increasingly popular due to its several specific health benefits. The present chapter focus on various fruit based functional beverages, their nutritional benefits, physiological benefits and functional attributes to human health.

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Chapter 1

CHEESE WHEY AS A SOURCE OF ACTIVE PEPTIDES: PRODUCTION, ANALYSIS, PURIFICATION AND ANIMAL AND HUMAN TRIALS

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ABSTRACT

Whey, a co-product in cheese manufacture, has been traditionally reused by its high content in soluble proteins. Complete, delactosed or low salts content powder whey are commonly used as food ingredients. Whey protein concentrates (WPC) or isolates (WPI) obtained by membrane technology (MT) with different proteins content are in fact one of the most important products in whey processing industries. These products are present in many human foods.

In recent years a number of bioactive peptides present in cheese whey have been described giving this co-product a new extra pharmaceutical and medical interest.

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In this chapter a review of possible biopeptides obtained from single whey proteins (mainly alpha-lactalbumin, beta-lactoglobulin and caseinomacropeptide - CMP) as well

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as from raw whey and WPC is made. The different biological and physiological activities of main peptides described in the literature are also included.

Enzymatic hydrolysis conditions, peptides fractionation by membrane technology, peptides analysis and animal and human trials to demonstrate the biological effects of these products are also reviewed.

1. CHEESE WHEY GENERALITIES

Cheese whey is the most important by-product from cheese and casein manufacture. Each kilogram of cheese produced generates between 8 and 9 litres of whey; that means that huge amounts of this product, that contains half of the total milk solids and 20% of the original milk proteins, is produced in world. At small cheese plants, whey continues being a disposal problem due to its high BOD (40,000-60,000 mg O_2/L) and the difficulty of managing small-medium amounts of whey.

In medium and large cheese and casein companies whey is converted in a number of valuable products oriented to animal or human food and in the last years new advances in protein hydrolysis and technological separations lead to products of high value that can be placed in the food-pharmaceutical industries interphase.

Most of the whey components have great value in human nutrition. Whey is the most important commercial lactose source. It is present in whey in percentages higher than 70% (on a dry basis) and permeates of ultrafiltered whey (PUF) shows even higher values.

Lactose favours the calcium and other minerals absorption in mammals and other oligosaccharides present in whey containing sialic acid promote the development of human brain (Park, 2009). Whey is an important natural source of Ca^{+2} and other minerals (K, Na, P, Zn, Mg, Fe, etc.) some of them playing important roles in metabolism of the human being (Hernández-Ledesma et al., 2011). The presence of these minerals usually causes technological problems when whey is processed at the industry (in most of the cases specific techniques are used to reduce mineral content in the final product) but they will be able to play important roles for human health in the next years.

However, more extensive research must be done at this point. Most of the water-soluble vitamins present in milk are in the whey fraction. Special interest has the B vitamin complex that is composed by different compounds with demonstrated bioactivity in metabolical rutes.

Concerning the protein fraction, the aminoacid composition of their soluble proteins is more valuable than others usually considered as reference (eggs proteins), and even more interesting than milk caseins. Considered as a whole, whey proteins have a number of technological properties (emulsion and foams stabilization, high water retention capacity, etc.) that are exploited in food industry using these proteins as additives in meat, baker and other food industries. From the point of view of human health, they seem to contribute to the regulation of body weight and improve the net muscle mass, among others benefits (Luhovyy et al., 2007; Phillips et al., 2009).

Most of the industrial products obtained from whey come from its protein fraction. Whey protein concentrates (WPC), whey protein isolates (WPI) with different levels of demineralization and lactose contents can be found in the market and are nowadays traditional ingredients in a number of adults and baby foods as well as they are present in special diets. In these protein products all proteins are concentrated maintaining the original

proportions between them, that is, proteins are concentrated but not fractionated. Only in some cases (lactoferrin, lactoperoxidase, etc.) the proteins are separated from the rest of whey proteins and commercialised with high purity due to their high added value.

The first part of this chapter describes briefly main characteristics and health effects of major proteins present in whey and the actual state of whey upgrading from an industrial point of view. The second part is focused on the production of biopeptides and their main bio-functionalities that is considered one of the most interesting research lines for the next years.

2. CHEESE WHEY: COMPOSITION

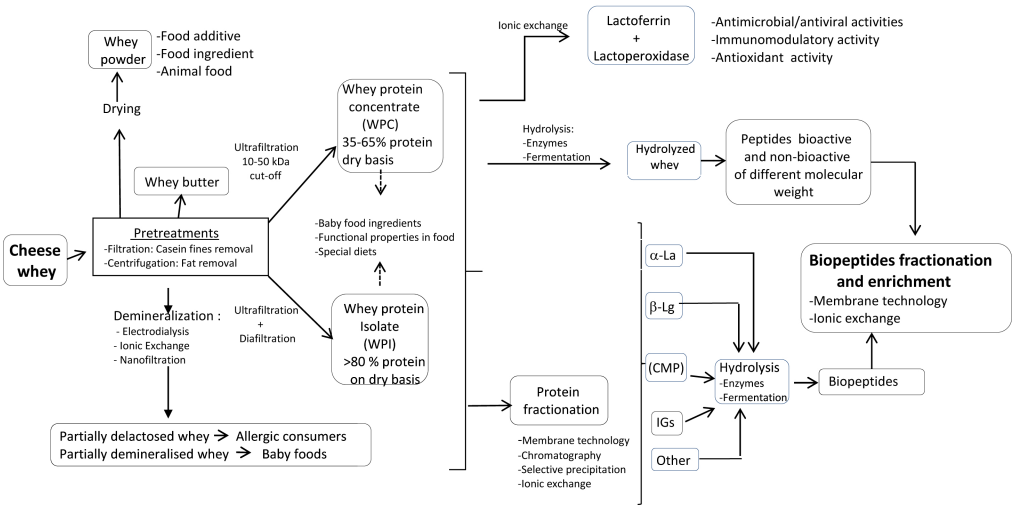
World whey production increases at the same rate as cheese consumption (around 2% per year**)** (Smithers, 2008). Around 80% of the total whey produced is rennet whey ("sweet whey") obtained by enzymatic attack of milk kappa-casein and the 20% remaining is acid whey (obtained in the manufacture of cottage cheese, caseins, etc.), obtained by acidification of milk (by adding acid or lactic bacteria cultures). Table I shows the composition of both types of whey.

As can be seen, main differences between them are related to the final pH of both liquids as well as the amount of salts (higher in acid whey) what affect the technologies used to reuse it (acid whey is difficult to dry in spray drying systems due to the sticky behaviour of concentrated whey). Protein fraction is similar in both types of whey except the presence of caseinomacropeptide (CMP or casein glycomacropeptide – GMP)(CMP is the soluble peptide obtained after the enzymatic action on the k-casein and then it is no present in acid whey). Lactic acid content in acid whey is due to the transformation of lactose by lactic bacteria.

Table I. Characteristics and composition of sweet and acid wheys (Goodall et al., 2008)

	Sweet whey (Liquid)	**Acid whey (Liquid)**
pH	5.9 -6.3	4.3-4.6
Total solids (%)	6.4	6.2
Fat (%)	0.5	0.04
Lactose (%)	4.6	4.0
Lactic acid (%)	0.05	0.8
Minerals (%)	0.5	0.8
Total Nitrogen (%)	0.8	0.7
Aprox. Protein proportions (% on total proteins)*		
- β-Lg	40-55	
- α-La	11-23	
- IGs	8-11	
- CMP	10-15	
- BSA	4-12	
- Lactoferrin	1	
- Lactoperoxidase	1	

*CMP is only present in rennet whey



α-La: α-Lactalbumin.
β-Lg: β-lactoglobulin.
CMP: Caseinmacropeptide.
IGs: Immunoglobulins.

Figure 1. Scheme of whey processing. Protein upgrading.

There are a number of whey derivatives that are commercialised at an industrial scale. Figure 1 shows some of the most important whey transformations that lead to final industrial products. Around 50% of the whey produced is transformed into dervivatives.

Whey powder is usually manufactured after evaporation and spray drying steps. The powder can be used as a complement in animal food or as an additive or ingredient in different human and baby foods (bakery, meat, confectionery, sauces, etc.). In some cases whey can be partially demineralised or delactosed in order to obtain products for specific diets. Common technologies to reduce minerals are nanofiltration, ion exchange and electrodialysis, being former the more commonly used due to its lower cost and efficiency in the reduction of monovalent ions (reductions between 50 – 80 % in monovalent ions and between 3-20% for divalent ones) (Suárez et al., 2006; Minhalma et al., 2007).

Whey butter obtained after whey centrifugation is being considered in the last years as a source of phospholipids with interesting effects on human health affecting several cell functions, such as growth, molecular transport system, memory processing, stress response, etc. (Astaire et al., 2003; Fong et al., 2007; Rezende et al., 2010).

Whey protein concentrates (WPC) are well known products obtained from whey that are commercialised by many companies. These products are produced by means of membrane technologies (ultrafiltration with spiral wound or flat organic membranes between 10 and 50 kDa cut-off). WPC can be commercialised with different level of protein purity (between 35 and 65% based on total solids- WPC35-WPC65). Higher protein concentrations, named whey protein isolate (WPI) showing more than 80 % on total solids basis, can also be manufactured by a combination of ultrafiltration + diafiltration, and they are excellent starting materials to later hydrolysis or protein fractionation. WPC can also be used as ingredient in adult food and in baby foods if some demineralization processes have been made previously. These products

are good as starting substrates to later protein fractionation and hydrolysis (Cheang and Zydney, 2004).

Technological and biofunctional properties of proteins can be upgrading by using these concentrated products. Theoretically these concentrates show certain biological activities due to their peptide composition (ACE-inhibitory, hypocholesterolemic, anticarcinogenic, etc.), but in most of the cases the intensity is too low due to that thye are in the sequence of the parent proteins (Bounous, 2000; Foegeding et al., 2002). For this, if the objective is to enhance all these properties (especially in medical applications) it is compulsory to fractionate major proteins and try to obtain single protein streams as pure as possible. Some methods developed in the past as salting out, precipitation in presence of solvents and strong thermal techniques, have been proved to be impractical from an industrial point of view. The techniques used to get these objectives must be rapid, non-denaturating and they must guarantee a high yield product with maximum purity, non-chemical addition, they might be easily scaled-up and not too much expensive.

Unfortunately there is not an unique technique that fulfils these prerequisites. Among the most widely used techniques are chromatographic methods, membrane technologies, selective precipitations, ionic exchange, etc. (El-Sayed and Chase, 2011). Chromatographic processes have the advantage of being less volume dependent because adsorbent capacity depends mostly on the mass of protein recovered and not on the volume of liquid processed, but production at industrial scale seems to be too much expensive. On the other hand, membrane techniques based on pressure driven methods (NF, UF, MF) produce reasonable yields but with low purities. Electrochemical processes (electrodialysis) have high electricity costs as main drawback. Selectivity precipitation shows some problems related to extreme pHs and difficulties to separate weak precipitates. As an alternative to single methods, coupling some of these techniques will be one of the major future challenges that protein fractionation industry must face (El-Sayed and Chase, 2011).

All proteins contained in whey have been studied in order to find biofunctional properties (Datta et al., 2008; Goodall et al., 2008; Saksena et al., 2010; Camfield et al., 2011; Hernández-Ledesma et al., 2011). The most interesting characteristics of main whey proteins are described below.

Lactoferrin and Lactoperoxidase

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein (673 amino acid residues) that belongs to the transferrin family. In bovine milk, LF is found in small amounts varying from 0.02 to 0.20 $g.L^{-1}$ and the protein remains quantitatively in cheese whey. LF has multiple biological properties that include antimicrobial, anti-inflammatory, anticarcinogenic, immuno-modulatory, and bone growth factor properties and it is considered an important host defense molecule (Wakabayashi et al., 2006). There is considerable interest for its use as a natural bioactive ingredient in food and in health and nutritional products (Wakabayashi et al., 2006; Branen and Davidson, 2009).

Lactoperoxidase (LP) is an enzyme present in milk and other external secretations that has antimicrobial, antiviral and immunomodulatory activities (Caccavo et al., 2002; Mercier et al., 2004; Shin et al., 2005; Wakabayashi et al., 2006). LP is present in cheese whey in amounts around 0.002 $g.L^{-1}$ (Zydney, 1998).

LF and LP are actually obtained with high purity (>90%) at industrial scale using pretreated whey, WPC or WPI as starting raw material. Both proteins have a strong basic character with an isoelectric point (IEP) between 8.0 and 9.0 that contrasts with the acidic IEP of the major proteins in milk (caseins IEP:4.6 and the two main whey proteins β-lactoglobulin IEP: 5.13 and α-lactalbumin IEP: 4.2–4.5). That distinct charge property allows isolation of LF and LP from whey by cation-exchange chromatography. However, this process has some limitations at industrial scale such as its high cost and relatively low throughputs. Membrane filtration could represent an interesting alternative to chromatography for LF and LP production by designing an accurate fractionation cascade. However, fouling and poor selectivity in protein separation have been associated with such membrane filtration processes. Different strategies have been investigated to overcome the limitations associated with LF and LP separation by membrane filtration including variation of the hydrodynamic parameters, modification of the physico-chemical environment, use of different membrane types and alteration of the membrane surface properties (Brisson et al., 2007; Fweja et al., 2010).

β-Lactoglobulin

This protein represents around 50% of the total protein content in whey. Its physicochemical properties have been published in many papers (Kamau et al., 2010). It has a globular structure and shows a molecular weight of 18.3 kDa and 162 amino acid residues (in its monomer form. β-Lg can be showed as dimer, trimer, tetramer and octomer mainly depending on the pH). This protein shows excellent gel formation and foaming properties and it can be used in a number of food industries as technical additive (Tolkach and Kulozik, 2005). Nutritional and functional properties of this protein are described as carrier of retinol and immunomodulatory and anti-carcinogenic activities (Chatterton et al., 2006; Sutton and Alston-Mills, 2006; Kamau et al., 2010), however it is not present in human milk and it has been described as one of the main allergen in infant nutrition (Lönnerdal and Lien, 2003). This protein is rich in cysteine (Cys) that stimulates anticarcinogenic peptides (Mcintosh et al., 1995). β-Lg can be obtained from whey or WPC by different technologies (ion-exchange, conventional filtration, ion-exchange membrane chromatography, selective precipitation and combined methods) (El-Sayed and Chase, 2011). β-Lg concentrates are produced as complement in sport man diets. Some attempts have been made to reduce the content of this protein in infant formulae (Lucena et al., 2006).

α-Lactalbumin

This bovine protein is similar to the human milk protein. Its main function is related with its participation in the lactose synthesis. It is described that the complex α-La and oleic acid has antitumoral activities (against bladder tumors and skill papillomas in human) (Chatterton et al., 2006; Hallgren et al., 2008). This protein shows a relatively thermal stability and low gel formation properties.

This protein is formed by 123 amino acids and shows to disulphide bonds. It has the ability of binding two Ca^{+2} that increase its stability. α-La represents around 20% of the total protein content in the whey and can be obtained from whey by different technologies: Chromatographic methods (Bordin et al., 2001); gel filtration (Neyestani et al., 2003); selective and reversible precipitation (Bramaud et al., 1997; Lucena et al., 2007; Fernández et al., 2011); enzymimatic hydrolysis (Konrad and Kleinschmidt, 2008). Among them, the reversible precipitation process is one of the most promising methods provided proper conditions are maintained. In the last years selective hydrolysis processes have been studied to separate β-Lg and α-La based on the higher resistance of the last one to enzymatic attack. After the hydrolysis step, membrane filtration retains α-la (less hydrolyzed) and most of the peptides from β-Lg and the rest of proteins permeate thorough the membrane (Kamau et al., 2010).

Caseinomacropeptide (CMP)

CMP concentration in whey varies between 0.55 and 0.70 $g.L^{-1}$. This peptide is only present in rennet whey and it is released by chymosin cleavage of k-casein during cheese manufacture. CMP has been extensively studied due to its properties as prebiotic and anticariogenic activity. The absence of phenylalanine in its composition makes CMP adequate to phenylketonuria patient diets as well as for patients with hephatic diseases due to its high concentration in valine and isoleucine. Other important properties are described in the literature (Abd el Salam et al., 1996; Setarehnejad et al., 2010). When whey is used as raw material for baby food manufacture CMP is a less desiderable component due to its high content in threonin (hyperthreoninemia is described in infants) (Rigo et al., 2001).

CMP isolation can be made by ultrafiltration processes due to the ability of this peptide of forming polymers higher than 50 kDa at neutral pH and its tendency to dissociate at acidic conditions (Kawasaki et al., 1996). Other fractionation processes are based on the aggregation of proteins at 90ºC for 1 hour, except CMP that can be concentrated by ultrafiltration membranes (MWCO 10 kDa) at pH around 7 (Martin-Diana and Fontecha, 2002).

Other Proteins

The rest of whey proteins are less studied. Bovine sero-albumin bovine (BSA) participates in synthesis of lipids (Choi et al., 2002) and has antioxidant properties (Hernández-Ledesma et al., 2011), but industrial methods to isolate it have not been developed until now. Proteose-peptone fraction is a very complex heat-stable protein mixture containing phosphoglycoproteins (lactorphin with immunomodulatory properties) (Campagna et al., 2004; Sugahara et al., 2005).

Immunoglobulins (IGs) are globular proteins present in cheese whey in concentrations between 0.5 and 0.7 $g.L^{-1}$. Cow IGs are an heterogeneous fraction with important immunogenic properties against animal pathogens. Some attempts have been made to use cow IGs against human diseases (rotavirus, *Campylobacter jejuni*, *Salmonella*, *Helycobacter pylori*, etc.) (Freedman et al., 1998). However, all methods need a previous cow´s injection

with the pathogen to produce milk with activity. Additionally, cow colostrum (which IGs concentration is more than one hundred times the concentration in milk), seems to be the best raw material to investigate possible applications of these proteins on human diseases (Sarker et al., 1998).

3. BIOACTIVE PEPTIDES FROM CHEESE WHEY

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions and may ultimately influence health (Kitts and Weiler, 2003). In recent years it has been recognized that dietary proteins, especially milk proteins, provide a rich source of biologically active peptides. These peptides are inactive within the sequence of the protein but they can be released through proteolysis by enzymes, be it via digestive enzymes or by means of enzymes derived from microorganisms or plants. The activity of these valuable peptides is based on their inherent amino acid composition and sequence, being some of them multifunctional, and their size may vary from two to twenty amino acid residues. Bioactive peptides were first mentioned by Mellander in 1950 (Mellander, 1950), when he suggested that casein-derived phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants. The knowledge of bioactive peptides has steadily increased since 1979 and, at present, numerous peptides exhibiting biofunctionality have been reported.

The investigation strategies concerning the production of bioactive peptides include the following steps:

1. Development of an assay system to determine biological activity.
2. Design the way to hydrolyze the proteins by means of different kind of enzymes or fermentation processes.
3. Peptides fractionation or isolation according to some of their physico-chemical properties (size, charge, reactivity, hydrophobicity, etc.).
4. Analysis of the structures and the verification of activity (*"in vitro"*, *""in vivo""* and human clinical tests).

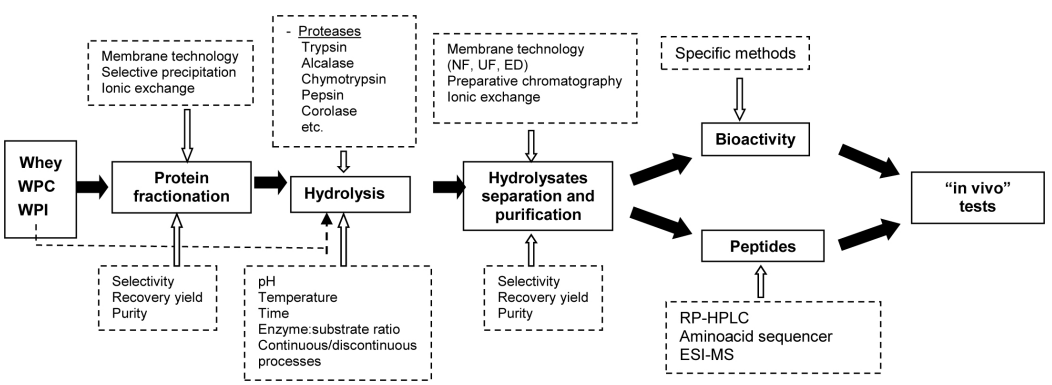


Figure 2. Steps in the production of biopeptides from whey and whey derivatives.

Table IIa. Bioactive peptides derived from α-La

Amino acid sequence	Bioactivity	Pharmacological properties	Production	*""in vivo""* studies	References
YGLF [α-La f(50-53)]: α-lactorphin	Opioid	[b]GPI inhibition	α-La + pepsin	Rats: reduction of blood pressure [Nurminen, 2000]	Antila, 1991; Pihlanto-Leppälä, 1997
	AHT[a] (ACE-inhibition)	[c]IC_{50} = 733 μM			
YGL [α-La f(50-52)]	AHT (ACE-inhibition)	IC_{50} = 409 μM	α-La + pepsin + trypsin and chymotrypsin		Pihlanto-Leppälä, 2000
VGINYWLAHK [α-La f(99-108)]	AHT (ACE-inhibition)	IC_{50} = 327 μM	α-La + trypsin		Pihlanto-Leppälä, 2000
WLAHK [α-La f(104-108)]	AHT (ACE-inhibition)	IC_{50} = 77 μM	α-La + trypsin		Pihlanto-Leppälä, 2000
LAHKAL [α-La f(105-110)]	AHT (ACE-inhibition)	IC_{50} = 621 μM	Fermentation of whey + pepsin and trypsin		Pihlanto-Leppälä, 1998
YG [α-La f(50-51)]	AHT (ACE-inhibition)	IC_{50} = 1522 μM	α-La + trypsin		Mullally, 1996
	Limphocyte proliferation		Synthetic peptide		Kayser, 1996
YGG [α-La f(18-20)]	Limphocyte proliferation		Synthetic peptide		Kayser, 1996
EQLTK [α-La f(1-5)]	Antimicrobial	Against G+[e]	α-La + trypsin		Pellegrini, 1999
GYGGVSLPEWVCTTF-ALCSEK [α-La f(17-31)S-Sf(109-104)]	Antimicrobial	Against G+	α-La + trypsin		Pellegrini, 1999

Table IIa. (Continued)

Amino acid sequence	Bioactivity	Pharmacological properties	Production	*""in vivo""* studies	References
VSLPEW [α-La f(21-26)]	Antioxidative	RAS[d]	α-La + thermolysin		Sadat, 2011
YDTQA [α-La f(36-40)]	Antioxidative	RAS	α-La + thermolysin		Sadat, 2011
IWCKDDQNPHSSNI [α-La f(59-72)]	Antioxidative	RAS	α-La + thermolysin		Sadat, 2011
INY [α-La f(101-103)]	Antioxidative	RAS	α-La + thermolysin		Sadat, 2011
INYW [α-La f(101-104)]	Antioxidative	RAS	α-La + thermolysin		Sadat, 2011
LDQW [α-La f(115-118)]	Antioxidative	RAS	α-La + thermolysin		Sadat, 2011
KGYGGVSLPEW [α-La f(16-26)]	AHT (ACE-inhibition)	IC_{50} = 0.8 µg/mL	whey + proteases from *Cynara cardunculus*		Tavares, 2011
DKVGINYW [α-La f(97-104)]	AHT (ACE-inhibition)	IC_{50} = 25.2 µg/mL	whey + proteases from *Cynara cardunculus*		Tavares, 2011
RELKDLK [α-La f(10-16)]	Limphocyte proliferation		Synthetic peptide		Jacquot, 2010

[a] AHT: antihypertensive effect.
[b] GPI : Effect on the contractions of guinea pig ileum *"in vitro"*.
[c] IC_{50} : concentration of an ACE-inhibitor needed to inhibit 50% of the ACE activity.
[d] RAS: Radical Scavenging activity.
[e] G+: Gram-positive bacteria; G-: Gram-negative bacteria.

Table IIb. Bioactive peptides derived from β-Lg

Amino acid sequence	Bioactivity	Pharmacological properties	Production	*""in vivo""* studies	References
YLLF [β-Lg f(102-105)]: β-lactorphin	Opioid	GPI stimulation	β-Lg + pepsin + trypsin or trypsin + chymotrypsin	Rats and mice: AHT and opioid effects [Nurminen, 2000; Ijäs, 2004]	Antila, 1991; Pihlanto-Leppälä, 1997
	AHT (ACE-inhibition)	IC_{50c} = 172 μM			
HIRL [β-Lg f(146-149)]: β-Lactotensin	AHT (ACE-inhibition)		β-Lg + chymotrypsin	Rats: hypocholesterolemic activity [Yamauchi, 2003]	Antila, 1991; Pihlanto-Leppälä, 1997 and Paakkari, 1994; Yamauchi, 2003
	Hypocholesterolemic				
	Opioid	GPI stimulation			
LAMA [β-Lg f(22-25)]	AHT (ACE-inhibition)	IC_{50} = 556 μM	β-Lg + trypsin		Pihlanto-Leppälä, 2000
LDAQSAPLR [β-Lg f(32-40)]	AHT (ACE-inhibition)	IC_{50} = 635 μM	β-Lg + trypsin		Pihlanto-Leppälä, 2000
VFK [β-Lg f(81-83)]	AHT (ACE-inhibition)	IC_{50} = 1029 μM	β-Lg + trypsin		Pihlanto-Leppälä, 2000
ALPMHIR [β-Lg f(142-148)]	AHT (ACE-inhibition)	IC_{50} = 43 μM	β-Lg + trypsin		Mullally, 1997
	Limphocyte proliferation		Synthetic peptide		Jacquot, 2010
VLDTDYK [β-Lg f(94-100)]	AHT (ACE-inhibition)	IC_{50} = 946 μM	β-Lg + pepsin + trypsin and chymotrypsin		Pihlanto-Leppälä, 2000
ALPMH [β-Lg f(142-146)]: Lactosin B	AHT (ACE-inhibition)	IC_{50} = 521 μM	β-Lg + pepsin + trypsin and chymotrypsin		Pihlanto-Leppälä, 2000
GLDIQK [β-Lg f(9-14)]	AHT (ACE-inhibition)	IC_{50} = 580 μM	Fermentation of whey + pepsin and trypsin		Pihlanto-Leppälä, 1998
VAGTWY [β-Lg f(15-20)]	AHT (ACE-inhibition)	IC_{50} = 1682 μM	Fermentation of whey + pepsin and trypsin		Pihlanto-Leppälä, 1998; Pellegrini, 1999; Jacquot, 2010
	Antimicrobial	Against G+			
	Limphocyte proliferation				
IPA [β-Lg f(78-80)]: Lactosin A	AHT (ACE-inhibition)	IC_{50} = 141 μM	whey + proteinase K	Rats: reduction of blood pressure [Abubakar, 1998]	Abubakar, 1998)

Table IIb. (Continued)

Amino acid sequence	Bioactivity	Pharmacological properties	Production	*""in vivo""* studies	References
YL [β-Lg f(102-103)]	AHT (ACE-inhibition)	IC_{50} = 122 μM	Synthetic peptide		Mullally, 1996
LF [β-Lg f(104-105)]	AHT (ACE-inhibition)	IC_{50} = 349 μM	Synthetic peptide		Mullally, 1996
SAPLRVY [β-Lg f(36-42)]	AHT (ACE-inhibition)	IC_{50} = 8 μM	whey + Protease N Amano		Ortiz-Chao, 2009
ALPM [β-Lg f(142-145)]	AHT	Not Measured		Rats: reduction of blood pressure [Murakami, 2004]	Murakami, 2004
AASDISLLDAQSAPLR [β-Lg f(25-40)]	Antimicrobial	Against G+	β-Lg + trypsin		Pellegrini, 1999
IPAVFK [β-Lg f(78-83)]	Antimicrobial	Against G+	β-Lg + trypsin		Pellegrini, 1999
IIAEK [β-Lg f(71-75)]: Lactostatin	Hypocholesterolemic AHT (ACE-inhibition)		β-Lg + Trypsin	Rats: hypocholesterolemic activity [Nagaoka, 2001]	Nagaoka, 2001; Janssen, 2006
WYSLAMAASDI [β-Lg f(19-29)]	Antioxidative	RAS	β-Lg + Corolase PP		Hernández-Ledesma, 2005
MHIRL [β-Lg f(145-149)]	Antioxidative	RAS	β-Lg + Corolase PP		Hernández-Ledesma, 2005
YVEEL [β-Lg f(42-46)]	Antioxidative	RAS	β-Lg + Corolase PP		Hernández-Ledesma, 2005
DAQSAPLRVY [β-Lg f(33-42)]	AHT (ACE-inhibition)	IC_{50} = 13 μg/mL	whey + proteases from *Cynara cardunculus*		Tavares, 2011
RLSFNP [β-Lg f(148-152)]	AHT (ACE-inhibition)	IC_{50} = 177.4 μm	Milk fermented by *Lactobacillus helveticus* LB10		Pan, 2010
EILLQK [β-Lg f(55-60)]	Limphocyte proliferation		Synthetic peptide		Jacquot, 2010
IDALNENK [β-Lg f(84-91)]	Limphocyte proliferation		Synthetic peptide		Jacquot, 2010
VLVLDTDYKKYLLF [β-Lg f(92-105)]	Limphocyte proliferation		Synthetic peptide		Jacquot, 2010
ALKALPMHIR [β-Lg f(139-148)]	Limphocyte proliferation		Synthetic peptide		Jacquot, 2010

Table IIc. Bioactive peptides derived from other whey proteins

Source protein	Amino acid sequence	Bioactivity	Pharmacological properties	Production	*in vivo* studies	References
BSA	AKFAWAVAR [BSA f(208-216)]: Albutensin A	Opioid	GPI Contraction	BSA + trypsin	Mice: digestive system effects [Ohinata, 2002]	Yamauchi, 1992
		Digestive system effects	Delay gastric emptying, elevates blood glucose levels			
	YGFGNA [BSA f(399-404)]: Serophin	Opioid				Tani et al., 1993; Meisel and Schlimme, 1996
GMP		Digestive function regulator		Present in sweet whey	Humans: satiety control [Lam et al., 2009]	Yvon et al., 1994; Beucher et al., 1994; Brody et al., 2000; Malkoski et al., 2001
		Gastric digestion supression				
		Antimicrobial				
		Dental caries prevention				
		Satiety promotion				
	MAIPPKKNQDK [κ-CN f(106-116)]	Antithrombotic activity	Inhibits platelets aggregation		[Maubois et al., 1991]	Jolles et al., 1986
LF	FKCRRWQWRM KKLGAPSICVR RAF [LF f(17-41)]	Antimicrobial	Against G-, G+, virus, yeast	LF + pepsin or chymosin		Bellamy et al., 1992; Hoek et al., 1997; Yoo et al., 1998; Wakabajashi et al., 2003; Samuelson et al., 2004
		Immunomodulatory properties				
		Anti-inflamatory properties				
Source protein	Amino acid sequence	Bioactivity	Pharmacological properties	Production	*in vivo* studies	References
	APRKNVRWCTI SQPEW-FKCRRWQWRM KKLGAPSITCV RRAFALECIRA [LF f(1-16)S-S(17-48)]	Antimicrobial	Against *Escherichia coli*	LF + chymosin		Hoek et al., 1997

Table IIc. (Continued)

	APRKNVRWCTI SQPEW-CIRA [LF f(1-16)S-S(45-48)]	Antimicrobial	Against *Micrococcus flavus*	LF + pepsin		Recio and Visser, 1999
	APRKNVRWCTI-FKCRRWQWRM KKLGAPSITCV RRAFALECIR [LF f(1-11)S-S(17-47)]	Antimicrobial	Against *Micrococcus flavus*	LF + pepsin		Recio and Visser, 1999
	FKCRRWQWRM KKLGAPSITCV [LF f(17-30)]	Antimicrobial	Against oral pathogenic bacteria	Synthetic peptide		Groenink et al., 1999
	SKCYQWQRRMR KLGAPSITCVRRT S [LF[d] f(17-41)]	Antimicrobial	Against *Escherichia coli*	Synthetic peptide		Vorland et al., 1999
	WKLLSKAQEKFG KNKRS [LF f(268-284)]	Antimicrobial	Against *E. coli, Candida albicans Bacillus subtilis and Pseudomonas aeruginosa*	Synthetic peptide		van der Kraan et al., 2004
	DLIWKLLSKAQE KFGKNKRS [LF f(263-284)]	Antimicrobial	Against *E. coli, Candida albicans Bacillus subtilis and Pseudomonas aeruginosa*	Synthetic peptide		van der Kraan et al., 2005
	LIWKL	AHT (ACE-inhibition)	IC_{50} = 0.47 μM	LF + pepsin	Rats: reduction of blood pressure	Ruiz-Giménez et al., 2012
	RPYL	AHT (ACE-inhibition)	IC_{50} = 56.5 μM	LF + pepsin	Study with rats: reduction of blood pressure	Ruiz-Giménez et al., 2012
	LNNSRAP	AHT (ACE-inhibition)	IC_{50} = 105.3 μM	LF + pepsin	Study with rats: reduction of blood pressure	Ruiz-Giménez et al., 2012
Proteose Peptone Fraction	Proteose-peptone component 3 (PP3)	Lipolysis inhibitor				Girardet et al., 1993
	C-terminal peptide of bovine PP3 f(113-135)	Antimicrobial	Against G- and G+ bacteria	Synthetic peptide		Campagna et al., 2004

In spite of that many studies have been made on the bioactivity of peptides from caseins (Meisel, 2004a; Phelan et al., 2009), it is well-known that whey proteins possess important nutritional and biological properties, particularly with regard to promotion of health and prevention of diseases (Madureira et al., 2007). They have and exceptional biological value and are a rich and balance source of sulphur amino acids (critical role as anti-oxidants) and essential amino acids, especially in the branched chain amino acids leucine, isoleucine and valine (metabolic regulators, weight control). Favorable combination of various biochemical and physiological features makes that whey peptides have the potential to play important roles in several areas of interest, namely as part of preventive and therapeutic health approaches (Meisel, 1997; Ko and Kwank, 2009). Controlled hydrolysis of all whey proteins, mainly β-Lg and α-La, leads to the production of bioactive peptides. Due to the relative difficulty in obtaining peptides by microbial fermentation, enzymatic hydrolysis has been the most common route to produce bioactive peptides from whey proteins and pancreatic enzymes, like trypsin, have been associated with efforts towards production, as well as characterization and identification of many peptides. After the hydrolysis step, peptides must be purified by means of different methods.

Some of these bioactive peptides have shown their functionality *"in vitro"* but their potential as effective health ingredients remains to be demonstrated in most of the cases due to the lack of clinical evidences and the limitation of some *""in vivo""* models.

Table II summarizes bioactive peptides derived from whey proteins that will be commented next.

3.1. Functionality of Whey Protein Peptides

Whey proteins provide a rich source of peptides, which are latent until released and activated by enzyme proteolysis (Hui, 2007; Korhonen, 2009). The potential bioactivities of the peptides released from these proteins are currently a subject of intensive research worldwide. The bioactivities most studied in the last years are:

1. Effects on the cardiovascular system (antihypertensive properties)
2. Hypocholesterolemic properties
3. Opioid and anti-opioid properties
4. Effects on the gastrointestinal system
5. Anticariogenic properties
6. Immunomodulation effects
7. Antibacteria properties
8. Antialergenic properties

The functions of peptides are being predominantly investigated in tissue culture systems and animal models. Whey peptides have so far been detected *"in vivo"* in blood or brain after ingestion of whole whey or whey proteins. These peptides can trigger physiological effects in the human body: on the nervous system via their opiate and ileum-contracting activities; on the cardiovascular system via their antithrombotic and antihypertensive activities; on the immune system via their antimicrobial and antiviral activities; and on the nutrition system via their digestibility and hypocholesterolemic effects. The physiological activity is measured by

the direct fuctional effect after oral administration of whey, ingestion of synthetic peptides and consume of food additives or pharmacologically with specific sequences (Guo et al., 2004; Townsend et al., 2004; Li et al., 2007; Quirós et al., 2007). Data prove the safety of fermented products by *Lactobacillus casei* strain *Shirota* (LcS) in healthy subjects and patients suffering from various diseases (Matsumoto et al., 2005). Also, the functionality of the peptides has been studied through simulations of gastrointestinal digestion of fermented products to obtain information about their route through the organism, further degradation due to digestive enzyme attack and the duration of the functional activity of the peptide (Didelot et al., 2006; Hernández-Ledesma et al., 2007; Wang et al., 2007a, b).

Currently *"in vitro"* and *"in vivo"* data on potential of whey peptides are relatively abundant. However clinical evidences are necessary to confirm their functionality (Saint-Sauveur et al., 2008).

3.1.1. Effects on the Cardiovascular System

The inhibition of the angiotensin-converting enzyme (ACE) by means of some whey peptides can exert an antihypertensive effect and the peptides that show this activity are usually called lactokinins (Wang et al., 2007b). At present, this is the most studied biological activity of whey derived peptides.

ACE is part of the renin-angiotensin system (RAS) and it has an important role in the regulation of blood pressure by converting angiotensin-I to a potent vasoconstrictor, angiotensin-II, which induces the release of aldosterone and therefore increases the sodium concentration and further blood pressure. By inhibiting ACE or by other still poorly known mechanisms, milk-derived peptides are able to low blood pressure in animal and humans (Jäkälä and Vapäätalo, 2010) and are believed to prevent cardiovascular diseases. Most of the ACE-inhibitory peptides derived from whey proteins are released by means of trypsin, being one of the most potent of them the heptapeptide ALPMHIR (IC_{50} = 43 μM) (Mullally et al., 1997). However, the use of a new food-grade proteolytic preparation, Protease N Amano, has resulted in the release of a novel peptide SAPLRVY which IC_{50} value is considerably lower (IC_{50} = 8 μM) (Ortiz-Chao et al., 2009). Nowadays, the main challenge in the production of these bioactive peptides is finding the suitable enzyme and hydrolysis conditions that enhance bioactivity and yield in their production.

Apart from ACE-inhibition, whey peptides may exert antihypertensive effects through other mechanisms such as inhibition of the release of endothelin-1 by endothelial cells (Maes et al., 2004), stimulation of bradykinin activity (Perpetuo et al., 2003), enhancement of endothelium-derived nitric oxide production (Sipola et al., 2002) and enhancement of the vasodilatory action of binding to opiate receptors (Nurminen et al., 2000).

Although there is a lot of information on the production and characterization of ACE-inhibitory peptides, their structure-activity relationship is not completely known. However, some general features have been found:

- ACE-inhibitory peptides usually contain between 2 and 12 amino acids residues, although active peptides with up to 27 amino acids have been identified. Amino acid sequences of peptides and the size of the peptides are associated to their biological activity or functional effect (Muro et al., 2011).

– The binding to ACE is strongly influenced by the C-terminal sequence. The presence of hydrophobic amino acids, e.g. proline, at each of the three C-terminal positions and the presence of a positive charge of lisine and arginine as the C-terminal residue may contribute to the inhibitory potency.

Amino acid sequences and the size of the peptides are associated to their biological activity or functional effect (Muro et al., 2011). In general, it is recognized that short peptides (<3 kDa) offer consistently higher ACE inhibitory activity (Hartmann and Meisel, 2007). Other researchers (Tsai et al., 2008; Miguel et al., 2009) indicated that the greatest level of ACE activity has been found in whey peptides up to 6 kDa. Sequences of short-chain with proline residues (Pro) are also identified as peptides with ACE inhibitory activity having hydrophobic amino acids such as tryptophan (Trp), tyrosine (Tyr) or phenylalanine (Phe), in at least one of the three C-terminal positions. For example, the sequence Pro-Glu-Trp has been found in four powerful ACE-inhibitory peptides derived from α-La hydrolysis by thermolysin (Vinderola et al., 2003; Foltz *et. al*, 2007; Otte et al., 2007a, b). Murakami et al. (2004) found that under hydrolysis conditions of β-Lg (60 to 80 °C) is possible to obtain a tetra-peptide with amino acid residues Ala-Leu-Pro-Met f(142-145) with hypotensive activity. The effect is attributed to proline residue in the tetra-peptide. Additionally, it has been proved that the power of inhibition can be increased with the presence in this terminal position of the positive charges of Lys (group e-amine) and Arg (guanidine group**)** (Hernández-Ledesma et al., 2008). Whey proteolysis by means of *Lactobacillus helveticus* extract release two recognized ACE-inhibitory tripeptides: Val-Pro-Pro and Ile-Pro-Pro (Sipola et al., 2001, 2002). Recently the amino acid sequence Leu-Leu (LL) has been proved to show high ACE-inhibitory characteristics. The molecular mechanisms, position, type, and energy of the LL/ACE interaction were investigated by using flexible molecule docking technology (Yamaguchi et al., 2009; Pan et al., 2012).

ACE inhibitory peptides are often produced by hydrolysis of β-lactorphins, that are peptides present in the primary amino acid sequence of bovine β-LG and they can be released by trypsin. Novel ACE inhibition has also been detected in synthetic peptides that corresponded to sequences within both β-LG and α-LA (Mullally et al., 1996; Li and Mine, 2004).

Peptide Quantitative Structure-Activity Relationship (QSAR) modeling has been used for predicting milk peptides structures with high ACE-inhibitory activities For peptides up to six amino acids, a relationship was found between this activity and some of the peptides characteristics (hydrophobicity and a positively charged amino acid at the C-terminal position). No relationship was found between the N-terminal structure and the ACE-inhibitory activity (Pripp et al., 2004).

In vitro, ACE inhibitory activity is measured through the absorbance of hippuric acid (HA) after the reaction of hydrolysates samples on hipuril-histidil-leucine (HHL). A variety of methods (e.g., spectrophotometry and fluorometry) by which ACE activity can be detected and analyzed are published (Cushman and Cheung, 1971; Gómez-Ruiz et al., 2002; Wu and Ding, 2002). High-performance liquid chromatography (HPLC) is widely used because of the effective separation of the substrate and product from the ACE reaction mixture to ensure accurate measurements. HPLC requires the use of a reverse phase C18 column and large amounts of organic solvents (acetonitrile or methanol). Capillary Electrophoresis (EC) is also used for ACE assays. The most common electrophoresis buffer assays used in EC system is

sodium borate buffer, due to the dual role the latter plays as the basal solution for the ACE assay mixture (Zhang et al., 2000). Electrophoretic experiments are carried out on a high voltage power supply. With HHL as the substrate, the enzymatic product (HA)is separated and quantified using capillary zone electrophoresis (CZE). CZE is performed with a 65 cm fused-silica capillary (55 cm effective length, 75 μm I.D.), using a 10 or 20 mM solution (pH 6.0) as the running buffer with an applied voltage of 20 kV at room temperature. Samples are applied in a 2 second electrokinetic injection and data are collected and peak migration time and area are analyzed (Chiang et al., 2005). This method can be applied directly to analyze an ACE reaction mixture without organic extraction prior to injection.

In all methods, the quantity of HA produced by ACE is measured spectrophotometrically at 228 nm using a UV Visible spectrophotometer. The ACE inhibiting activity is calculated and expressed in terms of IC_{50}, defined as the protein concentration required in the sample to inhibit 50% of the ACE (Mao et al., 2007). % ACE inhibition can be calculated with HA liberated by ACE and measured by RP-HPLC (Hyun and Shin, 2000). ACE inhibitory activity is calculated according to equation |1|. Percentage of ACE inhibition is expressed per 0.1 mg of protein in the fractions.

$$\% \text{ ACE inhibitory activity} = \frac{HA_{control} - HA_{sample}}{HA_{control}}.100 \quad (1)$$

The determination of ACE is very important since it constitutes a frequent test to evaluate antihypertensive drugs, by inhibition of angiotensin (Aleixandre et al., 2008). However, the IC_{50} value is not always directly related to the *"in vivo"* hypotensive effect, because some peptides can be susceptible to degradation or modification in the gut, the vascular system and the liver. By contrast, hypotensive activity of a long-chain candidate peptide can be caused by peptide fragments generated by gastrointestinal enzymes (Meisel et al., 2006). Active peptides must be absorbed in an intact form from the intestine after oral administration and further be resistant to degradation by plasma peptidases in order to reach the target sites. This is not always possible and, for example, peptide ALPMHIR is not sufficiently stable to gastrointestinal and serum proteinases (Walsh et al., 2004). However, proline-containing peptides are generally resistant to degradation by digestive enzymes (Masuda et al., 1996). On the other hand, only a few *"in vivo"* studies encompassing whey protein hydrolysates are available to date. In particular, peptides IPA and ALPM derived from β-Lg and YGLF derived from α-La were shown to reduce blood pressure *"in vivo"* (Abubakar et al., 1998; Nurminen et al., 2000; Murakami et al., 2004). Further studies are still required for a better understanding of the blood pressure reducing mechanisms of whey peptides and well controlled clinical human studies are needed to demonstrate the long-term physiological effects delivered by consuming such peptides.

Different "in vivo" studies on the antihypertensive effect of whey peptides show controversial results (Chen et al., 2003; Ijas et al., 2004; Muro et al., 2011). Hypotensive action is measured by lowering effect acute blood pressure after oral administration of whey, peptides, ingestion of whey or consume of dairy products. General therapeutic applications of whey proteins have been reviewed by Marshall (2004) and Saito (2008). Administration of pure peptides has produced a strong antihypertensive effect in spontaneously hypertensive rats (SHR) after a single-dose, but it has been found that IC_{50} values are even lower depending

on the substrate concentration used in the *"in vitro"* experiments (Lehtinen et al., 2010). Tretrapeptides such as α-Lactorphin (Tyr-Gly-Leu-Phe), β-lactorphin (Tyr-Leu-Leu-Phe) and β-lactosin (Ala-Leu-Pro-Met) showed strong antihypertensive effect in SHR (Sipola et al., 2002). Also proteinase K-digested whey was shown to decrease blood pressure in SHR after single-dose administration (Jauhiainen and Korpela, 2007b). From the digest, the peptide showing the strongest antihypertensive activity was found to be tripeptide Ile-Pro-Ala, originating from β-lactoglobulin.

Different methods to evaluate endothelial function have been used in clinical studies. Ambulatory arterial stiffness index (AASI) can be calculated from 24-hour blood pressure recordings, and it has been shown to be an independent predictor of cardiovascular mortality (Dolan et al., 2006). Another predictor of cardiovascular outcomes is aortic augmentation index (AAI), for which pulse waveform analysis is needed (Chirinos et al., 2005). In the study of Jauhiainen et al. (2007a), a significant improvement in AASI was observed after a 10-week treatment with *L. helveticus* fermented milk. In another study, AAI was decreased after 6 months' treatment with the same product.

On the other hand, various effects of tri-peptides (Ile-Pro-Pro) and (Val-Pro-Pro) in hypertensive subjects have been published (Xu et al., 2008; de Leeuw et al., 2009). Significant decrease of systolic blood pressure (SBP) and diastolic blood pressure (DBP) were found in the meta-analysis. In other clinical study, hydrolyzed whey protein supplement decreased blood pressure of prehypertensive or stage I hypertensive subjects by 8.0 mmHg of systolic and 5.5 mmHg of diastolic blood pressure after 6 weeks of treatment (Pins and Keenan, 2006). In contrast, milk drink supplemented with whey powder was not found to reduce blood pressure in mildly hypertensive subjects after 12 weeks' consumption (Lee et al., 2007). Thus, more intervention studies are needed to confirm the possible clinical benefits of the whey-derived peptides. Nevertheless, it´s mentioned that the results in human studies on antihypertensive peptides are usually quite small between the active and placebo treatment, so the study population should be enormously large to firmly prove the efficacy (Jäkälä and Vapäätalo, 2010). Alternative studies related to antihypertensive effect by consumption of dairy products have demonstrated a relationship between the intake of milk products and reduction in blood pressure. For example, the first National Health and Nutrition Examination Survey (NHANES I) showed that intake of dairy low-fat products, has consistently been associated with lower blood pressure levels and reduced risk of hypertension. A nine years' follow-up study of 6,912 white, non hypertensive men and women showed that subjects consuming three or more servings of low-fat milk per day had lower increase of blood pressure compared to those consuming less than one serving per week (Alonso et al., 2009).

Few studies did not find any significant effect either on SBP or DBP by treatment with whey peptide-containing products (van der Zander et al., 2008; van Mierlo et al., 2009).

Controversial human data on hypertensive peptides are discussed in a recent study (Jäkälä and Vapäätalo, 2010) where it´s presented that the following points affect the results found on antihypertensive peptides in human trials: administration of different products are not directly comparable, especially when the effects on a biological variable are small; the effective dose of lactotripeptides/day also affects the results; the concentrations of the peptides vary, especially if a regular product control during the long-term trial has not been conducted; the action of peptides can vary, because milk fermentation is produced by different species or strains; the reports on clinical trials have concentrated mainly on the changes in blood

pressure and vascular effects, however clinical chemistry as serum, fecal and urinary, could predict potential benefits relating to a target function in the body. The influence of subject background is discussed also in the review of Boelsma and Kloek (2009).

Another functionality related with the cardiovascular system is the antithrombotic activity showed for various peptides derived from the soluble C-terminal fragment of bovine κ-casein, CMP. The main antithrombotic peptide derived from CMP, MAIPPKKNQDK, is termed casoplatelin and this peptide inhibits both the aggregation of ADP-activated platelets as well as the binding of human fibrinogen γ-chain to its receptor region on the platelet surface. Smaller fragments of this peptide can also affect platelet function although they have much lower inhibitory activity that the complete fragment (Jollés et al., 1986). Three amino acid residues (Ile, Lys and Asn) of the CMP derived peptide seem to be important for its activity because they are homologous in position to the γ-chain sequence of human fibrinogen. It is important to mention that the undecapeptide has shown antithrombotic activity *"in vivo"* and could potentially be used to treat or prevent thrombosis (Maubois et al., 1991).

Regarding hypocholesterolemic effects numerous studies have shown that whey proteins, in contrast to milk casein, decreases serum cholesterol similar to soy protein. Peptide IIAEK derived from bovine β-Lg, also termed lastostatin, has shown this kind of functionality in both *"in vitro"* and *"in vivo"* studies (Nagaoka et al., 2001). This effect is supposed to be at least in part due to a decrease of micellar solubility of cholesterol which leads to lower intestinal cholesterol absorption but further results imply that the C-terminal side of lastostatin, in particular the glutamyl-lysine sequence, is crucial for the transcription of a human cholesterol-metabolizing enzyme.

3.1.2. Antioxidant Properties

Dietary consumption of antioxidants appears to provide extra benefits to the endogenous antioxidant defense strategies in the fight against oxidative stress. In addition to the well-know dietary antioxidants like vitamin C, vitamin E, polyphenols and carotenoids, recent studies have shown that peptides with antioxidant properties can be released from food sources like whey proteins. Various peptides derived from α-La and β-Lg hydrolyzed by means of thermolysin and Corolase PP respectively have demonstrated radical scavenging activity (Hernández-Ledesma et al., 2005; Sadat et al., 2011). Whey, hydrolysate proteins and free peptides have ability to scavenge free peroxyl radicals as well as to chelate transition metals such as calcium, iron, copper, and zinc. These properties of whey peptides could be used as antioxidants to prevent oxidative damage in muscle foods (Lindmark-Mansson and Akesson, 2000; Peña-Ramos and Xiong, 2003). The ability of peptides to inhibit deleterious changes caused by lipid oxidation appears to be related to certain amino acid residues in the peptides, such as tyrosine, methionine, histidine, lysine, and tryptophan, which are capable of chelating pro-oxidative metal ions (Bayram et al., 2008), capture free radicals and/or extinguish the reactive oxygen species (Murakami et al., 2004). Amino acid composition, sequence, and configuration of peptides affect their antioxidant activity (Muro et al., 2011). Other researchers have published that antioxidant activity of whey-derived peptides and whey itself is linked to the presence of cysteine-rich proteins which promote the synthesis of glutathione, a potent intracellular antioxidant (Meisel, 2005). Also, high amounts of histidine and some hydrophobic amino acids are related to the antioxidant potency (Peña-Ramos et al., 2004).

Antioxidant properties have been identified in whey peptides with typical structures (4 to 20 kDa) and between 5 to 11 aminoacids. Peng et al. (2009) obtained four fractions with different molecular weights by enzymatic treatment of whey protein isolate (WPI) using Alcalase. The peptide fraction in the range from 0.1 to 2.8 kDa showed a free radical lowering effect significantly higher than the other fractions with larger peptides. The presence of cysteine in the peptides promotes the synthesis of glutathione, a potent intracellular antioxidant (Erdman et al., 2008). Characteristic sequences (SerP-SerP-SerP-Glu-Glu), are effective cation chelators that form complexes with calcium, iron and zinc.

Antioxidative activity is not only related with the prevention of cardiovascular disease. Peptides that own this characteristic also contribute to the endogenous antioxidant capacity of foods and their use as preservatives is already revised in various studies (Elias et al., 2008). Furthermore, as cancer could be a consequence of oxidative DNA-damage, the application of antioxidative peptides to prevent and control this illness is being studied (Phelan et al., 2010). Oxidative stress, increase the production of reactive oxygen species (ROS) which in combination with outstripping endogenous antioxidant defense mechanisms, is a significant causative factor for the initiation or progression of several vascular diseases. ROS can cause extensive damage to biological macromolecules like DNA, proteins and lipids. Free radicals modify DNA, proteins and other small cellular molecules Antioxidants may decrease the oxidative damage and its harmful effects (Collins et al., 2005).

A variety of *"in vitro"* techniques have been developed for the detection of antioxidants on the basis of different antioxidative mechanisms under variable conditions reflecting the multifunctional properties of antioxidants in both physiological and food-related antioxidation processes. Antioxidant activity has been measured by means of three functions from peptides: antioxidant capacity, reduction capacity and metal chelating activity (Kim et al., 2007a). Antioxidant capacity can be determined through peroxyl radical of peptide using oxygen radical absorbance capacity (ORAC) assay based on quenching of fluorescence from the protein β-phycoerythrin by radicals utilizing the method of Cao et al. (1997). Final results are calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. Reduction capacity of peptide is determined through its capacity to reduce a metal such as Cu^{2+} to Cu^{1+} according to the method of Aruoma et al. (1998) or by the FRAP test, which consist in reduction of Fe^{+3} to Fe^{+2} (Benzie and Strain, 1999). Metal chelating activity is determined by the method of Decker and Welch (1990) using ferric ions. The metal chelating activity of the test sample is calculated using the formula |2| below:

$$\text{Metal chelating activity \%} = \frac{A_0 - A_1}{A_0} x100 \qquad (2)$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

Antioxidant capacity of peptides is evaluated using the FRAP test, which consists in reduction Fe^{+3} to Fe^{+2} (Benzie and Strain, 1999).

Other method used to determine antioxidant activity of peptides is based on the evaluation of the ability to inhibit or halt lipid oxidation in model systems. Test measures changes in the concentration of oxidized compounds, on depletion of oxygen or on formation of oxidation products. Quantification of the loss of reactants (oxygen, unsaturated fatty acids), formation of free radicals, and formation of oxidation products may be the most appropriate

markers depending on the stage of oxidation (Pihlanto-Leppälä 2006). The depletion of oxygen and the electron spin resonance (ESR) spectroscopy detection of radical, either directly or indirectly by spin trapping, can be used to follow the initial steps during oxidation (Andersen and Skibsted, 2006).

On the other hand, different *"in vivo"* trials have been conducted to assay the effect of antioxidant supplements of whey in clinical test and animal models.

In addition, antioxidant capacity from peptide is assayed with synthetic peptides by the structure-function relationship between the amino acid sequences (Rival et al., 2001). In this test is shown that peptides inhibit enzymatic and nonenzymatic lipid peroxidation, most likely by being a preferred target over fatty acid free radicals. Indirect evidence suggested that proteins/peptides can be oxidized during the process, according to a site- or sequence-specific mechanism. The extracellular release of superoxide or intracellular ROS and superoxide production, is measured by a plate reader assay with cells in situ or by cytometric flow analysis. Biomarkers, such as 8-hydroxy-20-deoxyguanosine, of oxidative damage in cellular DNA have been developed by Collins (2002), Halliwell and Whiteman (2004), Hoelzl et al. (2005) and Philanto and Keenan (2006).

3.1.3. Effects on the Nervous System

The opioid peptides derived from whey proteins, as well as the derived from other food proteins, are called "atypical" opioid peptides because their N-terminal sequence is different from that of the "typical" endogenous opioid peptides like enkephalins, endorphins and dynorphins. The tyrosine residue at the N-terminal and the presence of another aromatic amino acid at the third or fourth position form an important structural motif that fits into the binding site of the opioid receptors. Lack of the tyrosine residues results in a total absence of bioactivity. Proline residue in the second position of their structure is crucial for the three-dimensional orientation of the tyrosine and phenylalanine side-chains. The individual receptor at which these peptides are joined is responsible for specific physiological effects (Pihlanto-Leppälä, 2001; Wang et al., 2007a). Agonistic activity is comparable to that of endogenous ligands, whereas antagonistic peptides exert inhibitory effects similar to naloxone, a potent opiate receptor antagonist applied as a drug (in the case of heroin overdose) (Meisel, 2005).

α-La and β-Lg contain opioid-like sequences in their primary structure called α- and β-lacorphins that have pharmacological characteristics similar to morphine and they can be used then as analgesics. These peptides could be released by means of digestive enzymes like pepsin, trypsin and chymotrypsin. Both peptides are μ-type receptor ligands so they affect emotional behavior and suppression of intestinal motility. Furthermore, it has been shown that digestion of β-Lg with chymotrypsin produces β-lactotensin, which pharmacological activity is similar to that of β-lactorphin. These peptides also have antihypertensive functions and some of them showed hypocholesterolemic activity after administration to mice (Yamauchi et al., 2003). Proteolysis of α-La with pepsin produces some oligopeptides (up to 10 aminoacids) with opioid properties as the tetrapeptide (Tyr-Gly-Leu-Phe) and fragment f102-105 from β-Lg (Kamau, et al., 2010).

Tryptic digestion of BSA releases an opioid peptide, albutensin A (Tani et al., 1993). This peptide exhibits an ileum-contracting activity. It has been shown that the addition of this peptide on food intake in mice delays gastric emptying and elevates blood glucose too

(Ohinata et al., 2002), so it may eventually be used in human diets to promote weight loss and prevent obesity.

Opioid antagonists peptides derived from LF digestion, also called lactoferroxins, seem to be capable of antagonizing the inhibition of gut motility induced by casomorphins.

In spite of this intensive research, only some of these peptides have been proved their effects upon oral or intragastric administration and most of the tests have been performed in animals, hence much work remains to do in human clinical tests (Teschemacher, 2003).

3.1.4. Effects on the Gastrointestinal System

An improvement in whey proteins digestibility may easily be achieved via non-specific hydrolysis and this is beneficial for patients who suffer from digestion disorders such as cystic fibrosis, short bowel syndrome or pancreatitis (Hernández-Ledesma et al., 2004). Furthermore, whey protein hydrolysates could play important functions in the intestinal tract.

One of the most important peptides involved in these processes is GMP and a lot of studies have attempted to establish the potential role of this peptide and its non-glycosylated form (CMP) in regulation of intestinal functions. CMP has been reported to inhibit gastric secretions, slows down stomach contractions and stimulates the release of cholecystokinin, the satiety hormone involved in controlling food intake (Beucher et al., 1994). However, its clinical efficacy remains to be established.

Besides satiating effect of GMP and CMP, peptides with opioid-like activities affect food intake by the delay of gastric emptying and intestinal transit (Meisel, 1997).

GMP seems to contribute to anticariogenic effect by inhibiting the adhesion and growth of plague-forming bacteria in oral mucosa (Brody, 2000) and it has been used as ingredient in some dental care products. This κ-casein derived peptide may have a beneficial role in modulating the gut microflora due to its carbohydrate content (Manso and López-Fandiño, 2004) but its main nutritional role derives from its use as an ingredient in diets designed for people suffering from phenylketonuria who are unable to metabolize phenylalanine (Marshall, 1991).

3.1.5. Immune Modulation Peptides

Whey contains a number of immunomodulatory peptides that are naturally present or that are part of the primary sequence of whey proteins. The development of whey protein-based immunomodulating peptides is currently limited by the lack of their characterization and identification from bioactive hydrolysates and by the absence of clinical data on the physiological effects of these peptides. In fact, most studies on the immunomodulating effects of peptides have targeted the specific immune system by evaluating lymphocyte activation and proliferation, cytokine secretion and antibody production. Very few studies have addressed the impact of these components on the innate immune system (Gill et al., 2000; Gautier et al., 2006).

Little research on peptides derivates from β-Lg and α-La, lactoferrin, (LF), milk growth factors and immunoglobulin G (IgG) have also been reported to modulate lymphocyte proliferation *"in vitro"*. Tests are based on stimulation of superoxide anions such as ROS, which trigger non-specific immune defense systems (Kitts and Weiler, 2003). Other *"in vitro"* test consists in stimulation of phagocytosis of sheep red blood cells (SRBC) by murine peritoneal macrophages. Particularly LF is a potent modulator of inflammatory and immune responses, revealing host-protective effects not only against microbial infections but also in

inflammatory disorders such as allergies, arthritis, and cancer (Mcintosh et al., 1995). Crouch et al., (1992) isolated lactoferricin B peptide, obtained by hydrolysis of LF with pepsin; this peptide promotes phagocytic activity of human neutrophils via dual mechanisms that may involve direct binding to the neutrophil and opsonin-like activity. GMP has been discovered as a potent immunoenhancer even at low concentrations. This peptide enhances the proliferation and phagocytic activities of U937 cells (Li and Mine, 2004). Also, this peptide seems to play positive effects on patients with inflammatory bowel disease, but the mechanisms remains unknown (Kamau et al., 2010).

"In vitro" studies have demonstrated the potential of some whey components to modulate antibody production. For example, purified β-Lg enhances the production of IgM in cultures of murine spleen cells, but this effect can be abolished by trypsin hydrolysis of the protein.

Additionally, studies *"in vivo"* have shown that whey protein hydrolysates have an impact on the humoral immune system. The immune response has been evaluated in mice using the plaque forming cell (PFC) assay after systemic challenge with immune stimuli (SRBC). A study showing the effect of immunomodulatoy peptides by administration of LF to 95 elderly subjects before and after influenza vaccination in a double blind placebo controlled study. Before vaccination, the percentage of granulocytes of the total leukocytes was significantly higher in the lactoferrin group than in the placebo group. A similar effect was found for the monocytes 7 days after vaccination. The phagocytosis activity of the granulocytes was significantly enhanced in the lactoferrin group 1 day after vaccination. A small dipeptide, corresponding to the N-terminal end of bovine α-La (dipeptide) significantly increased proliferation of human peripheral blood lymphocytes. Bioactive peptides in yogurt preparations actually decreased cell proliferation. This report may explain, in part, why consumption of yogurt has been associated with a reduced incidence of colon cancer. Kayser and Meisel (1996) have described both stimulatory and suppressive immune responses of human lymphocytes to whey derived peptides.

An *"in vivo"* study of hydrolyzed whey proteins was used to evaluate inhibitory effects on the development of colon aberrant crypt foci (ACF) and intestinal tumors in azoxymethane (AOM). Pregnant Sprague-Dawley rats and their progeny were fed AIN-93G diets containing whey as the sole protein source. Colons and small intestines from the male progeny were obtained at 6, 12, 20 and 23 weeks after AOM treatment. At 6 and 23 weeks, post-AOM, WPH-fed rats had fewer ACF than did CAS-fed rats. Intestinal tumors were most frequent at 23 weeks, post-AOM. At this time point, differences in colon tumor incidence with diet were not observed; however, whey-fed rats had fewer tumors in the small intestine (7.6% vs. 26% incidence) (Xiaoa et al., 2006).

Yamauchi et al. (2006) reported that bovine LF reduces the number of infiltrating leukocytes during influenza virus infection (pneumonia) and suppresses the hyperreaction of the host. LF decreases the recruitment of eosinophils, reduces pollen antigen-induced allergic airway inflammation and it displays immunological properties influencing both innate and acquired immunities. Especially, oral administration of bovine LF seems to influence mucosal and systemic immune responses in mice (Sfeir et al., 2004).

Immunomodulatory peptides are also reported to influence cytokine production in cell culture experiments which are involved in immune and inflammatory actions of the body. Cross and Hill (1999) reported that a modified peptide rich in GMP suppresses the secretion of IL-4 and IFN-g in a ConA-induced murine splenic lymphocyte culture and that the effect is

partly abolished following enzymatic digestion of the extract with pepsin and pancreatin. GMP also induces the production of IL-1 family cytokines in murine macrophages and a macrophage cell line. The immunoenhancer property of this peptide at very low concentration seems to be due to the presence of terminal sialic acid and specific peptides at the end of its chain (Li and Mine, 2004). Chodaczek et al. (2006) demonstrated that a complex of LF with monophosphoryl lipid A is an efficient adjuvant of the humoral and cellular immune responses. Its stimulating effect on the immune system concerns mainly the maturation and differentiation of T lymphocytes, the Th1/Th2 cytokine balance and the activation of phagocytes.

In general, the mechanisms by which these milk-derived peptides exert either their immunopotentiating effects or influence proliferative responses are not currently known; however, is possible that the peptides exert an inhibitory effect on the proliferation of human lamina propria lymphocytes *"in vitro"* via the opiate receptor (Elitsur and Luk, 1991). This antiproliferative response was reversed by the opiate receptor antagonist, naloxone. Ohnuki and Otani (2007) reported that bovin IgG stimulates antibody responses in mouse spleen cell culture, whereas oral ingestion of bovine milk IgG suppresses the response in mice. It is unclear why bovine milk IgG has different effects on antibody responses *"in vitro"* and *"in vivo"*. Immunocompetent cells such as dendritic cells and macrophages possess several types of IgG receptors (Fc γ R) on their surface. Therefore, the different effects of bovine milk IgG on antibody responses may be due to the difference in Fc γ R for milk IgG on immunocompetent cells. The immunomodulating potential of peptide fractions isolated from β-Lg enzymatic hydrolysates has also been demonstrated (Prioult et al., 2004, Jacquot et al., 2010).

In addition, immunomodulatory peptides are naturally present or are part of the primary sequence of whey proteins. Particularly peptides derived from the N-terminal α-La from bovine milk significantly increase the proliferation of human peripheral blood lymphocytes. Short-chain peptides (<5kDa) obtained from the enzymatic hydrolysis of whey proteins, were identified as stimulators the proliferation of immune system cells (Mercier et al., 2004).

It has been shown that acidic and neutral peptides released from WPI digestion by means of a trypsin:chymotrypsin mixture stimulate splenocyte proliferation and cytokine secretion (Saint-Sauveur et al., 2008). The basic fraction of the aforementioned hydrolysate induces a sustained serum IgA secretion in mice infected with *Escherichia coli* while all the peptide fractions strongly stimulated total IgA production in non-infected mice (Saint-Sauveur et al., 2009).

Major discrepancies in the effect of some peptides on lymphocyte proliferation have been reported while the more limited literature on antibody production is less controversial. The effects of whey peptides on hypersensitivity, induction of oral tolerance and response to infections and diseases are currently topics for important researches.

To sum up, there is growing evidence that whey peptides may have some physiological activities on specific components of the immune response: lymphocyte activation and proliferation, antibody function and cytokine expression. However, a number of contradictory results have to be acknowledged maybe due to the use of different methodologies, raw materials and models.

3.1.6. Antimicrobial Effects

Proteolysis of α-La and β-Lg by means of digestive enzymes releases several antibacterial peptides that possess strong activity against Gram-positive bacteria (Pellegrini et al., 1999, 2000, Pellegrini, 2003).

GMP, as well as some of its derived peptides released by digestion with pepsin have also shown antibacterial effects (Kawasaki et al., 1992; Clare and Swaisgood, 2003; Campagna et al., 2004; El-Zahar et al., 2004).

Short-medium chain length bioactive peptides (10-50 amino acids) with cationic and hydrophobic properties have been known to be potent host-defense substances offering antimicrobial activity against a wide spectrum of pathogenic microorganisms such as Gram-negative and Gram-positive bacteria, fungi, viruses and parasites (Hancock and Sahal, 2006).

One of the most studied peptide derived from whey is lactoferricin. This peptide is released from LF by enzymatic digestion with pepsin or chymosin. It has shown not only bactericidal activity but also antiviral, antifungal, antiprotozoal, immunomodulating and anti-inflammatory properties (Wakabayashi et al., 2003; Gauthier et al., 2006).

The role of lactoferricin in tumor growth and metastasis has also been investigated (Yoo et al., 1998). Other potent antimicrobial peptides generated by degradation of LF with pepsin (lactoferricin B and lactoferrampine) have been effective against bacteria Gram positive, Gram negative, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Clare and Swaisgood, 2000; López-Expósito et al., 2006a, López-Expósito and Recio, 2006b).

The results show that the phosphorylation is essential for the antimicrobial activity. Some other studies have also demonstrated an antiprotozoal activity from LF (Omata et al. 2001) which has been treated in a review paper (Orsi, 2004), where it´s shown that LF is a multifunctional iron glycoprotein which is known to exert a broad-spectrum primary defense activity against bacteria, fungi, protozoa and viruses. Inhibiting influenza virus hemagglutination, inhibiting the binding of cholera toxin (CT) and *E.coli* enterotoxins and various oral pathogens as *Streptococcus mutans* were also studied by Malkoski et al. (2001) using whey peptides.

Other peptides from oligopeptides of whey, as kappacina, fragment f (138-158) of GMP characterized by having a phosphorylated residue serine (Ser) and accumulate on the cell membrane anion forming a pore, making it a powerful antibacterial peptide against Gram-positive (*Streptococcu mutans*) and Gram-negative (*P. gingivalis* and *E. coli*) (Rizzello et al., 2005).

Antimicrobial activity is also attributed to β-Lg and α-La. Digestion of these proteins produced some antimicrobial peptides. The activity was identified *"in vitro"*, acting against different Gram-positive and Gram-negative bacteria (*Escherichia*, *Helicobacter*, *Listeria*, *Salmonella and Staphylococcus*), yeasts and filamentous fungi (Fitzgerald and Murray, 2006).

The hydrolysis of whey proteins is especially important in the prevention of allergic diseases like, for example, in the design of hypoallergenic infant formulas (Exl, 2001).

Other effects as apoptosis of several types of cells and mineral binding properties have been described in several papers (Zhang et al., 2002; López-Expósito and Recio, 2008; Kim et al., 2007b; Kamau et al., 2010).

Table III shows the main human clinical studies of some commercial bioactive peptides.

Table III. Effects of whey proteins and whey hydrolyzed in human clinical studies

Whey components/peptides Human clinical study		Uses or commercial products	Reference
Whey proteins and whey hydrolysates	*Insulinotropic effects and reduces the postprandial glycemia in healthy subjects, and Type 2 diabetic patients * Decreases blood pressure of prehypertensive or stage I	"BioZate" 1; To low blood pressure and cholesterol	Kennedy, 1995 Rosaneli, 2002 Frid, 2005 ;Pins, 2006 Lee, 2007 ; Xu, 2008 de Leeuw, 2009
α-La and its hydrolysates	*Stress relief and reduced depressive mood *Improves cognitive functions in stress - vulnerable subjects by increased brain tryptophan and serotonin activity *Increases plasma tryptophan levels and its ratio to neutral amino acids *Inhibits the growth of skin papillomas in humans when topically applied	Ingredient for infant formulae "Vivinal alpha"	Markus, 2000, 2002, 2005 Gustafsson, 2004, 2005 Scrutton, 2007
3-hydroxyphthaloyl-β-Lg	Inhibits the human immunodeficiency virus type 1. Inhibits herpes simplex virus types 1	Not known	Oevermann, 2003
LF-enriched whey protein hydrolyzed and LF-bovine colostrum or probiotic bacteria	* Increases the number of bifidobacteria in fecal flora and the serum ferritin level *Decreases *Enterobacteriaceae*, *Streptococcus* and *Clostridium* * Decreases the incidence of bacteremia and severity of infection in neutropenic patients * Alleviates symptoms of hepatitis C virus infection and reduces small intestine permeability in drug - induced intestinal injury * Beneficial in stress - related neurodegenerative disorders and treatment of certain cancer types. * Induces apoptosis in human leukemia and carcinoma cell lines	Yogurt, baby foods and infant formulas. Oral care products: Toothpastes, mouth rinses, moisturizing gels and chewing gums. Oral products: "Modulen" and "Agennix"; treatment of cancer, asthma, wounds, and ulcers. "Talactoferrin product"; treatment of renal cell carcinoma, improve the healing of diabetic neuropathic ulcers in a phase I/II "Lactopharma"; decreases debilitating side effect of cancer chemotherapy and radiotherapy "Microlactin"; to alleviate the symptoms and dysfunction associated with osteoarthritis	Kayser, 1996 Iwasa, 2002 Ajello, 2002 Tamura, 2004 Mader, 2005 Zimecki, 2005 Hayes, 2006 Zimecki, 2007 Petitclerc, 2007 McBride, 2007 Lyons, 2007
GMP	*Alleviate hepatic diseases *Brain development and improvement of learning ability *Regulation of intestinal functions * Stimulate the release of cholecystokinin (CKK), the satiety hormone involved in controlling food intake and digestion in the duodenum * Promote the growth of bifidobacteria,	Fermented milk product "Calpis" or "Ameal S" and the Finnish fermented milk product "Evolus"	Wang, 2007b Manso, 2004

3.2. Production and Purification of Bioactive Peptides

Traditional therapeutic peptides were manufactured by transgenic, recombinant or synthetic methods but these approaches are known to be very expensive and thus are prohibitive for large-scale productions. Nowadays, the increasingly extensive knowledge about bioactive peptides encrypted in food proteins has open up the doors of new production processes starting from a relatively cheap raw material, especially in the case of whey and its industrial derivatives. This could result in a reduction in production costs with the added advantage of an efficient waste disposal.

Bioactive peptides can be incorporated in the form of ingredients into functional foods, novel foods and nutraceuticals, dietary supplements and even pharmaceuticals with the purpose of delivering specific health effects. In addition, bioactive peptides offer an interesting opportunity for personalized nutrition.

Some evidences suggest that bioactive peptides are produced naturally from dietary proteins during the gastrointestinal transit but that way they can´t be controlled and the amount of peptides released may be insufficient to generate a physiological response in adult humans. Suitable large-scale technologies for the production of bioactive peptides are needed. However, this is yet a non-resolved problem.

3.2.1. Protein Hydrolysis

Bioactive peptides released from food proteins are usually produced through *"in vitro"* enzymatic hydrolysis or "*in situ"* microbial fermentation but the first method is preferred due to the lack of residual organic solvents or toxic chemicals in the final products. Microbial fermentation products usually present a lack of uniformity and the risk of presence of inhibiting-fermentation compounds (Kamau et al., 2010). In fact, few studies have closely examined the microbial fermentation of whey proteins. ACE-inhibitory, immunomodulatory, antioxidative and antimicrobial peptides have been identified after microbial fermentation of milk but almost all of them result from casein hydrolysis (Korhonen and Pihlanto-Leppälä, 2006). The resistance of whey proteins to breakdown by bacterial peptidases has also been a controversial topic of discussion (Madureira et al., 2010).

The systematic use of parameters to describe hydrolysis reactions facilitates experimental work in protein hydrolysis. There are four parameters that define initial conditions for a given system enzyme-substrate (substrate concentration, enzyme/substrate ratio, pH and temperature) and other four that describe the reaction and the composition of the hydrolysate (degree of hydrolysis, protein solubilization index, average chain length of the soluble phase and percentage of trichloroacetic acid soluble peptides).

pH and temperature are the most important parameters to control in the case of enzyme reactions because they strongly affect the activity of these biocatalysts. Each enzyme works best at a certain pH and temperature, its activity decreases at values above and below that point. For example, the protease pepsin works best at pH 1-2 (found in the stomach) while the protease trypsin is inactive at this pH but it is very active at pH 8 (found in the small intestine as the bicarbonate of the pancreatic fluid neutralizes the arriving stomach contents). Furthermore, changes in pH alter the state of ionization of charged amino acids that may play a crucial role in substrate binding and/or the catalytic action itself. On the other hand, temperature increases the rate of enzymatic hydrolysis as with all chemical reactions. However, enzymes are biological molecules and they become denatured at high temperatures,

causing them to lose their catalytic activity. Strong research efforts have been made in the last years to get active enzymes at high temperatures.

In protein hydrolysis the degree of hydrolysis (DH), defined as the percentage of cleaved peptide bonds, is the key parameter for monitoring the reaction. Several methods for monitoring the DH during protein hydrolysis have been described in the literature: pH-stat, osmometry, soluble nitrogen content, the o-phthaldialdehyde (OPA) method and the trinitro-benzene-sulfonic acid (TNBS) method (Nielsen et al., 2001). Among them, the most used technique is the pH-stat. This method evaluates the progress of hydrolysis by titrating the released amino groups with an alkaline solution. The amount of base used is proportional to the DH (Adler-Nissen, 1986). Enzymes work at constant pH and temperature during the entire process, so that no buffering is needed. Furthermore, the method can be used to follow a hydrolysis reaction continuously, it is not laborious and does not include hazardous and unstable chemicals.

It has been shown that hydrolysis conditions influence enzymatic hydrolysis of proteins and peptide composition of hydrolysates depends on parameters like temperature, pH, concentration, buffer used and DH (Cheison et al., 2010, 2011). The study and control of these parameters is of vital importance to produce bioactive peptides in the desired extension. Response surface methodology, tool used in the design of experiments to obtain an optimal relationship between several explanatory variables and one or more response variables, has been used in recent studies to optimize hydrolysis conditions including enzyme-substrate ratio, pH and temperature with the purpose of obtaining the most powerful ACE-inhibitory peptides (Guo et al., 2009).

The proteases used for the production of bioactive peptides derived from whey can be from plants, microorganisms and animals. The most commonly used enzymes of animal origin are trypsin, pepsin, and chymotrypsin. Microbial proteases widely used are those obtainable from the *Bacillus* spp. *Bifidobacterium* and proteases from the Lactic Acid Bacteria. Microbial proteases provide some advantages over proteases from other sources because it costs relatively less to cultivate microorganisms and most of these proteases are expressed on the cell membrane, making harvesting and purification relatively cheap and less laborious (Agyei and Danquah, 2011). However, studies using digestive enzymes like trypsin or pepsin are more abundant in the case of whey proteins.

Trypsin specifically hydrolyses peptide bond with lysine of arginine residues on the carbonyl side. As others serin-proteases, it reaches its maximum catalytic activity at alkaline pH but its stability is limited by autolysis. This autolysis reaction is only important when handling the enzyme preparation before being added to the reaction mixture. The peptides resulting from trypsin hydrolysis of whey proteins, due to the high specificity of this enzyme, results in hydrolysate with excellent emulsifying properties (Chobert et al., 1988). ACE-inhibitory peptides are the most commonly produced by trypsin and they have been identified in tryptic hydrolysates of bovine β-Lg, α-La (Pihlanto-Leppälä et al., 2000) and in bovine, ovine and caprine κ-casein (Manso and López-Fandiño, 2003). Like trypsin, pure solution of pepsin is slowly degraded by autolysis reactions at pH values close to its optimum point. The enzyme is relatively stable to temperature and can be used up to 60ºC. Pepsin can release peptides from LF having antimicrobial activity (Recio and Visser, 1999; Ruíz-Giménez et al., 2012).

Batch reactors are commonly used at laboratory scale for enzymatic hydrolysis of proteins. The use of this kind of reactors is relatively simple and the main parameters which

need to be controlled being the temperature and the pH. However, they present disadvantages when they are going to be used under industrial scale conditions: low productivity, high operating costs, loss of catalytic activity due to inactivation, great variability of the quality of the products, etc. (Rios et al., 2004). Continuous processes are preferred at industrial scale conditions.

Another common approach is the use of immobilized enzymes onto highly activated supports (Lamas et al., 2001; Sousa et al., 2004). It allows enzymatic hydrolysis under mild and more controlled conditions. Additionally, immobilized enzymes can be recycled and the generation of secondary metabolites originating from autolysis of enzymes is avoided (Pedroche et al., 2007). Since there are loss of enzyme activity and constraints for diffusion into the support, the use of this technique is limited.

Use of enzymatic membrane reactors (EMR) for the production of bioactive peptides is gaining interest in the food industry because it´s a specific mode for running continuous processes in which enzymes are separated from end-products with the help of a selective membrane. That way it´s possible to obtain complete retention of the enzyme without deactivation problems commented for enzyme immobilization. Furthermore, EMR has been shown to improve the efficiency of enzyme-catalyzed bioconversion, to increase product yields and they can be easily scaled up (Perea and Ugalde, 1996; Martín-Orue et al., 1999; Prata-Vidal et al., 2001; Guadix et al., 2006). EMR operating in continuous mode has been applied for the production of bioactive peptides under lab-scale conditions: antithrombotic peptides derived from CMP (Bouhallab and Touzé, 1995); emulsifying peptides from β-Lg (Gauthier and Pouliot, 1996); opioid peptides from goat whey (Bordenave et al., 1999). The use of a multi-step recycling membrane reactor combined with and appropriate ultrafiltration membrane system has been used to separate marine-derived bioactive peptides (Kim and Wijesekara, 2010).

Because most of enzymes have molecular weight between 10 and 80 kDa, ultrafiltration membranes with cut-off between 1 and 100 kDa are frequently used to retain the enzyme.

It has also been reported that electrostatic or hydrophobic interactions between the biological molecules and the membrane surface could also influence the membrane process (Marshall et al., 1993; Lapointe et al., 2005). Permeate flux decline due to membrane fouling and enzyme activity decay are the main inconveniences of EMR. It has been shown that the presence a pre-hydrolysis step before the continuous operation minimizes fouling troubles due to the decrease of the viscosity of the initial solution (Darkono et al., 1989). In the specific case of protein hydrolysis, membrane fouling may, to a certain extent, be due to interactions peptide-peptide and membrane-peptide, especially if a charged membrane is used. In many occasions fouling problems are pH-dependent and the study of these interactions is very important to avoid or minimize it (Lapointe et al., 2005). It´s worth mentioning that enzymes are widely applied in the detergent industry to clean ultrafiltration membranes used in protein concentration (Argüello et al., 2005) as well as in EMR. However, little information is available on their role in simultaneous membrane cleaning during substrate hydrolysis. It´s of industrial interest to choose an enzyme with may confer on the EMR an auxiliary function of cleaning, hence reducing the gel layer on order to maintain permeate flux stability. On the other hand, the enzyme activity decay seems to be related with effects of temperature and denaturation of catalyst by adsorption/deposit onto the membrane (Paolucci-Jeanjean et al., 2001).

A membrane reactor operating in a cyclic batch mode is one of the latest developments regarding the selection of the operation conditions. This way of operation combines the advantages of both batch (easy operation and control) and membrane recycle reactor (enzyme reuse) (Prieto et al., 2007).

By the use of EMR a first peptide fractionation is achieved. Ultrafiltration using cut-off membranes of low molecular mass have been found as a possibility for separating out small peptides from high molecular mass residues and remaining enzymes. If a more purified permeate is required, EMR can be coupled with other separation techniques/units.

3.2.2. Fractionation and Isolation of Bioactive Peptides

Once the peptides are produced they remain in a mixture together with enzymes that must be deactivated by means of different techniques (heating, pH modification or chemical addition). Fractionation of peptides is a step that must be performed later in order to enrich the products in bioactive peptides and to assay their individual activity.

The methods commonly used for peptide fractionation and enrichment include ultrafiltration and nanofiltration, ion exchange, gel filtration technologies and liquid chromatography.

Liquid chromatography is the most extended isolation technique at lab-scale (Murakami et al., 2004; Tavares et al., 2011). In the same way, ion exchange chromatographic methods have been developed for the enrichment of casein phosphopeptides from casein hydrolysates. Most of these methods are effective under laboratory scale but they are prohibitive for large scale applications due to production costs. The high cost of purification techniques is a limiting factor to the commercialization of peptide-based products (Korhonen and Pihlanto-Leppälä, 2006). It has been estimated that separation and purification stages in industrial biotechnology processes can account for up to 70% of the capital and operating costs (Brady et al., 2008). Investigation into methodologies for the isolation of bioactive peptides under optimized conditions to reduce time and cost are ongoing.

A process-scale method for the isolation of casein phosphopeptides using acid precipitation, diafiltration and anion-exchange chromatography has been developed (Ellegård et al., 1999). The method was able to generate a process output of 40 kg casein phosphopeptides. Furthermore, a sequential chromatographic method involving gel filtration chromatography, ion-exchange chromatography and HPLC analysis has been used to isolate antioxidant peptides from porcine skin collagen (Li et al., 2007).

Ion exchange membrane chromatography has emerged as a promising technique for the enrichment of peptide fractions from protein hydrolysates. In this kind of technique the protein of interest is concentrated within the chromatographic medium and hydrolyzed *"in situ"*. The resulting active peptides are retained on the ion exchanger while the other peptides are washed out and finally the fraction containing the active peptides is eluted. This methodology was used to isolate cationic antibacterial peptides from lactoferrin (Recio and Visser, 1999).

As mentioned before, it´s established that pressure-driven membrane-based processes, such as ultrafiltration and nanofiltration, can be used to fractionate peptide mixtures and amino acids (Martín-Orue et al., 1998; Timmer et al., 1998; Lapointe et al., 2003; Lucena et al., 2006; Prieto et al., 2010). Membrane techniques are useful to obtain a permeate product enriched in small peptides and variations in operating conditions, may favour the permeation of bioactive peptides (Pouliot et al., 1999; Lapointe et al., 2005). Ultrafiltration cascades by

using different membranes cut-off combined with diafiltration steps have been demonstrated quite effective in α-La recovery from WPC (Lightfoot, 2006).

Nanofiltration separations are especially attractive for the fractionation of peptides contained in enzymatic hydrolysates. As surface charges (negative or positive) of nanofiltration membranes are their main distinctive feature, they offer the possibility of separating solutes through a combination of size and charge effects. Some researchers (Pouliot et al., 1999) have studied the fractionation of whey protein hydrolysates using charged membranes with a molecular weight cut-off between 1 and 5 kDa. They have shown, by changing the pH and the ionic strength of the hydrolysate, that the peptide separation is mainly governed by charge effects. Moreover, they reported that the best separation between acidic peptides (low isoelectric point) and basic peptides (high isoelectric point) was obtained at basic pH so it's interesting to further investigate the alkaline pH zone in order to identify optimal conditions for acidic and basic peptides separation. The same authors demonstrated that two peptides, differing by only one amino acid, were transmitted differently through the membrane. In a subsequent study it was found that the same peptide can be transmitted when issued from different hydrolysates, reflecting the importance of surrounding peptides, and, hence, the possible occurrence of peptide-peptide interactions (Pouliot et al., 2000). Additionally, there are peptides that can interact in the polarized layer during the filtration process and their transmission decreases with the time under specific conditions (Lapointe et al., 2005). Due to the aforementioned peptide-peptide and peptide-membrane interactions, it is difficult to predict the selectivity of the separation when using nanofiltration membranes and the process needs to be tailored to suit the peptide range aimed at and to minimize fouling by choosing the pH and the membrane material correctly (Butylina et al., 2006).

Ultrafiltration and nanofiltration seem to be the most promising techniques for peptide fractionation because of their low cost and easy scale-up. However, the process needs to be tailored to suit the peptide range aimed at and to minimize fouling by choosing the pH and the membrane material correctly (Butylina et al., 2006).

Electro-membrane filtration is an alternative method for the isolation of strongly charged biomolecules. It combines conventional membrane filtration with electrophoresis, making it more selective than membrane filtration alone and less costly than chromatography. By the manipulation of parameters like the type of membrane, electrical field strength, salination of hydrolysate and hydrolysate concentration, the product transfer and separation rate can be improved (Bargeman et al., 2002; Vanhoute et al., 2010). Lapointe et al. (2006) have been used this technique for the selective separation of cationic peptides from a Tryptic hydrolysate of β-Lg and special attention has been paid on peptide ALPMHIR (ahtihypertensive peptide). This peptide showed the highest transmission among all the peptides present in the hydrolysate during electrofiltration at basic pH.

The electro-membrane of electrodialysis process uses homopolar membranes (anionic or cationic) to allow the migration of ions mainly according to their charges. Ultrafiltration membranes can also be introduced in an electrodialysis stack, in addition to ion-exchange membranes, to improve electrodialysis applications and to concentrate specific molecules depending on their charge and size. In replacement of ion-exchange membrane, Galier and Roux-de Balmann (2004) investigated the use of porous membranes to separate poly (L-glutamic), α-La and bovine hemoglobin with good results. The use of electrodialysis with ultrafiltration membranes has been used to fractionate β-Lg hydrolysates during a batch recirculation process with the objective to separate acidic, basic and neutral peptides (Poulin

et al., 2006; Firdaous et al., 2009). This seems to be a very selective method of separation since amongst a total of approximately 40 peptides in the raw hydrolysate only 13 of them were recovered in the adjacent solutions. It has been shown that electrodialysis with ultrafiltration membranes would minimize the fouling of the ultrafiltration membrane. This promising technique could be applied to separate bioactive peptides and other charged molecules of interest from complex feedstocks in the food, pharmaceutical, fine chemistry and fermentation industries.

All the membrane techniques used to fractionate proteinaceous streams have fouling and polarization concentration as main drawbacks. Both of those effects reduce the permeate flow rate (increasing the installation size) and modify the membrane selectivity due to the effect of an additional "membrane" formed by the deposits on the membrane surface. Besides, proteins (especially hydrophobic proteins) usually show strong tendencies to be adsorbed on membrane surfaces. High lineal velocities, low protein concentration, low transmembrane pressures (lower that critical flux) and some mechanic techniques (as back-pulsing o back-flushing) can improve the efficiency of the membrane technology.

3.3. Analytical Techniques to Identify Biopeptides

Whey peptides produced by gastrointestinal digestion, in the fermentation process of milk and enzymatic hydrolysis, have different composition, and consequently different properties. Peptides usually contain 2-20 amino acid residues per molecule and their molecular structure and amino acid sequences are dependent of whey protein and enzyme used, as well as process conditions (temperature, pH, enzyme to substrate ratio and reaction time). Chemical measurements and analytical techniques are used to investigate correlations between these whey hydrolysate characteristics. Identification and quantification of peptides are based on description of their amino acid sequences, molecular mass, biological activity and functionally effects.

Electrophoresis (MCE), reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (ESI-MS) are the main analytical methods used to identify peptides resulting in a well-defined peak structure (Careri and Mangia, 2003; Muro et al., 2011). MCE method is often used for obtaining data from peptide fragments for sequence analysis. Peptides are electrophoretically separate by one-or two-dimensional polyacrylamide gel. The proteins-containing regions are detected by reversible staining and are cut out, and each protein is digested in situ by proteolytic enzymes. The resulting peptide fragments are separated by narrow-bore reverse-phase HPLC (according their polarity), collected, and sequenced by mass spectrometry methods (ESI-MS) and/or N-terminal sequencing. General tests of protein fractionation using gel electrophoresis (SDS-PAGE) have also been used to evaluate the protein profile after each step of hydrolysis (Gómez et al., 2002; Tauzín et al., 2002; Seppo et al., 2003; Matar et al., 2003; Meisel, 2001, Meisel and FitzGerald, 2004b; Fitzgerald et al., 2004; Gobbetti et al., 2004; Korhonen and Pihlanto-Lepääla, 2001; Yamamoto et al., 2003). Methods of Lowry et al. (1951) and the traditional Bradford method are used for measured proteins content in samples.

Monitorization of peptides formation throughout reverse phase RP-HPLC is described (Mota et al., 2006). Gradient elution of β-Lg and α-La hydrolysates was carried out with a mixture of two solvents (solvent A: 0.1% trifluoroacetic acid (TFA) in water and solvent B:

0.1% TFA in 80% aqueous acetonitrile, [v/v]). Peptides were eluted as follows: 0-1 min, 90% A; 1-10 min, 90–80% A; 10-15 min, 80-75% A; 15-20 min, 75-60% A; 20-30 min, 60-50% A; 30-33 min, 50-40% A; 33-36 min, 40-30% A; 36-39 min, 30-20% A; 39-41 min, 20-0% A. The flow-rate was 0.5 mL/min. The column was used at ambient temperature and detection at 215 nm. Total run time was 50 min. The chromatographic system was calibrated by the external standard method with solutions that contained bovine α-La in the range of 0.039-1.0 mg/mL and bovine β-Lg in the range of 0.0039-1.0 mg/mL. Major fractions are identified as peptides formation in HPLC profiles. Peptide recoveries and the absence of extraneous contaminants in the separation of the peptide fragment mixture allowed the generation of extensive internal sequence information. However the fractions usually contain multiple compounds that require further additional cycles of fractionation, concentration and assessment of bioactivity in order to identify the molecule responsible of the activity.

The molecular mass and amino acid sequences of peptides released by protease enzymes are studied by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) or electrospray (ESI-MS) techniques. The positive ions analysis function and 20 kV accelerating voltage are generally used. The adopted RP –HPLC, MALDI-MS and ESI-MS protocols provide molecular data of peptides and their concentrations. The methods are coupled to separate peptides and later fragment them by collision with an inert gas in the MS method.

The masses of the derived fragments are used to assay their composition according to their hydrophobicity, molecular size, and net charge. The results are compared with the theoretical values of the mass of fragments of protein sequences previously established. Similarly the potential of bioactive peptides is determined by comparison of the sequences previously reported (Mamone et al., 2009).

Standard electrospray ionization tandem mass spectrometry (ESI-MS/MS) for different detection methods is often used as a rapid preliminary verification of the identity of various molecular structures including ranging from 7 to 44 kDa with an accuracy of 0.01-0.03%. Intensity of characteristic picomol is showed in mass spectrums between m/z 0 to 1000 of each amino acid produced by digestion of whey proteins. ESI-MS/MS not only improves the speed but also the reliability of the structure determination when used in conjunction with other methods of peptides analysis. Charge states of peptides are identified by molecular mass determination via a charge deconvolution procedure. For example the hydrolysis of β-Lg by four enzymes (porcine trypsin (PT), Fusarium oxysporum trypsin (FOT), *Bacillus licheniformis* proteinase (BLP) and *Bacillus subtilis* proteinase, Neutrase®) was characterized by RP-HPLC, size exclusion high performance liquid chromatography, SE-HPLC, and by capillary electrophoresis (CE).

After 24 h of hydrolysis, all β-Lg was degraded by PT and BLP, but a large part of the protein was still intact after hydrolysis by FOT or Neutrase®. The hydrolysis catalysed by each enzyme resulted in different peptide profiles by RP-HPLC and CE. The main fraction of peptides was found to have MWs ranging from 1.0 to 3.0 kDa. Hydrolysates produced by PT or FOT were resolved into 18 peaks, while BLP hydrolysates were resolved into 25 peaks, corresponding well to the numbers of possible cleavage sites in β-Lg. Neutrase®, with a broad specificity, produced the largest number of peptides. However seven peptides from PT hydrolysis, eight from FOT hydrolysis and one from BLP hydrolysis were identified by mass spectrometry (MS) and Edman degradation (Madsen et al., 1997).

FINAL CONCLUSION

Cheese whey can be considered as a great protein source that is not completely exploited at industrial scale today. Whey, delactosed and desalting whey powders, whey protein concentrates and whey protein isolates are well know and all of them are being produced by whey processing companies and they are commonly used as functional ingredients in food and special diets. Other proteins as lactoferrin, lactoperoxisade, β-lactoglobuline and α-lactoalbumin can be found in the market with different purity degrees. Improvement of different technologies is necessary to get higher purity and recovery rates and probably the use of combined technologies is compulsory to develop economical processes.

Whey hydrolysates are being investigated nowadays in order to produce bioactive peptides that could be added in form of novel foods or even produced as pharmaceuticals products to deliver specific health benefits. Researchers are continuing to discover new whey protein peptides bioactivities. Only some of the peptides that can be obtained by enzymatic hydrolysis have been studied, then and extra work must be done to identify possible activities of many others. Technologies to separate, purify and concentrate these biopeptides must be developed at industrial scale. Membrane technologies (ultrafiltration, nanofiltration, electrofiltration, electrodialysis, etc.) have potentialities to be scaled-up, however better separation efficiencies are necessary to produce higher purity. For this, combination of different technologies will be probably useful to get these objectives. The development of other novel techniques as nanoencapsulation and nanoemulsions can help to increase the stability of these peptides after their isolation.

With respect to the activity of biopeptides there is a strong need for further human clinical tests so that most of the analysis conclusions have been obtained after *"in vitro"* or *"in vivo"* tests with animals.

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Chapter 2

TECHNOLOGICAL AND CHEMICAL CHARACTERIZATION OF PDO CHEESES OF ITALY

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ABSTRACT

Italy is one of the main cheese producers in the world, with over 30 Protected Denominations of Origin (PDO) awarded by the European Union, besides hundreds of typical and traditional cheeses. Italy has always supported a recognition policy, institutionalization as well as denomination of its productions, both due to Regional food traditions and for its interest to protect its food products. Therefore, PDO cheeses are a significant resource in Italy where approximately 50% of their milk production is destined for these products. Among these, Parmigiano Reggiano, Grana Padano, Gorgonzola and Mozzarella di Bufala Campana as well as Asiago, Taleggio or Ragusano are some of the best known in the world. Over 30 Italian PDO cheeses, all registered in the EU list (Reg. EC 510/2006), possess typical qualities strictly correlated to the environmental-production conditions. In particular, these cheeses are made using raw or pasteurized milk of cows, goats, sheep and buffalo, high-quality raw materials, in specific geographical areas of production and by using consolidated technologies linked to the 'art' of the cheesemaker. Such characteristics, consolidated over many years, led to obtain products with chemical, microbiological as well as distinctive sensory features, which are unique for each type of cheese.

The aim of this chapter is to summarize the knowledge about Italian PDO cheese, focusing the attention on their cheesemaking rules and particular traits that characterize them. For each cheese a card will be done with technological, chemical and sensory characteristics.

INTRODUCTION

Italy is the world leader in the dairy sector with a turnover of about 14.8 billion Euros. In Italy in 2010 about 1.75 million cows, 0.245 million buffalos, 7 million ewes and 0.8 million goats were counted. The production of milk by these animals has been estimated at 10.5 million tons of cow milk, 0.2 millions tons of buffalo milk, 0.4 millions tons of ewe milk and 0.2 millions tons of goat milk. About 2.9 millions tons of cow milk was used for direct consumption while the remaining milk was used to produce about 0.8 million tons of cow cheeses, 0.061 million tons of ewe cheeses, 0.009 million tons of goat cheeses, 0.05 million tons of buffalo cheeses, 0.08 million tons of mixed cheeses and 0.4 million tons of different dairy products such as butter, yogurt, powder milk, etc.

Among the 43 Italian cheeses registered as Protected Designation of Origin (PDO) and produced according to the European rules, the most important PDO cheeses are Parmigiano Reggiano, Grana Padano and Gorgonzola. Moreover data corresponding to the year 2012, registered Parmigiano Reggiano and Grana Padano as cheeses with the highest export both in Europe and in the World at volumes of 38,791 and 61,902 tons, respectively (ISTAT, 2013).

Information about the characteristics of all Italian PDO cheeses, their cheesemaking and composition will be described in this chapter. For each cheese, references (from SCOPUS source) concerning the chemical composition studies carried out over the past 10 years, and the website of the consortium, when available, will also be reported.

ASIAGO

Characteristics (PDO, June 12, 1996 modified on October 15, 2007)

There are two different types of cheeses, pressed Asiago and fostered Asiago. The pressed Asiago has a cylindrical shape (diameter 30-40 cm, height 11-15 cm and weight 11-15 kg) with a thin and elastic rind. The interior paste has marked and characterized by irregular eyes of white or slightly yellow color. It has a delicate and pleasant flavor. According to PDO requirements, the chemical composition of pressed Asiago at 20 days of maturation must be as follows: moisture 39.5±4.5%, protein 24.0±3.5%, fat 30.0±4.0%, sodium chloride 1.7±1.0%, fat in the dry matter must be not less than 44%. The fostered Asiago has a cylindrical shape (diameter 30-36 cm, height 9-12 cm and weight 8-12 kg) with a smooth and regular rind. The interior paste has small and medium eyes with a straw or slightly yellowish color. The taste is sweet in less ripened cheeses while fragrant in those having been aged. According to PDO requirements the chemical composition of fostered Asiago at 60 days of maturation must be as follows: moisture 34.5±4.0%, protein 28.0±4.0%, fat 31.0±4.5%, sodium chloride 2.4±1.0%, fat in the dry matter of not less than 34%.

Production Area

The geographical area of production is the whole territory of the provinces of Vicenza and Trento, and in some municipalities of the province of Padova and in a defined area of the province of Treviso.

Cheesemaking

Asiago is a semi-cooked cheese made with cow's milk. Cows are fed on pasture, with limited concentrate supplementations during the grazing season; during the indoor season the feeding is mainly based on conserved forages with notably higher amounts of concentrates. Feed based on fodder is not allowed in the case of the production of the cheese with the label "*product of mountain*". Production is divided into two different types of cheese, pressed Asiago and fostered Asiago. For the production of pressed Asiago cheese, raw or pasteurized (72°C, 15 seconds) milk (one or two milkings) can be used. Selected starters or milk cultures, possibly small amounts of sodium chloride and coagulated with bovine rennet at 35-40°C., are added to whole milk. After 15-25 minutes, the curd is cut to produce fine granules about the size of a walnut or hazelnut. The curd is cooked at 44°C and pressed for up 12 hours. For the production of fostered Asiago, raw or thermised (57/68°C, 15 seconds) milk from one or two milkings, is partially skimmed and selected starters (or milk cultures) and a small amount of sodium chloride are added. Lysozyme can be added (2.5 g/100 kg milk) except for the cheese with the label "*product of mountain*". The milk is coagulated by the addition of bovine rennet at a temperature of 33-37°C (15-30 minutes). The curd is cut to produce fine granules, the size of a hazelnut or less. The curd is semi-cooked at 47°C. After draining, the curd is put into molds. Before salting, the cheeses are kept for a minimum period of 48 hours at 10/15°C and 80-85% relative humidity (RU). The cheese may be dry salted or brine salted at 20°C, +/- 2°Be. The minimum ageing is 20 days for pressed Asiago, 60 days for fostered Asiago and 90 days, from the last day of production of fostered Asiago, and 30 days, from production of pressed Asiago, for the product with the label "*product of mountain*". Ripening is carried out at 10-15°C and 80-85% relative humidity. For the first 15 days after salting, fostered Asiago may be kept in storage at temperatures between 5 and 8°C. Asiago cheese, with the label "*product of mountain*" must be ripened in rooms located in mountain areas where temperature and humidity are determined by natural environmental conditions. The pressed Asiago cheese may also be labeled with the indication "fresh". Fostered Asiago may be labeled as "matured" or "aged" cheese if it has been ripened over 10 months while labeled "extra aged" if ripened more than 15 months. The Asiago cheese labeled as "*product of mountain*" cannot be treated on the surface with colorants and agents, which avoid the developing of molds.

http://www2.regione.veneto.it/videoinf/rurale/precedenti/anno%202006/33/Asiago_formaggi.htm

Figure 1. Asiago.

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Website

http://www.asiagocheese.it/

BITTO

Characteristics (PDO June 12, 1996 modified on November 25, 2009)

Bitto has a cylindrical shape (diameter 30-50 cm, height 8-12 cm and weight 8-25 kg) with a concave and sharp edge. The rind is solid with a depth of 2-4 mm. The color switches from yellowish to more intense yellow as it ripens. The interior paste is solid with rare eye-holes and its color changes from white to yellow depending on the age. At the beginning of the ripening process, the cheese taste is sweet and delicate. After the first year it develops a sharper and richer flavor, also due to the use of goat's milk.

The product can be grated as a condiment after one year of age. According to PDO requirements, fat in dry matter must be not less than 45% and moisture, at 70 days, must be 38%.

Production Area

The geographical area of production is the whole territory of the province of Sondrio, some municipalities in the Alta Val Brembana in the province of Bergamo, and some municipalities in the province of Lecco.

Cheesemaking

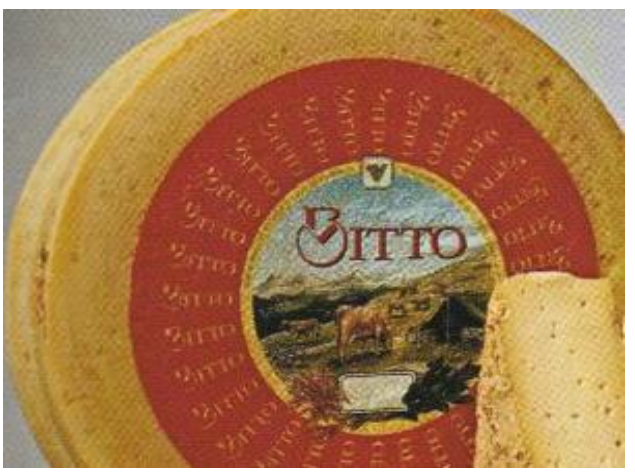
Bitto is a traditionally made, semi-cooked and semi-hard cheese produced at an altitude of at least 1,500 m only between June 1 and September 30. It is made with whole raw cow's milk of the Italian Brown breed, to which a supplement of raw goat's milk up to a maximum of 10% is allowable.

According to the guidelines on production, besides grazing pastures, the dairy cows' diet may be supplemented by feeds containing maize, barley, wheat, soybean and molasses (up to a maximum of 3 kg of dry matter per day). Milk must be processed within one hour from the end of the milking. Acidification is due to indigenous microflora, but the use of

autochthonous starters made up from indigenous Bitto microflora is allowed. Milk is coagulated with bovine rennet.

The curd is cooked at a temperature between 48 and 52°C for about 30 min and then is cut to produce granules with the size of a grain of rice. Once the curd is extracted, it is placed in traditional wooden molds, achieving the typical concave shape. Cheese can be dry salted or brine salted.

Cheese starts to ripen in the so-called Alpine "casera", a rural small hut near the pasture, and finishes in the factories down in the valley at the natural climatic conditions of the production area. This process lasts for at least 70 days.



http://www.visitfai.it/dimore/castelgrumello/la-tua-visita-132-Prodotti-tipici.

Figure 2. Bitto.

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Website

http://www.ctcb.it/

BRA

Characteristics (PDO 1 July 1996)

Bra can be found in two forms, soft and hard. Both types have a cylindrical form (diameter 30-40 cm, convex edge 7-9 cm, weight 6-8 kg). The color of the interior paste is white/ivory in the soft type and slightly yellow in the hard type, with very rare and small eyes. The structure is fairly solid and elastic in the soft type. The rind, not edible, is light grey, elastic, smooth and regular in the soft type while solid, hard and dark beige in the hard type. The soft type has a very soft and mainly sweet taste. The hard type is much more savory. The soft type is used as table cheese while the hard type is usually grated. For both types of cheeses, the fat in the dry matter must be a minimum of 32%.

Production Area

The geographical area of production is the whole territory of the province of Cuneo for both production and ripening, and the municipality of Villafranca Piemonte, in the province of Torino, for the ripening only. Soft and hard Bra cheese, produced and ripened in the mountain municipalities listed in the PDO, can be labeled "*di Alpeggio*".

Cheesemaking

Bra is a pressed, semi-fat cheese, made with cow's milk to which small amounts of sheep and/or goat's milk can be added. According to the procedural guidelines, the cattle must be fed with green forages or hay. Milk (from one or two milkings) is often partially skimmed. Coagulation is obtained by using liquid rennet at a temperature between 27 and 32 °C. The cheese must be produced with a typical technology involving a double curd cutting. The cheese is then appropriately pressed and put into apposite molds. Usually, the cheese is dry salted twice and in some cases brine salting is carried out as well. The ripening period must be at least 45 days for the soft type and 6 months for the hard type.

http://www.langood.it/public/shop/formaggi/formaggi-vaccini/formaggio-bra-tenero-giobrat.asp

Figure 3. Bra.

Website

http://www.assopiemonte.com/

CACIOCAVALLO SILANO

Characteristics (PDO 1 July 1996 modified on 4 July 2003)

Caciocavallo Silano has an oval or trunk-conical form with creeks. Each cheese can weigh from 1 to 2.5 kg. The rind is thin, smooth and straw yellow colored. The interior paste is white or straw yellow, regular, firm and it very rarely presents eyes. The taste is gentle and sweet in less aged cheeses becoming piquant in those aged. According to PDO requirements, the fat in dry matter must not be less than 38%.

Production Area

The geographical area of production is the whole territory of Calabria, Campania, Molise, Puglia and Basilicata regions.

Cheesemaking

Caciocavallo Silano is a semi-hard, pasta filata (or spun paste) cheese made with cow's milk. Milk is coagulated at 36-38°C by the addition of paste bovine or kid rennet. When the curd has reached the wanted firmness, its cutting is performed to produce granules with the size of a hazelnut. The ripening starts with a vigorous lactic fermentation, which lasts from 4 to 10 hours or more, depending on the acidity of the milk used. Cheese ripening is completed when the cheese is ready to be kneaded (or "spun"). In order to check the maturation time, a small amount of cheese is collected at very close time intervals and put in boiling water to test if it can be extended into elastic, shiny, continuous and strong fibers. Then, the paste is manually modeled until the required form is obtained and each single piece of cheese obtained is closed at the apex and quickly dunked in boiling water. The final form, with head where required, is given manually. The cheeses are individually cooled in cold water and salted using brine solution (resting not less than 6 hours). After the brining, Caciocavallo cheeses are hung in a couple on horizontal poles to be ripened for a minimum of 15 days.

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Website

http://www.parcosila.it/it/saperi-e-sapori/prodotti-dop/consorzio-caciocavallo-dop

http://www.parcosila.it/it/saperi-e-sapori/prodotti-dop/consorzio-caciocavallo-dop)

Figure 4. Caciocavallo Silano.

CANESTRATO PUGLIESE

Characteristics (PDO June 12, 1996)

Canestrato Pugliese has a cylindrical shape (diameter 25-34 cm, edge 10-14 cm, weight 7-14 kg) with a brownish, hard, roughness and a thick rind. The rind is treated with olive oil, possibly mixed with wine vinegar. The interior paste is solid and very friable with a slight or

more intense yellow according to the cheese age. The cheese has a characteristic piquant taste. According to PDO requirements fat in dry matter must be at least 38%.

Production Area

The geographical area of production is the whole territory of the province of Foggia and some municipalities in the province of Bari.

Cheesemaking

Canestrato Pugliese is a hard cheese made with sheep's milk. The herd must be fed with green and/or conserved forages or hay (only exceptionally with concentrate supplementation). Milk (from 1 or 2 milkings) is coagulated at 38-45°C with the addition of lamb rennet within 15-25 min. The following steps of cheese-making must be completed within 30-60 days, depending on the size and weight of the form. Cheese is pressed into particular containers, "canestri" or baskets, which give the characteristic roughness to the rind. Two-4 days after the production, cheese is still maintained in the baskets where it can be salted in dry conditions or using brine solution. Ripening lasts from 2 to 10 months and takes place in fresh and ventilated rooms. Cheese, aged for at least six months, can be consumed as table cheese or grated cheese.

http://www.arssa.abruzzo.it/atlanteprodotti/home.php?module=view_prodotto&id=34&idtipo=4

Figure 5. Canestrato Pugliese.

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CASATELLA TREVIGIANA

Characteristics (PDO 2 June 2008)

Casatella Trevigiana has a cylindrical shape (big form: diameter 18-22 cm, edge 5-8 cm, weight 1.8-2.2 kg; small form: diameter 18-12 cm, edge 4-6 cm, weight 0.25-0.70 kg). The interior paste is soft, shiny and a little bit creamy with a color ranging from milky-white to creamy-white. Small eyes can be present. The rind is almost absent. The cheese has fresh milky aroma and a sweet and a slightly acidic taste. According to PDO requirements the chemical characteristics must be as follows: moisture 53-60%, fat 18-25% and protein higher than 12%.

Production Area

The geographical area of production is the whole territory of the province of Treviso.

Cheesemaking

Casatella Trevigiana is a soft cheese made with cow's milk from the Italian Frisona, Pezzata Rossa and Bruna breeds. Cows must be fed with forages and territorial concentrates and the total fat content of the milk must be higher than 3.2%. Production starts within 48 hours from the milking. Milk can be pasteurized (70-75°C, 15-25 sec) and left to cool down at 34-40°C. Acidification is achieved by the addition of indigenous milk starters composed by *Streptococcus thermophilus* and thermophilic lactobacilli with a prevalence of *Lactobacillus delbrueckii* subsp. *lactis*. Milk is coagulated at 34-40°C by addition of liquid or powder bovine rennet within 15-40 min. The curd is cut crosswise and left to stand between 45 and 55 min, in order to obtain a more drained and firm curd. A second cut is carried out to produce granules with the size of a walnut. Curd is then extracted and put in molds, locally, at 25-40°C for a maximum of 3.5 hours (for bigger forms). During the draining, the forms are turned 2 to 4 times. Cheeses can be salted in brine solution (16-22°Be, 4-12°C) for 40-50 min for smaller forms and 80-120 min for bigger forms. Instead of using brine solution, dry salting can also be done. Ripening is carried out in molds at 2-8°C for 4-8 days, by turning the forms every two days. Casatella Trevigiana must be packed in the territory of production before commercialization.

http://www.veneto.to/veneto-qualita-dettaglio?uuid=144c7976-6ef4-4785-8b89-d8986ecb97e9&lang=it

Figure 6. Casatella Trevigiana.

Website

http://www.casatella.it/il-consorzio-di-tutela/

CASCIOTTA D'URBINO

Characteristics (PDO 12 June 1996)

Casciotta d'Urbino has a cylindrical shape (diameter 12-16 cm, edge 5-7 cm, weight 0.8-1.2 kg). The matured cheeses have a thin rind (1 mm thickness) and a straw yellow color. The interior paste is soft with characteristic small holes. The color is white-straw yellow. The characteristic taste, due to the particular cheesemaking process, is sweet and pleasantly acidic. It is consumed as table cheese. According to PDO requirements, fat in dry matter must be not less than 45%.

Production Area

The geographical area of production is the whole territory of the provinces of Pesaro and Urbino.

Cheesemaking

Casciotta d'Urbino is a semi-cooked cheese made from sheep (70-80%) and cow's milk (20-30%). Milk (from 2 milkings) is coagulated at 35°C with liquid and/or powder rennet. Cheese is manually pressed into special molds, using a typical procedure. Cheese is dry or brine salted. Cheese ripening lasts from 20 to 30 days at 10-14°C and 80-90% relative humidity, depending on the size of the forms.

http://www.guidaenogastronomia.com/casciotta-d-urbino.html

Figure 7. Casciotta d'Urbino.

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Website

http://www.casciottadiurbino.it/consorzio

CASTELMAGNO

Characteristics (PDO July 1, 1996 modified on December 14, 2012)

Castelmagno has a cylindrical shape (diameter 15–25 cm; edge: 12–20 cm; weight: 2–7 kg). These characteristics are referred to the minimum ripening period (60 days). Fresher forms have a not-edible, thin rind with a reddish yellow color, while the more matured forms have an aged aspect and a darker color. The structure of the cheese is very crumbly at the beginning of the ripening and becomes firmer as it is aged. The interior paste is white/ivory colored, tending towards ochre-yellow with streaks of greenish-blue color in the more matured forms. The presence of streaks is due to the development of natural molds, belonging to the *Penicillium* species. Its delicate taste gets stronger and sharper as the ripening season goes on. According to PDO requirements, fat in the dry matter must be a minimum of 34%.

Production Area

The geographical area of production is limited to three municipalities (Castelmagno, Pradleves and Monterosso Grana) in the province of Cuneo.

Cheesemaking

Castelmagno PDO cheese is a semi-hard cheese produced with raw cow's milk and a small amount of sheep and/or goat's milk (from 5 to 20%). Cattle must be fed on green forages or hay. Milk, which can be obtained from a maximum of four consecutive milkings and can be skimmed, has to be heated to 30-38°C and coagulated by the addition of liquid calf rennet (with at least 70% of chymosin). Pasteurization, thermisation and use of starter cultures are not allowed. Once the curd reaches an adequate firmness, it is turned and then cut under the whey, traditionally called "la laità". The following cutting is carried out to gradually reduce the size of granules to that of a hazelnut (10-15 min under continuous stirring). The curd is left to separate from the whey and then is transferred on a dry and clean cloth (called "risola") where it rests for 18 h. After the draining, the curd is put in suitable food containers, including wood containers, under the whey obtained from previous cheese making and left at 10°C for 2–4 days. The curd is then milled, salted, enveloped in a cloth and put in molds for at least one day, where it is manually or mechanically pressed. The cheese forms can further be dry salted to give color and consistency to the rind. Finally, the cheese is placed in natural caves where ripening takes place for at least 60 days at 5–15°C and 70–98% relative humidity, in order to allow the development of the natural molds.

http://www.castelmagno-oc.com/pres_cast/tesi_form_cast.htm

Figure 8. Castelmagno.

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Website

http://www.assopiemonte.com/

FIORE SARDO

Characteristics (PDO July 1, 1996)

Fiore Sardo cheese has a cylindrical shape, 12-18 cm high and 18-20 in diameter, and weight 1.5-4 kg. The rind changes color, from yellow to brown at the end of ripening and the interior has a white-yellow color and a Grana-like texture. During the ripening the cheese becomes piquant due to large lipolysis induced by the rennet enzymes. It could sell as "grated" cheese if it is ripened more than 6 months. According to PDO requirements, fat in dry matter must be higher than 40%.

Production Area

The geographical area of production is the whole territory of Sardegna Region.

Cheesemaking

Fiore Sardo is produced from whole raw ewe's milk. Sheep must be of Sardinian breed and must pasture in an environment rich in aromatic herbaceous (*Lolium rigidum*, *Trifolium subterraneum* and *Medicago arabica*) and shrubby (*Arbustis unedo*, *Pistacia lentiscus*, *Myrtus communis* and *Thymus*) species. The milk used comes from one (from June to September) or two (from October to May) daily milkings, and it can be thermized and natural starter cultures may be added. Fresh milk must have a pH of 6.6, a fat content of 6.2-7.5% and a protein content of 5.5-6.3%. Coagulation takes place in a copper vat using lamb or kid rennet paste, traditionally produced at the farm by shepherds themselves, and it is carried out in 12-28 minutes at 34°C during the spring and summer season, or at 36°C during the winter. After coagulation the coagulum is cut into millet-grain size and left to rest under the whey for at least 5 min. The curd is then transferred into stainless steel molds with a cut-down cone shape that confers to the cheese the typical "mule's back" form. After the molding, the cheese is turned upside down to promote the whey removal. At the end, cheeses are washed with hot water or whey to promote the formation of a smooth and resistant rind. The ripening is divided into three steps: the first step lasts 15 days during which cheeses are smoked 2 hours every day at 18-20°C using local wood or brush. The second step lasts 3 months at a temperature of 10-15°C during which cheeses are turned upside down. The last step takes place in a ripening room at a temperature lower than 15°C with a relative humidity of 80-85%. During this period cheeses are turned upside down and the surface can be rubbed with an olive oil-vinegar and salt emulsion.

http://www.sardissimo.it/public/photogallery/fiore_sardo_dop.jpg

Figure 9. Fiore Sardo.

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FONTINA

Characteristics (PDO June 12, 1996 modified on February 3, 2011)

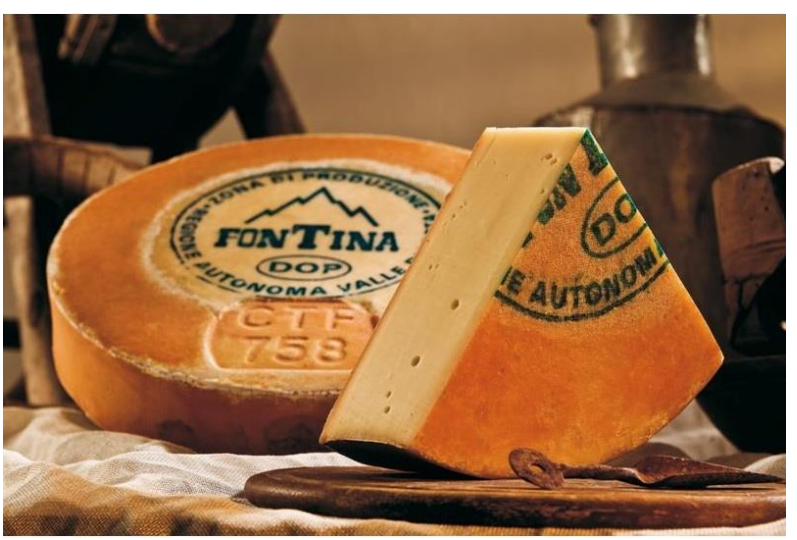
Fontina cheese has a cylindrical shape, 7-10 cm high and 35-45 in diameter, and weight 7.5-12 kg. The rind has a brown color at the end of ripening and the interior has a color ranging from ivory to pale yellow with an elastic texture. According to PDO requirements, the fat in the dry matter must be higher than 45%.

Production Area

The geographical area of production is the whole territory of Valle d'Aosta Region.

Cheesemaking

Fontina is produced from whole raw cow's milk exclusively obtained from native breeds (red pied, black pied and chestnut), fed with hay and green grass grown in the same Region. The milk used comes from one milking and it can be added with autochthonous starters that are preserved by the Consortium "Produttori e Tutela della DOP Fontina". Coagulation takes place in a copper or steel vat at 34-36°C for 40-60 min using calf rennet. After coagulation the coagulum is cut into maize-grain size and, stirring continuously, the curd is heated up to 46-48°C to promote the whey draining. Once the curd becomes sufficiently cleared it is left to settle under the whey for 10 min. The curd is then transferred into cloth bags that are put inside typical molds with concave-sides on which a small casein plate is applied with the cheese's progressive number and pressed. The pressing stage lasts 12 hours during which the cheese is turned upside down to promote the whey removal. Before the final turning the small number plate is applied with the producer's identification number which, together with the casein plate, guarantees the clear tracing of the product. Cheeses are brine-salted for no longer than 12 hours. Cheeses are ripened in caves dug out the rock or into a ripening room for at least 3 month at 5-12°C with a relative humidity higher than 90%, during which the cheese is periodically brushed and dry-salted on the surface. During the first month of ripening, cheeses are turned daily, salted one day and washed with a brine solution the day after to promote the formation of the typical red rind due to the growth of the microbial ecosystem of the surface.



http://vetrina.fontina-valledaosta.it/uploads/633/800_3.jpg

Figure 10. Fontina.

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Website

www.consorzioproduttorifontina.it

FORMAGGELLA DEL LUINESE

Characteristics (PDO April 11, 2011)

Formaggella del Luinese cheese has a cylindrical shape with fat sides, 4-6 cm high and 13-15 in diameter, and weight 0.7-0.9 kg. Molds can be present on the white rind with different colors. The interior has a white color with many eyes and an elastic texture. The cheese has a medium-mild, delicate, pleasant flavor that becomes stronger during the ripening. According to PDO requirements water content must be less than 55% and fat in dry matter must be higher than 41%.

Production Area

The geographical area of production includes numerous municipalities in the province of Varese.

Cheesemaking

Formaggella del Luinese cheese is made from whole, raw goat's milk coming from the Camosciata delle Alpi, Nera di Verzasca and Saanen breeds (and their crosses), which are typical of the Alpine area. In addition to grazing, the diet of the herds, which remain at pasture for 7/8 months, is based on the use of hay fodder from local mixed meadows supplemented with concentrates. During the grazing season, alongside the use of wild plants such as heather, spring pruning's from hazel, ash and chestnut trees and wild herbs, the diet is supplemented with concentrates and, in some cases, hay fodder. During the period in stalls, hay fodder and concentrates are primarily used. The hay fodder is mainly obtained from areas on the valley floor where mixed meadows with species such as *Dactylis glomerata*, *Poa pratensis*, *Poa trivialis*, *Avenula pubescens*, *Trifolium repens* and *Trifolium pratense* predominate. The use of grass silage is not permitted. The milk used, coming from a maximum of three milkings, is added with autochthonous starters or selected starters made of *Streptococcus* and *Lactobacillus* thermophilic strains and a small percentage of *Lactococcus* mesophilic strains. Coagulation is made at 32-34°C in 30-40 min using natural calf rennet. After coagulation the coagulum is cut into the size of maize-grains and, stirring continuously, the curd is heated up to 38°C to promote the whey removal. Once the curd becomes sufficiently cleared it is left to rest under the whey for 15 min. The curd is then transferred in 14 cm diameter molds and left on tables for 48 hours to promote the whey removal. During this period it is necessary to turn the cheese upside down at least 2-5 times. Cheeses are dry or brine-salted (solution at 18-20% NaCl at 14-20°C for 7 hours per kg of cheese). Cheeses are ripened in a room for at least 20 days at a temperature under 15°C with a relative humidity of 85-95%.

http://www.crsoresina.it/ricette/images/formagella_del_Luinese.jpg

Figure 11. Formaggella del Luinese.

FORMAGGIO DI FOSSA DI SOGLIANO

Characteristics (PDO November 30, 2009)

Formaggio di Fossa di Sogliano cheese has an irregular shape with typical bumps and depressions due to the pressure inside the pit. The surface is primarily wet and greasy and in some cases may be covered by butterfat and molds, which can be easily scraped off. Small cracks and possible yellow ochre stains of varying intensity on the surface are also typical of the product. There is minimal or no rind, and the cheese weighs 0.5-1.9 kg. The interior has an ivory white to a slightly yellowish color and a semi-hard texture. The flavor changes, depending on the composition of the aged cheese, in accordance with the following characteristics: the ewes' milk cheese has an aromatic aftertaste and a fragrant, full and pleasant flavor, slightly tangy to a greater or lesser extent; the cows' milk cheese is subtle and delicate, with a moderately salty and slightly acidic taste with a hint of bitterness; the mixed cheese has a pleasant flavor between salty and sweet, with a slightly bitter taste. According to PDO requirements the water content must be less than 55% and the fat in dry matter must be higher than 32%.

Production Area

The geographical area of production is the whole territory of the provinces of Forlì-Cesena, Rimini, Ravenna, Pesaro-Urbino, Ancona, Macerata and Ascoli Piceno and some municipalities in the province of Bologna.

Cheesemaking

Formaggio di Fossa di Sogliano is produced from 100% whole cow's milk or 100% whole ewe's milk or a mix of both milks with a ratio of 80:20 respectively. The bovine species used to produce the milk are Italian Friesian, Alpine Brown, Italian Red Pied and cross-breeds; the ovine species are Sarda, Comisana, Massese, Vissana, Cornella White, Fabrianese of Langhe and Pinzirita and cross-breeds. The animals may be housed or grazed. The basic feed for housed animals consists of fodder comprising grasses and legumes collected from monophyte, oligophyte and polyphite meadows. The milk used, coming from two daily milkings, must have the following properties: cow's milk (fat % by volume > 3.4; protein % by volume > 3.0; bacterial count UF/ml <100000; somatic cell/ml <350000; anaerobic sporigens/litre absent; inhibitors absent and cryoscopic point °C < 0.520); ewe's milk (fat % by volume > 6.0; protein % by volume > 5.4; bacterial count UF/ml < 1400000; somatic cells/ml < 1400000 and inhibitors absent). The milk can be used raw or pasteurized. Coagulation takes place at 30-38°C in 7-20 min using natural rennet. After coagulation the coagulum is cut into maize or kernel size and left to rest under the whey for 15 min. The curd is then transferred into molds (12-20 cm in diameter and 6-10 cm in high) and manually or mechanically pressed. The cheese can be subjected to a stewing process. Cheeses are dry or brine-salted. Cheeses are ripened for a minimum period of 2 months to a maximum period of

8 months. For the ripening, it is possible to use cells at 6-14°C with a relative humidity of 75-92%. Traditionally cheeses are ripened in a pit dug into tuffaceous rock (*fossa*). Before the cheeses, closed into cloth bags, are placed in the pits, the pits are suitably prepared according to the traditional method: when not in use the pits are kept closed by means of wooden covers with the addition of sand or rocks. When they are to be used, they are opened, aired and then sanitized by fire and smoke by burning small quantities of wheat-straw. When the fire is out, the pits are cleaned to ensure that no ash remains. A raised platform made of untreated wooden planks is placed at the bottom of each pit. This allows the fatty liquid, produced during the fermentation of the cheese as it ages, to drain away. The walls of the pit are lined with a layer of at least 10 cm of wheat-straw supported by vertical reed fencing. The pit is filled to the top with bags of cheese. After a period to allow settling, never more than ten days, more bags can be added to fill the pit. When the pit is full, the top is covered by uncolored cloths suitable for use with foodstuffs, and/or straw, to prevent transpiration. The pit is then closed by means of a wooden cover, which is sealed using plaster or lime sandstone mortar. Further covering using rocks, sandstone powder, sand and/or planks is permitted. This is when the aging starts. The pits may not be opened during the aging process. The cheeses remain in the pits from at least 80 to at most 100 days. Inside, the temperature remains constant, between 17 and 25°C, throughout the entire 80-100 days of ageing while the relative humidity increases from 80% to 95% in the first 24 hours and then remains stable for the entire ripening period.

http://www.comune.sogliano.fc.it/ilpaese/produzioni/formaggiodifossa/Img/Formaggio01.jpg

Figure 12. Formaggio di Fossa di Sogliano.

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Website

www.ilformaggiodifossa.com

FORMAI DE MUT DELL'ALTA VALLE BREMBANA

Characteristics (PDO June 12, 1996)

Formai De Mut Dell'Alta Valle Brembana cheese has a cylindrical shape, 8-10 cm high and 30-40 in diameter, and weighs 8-12 kg ± 10%. The color of the thin rind ranges from yellow to grey at the end of ripening, and the interior has a white ivory color with partridge eyes and an elastic texture.

Production Area

The geographical area of production includes some municipalities of Alta Val Brembana in the province of Bergamo.

Cheesemaking

Formai De Mut dell'Alta Val Brembana is produced from whole cow's milk obtained from the Alpine Brown breed. The animals are pastured during the summer period, and in winter are housed and fed with hay fodder mainly obtained from areas on the valley floor. The milk used comes from one or two consecutive milkings and it is characterized by a naturally low acidity. Coagulation takes place at 35-37°C in 30 min using rennet. After coagulation the coagulum is cut into rice-grain size and heated up to 45-47°C. When the temperature is reached, the curd is stirred for 30 min, out from the heat source, and left to rest under the whey for 20 min. The curd is then transferred into molds and manually or mechanically pressed for 48 hours to promote the whey removal. Cheeses are dry or brine-salted on alternate days for a period of 8-12 days. Cheeses are ripened for at least 45 days to 6 months at 8-13°C at a constant relative humidity.

http://www.originalitaly.it/blog/wp-content/uploads/2012/04/31100_19.jpg

Figure 13. Formai de Mut dell'Alte Valle Brembana.

Website

www.formaidemut.info

GORGONZOLA

Characteristics (PDO June 12, 1996 modified on February 3, 2009)

Gorgonzola cheese has a cylindrical shape, 13 cm high and 20-32 in diameter. Concerning the weight, it is possible to sell three different types of cheeses: large wheel, mild type: 10-13 kg, with a mild or slightly tangy taste, minimum maturation period 50 days; medium wheel, tangy type: 9-12 kg, with a pronounced tangy taste, minimum maturation period 80 days; small wheel, tangy type: 6-8 kg, with a pronounced tangy taste, minimum maturation period 60 days. The thin rind is grey due to the microbiota present and it has a white or pale yellow color with molds (marbling) producing characteristic blue-green veins and a creamy to firm texture. According to PDO requirements, the fat in dry matter must be higher than 48%.

Production Area

The geographical area of production is the whole territory of the provinces of Bergamo, Biella, Brescia, Como, Cremona, Cuneo, Lecco, Lodi, Milano, Monza, Novara, Pavia, Varese, Verbano-Cusio-Ossola and Vercelli and some municipalities in the province of Alessandria.

Cheesemaking

Gorgonzola is produced from whole pasteurized cow's milk obtained from the Italian Friesian breed. Milk is inoculated with natural milk cultures constituted by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* species and a suspension of *Penicillium* spores and selected yeasts, which characterize Gorgonzola as a blue cheese. Coagulation takes place at 28-36°C in 30 min using calf rennet. After coagulation the coagulum is cut and left to rest under the whey. The curd is then transferred into molds and the origin mark, with the identification number of the dairy farm, is placed on both flat ends and left 12-15 hours to promote the whey removal. Cheeses are dry-salted for several days at a temperature of 18-24°C. Cheeses are ripened for at least 50 days at 2-7°C with a relative humidity of 85-99%. During ripening cheeses are pierced two times: the first time after 15 days of ripening on one side, and after 25 days on the other side, so that the air can enter into the cheese leading to the growth of molds.

http://cdn.blogosfere.it/saporiericette/images/gorgonzola-dolce.jpg

Figur**e** 14. Gorgonzola.

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Website

www.gorgonzola.com

GRANA PADANO

Characteristics (PDO June 12, 1996 modified on June 17, 2011)

Grana Padano cheese has a cylindrical shape with slightly convex circumference rind, 18-25 cm high and 35-45 in diameter and weight 24-40 kg. The cheese has a hard and smooth rind with a thickness of 4-8 mm and golden yellow color. The interior has a white or straw-color, a hard but finely grained texture, flaky from the middle out and with barely visible eyes. The cheese can be sold as a "grated" product obtained exclusively from whole cheese that has already been certified. Grated cheese must have moisture not lower than 25% and not higher than 35%; the appearance should not be powdery, but even, particles with a diameter of less than 0.5 mm and not more than 25%.

The Grana Padano cheese produced in the Trento and Bolzano Provinces is named Trentingrana but the production discipline differs because the type of milk used must come from the Alpine Brown cow breed; in the fodder the use of silage is forbidden as well as the use of lysozyme. According to PDO requirements, fat in dry matter must be higher than 32%.

Production Area

The geographical area of production is the territory of the provinces of Alessandria, Asti, Biella, Cuneo, Novara, Torino, Verbania, Vercelli, Bergamo, Brescia, Como, Cremona, Lecco, Lodi, Mantova (only left of the Po River), Milano, Monza, Pavia, Sondrio, Varese, Trento, Padova, Rovigo, Treviso, Venezia, Verona, Vicenza, Bologna (only right of the Reno River), Ferrara, Forlì Cesena, Piacenza, Ravenna and Rimini and in the municipalities of Anterivo, Lauregno, Proves, Senale-S. Felice e Trodena.

Cheesemaking

Grana Padano is produced from raw milk obtained from cows of the Italian Friesian breed, fed primarily with feed produced on the home farm or in the Grana Padano production area. No less than 50% of the dry matter of the daily feed should be made up of feed with a ratio of fodder to feed of no less than 1, with reference to the dry matter, and at least 75% of the dry matter of the fodder in the daily ration should come from feed produced in the production area of the milk. The fodder could be fresh fodder, hay, straw, silage and it can contain cereals and their derivatives, oil seeds and their derivatives, tubers and root vegetables and products derived from them, dehydrated fodder, derivatives of the sugar industry, legume seeds, fats, minerals, additives. The milk, obtained by two daily milkings, is partially skimmed by a natural rising of the cream made in very large tanks for 12 hours at 12-15°C. After the creaming process the milk is put into a copper vat with an inverted cone shape and lysozyme (2.5 g/100 Kg of milk) could be added. After the addition of natural whey starter cultures, coagulation takes place in the vat using calf rennet. After coagulation the coagulum is cut and cooked at a temperature under 56°C. When the coagulum pieces reach an elastic texture they are then left to rest under the whey for a maximum of 70

minutes. The curd is then transferred into molds and left for 48 hours to promote the whey removal. Cheeses are brine-salted for 14-30 days. Cheeses are ripened for at least 9 months at 15-22°C with a relative humidity of 80-85%.

http://www.langhe.net/wp-content/uploads/2011/04/Grana-Padano.jpg

Figure 15. Grana Padano.

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Website

www.granapadano.com

MONTASIO

Characteristics (PDO June 12, 1996 modified on April 8, 2011)

Montasio cheese has a cylindrical shape, 8 cm high and 30-35 in diameter, and weighs 6-8 kg. The rind is smooth, regular and elastic with a yellow color and the interior has a slightly straw-yellow color with some eyes. The cheese has a piquant and pleasant taste. According to PDO requirements, water content must be higher than 36.72% and fat in dry matter must be higher than 40%.

Production Area

The geographical area of production is the whole territory of Friuli-Venezia Giulia Region, the whole territory of the provinces of Belluno and Treviso and the area along the boundary of the province of Padova from where it intersects with that of the province of Treviso to the Serenissima motorway, continuing to the motorway bridge over the Brenta River and then along the river to its mouth.

http://www.winecountry.it/assets/besideWine/cheese/montasio_young.jpg

Figure 16. Montasio.

Cheesemaking

Montasio is produced from raw cow's milk. The cow breeds used are Alpine Brown, Italian Red Pied and Black Pied. The breeds are fed with cereals (particularly maize and barley), green and dried fodder (polyphites and alfalfa) and silage (mainly from maize). These products make up about 80-85% of the total diet and come mainly from the production area. The concentrates and nucleus proteins are supplied by feed manufacturers generally located in the production area who mainly use local cereals, buy protein meal (e.g. soy and alfalfa, also produced on site) and mineral and vitamin supplements. The milk comes from a maximum of four milkings. The milk is then placed in copper vats heated at 32-36°C, with added natural milk starter cultures. Coagulation takes place using calf rennet for 20-25 min. After coagulation the coagulum is cut into rice-grain size and cooked at 42-48°C. When the temperature is reached the curd is stirred for 20-30 min out of the heat source. The curd is then transferred into molds and pressed to promote the whey removal. Cheeses are dry or brine-salted. Cheeses are ripened for at least 60 days at a temperature higher than 8°C.

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Website

www.formaggiomontasio.net

MONTE VERONESE

Characteristics (PDO July 1, 1996)

Denomination of Origin 'Monte Veronese' is reserved to the cheeses having specific characteristics and moreover, depending on the type of the milk used. In particular, the cheese is made in the same production area. Using the same cheese making rules, it may be produced starting from whole milk or semi-skimmed milk, and called "Monte Veronese" or "Monte Veronese d'allevo", respectively.

Production Area

The geographical area of production is located in the North of the province of Verona, beginning at the state highway n.11 (Padana Superiore) to enter the district of San Bonifacio, which is crossed, it passes through Villanova to reach San Martino Buon Albergo e San Michele center and Verona along the bypass, and it continues up to km 297 of the Croce bianca until Caselle and finishing in the district of Peschiera bordering on provinces of Verona and Brescia.

A) "Monte Veronese" PDO

Cheese has the following characteristics:

a) cylindrical shape
b) weight between 7 and 10 kg
c) size: heel height 6-10 cm, diameter 25-35 cm. The variations both of the weight and dimensions of the cheese depend by the manufacturing production as well as the ripening period
d) Thin and elastic rind, more or less intense straw yellow color
e) The paste is white lightly straw yellow with small holes equally distributed
f) The taste is mild and palatable. Ripening is carried out for a minimum of 90 days for the table cheese type and a minimum of six months for the grated cheese
g) fat in dry matter must be not less than 44%

B)"Monte Veronese "d'allevo PDO

Cheese has the following characteristics:

a) cylindrical shape with a flat or slightly bowed sides
b) weight between 6 to 9 kg
c) size: heel height 7-11 cm, diameter 25-35 cm. The variations both of the weight and dimensions of the cheese depend by the manufacturing production as well as the ripening period.
d) thin and elastic rind, more or less intense straw yellow color

e) The paste is white, lightly straw yellow with small holes equally distributed throughout and usually with a size 2 to 3 mm bigger than the cheese made from whole milk
f) The cheese has a perfumed taste typical for the ripened cheese which becomes more piquant when the ripening is longer
g) fat in dry matter must be not less than 30%

Cheesemaking

A) "Monte Veronese" from whole milk is a hard cheese exclusively made from raw whole cow's milk, coming from one or two consecutive milkings, and having a final acidity value ranging from 3.6 to 3.8 SH/50 ml. Milk acidity can be obtained naturally or by adding milk ferments derived from the dairy factory involved in cheese production or from other previous productions made from factories indicated in article 2 of the Regulation.

The milk is coagulated for 15-20 min by the addition of calf rennet and the breaking of the curd is carried out for few seconds to obtain clots with dimensions of a grain of rice.

The curd is heated to reach the final temperature of 43-45°C for 10 min and left to cook for 25-30 min. Salting is carried out in dry conditions or in brine solution after the draining off of the curd, about 24 hours. Ripening finishes within 30 days with a minimum of 25 days.

http://www.venlat.it/prodotti.html

Figure 17. Monte Veronese PDO.

B) “Monte Veronese d’allevo” is either a table or grated cheese type made from semi-cooked pasta exclusively by using partially skimmed cow’s milk obtained from one or two consecutive milkings. Final acidity value of the milk ranging from 3.8 to 4 SH/50 ml, may be obtained naturally or by adding milk ferments derived from the dairy factory involved in cheese production or from other previous productions made from factories indicated in article 2 of the Regulation.

The curd is obtained using rennet made from calves' stomachs for 25-30 min and breaking is carried out for few seconds up to obtain the clot with dimensions of rice grains.

The curd is heated to reach the final temperature of 46-48°C and left to cook for 15 minutes.

The curd is then allowed to stand in a warm condition for about 25-30 min.

Salting is carried out in dry conditions or in brine solution after the draining off of the curd of about 24 hours. Ripening finishes at a minimum of 90 days for the cheese used as table cheese type while the grated cheese must ripen for a minimum of six months.

Website

http://www.monteveronese.it/

MOZZARELLA DI BUFALA CAMPANA

Characteristics (PDO June 12, 1996, modified on February 4, 2008)

Mozzarella di Bufala Campana is a steamed cheese exclusively produced with buffalo’s milk and marketed inside its preserving liquid or “accompanying” liquid. Mozzarella di Bufala Campana must have a characteristic and delicate taste with fat (on dry matter) of a minimum of 52% and maximum moisture content of 65%.

Production Area

The geographical area of production is the whole territory of the provinces of Caserta and Salerno and some municipalities in the provinces of Napoli, Benevento, Frosinone, Latina, Roma, Isernia and Foggia (only some of the area for each municipality).

Cheesemaking

Mozzarella di Bufala Campana is a cheese made from the milk of the Mediterranean Buffalo breed signed on a specific registry. In the past, pasta filata cheeses were obtained from raw milk. Nowadays, to avoid sanitary problems linked to the animal, for example the possible presence of *Brucella* spp., the sanitary regulations require the pasteurization of the milk before its transformation.

Procedural guidelines have been modified to permit the production of Mozzarella di Bufala Campana either with raw milk or thermally treated milk or pasteurized milk (GU n.258 of 6th November 2003).

Milk used must have a fat and protein content not less than 7.2% and 4.2%, respectively. It is exclusively supplied by the production obtained from one or two milkings, and sent for transformation within sixteen hours from the first milking. The milk is coagulated after the addition of natural whey starters because the regulation does not allow the use of commercial starters.

Milk is coagulated at the temperature range of about 33-39°C by using calf rennet (about 30% of pepsin) for 20-30 minutes and left to stand for about five hours. Curd obtained is then cut in slices therefore being grinded and heated by addition of hot water at 95°C. Curd is then carried out to 'filatura' therefore 'mozzata' or shaped into a particular size and form. After the shaping, the Mozzarella is rapidly cooled at the temperature of about 28-25°C and kept in its acidulous, eventually salted, preserving liquid. Mozzarella di Bufala Campana PDO may be smoked using only natural and traditional processes and in this case the designation of origin must be followed by the term "smoked".

http://www.taccuinistorici.it

Figure 18. Mozzarella di Bufala Campana.

REFERENCES

Romano, R; Borriello, I; Chianese, L; Addeo, F. Quali-quantitative determination of triglicerydic, fatty acids and CLA in "Mozzarella di Bufala Campana" by high resolution gascomatography (HRGC) | La Mozzarelle di Bufala Campana (DOP): Caratterizzazione quali-quantitiva della componente trigliceridica ed acidica (CLA) nell'arco dell'anno mediante gascromatografia ad alta risoluzione (HRGC*). Progress in Nutrition*, 2008 10 (1), 22-29.

Website

http://www.mozzarelladop.it/

MURAZZANO

Characteristics (PDO June 12, 1996)

Murazzano is a fresh cheese produced exclusively with milk from ewes of Langhe's breed (or a minimum of 60%) or eventually adding cows' milk but no more than 40%. Animals must be fed with fresh and/or stored pasture coming from the production area. Murazzano PDO cheese has a cylindrical shape with flat sides and diameter of 10-15 cm, heel height of 3-4 cm and a weight of 300-400 g. The ripening time is long, from 4 to 10 days. The cheese has no rind but matured cheese has a yellowish film. The paste is white or ivory-white with few small holes. The texture is soft. The odor is fine and delicate. The taste is mainly sweet and fine but becomes more savory and intense in aged products. Murazzano PDO cheese has a minimum fat content (on dry matter) of 44% but if it is produced with 100% of ewe's milk (minimum fat content on dry matter of 47%) the label may say "pure ewe". The cheese must have cylindrical shape.

Production Area

The geographical area of production includes all the municipalities in the 'Comunità Montana Alta Langa' in the province of Cuneo and also some municipalities in the province of Cuneo.

Cheesemaking

Murazzano is produced with raw or pasteurized milk. The milk from two daily milkings is coagulated by the addition of bovine liquid rennet at a temperature of 37°C (with a tolerance of +/- 3°C) for 30 minutes. Commercial starter cultures containing thermophilic and mesophilic lactic bacteria may be used. Generally, no starter cultures are used but producers may use a selected starter to characterize their production. The curd is cut twice: the first produces grain of large size, like an orange, while the second produces granules with the size of a hazelnut. The curd is collected and drained in molds during the first 24 hours. The cheese is dry-salted. Ripening may be 4-10 days long or may otherwise reach the maximum of 2 months.

REFERENCES

Pirisi, A; Comunian, R; Urgeghe, PP; Scintu, MF.Sheep's and goat's dairy products in Italy: Technological, chemical, microbiological, and sensory aspects. *Small Ruminant Research*, 2011 101 (1-3) , 102-112.

http://www.cooperativalapoiana.it

Figure 19. Murazzano.

Website

http://formaggiomurazzanodop.com/consorzio-tutela.php

NOSTRANO DI VALTROMPIA

Characteristics (PDO July 6, 2012)

Nostrano di Valtrompia is a hard cheese made from partially skimmed raw cow's milk from cows of the Bruna Alpine breed, with the addition of saffron in a quantity between 0.05 and 0.2 g/100 kg of milk. The cheese has a cylindrical shape with flat sides, diameter of 35-45 cm, heel height of 10-15 cm and a weight of 8-18 Kg. The cheese rind ranges in color from yellow-brown to a reddish color. The paste is firm, straw yellow sometimes deep, with small holes equally distributed. Taste is intense, sometimes spicy. The fat content of the cheese ranges from 18 to 28% while the fat content in the dry matter ranges from 27.5 to 42%. Moisture of the cheese is 36%.

Production Area

The geographical area of production is the province of Brescia located in Trompia Valley.

Cheesemaking

The milk from two daily milkings, sometimes four during the periods of low lactation, is coagulated by the addition of bovine liquid rennet (chymosin minimum at 70%) at a range of temperature from 35°C to 40°C for 60 min. No starter or culture additions are used but commercial starter cultures containing thermophilic lactic bacteria may be employed. The curd is cut in a range time of 10-30 min and produces granules with the size of a rice grain. The curd is collected and saffron is added. Hence, the curd is cooked at a temperature of 47-52°C and it is left to stand in the whey for 15-60 min and then carried out for draining off for the first 24 hours. After 24 hours, not subjected to the pressing process, it is turned for 4-5 times at room temperature. Brining is carried out in dry conditions for 5-20 days depending by the cheese size, room temperature, and ability of the cheese to absorb the salt, moisture and pH of the cheese. Ripening finishes for a minimum of 8 months to 24 months at a temperature of 5-18°C and humidity of 60-90%. During the ripening, the cheese rind is frequently oiled to prevent the presence of acarus and mold contamination with the possible result of pitting of the rind.

http://www.bresciaoggi.it

Figure 20. Nostrano di Valtrompia.

Website

http://www.ruralpini.it/Inforegioni09.09.11-Nostrano-Valtrompia.htm

PARMIGIANO REGGIANO

Characteristics (PDO June 12, 1996 modified on September 5, 2003 and August 8, 2011)

Parmigiano Reggiano is a hard semi-fat cheese made from raw cow's milk partially skimmed by natural surface skimming. Cow's milk, exclusively derived from animals fed with forages originated from the area of origin, must be used as raw milk and it is not subjected to any thermal treatments. The use of additives is forbidden.

Cheese has a cylindrical shape with flat sides and the heel is more or less slightly convex with flat sides. The diameter is 35-45 cm, heel height is 20-26 cm and it has a minimum weight of 30 Kg. The color of the rind is natural straw yellow with a thickness of about 6 mm. The paste has a structure with grained and flaky texture. The cheese has a fragrant aroma and delicate taste, flavorsome without being too pungent. Fat in the dry matter must not be less than 32%.

Production Area

The geographical area of production is the whole territory of the provinces of Parma, Reggio Emilia, Modena, Bologna (on the left side of Po river), and Mantova (on the right side of Po River).

Cheesemaking

The milk from two daily milkings, evening and morning, is delivered to the cheese dairy within two hours from each milking. Each milking must be conducted within four daily hours and the milk must be immediately refrigerated and stored at a temperature not less than 18°C.

The milk is partially skimmed by natural surface skimming in steel tanks in open ambient. Milk from the morning milking, immediately after its delivery to the cheese dairy, is added with the partially skim milk of the previous evening milking. It is possible to stock a portion of milk, no more than 15%, for the cheese making of the day after. In this case the milk must be collected in appropriate refrigerated tanks equipped with suitable agitators at a temperature not less than 10°C and collected inside the tanks of natural surface skimming the same evening. Starter inoculum for the milk is constituted by natural starter whey obtained from the spontaneous acidification of the whey left from the cheese making of the day before. Coagulation of the milk is carried out in a copper truncated cone vat and it is obtained using calf rennet. Once the milk is coagulated, the curd is broken and cooked at 55-56°C, left to settle at the bottom of the vat to obtain a compact mass.

Afterwards, the curd is transferred in specific molds for shaping and after some days it is immersed into the salt solution for brining.

Ripening is carried out for at least 12 months and during the summer period the temperature of the ripening room must not be less than 16°C.

REFERENCES

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Zannoni, M. Evolution of the sensory characteristics of Parmigiano-Reggiano cheese to the present day. *Food Quality and Preference* 2011, 21 (8), 901-905.

Malacarne, M; Summer, A; Fossa, E; Formaggioni, P; Franceschi, P; Pecorari, M; Mariani, P. Composition, coagulation properties and Parmigiano-Reggiano cheese yield of Italian Brown and Italian Friesian herd milks. *Journal of Dairy Research* 2006, 73 (2), 171-177.

Website

http://www.parmigiano-reggiano.it/

http://www.idealcheese.com

Figure 21. Parmigiano Reggiano.

PECORINO DI FILIANO

Characteristics (PDO December 14, 2007)

Pecorino di Filiano is a hard cheese made with whole milk exclusively obtained by sheep of Gentile di Puglia and Lucania, Leccese, Comisana, Sarda and their crossbreed coming from an area indicated by procedural guidelines. Milk used for the cheese making comes from one or two milkings (generally evening milkings and that of the morning after).

The cheese has a cylindrical shape with flat sides, a flat or slightly convex heel, a diameter from 15 to 30 cm and heel height from 8 to 18 cm.

The rind has a typical color left by the cane cheese-sieves called '*fuscella'*. In the most ripened cheeses, the color ranges from yellow golden to dark brown due to the treatment with extra virgin oil and vinegar produced in Basilicata. Cheeses are manually rubbed on the side.

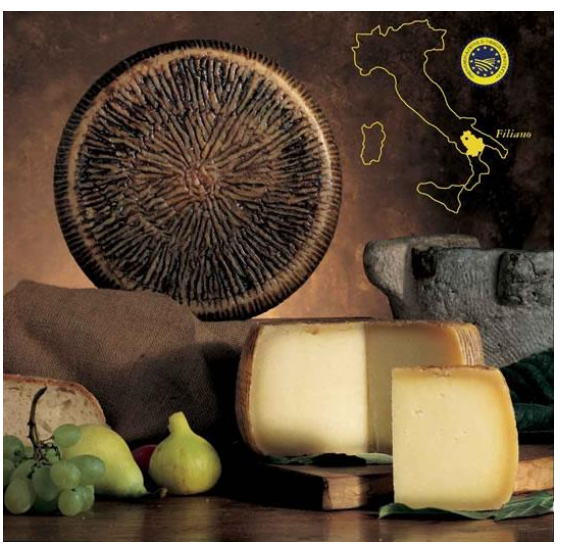
Cheese texture is compact presenting very small holes not equally distributed and a color ranging from white to straw yellow in the aged cheese.

Flavor is initially sweet and mild and it becomes lightly spicy in taste during the maturation.

Fat in dry matter must not be less than 30%.

Production Area

The geographical area of production is the whole territory of the province of Potenza.



http://www.basilicatanet.com

Figure 22. Pecorino di Filiano.

Cheesemaking

Pecorino di Filiano is made from milk derived by one or two milkings (evening and morning milkings) within 24 hours. Raw milk opportunely filtered, using sieves, is heated up to the maximum temperature of 40°C with added kid or lamb rennet derived from animals artisanally breeded in that production area.

Once the milk is coagulated, the curd is broken by using a wooden spoon called '*scuopolo*' or '*ruotolo*' to obtain the clot with dimension as grain of rice. After the rest of the curd under the whey for few minutes, it is put inside the molds to obtain the typical shape of the cane cheese-sieves *fuscella*. Hence, the curd is pressed by the hands for the draining of the

whey and heated at 90°C for a maximum of 15 minutes. Salting is carried out either dry or brined and repeated many times every day depending by the shape of the cheeses or in a saturated brine solution where the cheese is directly immersed and left for 10-12 hours per kg of cheese. Ripening takes place in natural tufa caves or in specific maturing rooms at the temperature of 12-14°C and a relative humidity of 70-85% for at least 180 days. Starting from the day 20 of ripening, the rind of the cheese may be treated with extra virgin oil produced in Basilicata and vinegar. Pecorino di Filiano cheese is produced all year round.

Website

www.pecorinodifiliano.it

PECORINO ROMANO

Characteristics (PDO June 12, 1996 modified on October 29, 2009)

Pecorino Romano is a hard, cooked cheese produced exclusively from fresh full-fat sheep's milk obtained from sheep bred in the area indicated in the procedural guideline. Cheese has a cylindrical shape with flat side, diameter between 25 to 35 cm and heel high from 25 and 40-cm. Cheese weight ranges from 20 to 35 Kg according to the size of the cheese wheel. The rind is thin, ivory color or natural straw yellow. Cheese texture is compact or presenting little holes; color ranges from white to straw yellow more or less intense. Flavor is aromatic and lightly spicy for the table cheese; or very spicy, intense and pleasant taste. Fat in dry matter must not be less than 36%.

Production Area

The geographical area of production is the whole territory of Sardegna and Lazio region and the whole territory of the province of Grosseto.

Cheesemaking

Pecorino Romano is a hard cheese made with whole milk exclusively obtained by sheep of the Sarda breed coming from area indicated by procedural guidelines. Milk used for the cheesemaking comes from one or two daily milkings.

Milk may be heat-treated and eventually inoculated with autochthonous lactic cultures sometimes integrated with cultures, which come from the area of production. Milk is heated at 38-40°C with added lamb paste rennet coming from the animals bred in that specific area. Cooking of the curd must take place at variable temperatures from 45 to 48°C. Salting is carried out in dry condition or in brine solution and it may be as long as 120 days. Ripening is long, at least five months, for the table cheese, or eight months for the grated cheese. Cheese

may be enveloped with neutral or colored plastic bags. Cheese is produced during the period from October to July.

http://www.cheesemaking.com/Romano.html

Figure 23. Pecorino Romano.

REFERENCES

Mangia, NP; Murgia, MA; Garau, G; Deiana, P. Microbiological and physiochemical properties of Pecorino Romano cheese produced using a selected starter culture. *Journal of Agricultural Science and Technology*, 2011 13 (4) , 585-600

Website

http://www.pecorinoromano.com/

PECORINO SARDO

Characteristics (PDO July 1, 1996 modified on May 1, 2011)

Pecorino Sardo is a cooked cheese made exclusively from whole sheep's milk, eventually heat-treated or pasteurized, separated into two different categories:

- 'sweet' of short maturation (20-60 days) and
- 'mature' ripened not less than 2 months. Cheese has a cylindrical shape with flat sides and flat or slightly convex heel.

Pecorino Sardo "*sweet*" type has a cylindrical shape with flat sides, diameter between 15 to 18 cm and heel height from 8 and 10 cm. Cheese weight ranges from 1 to 2.3 kg. Rind is

smooth, thin, white in color or lightly straw yellow. Cheese texture is compact, soft and elastic, firm or presenting little holes. Flavor is sweet and aromatic or lightly acid. Fat in dry matter must not be less than 40%.

Possible variations of the cheese sizes and weight may depend both by the technical conditions of production and by the ripening period.

Pecorino Sardo "*mature*" type has a cylindrical shape with flat sides, diameter between 15 to 22 cm and heel height from 10 and 13 cm. Cheese weight ranges from 1.7 to 4.0 kg. The rind is smooth, thin, lightly straw yellow in color in its early shape; while hard and fine grained in the more ripened cheese tending to straw yellow during the ripening, firm or with eyes irregularly distributed.

Flavor is lightly spicy. Fat in dry matter must not be less than 35%.

Production Area

The geographical area of production is the whole territory of the Sardegna region.

Cheesemaking

Whole sheep's milk inoculated with lactic starter cultures coming from the origin area and indicated as "*Streptococcus thermophilus"* is coagulated at a temperature between 35-39°C with calf rennet at a quantity to ensure the complete coagulation after 35-40 minutes.

Once the milk is coagulated, the curd is broken up to reach the dimension of hazelnuts for the "*sweet*" type, while the dimension of a corn grain is for the "*mature*" type. Curd is then semi-cooked at a temperature not higher than 43°C. Cheese is subjected to steaming and pressing at temperature conditions to guarantee the optimal acidification and draining of the cheese.

http://www.italos.it

Figure 24. Pecorino Sardo.

Once the draining is completed, brining of the cheese is made in a wet or dry condition for a short time using salt at a concentration of no more than 2% of the cheese.

Ripening is carried out in an appropriate room at a temperature between 6 to 12°C and humidity between 80-95%.

REFERENCES

Mandrau, MA; Mangia, NP; Murgia, MA; Sanna, MG; Garau, G; Leccis, L; Caredda, M; Deiana, P. Employment of authochthonous microflora in Pecorino Sardo cheese manufacturing and evolution of physiochemical parameters during ripening *International Dairy Journal*, 2006, 16 (8), 876-885.

Website

http://www.pecorinosardo.it/

PECORINO SICILIANO

Characteristics (PDO June 12, 1996)

Pecorino Siciliano is a hard cheese exclusively made from fresh whole raw sheep's milk without the addition of any starter cultures and coagulated using lamb rennet.

Cheese has a cylindrical shape with a flat side, diameter between 14 to 38 cm and heel height from 10 and 18 cm. Cheese weight ranges from 4 to 12 kg. The rind is smooth, thin, white color or lightly straw yellow. The cheese texture is compact, soft and elastic, firm or presenting little holes. Flavor is sweet and aromatic or lightly spicy. Fat in dry matter must not be less than 40%.

Production Area

The geographical area of production is the whole region of Sicilia.

Cheesemaking

Raw whole sheep's milk is coagulated by using rennet at a temperature between 37-40°C for about 40 minutes.

Once the milk is coagulated, the curd is broken up to reach the dimension of a grain of rice. Cheese is subjected to steaming, using hot water at a temperature of 40-45°C and the curd is left to rest for 10-20 minutes. The curd is then transferred in the cane cheese-sieves called "*fascedde*", which confer to the cheese the typical shape, and lightly pressed to guarantee the optimal draining of the whey. Once the whey is recovered, a part of it is employed for making the Ricotta cheese while other part is heated at 85°C, used for the

heating of the curd (blanching). Heating of the curd goes on for 2-4 hours until the temperature drops to 45-50°C. Cheese is then placed on a table for 24-48 hours turning it upside down many times.

Salting of the cheese is carried out in dry conditions and the ripening takes place in an appropriate room with good ventilation for a minimum of 4 months (for the semi-matured cheese) or up to 12 months.

http://www.consorziopecorinosiciliano.it

Figure 25: Pecorino Siciliano.

REFERENCES

Vernile, A; Baresford, TP; Spano, G; Massa, S; Fox, PF. Chemical studies of Pecorino Siciliano cheese throughout ripening. *Milchwissenschaft*, 2007, 62 (3), 280-284.

Website

http://www.consorziopecorinosiciliano.it/

PECORINO TOSCANO

Characteristics (PDO July 1, 1996 modified on April 14, 2010)

The name Pecorino is a generic term to indicate a cheese produced only from sheep's milk. Pecorino Toscano is a cheese produced in two typologies: soft cheese (fresh) and semi-hard (ripe) cheese, only from whole sheep's milk from the production area.

It has a cylindrical shape with slightly convex outer sides; dimensions: diameter of the faces from 15 to 22 cm, height of the heel from 7 to 11 cm, weight: from 0.75 a 3.50 kg.

Production Area

The geographical area of production is the whole territory of the Toscana region and some municipalities in the Umbria and Lazio regions, and can be produced all year.

Cheesemaking

Whole raw or pasteurized ewe milk, that can be inoculated with cultures of autochthonous or selected starter, must be coagulated at a temperature ranging between 33 and 38 °C with the addition of calf rennet in order to obtain the curd within 20-25 minutes. The cheesemaking and processing between the two cheese typologies of Pecorino Toscano differ, in particular, in the breaking mode of the curd. The curd for the preparation of the soft cheese (fresh typology) is broken into grains of a hazelnut size, while to produce hard and ripened cheese the curd is broken more finely until it is the size of grain to immediately dry off the whey. In this last cheesemaking, it is possible to semi-cook the curd until it reaches 40-42 °C for 10-15 min. After breaking and possibly cooking, the curd is put into suitable forms to drain the whey. The syneresis is carried out by manual pressing or stewing in steel containers heated by an injection of steam until 50 °C. Duration of this stewing is variable and longer (up to 10 hours) for hard cheeses. The obtained product is commonly dry salted for an average of 32 hours or in a brine solution at 12-15 °C for 10-36 hours.

Ripening of soft cheese typology is at least 20 days at low temperatures (> 5 °C) while semi-hard cheese should be left for at least four months of ripening at <12 °C, both with a humidity between 75-90%. This last type is frequently used for grating.

According to the Disciplinary of Production, Pecorino Toscano cheese must have at least a fat content (on dry matter) of 45% for soft cheese and 40% for semi-hard cheese.

http://www.pecorinotoscanodop.it/

Figure 26. Pecorino Toscano.

REFERENCES

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Buccioni, A; Minieri, S; Conte, G; Benvenuti, D; Pezzati, A; Antongiovanni, M; Rapaccini, S; Mele, M. Changes in conjugated linoleic acid and C18:1 isomers profile during the ripening of Pecorino Toscano cheese produced with raw milk. *Italian Journal of Animal Science*, 2012, 11 (4), 426-430.

Website

http://www.pecorinotoscanodop.it/

PIACENTINU ENNESE

Characteristics (PDO February 15, 2011)

Piacentinu Ennese is a particular semi-cooked, hard, pressed cheese produced from whole raw ewe's milk. It has a natural acidity of fermentation at pH 6.5-6.8 of autochthonous Sicilian breed of sheeps (Comisana, Pinzirita, Valle del Belice) characterized by the addition of saffron (*Crocus sativus*) produced only in the same area and whole black peppercorns (*Piper nigrum*). It has a cylindrical shape, with slightly convex or almost straight outer sides.

Dimensions, obtainable by the use of a rattan basket, are: outer sides minimum 14 cm up to 15 cm, with a diameter of the pot minimum 20 cm, maximum 21 cm. Weight is between 3.5 and 4.5 kg.

The cheese smell is delicate with a slight aroma of saffron, salt is barely perceptible, and is slightly spicy in the first phases of ripening, which could increase.

Production Area

The geographical area of production is the whole territory of the province of Enna.

Cheesemaking

In the discipline of production of this particular cheese, much attention is focused on the animal feed. In fact, whole raw ewe's milk is used in the cheesemaking and produced from ewes bred at natural pastures, located at an altitude between 400 and 800 m above sea level, and/or fodder, hay and straw obtained in the production area. Wheat stubble and vegetative byproducts from, for example, cladodes of prickly pear, non-OGM cereal grains and concentrates are also allowed as animal feed.

Whole raw ewe milk must come from two manufacturing area milkings within 24 hours. After heating the milk to a maximum temperature of 38 °C, it is filtered, saffron (maximum 5 g/100 l of milk) is added and solubilized and then coagulated, after the addition of lamb or kidskin rennet, into a wooden vat within 45 minutes. The curd is cut into particles the size of rice grains, then summarily separated from the serum and collected in rush baskets ("*fascedde* "), which leaves a particular modeling on the surface of the rind. During this phase, whole black peppercorns are added into the curd. At each pepper addition, the curd is strongly pressed, and burned for 3-4 hours. Then the curd is set on a wooden board to dry at room temperature. The curd is then dry salted with coarse salt, repeating the operation twice in ten days. The minimum time for Piacentino Ennese ripening is 60 days in ventilated rooms, or locally, at a temperature of 8-10 °C and humidity between 70-80%.

From a point of view of the chemical characteristics of Piacentinu Ennese PDO cheese, the discipline describes that the fresh forages from area of production, eaten by the sheep, have characteristic terpenic fingerprints, which can be found in the milk and cheeses produced during the summer pasture. These terpenoidic molecules, such as the α-terpineol, are thus considered chemical biomarkers, found in the cheese.

According to the Disciplinary of Production, Piacentino Ennese PDO must have at least a fat content (on dry matter) of 40%, protein content (dry matter) of 35%, salt content (dry matter) of 5%, and a pH between a 4.8-5.7.

http://www.blogsicilia.eu/

Figura 27. Piacentinu Ennese.

REFERENCES

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Horne, J; Carpino, S; Tuminello, L; Rapisarda, T; Corallo, L; Licitra, G. Differences in volatiles, and chemical, microbial and sensory characteristics between artisanal and industrial Piacentinu Ennese cheeses. *International Dairy Journal*, 2005, 15 (6-9), 605-617.

PIAVE

Characteristics (PDO May 21, 2010)

Piave is a cooked, hard and ripened cheese. It is produced using 80% of Italian Bruna, Pezzata Rossa and Frisona cow's milk, and comes from the mountains of the production area. Cheese is made inoculating autochthonous starters and it is produced in dairies located within the same territory. Piave is made from milk of cows fed with a minimum of 70% of local forages and industry feed, vegetables, fruit and urea. Biuret as feed must be excluded.

Piave cheese is produced in five different aged typologies:

1) *Fresh* with a ripening between 20 and 60 days; diameter of 320 mm ± 20 mm; heel height of 80 mm ± 20 mm; weight of 6.8 kg ± 1 kg.
2) *Medium* (‘*Mezzano’*): ripening between 60 and 180 days; diameter of 310 mm ± 20 mm; heel height of 80 mm ± 20 mm; weight of 6.6 kg ± 1 kg.
3) *Old* with a ripening aged more than six months; diameter of 290 mm ± 20 mm; heel height of 80 mm ± 20 mm; weight of 6.0 kg ± 1 kg.
4) *Old gold selection* with a ripening aged more than 12 months; diameter of 280 mm ± 20 mm; heel height of 75 mm ± 20 mm; weight of 5.8 kg ± 1 kg.
5) *Old reserve* with a ripening aged more than 18 months; diameter of 275 mm ± 20 mm; heel height of 70 mm ± 20 mm; weight of 5.5 kg ± 1 kg.

Piave presents these sensorial characteristics:

- Flavor: sweet for Fresh and Medium typology cheese that, with the ripening, becomes more intense until it is slightly spicy in aged typologies.
- Rind: clear and tender in the Fresh typology, during the ripening it becomes darker and thicker tending towards an ocher color.
- Paste: blank in Fresh typology without holes while, in aged cheese, the paste is more granular and crumbly with a slight yellowish color.

Production Area

The geographical area of production is the whole territory of the province of Belluno.

Cheesemaking

Whole raw cow’s milk, coming from two or four milkings within 72 hours, is titrated by centrifugation to have a final fat content of 3.5 ± 0.3% w/w. Milk is then pasteurized at 72 °C for 16 seconds, lysozyme and starter are added to reach a final acidity of 10°SH/50 ± 3. Afterwards milk is inoculated with whey starter (acidity of 27°SH/50 ± 3), heated at 34-36 °C and coagulated after the addition of rennet (at least 50% chymosin). The curd is cut into particles the size of rice grains, cooked at 44-47 °C and subjected to agitation for 1.5-2 hours. The curd is pressed inside the mold, marked with the name ‘Piave’ and then salted in a brine solution at for at least 48 hours. Ripening of the cheese is carried out locally with a temperature of 8-14 °C and a humidity of 70-90%.

The disciplinary code of Piave ensures the traceability of the product by means of a control plan on breeders, cheese-makers and packers, and compliance monitoring is carried out by a Control Organization.

http://www.agraria.org

Figure 28. Piave.

Website

http://www.formaggiopiave.it/

PROVOLONE DEL MONACO

Characteristics (PDO February 9, 2010)

Provolone del Monaco is a semi-hard '*pasta filata'* (stretched-curd) seasoned cheese, produced only with raw cow's milk from autochthonous cows fed in the production area of the Napoli province. Disciplinary pays particular attention to the production of the raw materials such as milk, as well as to the welfare of the animals. Animal feed can be represented by at least 40% (on dry matter) of forages and/or brushwood produced, for example, by pruning of citrus and olive trees. A list of not permitted animal feed is indicated, such as genetically modified crops or animal origin feed.

The cheese has a slightly elongated shape, a weight between 2.5 and 8 kg, a thin rind of slightly yellowish color with slight longitudinal bays, like raffia strings, which divides the cheese into at least in six sections and are used as support for a pair of cheeses. The interior paste is cream-colored, elastic and compact, with typical holes of diameters up to 5 mm. It has a sweet and buttery flavor and a light and pleasant spicy taste that will become spicier after seven-eight months of ripening.

Production Area

The geographical areas of production are some municipalities in the province of Napoli.

Cheesemaking

Whole raw cow's milk, coming from one milking, after heating at a temperature of 34-42 °C, is coagulated with the addition of kid or calf rennet. The curd is cut into particles the size of maize' grains, and then allowed to stand for 20 minutes. The curd is then heated at a temperature between 48-52 °C and left to stand for 30 minutes allowing the whey to be extracted. After spinning trials of the curd to have a positive elasticity and resistance, the curd is cut into ribbons in hot water (85-95 °C) and then refrigerated in cold water for firming. The product is then salted in brine at for 8-12 hours/kg of product.

Provolone del Monaco has to be ripened for the first 20 days at room temperature for lactic-proteolytic maturation and then at 8-15 °C for at least for six months.

According to the disciplinary code, traceability is guaranteed documenting incoming and outgoing products.

Cheese needs to have a fat content (dry matter) of at least 40.5%.

http://www.agricoltura.regione.campania.it/tipici/provolone_monaco-new.html.

Figure 29. Provolone del Monaco.

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PROVOLONE VALPADANA

Characteristics (PDO June 12, 1999 modified on November 7, 2012)

Provolone Valpadana is a semi-hard cooked '*pasta filata'* cheese produced exclusively by whole cow's milk coming from the production area. Cheese can be distinguished in two typologies, one sweet and one spicy, if they are made using pasteurized or thermized milk respectively.

Spicy cheese, after eight months of ripening, can be marked as P.V.S. (Provolone Valpadana Stagionato i. e. Provolone Valpadana Ripenend) exclusively after a technical

verification about the external appearance of the form, the texture of the paste, the color, the flavor and the taste.

Provolone Valpadana is made from milk of cows fed with at least 70% of local forages, which are guaranteed according to the disciplinary code, with incoming and outgoing documents.

Different shapes are produced such as salami, melon or pear forms with a smooth and thin rind that can be absent for the sweet typology. The paste is generally compact, but not dry, and may have sparse holes. Flavor is delicate up to to three months of maturation, then it becomes spicier with more advanced ripening.

Production Area

The geographical area of production is the whole territory of the provinces of Cremona, Brescia, Verona, Vicenza, Padova, Rovigo, Piacenza and some municipalities in the provinces of Bergamo, Mantova, Lodi and Trento.

Cheesemaking

In cheesemaking rules, the disciplinary code describes only the requirement for using natural whey starters, obtained by incubation of the residual whey of the previous cheesemaking until an acidification of a maximum of 26°SH/50 mL. This choice in the disciplinary code could result in a reduction of the microbial diversity of natural whey starter but at the same time, the advantage of less variability of the lactic fermentation. Whole raw cow's milk, coming from one milking, after heating to a temperature of 36-39°C, is coagulated with the addition of calf rennet for sweet cheese typology, and lamb and/or kid rennet for the spicy typology without lipases addition.

The practice of cooking is not described in the disciplinary code, however the spinning of the paste (‘*filatura della pasta’*) is reported. It must be carried out manually, after the natural lactic fermentation, on a clot of the curd with spinning pH of between 4.70 and 5.20. Cheese is then allowed to stand in cold water, and the forms of cheese are salted in a brine solution for a period from a few hours to 30 days, depending on the weight of the forms. Before the ripening, performed in suitable local areas suspended from a support, forms are subjected to the drying. During the maturation, the cheese could be coated with additives such as E202 (potassium sorbate), E203 (calcium sorbate), E235 (pyramicin) or paraffin.

The period of ripening depends on the weight of the forms:

- up to 6 kg, a minimum ripening of ten days
- over 6 kg, minimum ripening of 30 days
- over 15 kg and for spicy typology, a minimum ripening of 90 days
- over 30 kg and spicy typology, ripening over 8 months.

The cheese can be smoked.

Cheese needs to have a fat content (dry matter) between 44-54% and a water content over 46% in sweet and spicy typology up to 6 kg, and a water content over 43% in spicy typology over 6 kg of weight.

http://www.consorziotutelaprovolone.it/

Figure 30. Provolone Valpadana.

Website

http://www.consorziotutelaprovolone.it/

QUARTIROLO LOMBARDO

Characteristics (PDO 12 June 1996)

Quartirolo Lombardo is a soft table cheese produced by whole or semi-skimmed cow's milk, coming from two milkings obtained from production area.

Quartirolo Lombardo cheese has a quadrangular parallelepiped shape with flat sides, 4-8 cm high, 18-22 in diameter and weight 1.5-3.5 kg. The rind is thin and soft, pinkish white in cheeses during the initial stage of ripening, becoming reddish gray-green in the mature product. The texture of the paste is crumbly and becomes more compact and soft during the maturation of white color. The taste is slightly sour in fresh cheese and more aromatic in ripened cheese.

Production Area

The geographical area of production is the whole territory of the provinces of Brescia, Bergamo, Como, Cremona, Milano, Pavia and Varese.

Cheesemaking

Coagulation is performed by adding calf rennet during the heating at 35-40°C within 25 minutes with the possibility of adding natural starter cultures. The curd is cut twice to reach a grain size of a hazelnut and then mixed with the whey in special forms. After the stewing at 26-28°C for a minimum of four hours to a maximum of 24 hours, the cheese is shaped. It is salted in dry conditions or in brine solutions in the local area at 10-14°C. The ripening is carried out locally at 2-8°C and humidity of 85-90% for a time between five to 30 days for soft cheese. After 30 days of aging, it is marked 'Qualtirolo Lombardo maturo' (i.e. Quartirolo Lombardo ripened).

No treatments of the rind are allowed.

The disciplinary code allows a fat content (dry matter) at least 30% for the cheese produced by semi-skimmed milk.

www.quartirololombardo.com/

Figure 31. Quartirolo Lombardo.

Website

http://www.quartirololombardo.com/

RAGUSANO

Characteristics (PDO July 1, 1996)

Ragusano is a traditional *pasta filata* cheese, produced from cow's whole raw milk, fed with wild plants, herbs and hay, from the mountain plateau of the production area.

The cheese has a quadrangular parallelepiped shape, dimensions: sides of the square cross-section from 15 to 18 cm, length of the parallelepiped from 43 to 53 cm, weight from 10 to 16 kg. Cheeses have thin, smooth, yellow-brown rind and a compact and yellow body with small holes.

Milky, sweet and delicate notes characterize young cheese while the aged cheese presents a spicy, butyric and floral aroma.

Production Area

The geographical area of production is the whole territory of the province of Ragusa and some municipalities of Siracusa.

Cheesemaking

Milk, coming from one or more milkings, is coagulated with natural lamb or kid rennet paste likely to entail a time of setting and hardening from 60 to 80 minutes. Cut of the curd is made, following the addition of water at 80 ± 5°C, to obtain a rice-grain size. Then the curd is drained by sedimentation and pressed to facilitate the purging of the whey, and a mass of solid curd is obtained. The curd is then reheated under the whey or water at 80°C for at least 85 minutes which allows the fusion. A drying stage is conducted leaving the paste for approximately 20 hours and then it is cut into slices, that are covered again with water about 80°C for 8 minutes. Paste then is stretched to obtain a spherical mass in shape with a smooth outer surface and then in order to assume a parallelepiped shape. The technology of this '*pasta filata*' cheese differs drastically from other cheese varieties in using hot water (around 85°C) to stretch the curd. Typically, these forms are immersed in saturated salt brine in order to obtain a cheese with interior content (dry matter) of 6% sodium chloride. Then it is ripened in two blocks of cheese tied together with a rope and hung over the rafters to facilitate moisture loss in ventilated rooms at 14-16°C. A cover of olive oil is allowed for smoked aged cheeses, which are marked as smoked cheese.

A minimum period of ripening is not mentioned in the disciplinary code, even if two levels of minimum fat content (dry matter) are dependent on time ripening: at least 40% for table cheeses and a minimum of 38% for cheeses ripened over six months. The maximum humidity content is 40%, without differentiating the cheeses.

http://en.wikipedia.org/wiki/

Figure 32. Ragusano.

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RASCHERA

Characteristics (PDO 1 July 1996)

Raschera is a semi-hard, pressed, medium fat or fat cheese, produced from raw or pasteurized cow milk, sometimes supplemented with small additions of sheep and/or goat milk. Raschera is a cylindrical or quadrangular cheese with flat surfaces. The cylindrical

shape has a diameter of 30-40 cm, a convex edge of 7-9 cm and a weight of 7-9 kg. The square shape has a side of 40 cm, an irregular edge of 12-15 cm and a weight of 8-10 kg. The rind is thin, elastic and regular, reddish-grey with yellow highlights and red patches. The paste is white or ivory-white with few small and sparse holes. The texture is consistent and elastic.

From a sensorial point of view, the odor must be fine, delicate, and typically fragrant in fresher cheeses and the taste is slightly spicy and savory when seasoned. The ripening lasts at least 30 days.

Production Area

The geographical areas of production are some municipalities in the Monregalese Valley (Italian Maritime Alps, NorthWest Italy) in the province of Cuneo. This cheese can be named "*d'Alpeggio*" when it is produced in summer in mountain pastures from the Italian Alps at an altitude of over 900 meters above sea level.

Cheesemaking

In the traditional Raschera PDO cheesemaking, raw cow milk, or partly skimmed milk, coming from two manufacturing area milkings, is warmed to 27–30°C and left resting for 20–30 min after the addition of liquid bovine rennet. Clotting time is established visually by the cheesemaker. When pasteurized milk is used, a commercial starter, generally made with thermophilic and mesophilic lactic bacteria, is used. The curd is cut into particles, then separated from the serum and collected in a hemp cloth, which is left for approximately 10 minutes of drainage. Finally, the curd is wrapped in the cloth is put into cylindrical or quadrangular forms and pressed to allow the remaining whey to drain away for at least 12 h. At the end, the product is ready to be dry salted for three days for sides and ripened in natural cells dug underground at 12-15°C for at least 30 days. Here the constant temperature and humidity create the optimal conditions for the 1–6 months of ripening and consequently for the formation of the reddish rind characteristic of this cheese. According to the Disciplinary of Production, Raschera PDO cheese must have at least fat (dry matter) of 32%.

http://www.raschera.com/

Figura 33. Raschera.

Website

http://www.raschera.com/

ROBIOLA DI ROCCAVERANO

Characteristics (PDO July 1, 1996)

The Robiola di Roccaverano is a fresh cheese produced from ewe's or goat's milk or a mixture of ewe, goat and cow's milk. The quantity of cow's milk must be less than 85%. The Robiola di Roccaverano cheese is cylindrical with flat surfaces. The cylindrical shape has a diameter of 10-15 cm, an edge of 4-5 cm and a weight of 250-300 g. There is no rind and the paste is white or ivory-white without holes. The texture is soft and slightly consistent. The odor is fine, delicate, and rarely pungent. The taste is mainly acid and fine.

Production Area

The geographical areas of production are some municipalities in the provinces of Asti and Alessandria.

Cheesemaking

The Robiola di Roccaverano is produced with raw or pasteurized milk. The milk is curdled by the addition of bovine liquid rennet at a temperature of 30-35°C. A commercial starter is also used, generally made with mesophilic lactic bacteria. Generally each producer uses a different starter to characterize his production. The curd is cut to produce granules with the size of a hazelnut. The curd is collected and drained inside specific molds. The cheese is dry salted. Natural aromas such as truffle, hot pepper, herbs or rosemary may be added.

According to the Disciplinary of Production, Robiola di Roccaverano must have at least a 50% fat content (in dry matter), a protein content (in dry matter) of 34% and ash (in dry matter) of 3%.

(http://www.robioladiroccaverano.com)

Figure 34. Robiola di Roccaverano.

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Website

http://www.robioladiroccaverano.com/
http://www.assopiemonte.com/

SALVA CREMASCO

Characteristics (PDO December 20, 2011)

The Salva Cremasco is a quadrangular cheese with flat surfaces. The square shape has a side of 11-13 cm or 17-19 cm, an edge of 9-15 cm and a weight of 1.3-1.9 or 3-5 kg. The ripening is carried out for at least 75 days. The rind is soft and regular with typical surface bacteria and a yellow-brown color. The paste is white or pale-yellow, with small, irregular and sparse holes. The texture is consistent and springy, very weak above the rind. The taste is aromatic, intense and piquant in the more ripened cheese.

Production Area

The geographical area of production is the whole territory of the province of Bergamo, Brescia, Cremona, Lecco, Lodi, and Milan.

Cheesemaking

The Salva Cremasco is produced with whole raw or pasteurized cow’s milk. The milk is curdled by the addition of bovine liquid rennet at a temperature of 32-40°C. Autochthonous

starters could be used. Generally each producer uses a different starter to characterize his production. After 10-20 min the curd is cut two times to produce granules the size of a hazelnut and then put in typical square molds. After a period at 21-29°C for 8-16 hours, the cheese is salted in dry condition or in brine solution. The ripening is performed at 2-8°C with 80-90% humidity. During the ripening, the rind is washed with brine to obtain a thin and colored rind and a strong proteolysis of the paste.

According to the Disciplinary of Production, Salva Cremasco must have at least 48% fat in dry matter and water content lower than 47%.

(http://www.mondodelgusto.it)

Figure 35. Salva Cremasco.

Website

http://www.salvacremasco.com/

SPRESSA DELLE GIUDICARIE

Characteristics (PDO December 22, 2003)

The Spressa delle Giudicarie is a cylindrical cheese with a diameter of 30-35 cm, an edge of 8-11 cm. and a weight of 7-10 kg. The ripening lasts at least 90 days for the "fresh" cheese and at least 180 days for the "ripened" cheese. The rind is soft and regular with a grey or yellow-brown color. The paste is white or pale-yellow, with small, irregular and sparse holes. The texture is consistent and springy with aromatic odor. The taste is sweet in the "fresh" cheese and salty and piquant in the "ripened" cheese. The Spressa delle Giudicarie can be produced between September 10 and July 30.

Production Area

The geographical areas are the municipalities in the Giudicarie, Chiese, Rendena and the Ledro Valley.

Cheesemaking

The Spressa delle Giudicarie is produced with partially skimmed cow's milk obtained with natural surfacing. The milk is curdled by the addition of bovine liquid rennet at a temperature of 33-37°C. Starters could be used. Generally each producer uses a different starter to characterize his production. After 20-50 min the curd is cut to produce granules with the size of rice and then cooked at 40-44°C for at least 20-30 min. The curd is mixed for a further 35-65 min then removed and put into cylindrical molds. The curd is then left to stand for 24 hours then salted in brine for 4-6 days or dry for 8-12 days. Ripening is performed at 10-20°C with 80-90% of humidity. According to the Disciplinary of Production, Spressa delle Giudicarie must have at least 33% fat content in dry matter and a water content ranging from 32 and 40% for the fresh cheese, and 28 and 38% for ripened cheese.

(http://www.agraria.com)

Figure 36. Spressa delle Giudicarie.

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SQUAQUERONE DI ROMAGNA

Characteristics (PDO July 24, 2012)

The Squaquerone di Romagna is a spreadable cheese with a weight of 0.1-1 kg. The ripening is carried out for 1-4 days. There is no rind and the paste is white, without holes and

a defined shape. The texture is soft, creamy, and spreadable. The odor is soft and milk flavored. The taste is sweet.

Production Area

The geographical area of production is the whole territory of the province of Ravenna, Forlì-Cesena, Rimini, Bologna and Ferrara (only the area between the SS24 and the Po River).

Cheesemaking

The Squacquerone di Romagna is produced with whole pasteurized cow's milk. The cheesemaking must be done within 48 hours from milking. The milk is curdled by the addition of bovine liquid rennet at a temperature of 35-40°C. Starters could be used. Generally each producer uses different starters to characterize his production. After 10-30 min the curd is cut to produce granules the size of a nut and left to stand to obtain a pH of 5.9-6.2. The curd is put into typical cylindrical molds and left to stand for 24 hours at 15°C. The curd is then salted in brine for 10-40 min for each kilogram of cheese. Ripening is performed at 3-6°C for 1-4 days. According to the Disciplinary of Production, Squacquerone di Romagna must have a fat content in dry matter ranging from 46 and 55% and a water content ranging from 58 and 65%.

(http://www.ravennaintorno.it)

Figure 37. Squaquerone di Romagna.

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STELVIO O STILFSER

Characteristics (PDO February 15, 2007)

The Stelvio o Stilfser is a cylindrical cheese with a diameter of 36-38 cm, an edge of 8-10 cm and a weight of 8-10 kg. The ripening is carried out for at least 60 days. The rind is soft and regular with a yellow-orange color for fresh cheese or yellow-brown color for ripened cheese. The paste is pale-yellow with irregular and sparse holes. The texture is consistent and springy with an aromatic odor.

Production Area

The geographical area of production is the whole territory of the province of Bolzano.

Cheesemaking

The Stelvio is produced with whole pasteurised cow's milk. The cheesemaking must be done within 48 hours from milking. The milk is curdled by the addition of bovine liquid rennet at a temperature of 32-33°C. Starters and lysozyme could be used. Generally each producer uses a different starter to characterize his production. After 20-30 min the curd is cut to produce granules the size of maize, and then about 25% of the whey is removed and water at 50-70°C is added to obtain a final temperature of 36-40°C. The curd is mixed for some minutes then removed and put into cylindrical molds. The curd is then pressed from 30 min to 2 hours. After a period in a hot room, the cheese is salted in brine solution for 36-48 hours. Ripening is performed locally at 10-14°C and 85-95% of humidity. During the ripening the rind is washed with brine containing a mix of chromogenic bacteria to obtain a colored rind. According to the Disciplinary of Production, Stelvio o Stilfser must have a fat content in dry matter higher than 50% and a water content lower than 44%.

http://www.turismo-trentino-alto-adige.com

Figure 38. Stelvio o Stilfser.

Website

http://www.stilfser.it

TALEGGIO

Characteristics (PDO June 12, 1996)

The Taleggio is a quadrangular cheese with flat surfaces. The square shape has a side of 18-20 cm, an edge of 4-7 cm and a weight of 1.7-2.2 kg. The ripening is carried out for at least 40 days. The rind is thin and soft, and regular with a light yellow-red color. The paste is white or ivory-white with small and sparse holes. The texture is moderately consistent and elastic. The odor is soft with milk and cream characteristics. The taste is very soft and mainly sweet.

Production Area

The geographical area of production is the whole territory of the province of Bergamo, Brescia, Como, Cremona, Lecco, Lodi, Milano, Pavia, Novara, and Treviso.

Cheesemaking

The Taleggio is produced from whole pasteurized cow's milk. The milk is curdled by the addition of bovine liquid rennet at a temperature of 36-38°C. Starters are used. Generally each producer uses a different starter to characterize his production. After 15 min the curd is cut to produce granules the size of a hazelnut and put in typical square molds. After a period at 22-28°C for at least 10 hours, the cheese is salted in a dry condition or in a brine solution. During the ripening the rind is washed with brine to obtain a thin and colored rind and a strong proteolysis of the internal paste. According to the Disciplinary of Production, Taleggio must have a fat content (in dry matter) ranging from 48 and 53%, a protein content ranging from 36 and 44%, an ash content ranging from 3 and 4%, and a water content ranging from 45 and 50%.

(http://www.italos.it)

Figure 39. Taleggio.

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Website

http://www.taleggio.org

TOMA PIEMONTESE

Characteristics (PDO July 1, 1996)

The Toma Piemontese is a cylindrical cheese produced in two sizes: large (6-8 kg) with a diameter of 24-34 cm and small (1.8-5.9 kg) with a diameter of 15-25 cm. The ripening lasts at least 15 days for the small size and 60 days for the large size. There are two types of Toma Piemontese:

- Toma Piemontese: the rind is smooth, elastic and regular. The color ranges from light straw yellow to reddish brown depending on its ageing. The paste is white or ivory-

white with small and sparse holes. The texture is moderately consistent and elastic. The odor is soft with milk and cream characteristics. The taste is also very soft and mainly sweet. It is produced in small and large size.

- Toma Piemontese semigrassa (semi-skimmed): the rind is smooth and regular but not elastic. The color is a wrinkled, hard, reddish-grey with yellow and white highlights depending on its ageing. The paste is yellowish with small and sparse holes. The texture is consistent and not elastic. The odor is strong and persistent. The taste is very savory and mainly salty in ripened products. It is produced only in large size.

Production Area

The geographical area of production is the whole territory of the province of Cuneo, Biella, Novara, Torino, Verbania and Vercelli and some municipalities in the provinces of Alessandria and Asti.

Cheesemaking

The Toma Piemontese is produced with whole raw or pasteurized cow's milk. The milk is curdled by the addition of bovine liquid rennet at a temperature of 32-35°C. A commercial starter generally made with thermophilic lactic bacteria is added to pasteurized milk. Generally each producer uses a different starter to characterize his production. The curd is cut twice to produce granules the size of maize. After draining, the curd is put in molds and pressed for at least 5-6 hours. The cheese is then salted in brine for about 36 hours. Dry salting is also used. The Toma Piemontese semigrassa (semi-skimmed) is produced with skimmed raw or pasteurized cow's milk. The cheesemaking is similar to that used for Toma Piemontese. According to the Disciplinary of Production, the chemical composition of Toma Piemontese is correlated to the size. For Toma Piemontese large size the water content is 43-51%, protein content (in dry matter) is 37-4% and fat content (in dry matter) is 44-54%. For Toma Piemontese small size the water content is 42-48%, protein content (in dry matter) is 40-46% and fat content (in dry matter) is 50-54%. For semi-skimmed type, the water content is 46-54%, protein content (in dry matter) is 50-60% and fat content (in dry matter) is 30-38%.

http://www.langhe.net

Figure 40. Toma Piemontese.

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Website

http://www.assopiemonte.com/

VALLE D'AOSTA FROMADZO

Characteristics (PDO July 1, 1996)

Valle d'Aosta Fromadzo is a cylindrical cheese with a diameter of 15-30 cm, an edge of 5-20 cm and a weight of 1-7 kg. The ripening lasts at least 60 days. The rind is soft and regular with a yellow-grey or yellow-brown-grey color. The paste is pale-yellow, with irregular, small and sparse holes. The texture is consistent and springy with aromatic odor. The taste is sweet if fresh and salty-piquant if ripened. In 2011 the production was about 6 tons.

Production Area

The geographical area of production is the whole territory of Valle d'Aosta region.

Cheesemaking

The Valle d'Aosta Fromadzo is produced with whole or partially skimmed raw cow's milk. The milk is curdled by the addition of bovine liquid rennet at a temperature of 34-36°C. Autochthonous starters could be used. The curd is cut, cooked to 45°C, mixed for some minutes then removed and put into cylindrical molds. The curd is pressed and salted in brine or in dry conditions. Ripening is performed at 8-14°C with a humidity higher than 60%. According to the Disciplinary of Production, valle d'Aosta Fromadzo must have a fat content (in dry matter) lower than 20% for skimmed cheese and ranging from 20 and 35% for whole cheese.

(http://www.originalitaly.it)

Figure 41. Valle D'Aosta Fromadzo.

Website

http://www.fromadzo.com/

VALTELLINA CASERA

Characteristics (PDO July 1, 1996)

The Valtellina Casera is a cylindrical cheese with a diameter of 30-45 cm, an edge of 8-10 cm and a weight of 7-12 kg. The ripening lasts at least 70 days. The rind is soft and regular with a yellow-pale color. The paste is pale-yellow, with irregular, small and sparse holes. The texture is consistent and springy with an aromatic odor. The taste is sweet and piquant for the long ripened cheeses. In 2011 the production was about 1,200 tons.

Production Area

The geographical area of production is the whole territory of the province of Sondrio.

Cheesemaking

The Valtellina Casera is produced with partially skimmed raw cow's milk. The milk is curdled by the addition of bovine liquid rennet, then the curd is cut to produce granules the size of maize and cooked between 40 and 45°C for about 30 min. The curd is put into cylindrical molds and pressed for 8-12 hours. The cheese is salted in brine or in dry conditions. Ripening is performed at 6-10°C with a humidity higher than 80%. According to the Disciplinary of Production, Valtelina Casera must have a fat content (on dry matter) higher than 34%.

(http://www.valsana.it)

Figure 42. Valtellina Casera.

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Website

http://www.ctcb.it/

VASTEDDA DELLA VALLE DEL BELICE

Characteristics (PDO October 28, 2010)

The Vastedda della Valle del Belìce is a cheese with a typical round loaf shape with a diameter of 15-17 cm, high 3-4 cm and a weight of 0.5-0.7 kg. The ripening is carried out for

1-2 days. The rind is absent and the paste is white, smooth, and without holes. The texture is soft and soluble. The taste is sweet and sour.

Production Area

The geographical areas of production are some municipalities in the province of Agrigento, Trapani and Palermo.

Cheesemaking

The Vastedda della Valle del Belìce is produced with whole raw ewe's milk. The ewe must be of Valle del Belice race. Cheesemaking must be done within 48 hours from milking. The milk is curdled in a copper boiler by the addition of lamb rennet at a temperature of 36-40°C. After 40-50 min the curd is cut to produce granules the size of rice and washed with hot water. The curd is then removed and put into typical rush molds. After 24-48 hours the curd is cut, put in a typical basin on wood and mixed with hot water (80-90°C). The curd is hand worked to obtain a sphere. This sphere is put in a pottery plate where the curd obtains the typical shape. After 6-12 hours the cheese is salted in brine from 30 min to 2 hours. According to the Disciplinary of Production, Vastedda della Valle del Belìce must have a fat content (in dry matter) higher than 35%.

(http://www.gentedelfud.it)

Figure 43. Vastedda della Valle del Belice.

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http://www.consorziovastedda.it

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Chapter 3

BIOTECHNOLOGICAL CONVERSION OF WHEY INTO HIGH-VALUE PRODUCTS

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ABSTRACT

Whey is an important surplus product of food industry. Based on the point of view, huge quantities of whey produced worldwide can represent an environmental problem with its disposal, or, due to the fact that whey is rich in fermentable nutrients, it can be considered as an attractive substrate for biotechnological production of various industrially interesting products. Therefore, this chapter is intended to summarize possible up-stream processing methods, general principles and fermentation strategies for the microbial productions using whey as a substrate. Further, among huge amount of potential products, this chapter focuses on the production of high value substances and materials which find applications especially in the fields of health care, medicine and pharmacy. At first, many biologically active substances such as vitamins, carotenoids, antibiotics etc. can be produced from whey employing various microorganisms and cultivation strategies. Moreover, also number of biopolymers which can be used in the form of drug carriers, scaffolds, sutures, adhesives *etc.* can be produced by using whey as a cheap complex substrate. Apart from poly (lactic acid), these materials include polyhydroxyalkanoates - bacterial polyesters and also several polysaccharides such as xanthan, alginate, hyaluronic acid, gellan, pullulan, dextran or chitosan. In addition, the aim of this review is also to provide basic economical consideration of fermentation processes. In conclusion, utilization of whey as a substrate for microbial productions of high value products could result in a very promising process meeting both economic and ecological requirements.

1. INTRODUCTION

Cheese whey is a by-product of dairy industry; particularly, it represents the watery portion which is formed during coagulation of major cheese proteins – casein – in cheese manufacturing or caseins production (Guimaraes et al., 2010). Because cheese whey constitutesapproximately80-90% (v/v) of the total milk used and, moreover, whey retains about 55% of total milk nutrients, huge amounts of liquid waste with high organic compounds content (BOD_5 = 30 000–50 000 ppm, COD = 60 000–80 000 ppm) are formed. Apart from lactose, which is predominantly responsible for high BOD_5 and COD values, cheese whey also contains soluble proteins, lipids and mineral salts. Although several possibilities of cheese whey utilization have been explored, a major portion of the world cheese whey production is discarded as an effluent. Its disposal as a waste is serious pollution problem for the surrounding environment since it affects the physical and chemical structure of soil which results in decreased potential of crop yields. In addition, release of whey into the water bodies harms aquatic life due to depleting the dissolved oxygen (Siso, 1996; Panesar et al., 2007). Therefore, to overcome problems associated with whey disposal in both economic and ecological meanings, it would be very reasonable to use cheap whey as a substrate for production of high value product (s). Availability of the utilizable lactose in whey as well as the presence of other essential nutrients supporting the growth of various microorganisms make the whey potential excellent raw material for biotechnological productions of high value commodities.Additionally,the process would significantly decrease BOD_5 and COD values of entering waste and, thus, minimize the impact of the whey on the environment. This chapter is intended to provide an overviewof the potential processes enabling transformation of waste whey into the high value materials and substances which find applications especially in the fields of pharmaceutical industry and medicine.

2. WHEY TYPES AND THEIR COMPOSITION

Whey contains most of the water-soluble components (lactose, whey proteins, milk salts, *etc.*) of milk transformed and, of course, water. The precise whey composition is dependent on the method of cheese or caseins manufacturing and it also varies during the season of milk production. Generally, whey is often classified as "*sweet*" if derived from precipitation of caseins by rennet, or as "*acid*", when casein coagulation was carried out by acidification of milk either by partial fermentation of lactose to lactic acid or by the addition of mineral acids (HCl, H_2SO_4, *etc.*) (Mawson, 1993). In general, whey produced from rennet-coagulated cheeses is low in acidity (pH about 6.5), while the production of acid cheeses (such as ricotta or cottage cheese) or caseins yields acid whey with pH lower than 5. Cheese-whey salts are comprised of NaCl and KCI (more than 50% of total salts), calcium salts (primarily phosphate) and others. Cheese whey also contains appreciable quantities of other components, such as lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid), some of vitamins belonging to B group, free amino acid *etc.* (Siso, 1996). Table 1 summarizes typical composition of sweet and acid whey.

Whey proteins represent about 20% of the milk proteins. The most abundant of whey proteins are β-lactoglobulin (50%), α-lactoalbumin (12%), immunoglobulins (10%), serum

albumin (5%) and proteose peptones (0.23%). The Protein Efficiency Ratio value and essential amino acid content of whey proteins are higher than those of caseins. Moreover; their biological value exceeds even that of whole egg proteins (Kilara et al., 2005).

The main differences between the two types of whey are in the mineral content, pH and composition of whey protein fraction. The acid coagulation approach results in the decrease of pH to the value about 4.5, which is necessary for casein precipitation. At this low pH, calcium presented in casein micelles is partially solubilized and released into the solution. This causes higher calcium content of acid whey.

Table 1. Typical composition of sweet and acid whey (Jelen, 2003)

Component	Sweet whey [g/l]	Acid whey [g/l]
Total solids	63-70	63-70
Lactose	46-52	44-46
Protein	6-10	6-8
Calcium	0.4-0.6	1.2-1.6
Phosphate	1.3	2.4.2005
Lactate	2	6.4
Chloride	1.1	1.1

On the other side, precipitation of casein *via* action of rennet results in releasing of specific fragment of κ-kasein molecule. This fragment is termed glycomacropeptide (GMP) and due to its solubility it ends up in the whey. GMP is important protein of sweet whey, because it constitutes approximately 20% of its protein content, however, it is not present in acid whey (Panesar et al., 2007).

3. CHEESE WHEY AS A FERMENTATION MEDIUM

Dairy industry all over the world generates huge amount of whey per litter of milk processed. About 50% of total world cheese-whey production is treated and transformed into various food products (of which 45% is used directly in liquid form, 30% in the form of powdered cheese whey 15% as lactose and byproducts from its removal, and the rest as cheese-whey-protein concentrates) the remaining 50% is disposed as a waste (Marwaha and Kennedy, 1988). This potentially danger waste can be used for production of various biotechnological products *via* microbial fermentation. Generally, the processes based on microbial cultures on cheese whey are considered as the most profitable alternatives for the transformation of cheese whey surplus (Siso, 1996). However, there are many potential facts and risks that should be taken into account prior utilization of cheese whey as a fermentation medium.

Whey is foremost a dilute solution and many difficulties encountered in whey handling stem from this fact. First of all, it complicates the transportation of whey and, secondly, cheese whey tends to spoil by action of naturally occurring microorganisms which significantly devaluates it potential application as a fermentation medium. Therefore, it is worthy decreasing concentration of water resulting in preparation of cheese whey concentrate. During the procedure of pre-concentration, the cheese whey is, at least, partially sterilized.

Moreover, due to high osmotic pressure, this form of cheese whey is much more microbially stable and, in addition, because of the decrease of the whey weight it also simplifies the process of whey transportation.

Since most of biotechnological productions employ microbial monoculture, it is necessary to overcome any possible contaminations of fermentation medium by undesired microorganisms. Hence, the medium must be sterilized prior cultivation. Sterilization of whole cheese whey is problematic, because heating of whey to as high temperature as 121°C results in precipitation of whey proteins (Parris et al., 1993). The protein precipitate seriously complicates the fermentation as well as recovery of the biomass or the down-stream processing of the fermentations products. Therefore, it is usually needed to remove the excessive proteins prior sterilization. This can be done by decreasing pH by mineral acids and boiling (Obruca et al., 2011). On the other side, the whey proteins dispose with high nutritive value which would be reduced by any improper treatment. It is reasonable to remove proteins by more gentle method which allows their subsequent application in human diet or in animal feeding. The most commonly used methods nowadays are ultrafiltration and diafiltration because of their advantages of cost reduction, high processes speed, the absence of denaturation or protein structure modification and the fact that protein concentrate is free of salts, thereby making it suitable for all kinds of human foods (Evans and Gordon, 1980). The process of protein removal ends up with two products. The first is called whey protein concentrate and it contains almost exclusively protein fracture of whole whey. The second products, termed whey permeate, contains lactose and all the other soluble substances. Protein recovery can be highly profitable and reduces effluent load of the whey about 10 000 ppm of COD. However; the whey permeate still contains about 50 000- 60 000 ppm of COD, mostly represented by lactose (Mawson, 1993).

Another option how to overcome the precipitation of cheese whey proteins during sterilization is their hydrolysis *in situ* by proteolytic enzymes or microbes (Vasala et al., 2005). Whey protein hydrolysis yields peptide mixture with increased solubility and altered foaming characteristics. In addition, the peptides can be utilized by microbes during fermentation as carbon, nitrogen and essential amino acids source and, thus, they could represent very important factor supporting the growth of microorganisms and the fermentation process (Margot et al., 1994; Senthuran et al., 1997).

Some microbial strains suitable for production of various substances do not dispose with β-galactosidase activity, in other words, these microbes are not capable of utilizing lactose directly. On the other side, they can utilize glucose and/or galactose and, hence, they can be used for fermentation using whey with pre-hydrolyzed lactose. The lactose can be cleaved by acids or by simple application of β-galactosidase into the cheese whey prior or after sterilization (Kohler et al., 2008; Marangoni et al., 2001) or by using immobilized enzyme or whole cell system (Kosseva et al., 2009).The question is whether the process of biotechnological production remains profitable even after inclusion of relatively expensive process of whey lactose hydrolysis.

Whole whey or whey permeate are usually not sufficient medium for optimal microbial growth and production of desired metabolites. Hence, whey medium is supplemented with various substances serving as additional nitrogen source and by specific growth factors required by particular microorganism. Generally, yeast extract and yeast autolysate are used as complex nitrogen sources (Amrane, 2005; Obruca et al. 2011, Marova et al., 2011) or

nitrogen can be also added in the form of mineral salts such as $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl *etc*. (Aksu and Eren, 2005; Nath et al., 2008; Obruca et al., 2011) depending on preferences of the particular microbe. Introduction of inorganic phosphate was also found to be beneficial for some biotechnological productions using whey as a substrate (Amrane, 2000; Liu et al., 2003; Nikel et al., 2005). Besides mentioned supplements, addition of other nutrients (molasses, corn steep molasses), vitamins, minerals, amino acids and inorganic supplements to whey or whey permeate were also investigated (Amrane, 2000; Liu et al., 2003; Nikel at al. 2005; Khanafari and Sepahei, 2007; Obruca et al. 2011). Of course, it is crucial to take into account demands of the microbes to be employed. The supplementation strategies of whey based medium for particular microorganisms will be mentioned in the following parts of this chapter.

4. FERMENTATION STRATEGIES

Generally, there are few fermentation strategies which can be applied for microbial utilization of whey or any other substrate and production of various commodities. This part of the book chapter is intended to briefly describe individual strategies with respect to particular details stemming from the utilization of cheese whey as a culture medium.

Batch Cultivation

At first, probably the simplest and the most often applied fermentation strategy is a batch cultivation, which is characterized by the growth of microorganisms without supply of additional substrate after inoculation (Figure 1A). While the substrate is metabolized, biomass and products are formed during cultivation. The batch is stopped and harvested when the desired product concentration is maximal. Thereafter, the reactor is cleaned and sterilized for the next batch. When the process is carried out in the bio-reactor (also termed fermenter), during batch cultivation several components other than substrate might be added. In aerobic cultivation air or O_2 is continuously added through a sparger. To maintain constant pH, alkalior acid components are added to the broth. Antifoam agents are required for foam control and other agents might be added to the broth to induce the production of a desired metabolite (Ullmann; 2005).

Batch cultivation reveals typical stages of microbial growth and metabolites production. First phase is called "*lag-phase*" and it is characterized by adaptation of microorganisms to conditions within bioreactor. During lag-phase neither growth nor production occurs and, therefore, there is an attempt to maintain this phase as short as possible. Whenever using cheese whey as fermentation medium, there is a risk that antibiotics and other undesired substances stemming from milk will inhibit growth of microorganisms and prolong the lag phase or even stop the fermentation (McEwen et al., 1992). Thereby, the composition of the whey should be precisely analyzed in order to prevent these obstacles. Furthermore, due to the high lactose and salt content, whey possesses relatively high osmotic pressure which could have inhibiting effect on microbes as well. Taking into account abilities of microbes to

face high osmotic pressure and also economic demands of process, whey medium can be diluted or desalted.

When microorganisms fully adapt to environment, they start to growth very intensively, usually the biomass concentration increases exponentially. This stage of cultivation is termed "*growth phase*". Besides biomass, also primary metabolites (these which production is growth associated such as ethanol, lactic acid *etc.*) are produced (Ullmann; 2005).

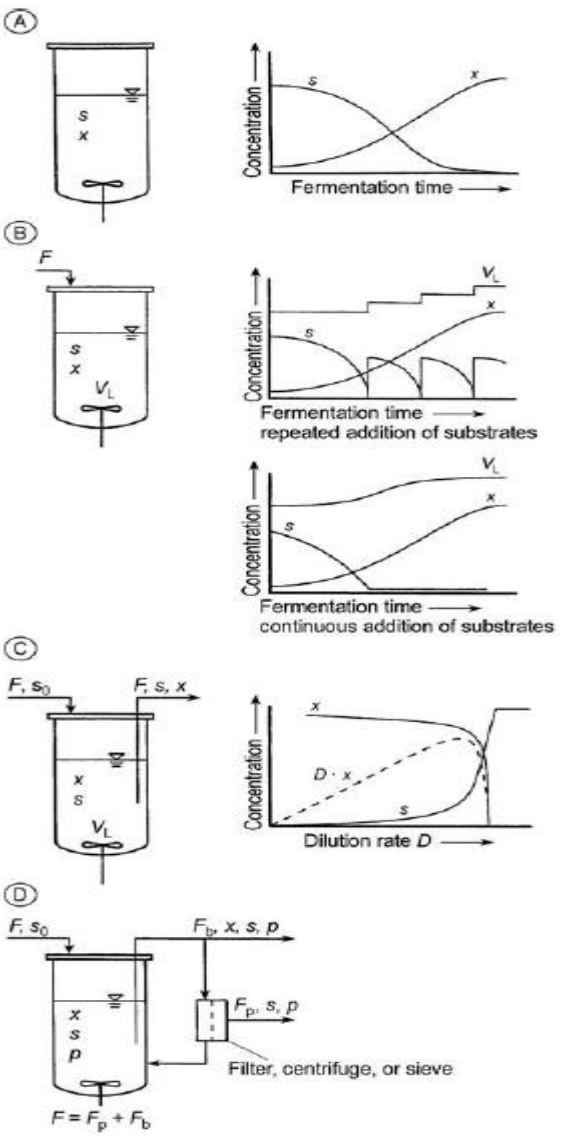


Figure 1. Standard fermentation stretegies. A) Batch cultivation; B) Fed-Batch; C) Continuous cultivation; D) Continuous cultivation with cell recycle.V_L = Working volume; F = flow of fresh substrate; Fp = Permeate flow, cells free; F_b = Bleed flow with cells; s = substrate concentration; x = biomass concentration, p = product concentration; s_0 = concentration of substrate in the flow. Ullmann (2005).Reproduced with permission.

Finally, when either the substrates of the medium are depleted or accumulation of metabolism products possessing inhibiting effect on microbial culture reaches certain level, the growth of microorganism is stopped and so called "*stationary phase*" is about to begin. During stationary phase no biomass growth occurs, however, production of secondary metabolites starts. Because number of desired products such as carotenoids, polyhydroxyalkanoates *etc.* are traditionally considered as secondary metabolites, it is crucial to precisely optimize the time, when concentration of desired products reaches its maximum. Any unnecessary prolongation of fermentation process could result in the decrease of yields and in the increase of production cost (Oka, 1999).

Fed-Batch Cultivation

To overcome the growth limitations due to the initial substrate concentration, substrates can be added to the broth during cultivation. This mode of operation is termed as fed-batch cultivation. Substrates can be either supplied stepwise during the cultivation or continuously (see Figure1B). Fed-batch technique is the state of the art for the efficient production employing bacteria, fungi as well as yeasts. Modern biotechnology processes with genetically engineered microorganisms often use fed-batch techniques to reach high cell densities before adding the inducing agent to start the production of the desired product. Nevertheless, fed-batch mode of fermentation is also commonly used for cultivation of non-genetically engineered strains (Ullmann, 2005).

To overcome significant volume increases during fermentation, the feeding solution usually contains high concentration of carbon substrate. There are many reports on utilization of whey concentrate for fed-batch feeding (Ahn et al., 2000; Ahn et al., 2001;Viitanen et al., 2003). To counter this, the lactose concentration is increased by pre-concentration of the whey for instance by the addition of concentrated lactose or by using reverse osmosis or other technique (Mawson, 1993). However, it should be kept in mind that apart from lactose also salts are concentrated. This can easily result in a significant increase of osmotic pressure within the bioreactor inhibiting both the cell growth and production of the metabolites of interest. Therefore, whey should be at least partially desalted prior pre-concentration.

In comparison with batch cultivation, fed-batch process can provide improved productivity as a whole because of the enhanced yields and reduced fermentation time. On the other side, production of metabolites which enhance viscosity (such as extracellular polysaccharides) of media results in significantly decreased oxygen availability in fermentation media. This could be serious complication especially in high density cell cultivations obtained in fed-batch mode. Therefore, batch-cultivation is not the mode of choice for all the fermentation productions (Oka, 1999).

Continuous Fermentation

In continuous fermentation, a complete medium is fed to a fermentor after an appropriate period of batch fermentation, and the same quantity of broth is continuously taken from the fermentor to maintain the fermentation broth at a fixed volume (Figure 1C and D). This may be performed either by the "chemostat" method using a substrate or limiting substance at

certain concentration level, or by the “turbidostat" method in which the cell level is adjusted to maintain constant cell mass. Because the continuous fermentation process allows improvement of productivity compared with the ordinary fermentation, the initial investment in equipment is small relative to the production volume, and operation cost is low. However, the first drawback is that it is not suitable for small-scale production. Secondly, the challenges of sterile operation and equipment maintenance are more necessary than they are for batch and fed-batch fermentation. Finally, it is rather difficult to set conditions of continuous fermentation for production of metabolites which are not growth-associated (Oka, 1999). The utilization of whey as a medium in continuous fermentation systems has been recently reported for ethanol (Agustriyanto and Fatwanti, 2009), bio-hydrogen (Azbar et al., 2009; Davila-Vazquez et al., 2009) or methane (Venetsaneas et al., 2009) productions.

An alternative mode of fermentation which can be in principal also considered as a variety of continuous cultivation is application of immobilized cells system. The immobilized cells are defined as “the microbial cells physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and/or continuously”. The medium flow through the reactor with immobilized cells and is converted into desired product(s). Immobilization technology has several advantages, beacause it permits higher cell densities in bioreactors, improves stability, makes re-utilization and continuous operation possible, and precludes the need to separate the cells from the substrate products following processing (Kosseva et al., 2009). Of course, immobilized cells systems are suitable for production of extracellular products. There is great number of the published articles dealing with production of ethanol and lactic acids by cells immobilized systems of various microorganisms using cheese whey medium (Goksungur et al., 2005;Schepers et al., 2006; Panesar et al., 2007;Mirdamadi et al., 2008; Staniszewski et al., 2009;Kosseva et al., 2009, Guo et al., 2010). In addition, also some antibiotics such as nisin and pediocin PA-1 were produced from cheese whey using immobilized cells of *Lactococcus lactis* and *Pediococcus acidilactici* UL5, respectively (Liu et al., 2005; Naghmouchi et al., 2008).

5. PRODUCTION OF SELECTED BIO-SUBSTANCES

Vitamins

According to a recent World Health Organisation (WHO) review, vitamin B_{12}(Figure 2) and folate (also called vitamin B_9) (Figure 3) deficiencies may be a public health problems affecting millions of people (WHO, 2008). Anemia and increased risk of birth defects in newborn are some of the health consequences of low folate and vitamins B_{12} status. The low level of folate may also cause higher risk of cardiovascular diseases, dementia and Alzheimer´s disease (Gisondi et al.2007), moreover, there is some evidence for an increased cancer risk (Mitchell et al., 2004). Vitamins B_{12} deficiency additionally leads to neurological damage and has been linked with psychiatric disorders (Truswell, 2007).

Folate can be produced by plants and microorganisms, whereas vitamin B_{12} is only synthetized by some bacteria and archae. Moreover, folates can be also produced by chemical synthesis by reacting malondialdehyde derivatives with aminobenzoyl-L-glutamic acid and

triaminopyrimidinone derivatives, however, a number of studies have reported possible adverse effects caused by high intake of synthetic form of folic acid (Osterhues et al., 2009). Both vitamins can be produced *via* fermentation using either lactic acid bacteria or propionic acid bacteria for production of folate. Vitamin B_{12} can be biotechnologically produced by propionic bacteria and some pseudomonas (Hugenschmidt et al., 2010).

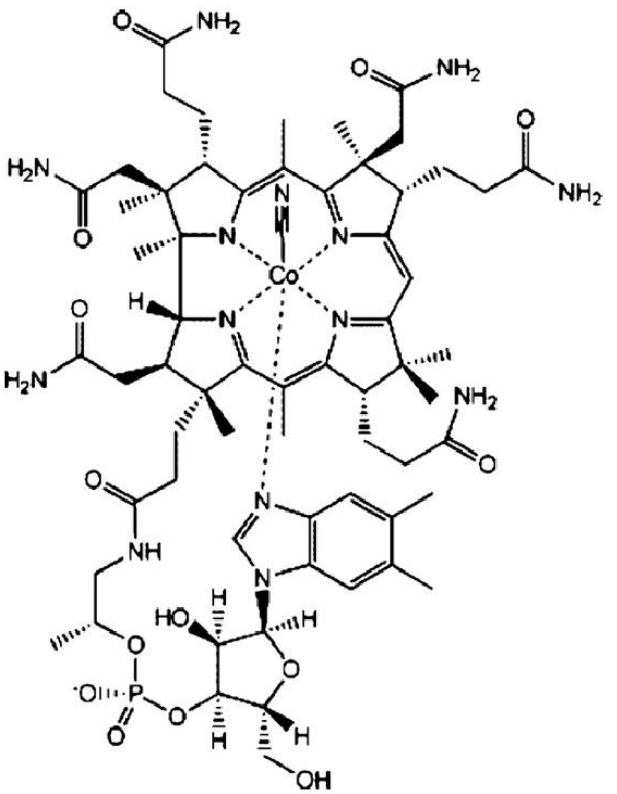


Figure 2. Stucture of Vitamin B_{12}(Jin et al., 2009) Reproduced with permission.

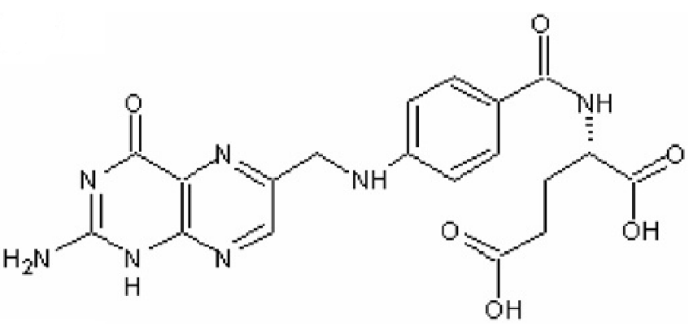


Figure 3. Structure of folic acid (Mote at al., 2011). Reproduced with permission.

There have been several reports on biosynthesis of both folate and vitamin B_{12} by bacteria fermentation using whey as a substrate. Study was carried out in Erlenmeyer flasks and focused on optimization of cultivation conditions. Vitamin yields were significantly increased by medium supplementation with 1.5% of yeast extract and 5 ppm of cobalt was

adequate to give good levels of vitamin B_{12}. The vitamin B_{12} precursor 5,6-dimethylbenzimidazole was found to be adequate at 10 ppm in the absence of aeration, while in the presence of aeration, a zero level of precursor was found to be most desirable. The vitamin formation was observed during the latter part of the fermentation after the organism approached maximal growth. Staniszewski and Kujawski (2007) screened capabilities of selected propionic bacteria to produce vitamin B_{12} in single culture as well as the possibility of strain co-operation in a mixed culture on whey medium. The most significant changes in the content of vitamin B_{12} were observed in the early phase of logarithmic growth up to 72 h of incubation, in all cultures examined.

Propionibacteria produce vitamin B_{12} intracellularly when cultivated anaerobically and simultaneously excrete mainly propionic acid and acetic acid extracellularly. The primary problem for vitamin B_{12} production using propionic bacteria is that the end products such as propionic acid *etc.* inhibit cell growth. It is, therefore, crucial to remove the end products *in situ* and, thus, improve the cell growth which results in the significantly improved vitamin yields (Hatanaka et al., 1998). Under conditions of aerobic growth the synthesis of propionic acid is suppressed; however, oxygen presence inhibits vitamin B_{12} production as well. The propionic acid level can be kept at low level by using cell recycle system. Nevertheless, the major drawback of employing the cell recycle system is that part of the culture medium is drawn off through hollow fiber membrane without efficient use. This problem has hindered the practical application of the cell recycle system. Interesting solution was suggested by Miyano et al. (2000). The propionic acid bacterium *Propionibacterium freudenreichii* was cultivated in mixed culture with *Cupriavidus necator* to keep propionic acid at low level noting that propionic acid produced by propionic bacteria can be assimilated by the second microorganism.

Hugenschmidt et al. (2010) screened intracellular production of vitamin B_{12} and extracellular production of folate by 151 lactic acid bacteria and 100 propionic acid bacteria of different origin using supplemented whey permeate as a substrate. Five lactic bacteria strains belonging to the species *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus brevis* and *Lactobacillus fermentum* exhibited high extracellular folate productions, with a maximum yield of 397 ± 60 ng mL^{-1} for *L. plantarum* SM39. The highest vitamin B_{12} production was measured for *Propionibacterium freudenreichii*DF15with 2.5 mg mL^{-1}.

Vitamin B_{12} and folate can be produced in one fermentation employing co-culture of *Lactobacillus plantarum* SM39 and *Propionibacterium freudenreichii* DF13 on whey permeate supplemented with yeast extract. The cultivations were carried out in two-step mode (3 days anaerobic followed by 4 days aerobic) which led to high vitamin B_{12} and folate yield, 751 and 8399 ng mL^{-1}, respectively (Hugenschmidt et al., 2011).

Riboflavin is another vitamin which can be prepared by fermentation. Buzzini and Rossi (1997) reported riboflavin production by yeasts using various agro-industrial residues including whey as substrates. The highest yields (1.135 mg L^{-1} in 10 days) were reached using *Torulopsis candida* 4253 in fed-batch mode. Cheese whey can be also used for production of riboflavin employing *Ashbya gossypii*. The production can be increased by addition of sugar alcohol and vitamins into the fermentation media (Ozbas and Kutsal, 1991).

Carotenoids

Carotenoids are naturally occurring lipid-soluble pigments, the majority being C40 terpenoids. They act asmembrane-protective antioxidants that efficiently scavenge $^1O_2$ and peroxyl- radicals. Their antioxidative efficiency is apparently related to their structure. The most significant part in the molecule is the conjugated double bond system that determines their color and biological action. Carotenoid pigments occur universally in photosynthetic systems of higher plants, algae and phototrophic bacteria. In non-photosynthetic organisms, carotenoids are important in protecting against photooxidative damage. Thus, many non-phototrophicbacteria, yeasts and fungi rely on carotenoids for protection when growing in light and air (Marova et. al., 2010).

Commercially, carotenoids are used as precursors of vitamin A, in nutrient supplementation, for pharmaceutical purposes, in various cosmetic products, as food colorants and in animal feeds. There is an increased interest in carotenoids as natural antioxidants and free radical scavengers because of their ability to reduce and alleviate chronic diseases, various pathological stages and aging. Furthermore, epidemiological evidence and experimental results suggest that dietary carotenoids inhibit the onset of many diseases in which free radicals are thought to play arole in initiation, such as arteriosclerosis, cataracts, multiple sclerosis and cancer (Hughes, 1999).

Due to their wide application in pharmacy, the demand and market for carotenoids is constantly growing. However, the application of chemical synthetic methods to prepare carotenoid compounds as food additives has been strictly regulated in recent years. Therefore, attention is paid to the finding of suitable natural methods for its production. One possibility lies in biotechnological techniques employing the potential of microorganisms that are able to convert various substrates into carotenoid pigments, even if this approach is restricted by a number of useful species and also the carotenoid yield can hardly compete with the chemical synthesis or with plant material extraction (Lee and Schmidt-Dannert, 2002; Marova et al., 2010).

Since an important aspect of the fermentation process is the development of a suitable culture medium to obtain the maximum amount of desired product, cheap raw materials and by-products of agro-industrial origin have been proposed as a low-cost alternative carbohydrate sources for microbial carotenoids production. This is beneficial strategy also from the point of view of minimizing environmental and energetic problems related to residues and effluent disposal. During the product recovery process, the biomass is isolated from the fermentation broth and transformed into a form suitable for carotenoids isolation. Recovery involves extraction of carotenoids from biomass by appropriate solvent, their concentration and purification. Generally, cell disruption prior extraction is recommended to increase efficiency of recovery process (Marova et al., 2011).

There are many reports on utilization of cheese whey for carotenoids production. At first, Frengova et al. (2004) studied carotenoids production by lactose-negative strain yeast *Rhodotorula rubra* GED5 in co-culture with lactose-positive yeast strain *Kluyveromyces lactis* MP11 in whey permeate supplemented with $(NH_4)_2SO_4$, $MgSO_4$, KH_2PO_4, $MnSO_4$ and yeast extract. Maximum yields of cell mass (24.3 g L^{-1}) and carotenoids (10.2 mg L^{-1} of culture fluid or 0.421 μg g^{-1} of dry cells) were obtained by growing the microbial association in whey permeate containing lactose at concentration of 50 g L^{-1} in a fermentor with an airflow rate of 0.8 L $L^{-1}min^{-1}$, agitation of 220 rpm, and temperature of 30°C. The identified

carotenoid pigments were β-carotene, torulene, and torularhodin. In another paper the same group reported successful production of carotenoids from whey permeate (supplemented as described above) by co-cultivation of *Rhodotorula rubra* GED5 and yoghurt starter cultures *Lactobacillus bulgaricus* 2–11 and *Streptococcus thermophilus* 15HA. In associated cultivation under intensive aeration (1.3 L min^{-1} airflow rate), initial pH 5.5, 30° C,the lactose-negative strain *R. rubra* GED8 synthesized large amounts of carotenoids (13.09 mg L^{-1} of culture fluid). The carotenoid yield was approximately two-fold higher in association with a mixed yoghurt culture than in association with single yoghurt bacteria. The major carotenoid pigments comprising the total carotenoids were β-carotene (50%), torulene (12.3%) and torularhodin (35.2%) (Simova et al., 2004; Frengova et al., 2006).

Aksu and Eren (2005) focused on carotenoids production employing different yeast strain - *Rhodotorula mucilaginosa*. This yeast is lactose positive, hence, no co-culture is needed to be added. Optimum pH and temperature for total carotenoids production were determined as 7.0 and 30°C, respectively. Total carotenoids concentration and pigment production yield were significantly increased with increasing aeration rate up to 2.4 vvm. An initial ammonium sulphate concentration of 2 g L^{-1} gave the maximum carotenoids production. Further, cotton seed oil significantly activated carotenoids accumulation in the yeast cells. Among carbon sources tested, the highest product yield (35.0 mg total carotenoids per gram of dry cells) was achieved when 13.2 g L^{-1}lactose containing whey was the carbon source in the broth. One year later, the same authors published very similar study with another member of *Rhodotorula* yeast genus – *Rhodotorula glutinis*. Also this yeast possesses sufficient β-galactosidase activity and is, therefore, capable of utilizing lactose directly. The optimal pH was determined as 6 and temperature was optimized at 30°C. Again, ammonium sulfate and cotton seed oil addition significantly supported carotenoids biosynthesis. Cultivation of *R. glutinis* in whey containing 13.2 g L^{-1} of lactose yielded 56.8 mg L^{-1} of carotenoids (Aksu and Eren, 2006).

Valduga et al. (2009) tested acid and enzymatic hydrolysis of whey lactose and application of hydrolysate for subsequent bio-production of carotenoids by *Sporidiobolus salmonicolor* CBS 2636. The carotenoids were recovered using liquid N_2 combined withdimethyl sulfoxide for cell rupture and an acetone/methanol mixture (7 : 3 v/v) for extraction. The maximum concentration of total carotenoids obtained was 0.59 mg L^{-1}in pre-hydrolyzed cheese whey supplemented with 4 g L^{-1} of K_2HPO_4 at180 rpm, 25° C and pH4.The use of enzyme-hydrolyzed cheese whey was more effective in carotenoid bio-production by *S. salmonicolor* CBS 2636 than use of acid-hydrolyzed cheese whey. Hydrolyzed cheese whey was also used as carbon substrate for carotenoids production by *Blakeslea trispora*. The aim of the study was to optimize introduction of non-ionic surfactants and β-ionone as activators of carotenoids accumulation. Maximum production was obtained when whey medium was supplemented with Tween 80 (33.6 g L^{-1}), Span 80 (68.7 g L^{-1}) and β-ionone (2.6 g L^{-1}) (Varzakakou et al., 2010; Varzakakou and Roukas, 2010).

Nasrabadi abd Razavi (2011) employed random mutagenesis in order to isolate efficient β-carotene producing strain of *Rhodotorula acheniorum*. The lactose-positive mutant strain named MRN was tested on its ability to produce carotenoids on whey. Conditions for carotenoids production were optimized as follow: lactose concentration 55 g L^{-1}, pH 5.85, $(NH_4)_2SO_4$ 3.5 g L^{-1}, temperature 23°C and aeration 1.56 vvm. Under these conditions, the strain produced 262.1 mg L^{-1} of β-carotene.

Due to the fact that stress response of red yeasts is usually associated with carotenoids overproduction, very efficient strategy to enhance carotenoids accumulation in various red-yeast strains is an introduction of exogenous stress factors such as ethanol, hydrogen peroxide or high osmotic pressure into the fermentation media (Marova et al., 2010).

Table 2. Summary of carotenoids productions from whey media reported in literature

Microorganism	Fermantation strategy	Substrate	Biomass [g L^{-1}]	Yields* [mg L^{-1}]	Reference
Rhodotorula rubra GED5	Batch, co-culture with *Kluyveromyces lactis*	Whey permeate, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, yeast extract	24.3	10.2	Frengova et al. (2004)
Rhodotorula rubra GED5	Batch, Co-culture with *Lactobacillus bulgaricus* 2–11 and *Streptococcus thermophilus* 15HA	Whey permeate, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, yeast extract	26.0	13.1	Simonova et al. (2004); Frengova et al. (2006)
Rhodotorula mucilaginosa	Batch culture	Whey, yeast extract, malt extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$	1.8	63.0	Aksu and Eren (2005)
Rhodotorula glutinis	Batch culture	Whey, yeast extract, malt extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$	1.6	56.8	Aksu and Eren (2006)
Sporidiobolus salminicolor CBS 2636	Batch culture	Hydrolyzed cheese whey, KH_2PO_4	[2]*n.a.*	0.6	Valduga et al. (2008)
Blakeslea trispora	Batch culture, surfactants and b-ionone	Hydrolyzed cheese whey	*n.a.*	*n.a.*	Varzakakou et al., (2010); Varzakakou and Roukas (2010)
Rhodotorula achenior MRN	Batch culture	Whey permeate, $(NH_4)_2SO_4$	24.5	262.1	Nasrabadi and Razavi (2011)
Rhodotorula glutinis CCY 20-2-26	Bath culture, fermentor, salt stress	Deproteinized whey	44.7	45.7	Marova et al. 2011
Rhodotorula mucilaginosa CCY 20-7-31	Batch culture, fermentor	Deproteinized whey	29.8	11.3	Marova et al. 2011
Sporobolomyces roseus CCY 19-4-8	Batch culture, fermentor	Deproteinized whey	10.2	29.4	Marova et al. 2011

Note: [1] Total yields of carotenoids expressed as mg L^{-1}, [2]*n.a.* stands for: data not available.

Therefore, several red yeast strains (*Sporobolomyces roseus*, *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*) were enrolled into a comparative screening study. To increase the

yields of these pigments, several types of exogenous as well as nutrition stress were tested. As inexpensive substrates, whey and potato extract were tested. Stress conditions induced by peroxide and salt stresses were applied too. The production of carotene enriched biomass was carried out in flasks as well as in laboratory bio-reactor. The highest yields were obtained in whey medium using *Rhodotorula glutinis* CCY 20-2-26 (45 g L^{-1} of biomass and 46 mg L^{-1} of β-carotene) in laboratory fermentor (Marova et al., 2011).

The table 2 provides summary of fermentation strategies and yields of carotenoids from cheese whey-based substrates. According to the number of studies aimed at biotechnological production of carotenoids using cheese whey, it seems that whey represents very attractive substrate. This intensive research demonstrates the enormous potential in its application and creates new economic competitiveness and market of microbial carotenoids.

Antibiotics

Antibiotics are wide family of diverse compounds enabling efficient inactivation of various microorganisms, mostly bacteria but also yeasts and fungi. It is the reason why antibiotics find many applications especially in medicine, but also in food industry or agriculture. Many of these compounds are naturally produced by some bacteria and fungi. Therefore, there have been a lot of attempts to improve the antibiotic production by fermentation in terms of process yields and economics and also in these cases, cheese whey and cheese whey permeate were considered as attractive substrates for fermentation.

Probably the most deeply studied antibiotic is penicillin which is commercially produced by fungal strain *Penicillium chrysogenum*, a filamentous microorganism consisting of multicompartment structure of morphologically heterogenous hyphae (Mou and Cooney, 1983; Paul et al., 1998). *Penicillium chrysogenum* is able to utilize lactose directly, thus, Paul et al. (1998) studied its growth and penicillin production in fed-batch mode using cheese whey with glucose as substrates. Furthermore, cheese whey can be used as an additional co-substrate and moisturizer when penicillin is produced in solid state fermentation mode by *Penicillium* sp. (Hamzan et al., 2009).

Bacteriocins are bacterial peptides that inhibit or kill microorganisms that are usually, but not exclusively, closely related to producer strain. Among producing strains, especially lactic bacteria and members of *Bacillus* genus are in the middle of the attention, since both bacterial groups include a wide variety of industrially important species and have history of safe use in both food and industry (Paik et al., 1997). Although bacteriocin production is often performed in complex media which promote abundant growth and relatively high bacteriocin levels it seems more economical to use some of the wastes of food industry as the raw material for the basis of culture media (Lee et al., 2003). Hence, Cladera-Olivera at al. (2004) produced bacteriocin employing *Bacillus licheniformis* P40 in cheese whey. The medium composition and cultivation conditions were optimized using response surface methodology. Data showed maximum bacteriocin production at initial pH between 6.5 – 7.5 and temperature in range 26-37°C when the cheese whey concentration was 70 g L^{-1}. Sharma and Gautam (2008) isolated, from cheese whey, strain of *Bacillus mycoides* able to excrete bacteriocin which showed strong inhibition activity against food borne serious pathogen *Listeria monocytogenes* and *Leuconostoc mesenteroides*. The bacteriocin withstood temperature up to 100°C and was active at wide pH range. *Enterococcus faecalis* A-48-32 is also capable of production of

bacteriocin using cheese whey as a substrate. Medium composition was optimized and various factors, such as whey concentration, glucose, pH and inoculum size were adjusted. The critical factors for maximal production were stabilization of pH at 6.55 and addition of 1% glucose (Ananou et al., 2008). Another very promising peptide antibiotic is mutacin that is naturally produced by common oral bacterium *Streptomyces mutans*. Mutacin disposes with antimicrobial mode action towards a wide range of pathogenic Gram-positive bacteria due to inhibition of their cell wall synthesis. Since lactose was observed to be optimal carbon source for mutacin production (Dahal et al., 2010), it is possible to produce this antibiotic in cheese whey as well. Pediocin PA-1 is peptide antibiotic produced by *Pediococcus acidilactici*. Naghmouchi et al. (2008) aimed at its production by cells immobilized in κ-carrageenan/locust bean gum gel beads. The production was studied during repeated-cycle batch (RCB) culture with pH control in Man Rogosa and Sharpe broth supplemented with either 1% glucose or sweet whey permeate medium. The maximum pediocin PA-1 activity obtained during RCB fermentation was 4096 AU mL^{-1}; it was attained after only 2 h of cultivation in sweet whey medium. Pediocin PA-1 production in the repeated-cycle batch culture was highly stable over 12 fermentation cycles.

Finally, nisin is peptide antibiotic produced by *Lactoococcus lactis*. Nisin´s antimicrobial activity against Gram-positive bacteria is due to pore formation in the membrane, which cases leakage of cytoplasmic contents. Continuous production of nisin in laboratory media and whey permeate was investigated by Liu et al. (2005) using a packed-bed bioreactor. Optimal conditions for continuous nisin production in whey permeate were pH 5.5, 31°C, 10-20 g L^{-1} casein hydrolysate, and $0.2h^{-1}$ dilution rate. Under these conditions, a maximum nisin titre of 5.1×10^4 AU mL^{-1} was observed. The bioreactor was operated continuously for 6 months without encountering any clogging, degeneration, or contamination problems. De Aurazs et al. (2008)also produced nisin in whey medium. The results showed that *L. lactis* preferred not-filtrated whey where nisin yields reached 11120 AU L^{-1}. In another study concerning nisin production from cheese whey, medium composition was optimized using statistical approach. Optimized conditions were used for constant pH fermentations, where a maximum activity of 575 AU mL^{-1} was achieved at pH 6.5 after 12 h (Gonzalez-Toledo et al., 2010).

Lactic Acid

Lactic acid is considered as one of the most useful chemicals, it is used in the food industry as a preservative, acidulant and flavouring, in the textile and pharmaceutical industries, and in the chemical industry. Moreover, lactic acid consumption has increased considerably because of its role as a monomer in the production of biodegradable polymer – poly (lactic acid) (PLA), which is well-known as a sustainable bioplastic material with many applications in medicine (Datta et al., 1995; Wee et al., 2006).

Of the80 000 tonnes of lactic acid produced worldwide every year, about 90% is made by lactic acid bacteria fermentation and the rest is produced synthetically by the hydrolysis of lactonitrile (Hofvendahl and Hahn-Hagerdal, 2000). Microbial fermentation has a significant advantage in that by choosing a bacteria strain only one enantiomer, an optically pure product can be obtained, whereas synthetic production results in a racemic mixture of DL-lactic acid. As the physical properties of PLA strongly depend on the enantiomeric composition of lactic acid, the production of optically pure lactic acid is essential. For example, optically pure L-

lactic acid is polymerized to a high crystal polymer suitable for fiber and oriented film production and is expected to be useful in production of liquid crystal as well (Hofvendahl and Hahn-Hagerdal, 2000; Panesar et al., 2007).

Lactic acid offers natural ingredients for cosmetic applications. Although primarily used as moisturizers and pH regulators, it possesses multiple other properties such as antimicrobial activity, skin lightening, and skin hydration. The moisturizing effect is related directly to lactate's water retaining capacity, and the skin-lightening action of lactic acid is produced by the suppression of the formation of tyrosinase. Lactic acid is also used in the pharmaceutical industry as an electrolyte in many intravenous solutions that are intended to replenish the bodily fluids or electrolytes. In addition, lactic acidis used in a wide variety of mineral preparations, which include tablets, prostheses, surgical sutures, and controlled drug delivery systems. Finally, as the fully biodegradable and biocompatible material, PLA finds wide range of potential applications in medicine in the form of scaffolds, implants, sutures, bone fixation, fibers, or micro-particles for targeted drug delivery. The polymer is derived from monomers that are natural metabolites of the body, thus, degradation of the material yields the corresponding hydroxyl- acids, making it safe for *in vivo* use. Because the naturally occurring lactic acid is in L-form, polymer consisting of L-lacticacid is considered being more biocompatible (Nampoothiri et al., 2010).

Lactic acid is excreted as a final product of anaerobic metabolism by number of microorganisms. Probably the most important group of these microbes is termed as lactic acid bacteria. Lactic acid bacteria are recognized as "generally regarded as safe" bacteria. This status underlines their increasing use in traditional foods and in an expanding range of novel foods and products designed to have specific nutritional or other health-enhancing benefits (nutriceuticals, prebiotics, probiotics, *etc.*).The key property in defining lactic acid bacteria is that these bacteria produce lactic acid as the major or sole fermentation product. A typical lactic acid bacterium can be described as Gram positive, non-spore forming, catalase-negative, devoid of cytochromes, of non-aerobic habit but aero-tolerant, fastidiously acid tolerant, and strictly fermentative, with lactic acid as the major end products during sugar fermentation (Panesar et al., 2007).

Batch, fed-batch, repeated batch, and continuous fermentations are the most frequently used methods for lactic acid production (Wee et al., 2006). Moreover, employment of cell immobilized techniques attracts attention of many researchers. Various matrixes such as agar, polyacrylamide, alginate, κ-Carrageenan or glass beads were used for cell entrapment (Panesar et al., 2007; Kosseva et al., 2009). The fermentative production of lactic acid is associated with serious complication – lactic acid reveals significant inhibitory effect on microbes to be employed. The inhibitory have been alleviated to a certain extent by conducting fermentation in a continuous dialysis process, in a hollow fiber fermenter or inan electro dialysis system (Panesar et al., 2007).

In order to make the biotechnological production of lactic acid economically feasible, cheap raw materials are necessary, because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for lactic acid productions should have the following characteristics: cheap, low levels of contaminants, rapid production rate, high yield, little or no by-product formation, ability to be fermented with little or no pre-treatment and year-round availability. When refined materials are used for production, the costs of product purification should be significantly reduced.

Table 3. Productions of lactic acid from cheese whey

Microorganism	Fermentation	Lactic acid[1] [g L^{-1}]	Qv[2] [g L^{-1} h^{-1}]	Reference
Streptococcus thermophilus	Immobilized cell system (k-Carrageenan)	18.0	5.9	Audet et al., 1988
Streptococcus thermophilus	Immobilized cell system (k-Carrageenan)	19.0	6.0	Audet et al., 1989
Lactobacillus rhamnosus ATCC 7469	Batch culture	30.0	1.9	Mulligan and Gibbs, 1991
Lactococcus lactis sp. *cremoris* 2487	Batch culture	37.0	4.6	Mulligan and Gibbs, 1991
Lactobacillus casei	Immobilized cell system (agar), continuous cultivation	22.0	7.3	Mostafa, 1996
Lactobacillus casei	Immobilized cell system (agar), continuous cultivation, cell recycle	28.0	9.4	Mostafa, 1996
Lactobacillus casei and *Lactococcus lactis* co-culture	Immobilized cell system (Ca-alginate beads), continuous cultivation	20.0	2.0	Roukas and Kotzekidou, 1996
Lactobacillus casei and *Lactococcus lactis* co-culture	Immobilized cells system (Ca-alginate), fed-batch cultivation	46.0	1.9	Roukas and Kotzekidou, 1998
Lactobacillus helveticus R211	Batch culture, pH control	66.0	1.4	Schepers et al., 2002
Lactobacillus casei NRRL B-441	Batch culture	46.0	4.0	Buyukkilci and Harsa, 2004
Lactococcus lactis spp. *lactis* ATCC 11454	Batch culture. Nisin-coproduction	19.3	*n.a.*	Liu et al., 2004
Lactobacillus casei	Immobilized cell system (pectate), Batch culture	33.0	0.7	Panesar et al., 2007
Kluyveromyces marxianus IFO 288, *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 and *Lactobacillus helveticus* ATCC 15009 co-culture	Batch culture	19.8	0.5	Plessas et al., 2008
Entercoccus flavescens	Batch culture, pH control	12.6	0.4	Agarwal et al., 2008
Lactobacillus bulgaricus	Batch culture	20.8	0.3	Ghasemi et al., 2009
Lactococcus lactis TISTR 1401 and *Lactobacillus casei* TISTR 1341 co-culture	Immobilize cell system (Na-alginate), repeated batch cultivation	29.9	0.7	Choojun and Suttisuwan, 2010
Lactobacillus sp. LMI8	Batch culture	52.4	1.7	de Lima et al., 2010
Lactobacillus plantarum	Batch culture	120.0	2.4	Brinques et al., 2010
Lactobacillus casei	Batch culture, no-pH control	33.7	0.9	Panesar et al., 2010
Lactobacillus delbrueckii NCIM2025 and *Lactobacillus pentosus* NCIM 2912 co-culture	Immobilized cell system (pine needles),	44.9	*n.a.*	Ghosh and Ghosh, 2011

Notes: [1] concentration [g L^{-1}] of lactic acid produced by the process; [2]productivity of the process.

However, it is still economically unfavorable because the refined carbohydrates are so expensive that they eventually result in higher production costs (Wee et al., 2006). Among the cheap raw materials, cheese whey is probably the most widely used for lactic acid production. Therefore, a lot of recent works deals with bioconversion of whey into the lactic acid.

Different lactobacilli cultures (*L. helveticus*, *L. delbrueckii*subsp. *bulgaricus*, *L. acidophilus*, *L. casei*, *etc.*) have been used for the utilization of whey for lactic acid production. Generally, *L. heleveticus* is the generally preferred microorganism, as it produces almost twice the amount of lactic acid from milk, compared to other common lactic acid bacteria. In addition, *L. heleveticus* is homolactic fermentative and produces a racemic mixture (DL) as compared to only dextrorotatory lactic acid (D) produced by *L. delbrueckii*. Moreover, it also provides an alternative solution to the phage contamination in dairy industries, which is generally encountered during *L. delbrueckii* subsp. *bulgaricus* fermentation. Employment of *S. thermophilus* has some drawbacks, because only a few strains of this bacterium are capable of fermentation of galactose, and it requires some growth factors for lactic acid production in milk-based medium (Roy et al., 1986). In recent studies other organisms such as *L. delbrueckii* and *Bifidobacterium longum* have also shown considerable promise (Satyanarayana and Venkateshwar, 2004; Li et al. 2006).

The table 3 sums up recent studies on whey transformation into lactic acid employing various bacteria strains as well as fermentation strategies. Generally, it can be stated that higher lactic acid concentrations may be obtained in batch and fed-batch cultures than in continuous cultures, whereas higher productivity may be achieved by the use of continuous cultures.

6. PRODUCTION F SELECTED BIO-MATERIALS

Polysaccharide Based Materials

Xanthan Gum

Xanthan gum is a natural polysaccharide and an important industrial biopolymer. It was discovered in 1963 at Northern Regional Research Center (now called The National Center for Agricultural Utilization Research) of the United States Department of Agriculture (USDA). Xanthan gum, produced by the bacterium *Xanthomonas campestris* was extensively studied because of its properties that would allow it to supplement other known natural and synthetic water-soluble gums. Substantial commercial production began in early 1964 (Palaniraj and Jayaraman, 2011).

The primary structure of xanthan gum shown in Figure 3 is a linear (1→4) linked β-D-glucose backbone (as in cellulose) with a trisaccharide side chain on every other glucose at C-3, containing a glucuronic acid residue linked (1→4) to a terminal mannose unit and (1→2) to a second mannose that connects to the backbone. Approximately 50% of the terminal mannose residues are pyruvated and the non-terminal residue usually carries an acetyl group at C-6 (Becker et al., 1998; Palaniraj and Jayaraman, 2011).

Besides many applications in food manufacturing and various industry fields, xanthan is used also in medicine, pharmacy and cosmetics. It serves as a stabilizer of a variety of insoluble drugs and materials such as barium sulfate (X-ray diagnoses), complexed

dextromethorphan (for cough preparations) or thiabendazol. Further, xanthan can be also employed for preparation of hydrogels for sustained release of encapsulated drugs (Coviello et al., 2007).

As was mentioned above, xanthan is produced extracellularly by *Xantomonas campesteris*. Because this bacterium possesses very low level of β-galactosidase activity, there have been a lot of efforts to construct strains that are able to utilize lactose directly and produce xanthan using inexpensive medium. Schwartz and Bodie (1985) isolated lactose-utilizing strain after serial sub-culturing the wild-type strain in lactose medium, although the strain was unstable.Thorne et al. (1988),Fu and Tseng (1990) and Papoutsopoulou et al. (1994) exploited insertion of *Escherichia coli*β-galactosidase gene into the *Xantomonas campesteris* and studied its xanthan production abilities in whey-based medium. The xanthan yields were comparable with those obtained in glucose medium.

Slightly different strategy was used by Ekateriniadou et al. (1994) who transferred phospho-β-galactosidase, maturation protein and proteinase P genes from *Lactobacillus lactis* into *X. campesteris*. The transgenic strain was stable and capable of xanthan production on whey. This constructed strain was used for production of xanthan gum in batch reactor using whey medium. Very high xanthan yields (17.3 g L^{-1}) were observed when pH was maintained at 8.0 (Liakopoulou-Kyriakides et al., 1997).

To overcome application of genetically modified strain Yang et al. (2002) focused on induced mutagenesis using nitrous acid as a mutagen and consequently selected mutant strains based on their ability to utilize lactose. Mutants encoded as *Xantomonas campestris* Xc17L possessed 3.5-fold higher β-galactosidase activity than wild strain and, in addition, the ability of β-galactosidase expression was stable for at least 100 generations. The amounts of xanthan produced in whey medium were comparable with those obtained in glucose-based medium (about 2.0 g L^{-1}).

Finally, Mesomo et al. (2009) as well as Silva et al. (2009) employed *Xanthomonas campestri s*p. *magiferaeidicae* for xanthan production on whey. This strain featured sufficient β-galactosidase activity to utilize lactose as the only carbon substrate. The whey-based medium composition and cultivation conditions were optimized in terms of maximal production by addition of 1 g L^{-1} magnesium sulphate, 20 g L^{-1} potassium phosphate, 28° C temperature; initial pH was set at 7.2 and agitation at 390 rpm with 1.5 vvm aeration. These condition yielded 36 g L^{-1} of xanthan at the 72nd hour of cultivation (Mesomo et al., 2009). Silva et al. (2009) tested supplementation of whey medium with sucrose, however, it resulted in lowered xanthan yields as compared to cheese whey as a sole carbon source. The strain produced the highest yields of xanthan (25 g L^{-1}) on whey with 0.1% $MgSO_4$ $7H_2O$ and 2.0% K_2HPO_4.

Dextran

Dextrans can be defined as glucose homopolysaccharides that feature a substantial number of consecutive α-(1→6) linkages in their major chains, usually more than 50% of the total linkages. These α-D-glucans possess also side chains stemming from α-(1→2), α-(1→3), or α-(1→4) branch linkages (Coviello et al., 2007).

Dextrans, mostly in partially hydrolyzed form, find a great number of applications in the fields of medicine and pharmacy. This biopolymer has significant commercial values in blood plasma substitute formulations. Anemia associated with chronic kidney disease is being

treated with parenteral iron-dextran complex formulations such as DexFerrum® (Sawale and Lele, 2010). Furthermore, it is also very promising material for drug delivery systems. Dextran and its derivatives are among the main promising candidates for the preparation of networks capable of giving a sustained release of proteins (Coviello et al., 2007).

Dextran for human applications is usually produced by *Leuconostoc mesenteroides*. The bacterium growing in sucrose rich media releases enzyme, dextransucrase, which converts excess of sucrose into dextran and fructose. Schwartz and Bodie (1984) developed fermentation system with sucrose-supplemented whey medium for dextran production employing *Leuconostoc mesenteroides* ATCC 14935. The optimal whey-based medium consisted of4% Teklac, 10% sucrose, 0.1% K_2HP0_4, and 0.05% yeast extract. Also Santos et al. (2005) reported dextran production by *Leuconostoc mesenteroides* in cheap medium consisting of carob extract and cheese whey. The process yielded 7.23 g $L^{-1}$of dextran with mean molecular weight of 326 kDa (Santos et al., 2005).

Pullulan

Pullulan is a linear homopolysaccharide of glucose that is often described as α-(1→6) linked maltotriose (Figure 4), synthesized primarily by strains of the yeast-like microorganism *Aureobasidium pullulans*. The unique linkage pattern of pullulan endows the polymer with distinctive physical traits, including adhesive properties and the capacity to form fibers, compression moldings, and strong, oxygen-impermeable films. Unlike bacterial dextrans, which are synthesized extracellularly by secreted enzymes, pullulan is synthesized intracellularly and secreted by *A. pullulans* into fermentation broth. Culture conditions and strain selection are important in obtaining high molecular weight pullulan that is relatively free of melanin (black stain co-produced in *A. pullulans*) (Leathers, 2003).

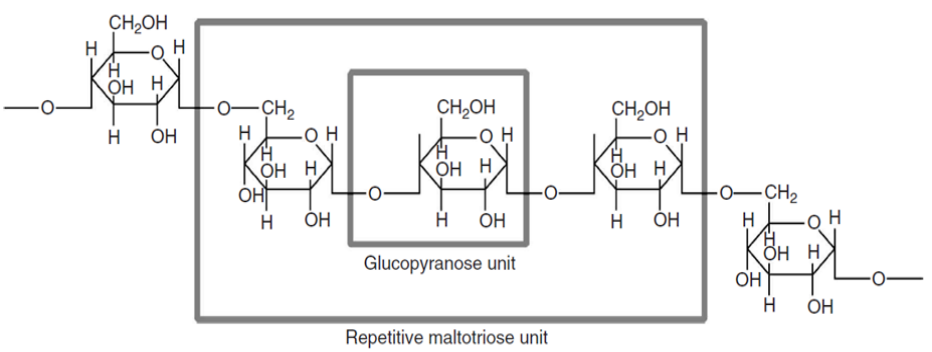


Figure 4. Chemical structure of pullulan. Lin et al. (1999). Reproduced with permission.

Pullulan is now extensively studied forvarious applications in biomedical field. This is mainly due to its non-toxic, non-immunogenic and biodegradable properties. In comparison with a similar but more popular polysaccharide, dextran, the degradation rate of pullulan in serum is faster than that of dextran (Rekha and Sharma, 2007). Due to its adhesive properties, pullulan and its derivatives can be used in wound-healing compositions. Further, pullulan can be used in pharmaceutical coatings, including sustained-release formulations. For instance, Akiyoshi et al. (1998) developed insulin delivery system of the size in range of 20-30 nm by complexing the hydrogel nanoparticle of cholesterol bearing pullulan. These nanoparticles

were stableand protected insulin from the enzymatic degradation and, moreover, they suppressed insulin aggregation. It was proved *in vivo* that thebiological activity of the entrapped insulin remained intact. Furthermore, several oral care products (such as Listerine®) based on pullulan films have recently been commercialized (Leathers, 2003; Coviello et al., 2007).

In order to reduce cost of fermentation medium, Hafez et al. (2007) studied bioconversion of various industrial by-products into pullulan. When whole sweet whey containing 5% of lactose was used, *A. pullulan* produced 5.5 g L^{-1} of pullulan. The yields were more than doubled by using hydrolyzed sweet whey supplemented with 0.05% glutamic acid and 0.298 KH_2PO_4 as a fermentation medium. After that, pullulan yields reached 12.4 g L^{-1}.

Roukas (1999) studied bio-production of pullulan from deproteinized whey. Different fermentation strategies were used, however, neither adaptation technique nor mixed culture system of *A. pullulans* and/or *Lactobacillus brevis* X20, *Debaryomyces hansenii* 194 and *Aspergillus niger* increased production of desired polysaccharide. The maximal yields were achieved when lactose was enzymatically hydrolyzed and medium was supplemented with 0.5% KH_2PO_4, 1% L-glutamic acid, 2.5% olive oil and0.5% Tween 80. In this medium, pullulan yield was 11.0 g L^{-1} and sugar utilization reached 93.2%.

Alginate

Alginates form an important family of biopolymers of both technological and scientific interest. These polymers are linear polysaccharides, which are composed of variable amounts of (1–4)-β-D-mannuronic acid and itsepimer, α-L-guluronic acid. Alginates present a wide of applications, acting for example as stabilizing, thickening, gel or film-forming agents in various industrial fields. Currently, commercial alginates are extracted from marine brown algae and are used for a wide variety of applications, mainly in the food and pharmaceutical industries. Alginates extracted from algae are relatively cheap products, having selling prices in the range of 5–20 \$ kg^{-1} for the majority of the application, however, alginates of very high purity are used in the pharmaceutical field and these are sold for up to 40 000 \$ kg^{-1}. The algal alginates have several problems concerning their production which may limit their use in many interesting contexts, especially in the pharmacy where polymers with a very well defined composition are required (Rehm and Valla, 1997; Galindo et al., 2007).

Aside from algae, alginates are also produced by bacteria and many of their physicochemical characteristics are similar to those ofalgae, so that they can be used for the same applications as algal alginates, as well as in other more sophisticated contexts. Alginates produced by microorganisms differ from those of algae because bacterial polymers are acetylated. In addition, bacterial alginates usually have a higher molecular mass than the algal polymers (ranging from 48 to 186 kDa). A molecular mass as high as 4 000 kDa for the polymer synthesized by a mutant strain of *A. vinelandii* has been reported. Both acetylation and molecular mass directly affect the viscosity and other rheological properties of alginate solutions and, therefore, this would determine its utility in specific applications of alginate in the food and pharmaceutical fields (Galindo et al., 2007).

Khanafari and Sepahei (2007) reported production of alginate employing *Azotobacter chroooccum*. The genus *Azotobacter* seems to be the best candidate for the fermentative production of alginate molecules characterized by a chemical composition, molecular mass and molecular mass distribution suited to well-defined applications, especially required in the

biotechnological, biomedical and pharmaceutical fields. Of the three strains tested for whey degradation ability, only *Azotobacter chroococcum* 1723 produced significant apparent growth on the whey broth. After optimizing environmental factors such as pH, salt concentration and temperature, this strain was able to produce exopolysaccharidegreater than 5 gL^{-1}. Optimum results were obtained when using whey broth as a fermentation medium without extra salt, temperature at 35 °C and pH 7. Increasing inorganic and organic nitrogen sources (yeast extract and NH_4NO_3) reduced whey utilization and alginate production at least 30%.

Chitosan

Chitosan, a linear hydrophilic polysaccharide of β–(1→4) glucosamine (Figure 5), is obtained by thermo-chemical deacetylation of chitin, which is found in the exoskeleton of crab, shrimp, lobster, crawfish and insects. Chitosan can also be isolated from the cell wall of certain groups of fungi, particularly zygomycetes. Chitosan isolated from fungi is of more uniform physicochemical properties than obtained by deacetylation of crustacean chitin. Recent research is therefore focused on the production of chitosan by fermentation of fungus. Another advantage of fungal chitosan is that the production as well as physico-chemical properties *e.g.* molecular weight of this chitosan can be manipulated by changing the parameters of the fermentation (Chatterjee et al., 2005; Chaterjee et al., 2008).

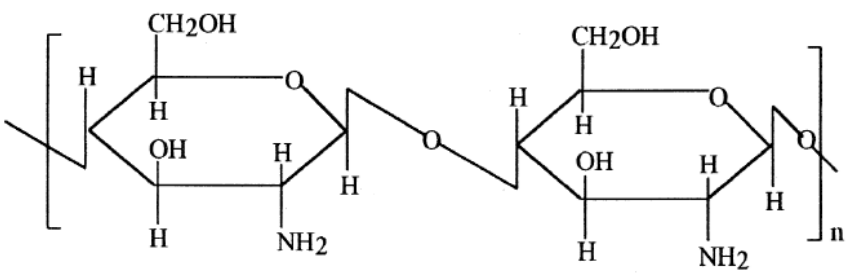


Figure 5. Structure of chitosan, Kumar (2000). Reproduced with permission.

A wide variety of applications for chitosan and its derivatives have been reported over the last three decades. For instance, it has been suggested that chitosan may be used to inhibit fibroplasia in wound healing and to promote tissue growth and differentiation in tissue culture. Fibers made of chitosan are useful as absorbable sutures and wound-dressing materials. Chitosan may be also applied as an artificial skin for persons who suffered extensive losses of skin (Kumar, 2000).

Chatterjee et al. (2007) induced chitosan production by introduction of plant growth hormones such as gibberellic acid, indole-3-acetic acid, indole-3-butyric acid into cultivations of *Rhizopus oryzae* in deproteinized whey. Hormones, at different concentrations, increased the mycelial growth by 19–32%. Howeve, the increase of chitosan content of the mycelia was relatively small (1.7–14.3%) over the control. Maximum enhancement was observed with gibberellic acid that at concentration of 0.1 mg L^{-1} enhanced chitosan production about fifty percent. Nevertheless, hormones, at higher doses, instead of stimulation inhibited both growth and mycelial chitosan content.

Hyaluronic Acid

Hyaluronic acid is an unbranched unsulfated glycosaminoglycane composed of repeating disaccharide units of D-glucuronicacid and N-acetylglucosamine linked α-(1→4)and β-(1→3), respectively. While hyaluronicacid is produced from streptococci for industrial purposes, it is also present in human connective tissues, where it plays an important role in many biological mechanisms. Hyaluronic acid is mainly found in the cartilage and in thevitreous, where it primarily lubricates body tissue and blocks the spread of invading microorganisms (Coviello et al., 2007).

Hyaluronic acid is used as a viscoelastic material in ophthalmologic surgery and as an injectable solution for the treatment of joint diseases in orthopedics. The remarkable viscoelastic properties of hyaluronic acid and its complete lack of immunogenicity make it an attractive biomaterial. Hyaluronic acid also possesses several pharmacological properties, as it inhibits platelet adhesion and aggregation, and stimulates angiogenesis, making it suitable for vascular applications. Its efficiency as a drug carrier matrix has been demonstrated through the vestibular delivery of gentamicin, ocular delivery of pilocarpine, intranasal insulin release, and vaginal delivery of calcitonin (Larsen and Balasz, 1991;Coviello et al., 2007).,

The bacteria employed for biosynthesis of hyaluronic acid are nutritionally fastidious microorganisms which require complexnutrients due to their limited ability to synthesize specific amino acids and B-group vitamins. Additionally, there is the nutritionalrequirement with respect to organic nitrogen, which also suppliesa large portion of carbon for their cellular biosynthesis.In general, the culture media used for the microbial productionof hyaluronic acid contain glucose as a carbon source and appreciable amounts of complex nitrogen sources andgrowth factors like yeast extract and bacterial peptones (Gao et al., 2006; Pires et al., 2010). In order to find cheaper alternative to these expensive complex substrates, Pires et al. (2010) tested hydrolysate soy protein concentrate, whey protein concentrate and cashew apple juice with focus on the production of hyaluronicacid by *Streptococcus zooepidemicus.* Application of whey protein concentrate strongly increased biomass growth; however, the yields of hyaluronic acids were lower than in control culture containing yeast extract.

Gellan

Gellan gum is a bacterial exopolysaccharide commercially prepared by aerobic submerged fermentation of *Sphingomonas*sp. From chemical point of view, gellan gum is a linear tetrasaccharide built up by →4)-L-rhamnopyranosyl-(α-1→3)-D-glucopyranosyl-(β-1→4)-Dglucuronopyranosyl-(β-1→4)-D-glucopyranosyl-(β-1→ withO(2) L-glyceryl and O(6) acetyl substituents on the 3-linkedglucose (Figure 6). It consists of about 50000 residues and it is normally de-esterified by alkali treatment before use. Gellangum forms a 3-fold double helix from two left-handed chains with the acetate residues on the periphery, and glyceryl groups and hydrogen-bonds stabilizing the inter-chain associations (Coviello et al., 2007).

Besides wide range of applications in food industry such as manufacturing of dairy products, fabricated foods, jams or pet foods, gellan attracts attention of pharmaceutical industry as well. It seems to be very promising material for formulation of controlled release systems controlling bioavailability of administrated drugs for instance in ophthalmology (Bajaj et al., 2007).

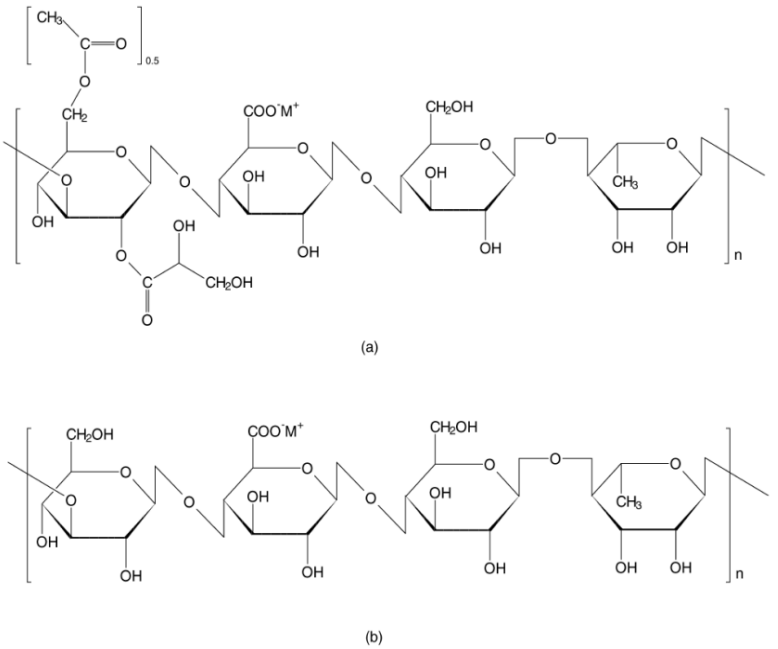


Figure 6. The chemical structure of gellan gum: (a) native form (high acyl); (b) deacylated form (low acyl) (Mao et al., 2000). Reproduced with permission.

In order to produce gellan from lactose base substrates including sweet cheese whey, Dlamini and Peirs (1996) employed improved strain of *Sphingomonas elodea* ATCC 31461 capable of producing high broth viscosities when grown in enriched whey permeate and enriched sweet whey media. Maximum biopolymer productions were observed in 25% whey solution (v/v). Study on enzyme activities suggested that transport of lactose into the cells is by permease system as opposed to phosphotransferase system. Fialho et al. (1999) aimed at gellan production from whey and lactose employing other bacterial strain, *Sphigomonas paucimobilis* ATCC 31461.They found that altering the growth medium can markedly affect the polysaccharide yield, acyl substitution level, polymer rheological properties, and susceptibility to degradation. Depression of gellan production from lactose compared with gellan production from glucose (approximately 30%) did not appear to occur at the level of synthesis of sugar nucleotides, which are the donors of monomers used for biosynthesis of the repetitive tetrasaccharide unit of gellan. The lactose-derived biopolymer had the highest total acyl content, the glucose- and whey-derived gellans had similar totalacyl contents but differed markedly in their acetate and glycerate levels. The process of gellan production resulted in production of 8 g L^{-1} of gellan at the 100^{th} hour of cultivation using 25% (v/v) cheese whey as a medium.

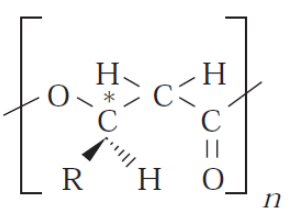
Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are a family of linear polyesters of usually 3- but also 4, 5 and 6-hydroxyacids, synthesized by a wide variety of variety of fermentation of sugars, lipids, alkanes and alkanoic acids. Bacteria accumulate PHA as a source of carbon, energy and

reducing power intracellularly in form of cytoplasmic inclusion termed PHA granules. Once extracted from the cells, PHA exhibit thermoplastic and elastomeric properties similar to polypropylene or polyethylene *etc*. Nevertheless, unlike the plastics of petrochemical origin, PHA can be easily biodegraded to carbon dioxide and water. In addition, these polymersare biocompatible and hence have a number of medical applications. Furthermore, all of the monomeric units of PHAs are enantiomerically pure and in the R-configuration. R-hydroxyalkanoicacids produced by the hydrolysis of PHA can also be used as chiral starting materialsin fine chemical, pharmaceutical and medical industries (Sudech et al., 2000; Philip et al., 2007).

Mechanical and physical properties of PHA strongly depend on the monomer composition. From chemical point of view, PHA are polyesters of hydroxyalkanoic acids. PHA can be classified according to number of carbon atoms in monomer unit: short chain lengths (scl) PHA consist of 3-5 carbon atoms and medium-chain length (mcl) PHA contain 6-14 carbon atoms per monomer unit. More than 100 monomers have been found in the naturally synthesized polyester polymers (Steinbuchel and Valentin, 1995; Sudech et al., 2000).

Hence, a range of designer PHA with desirable properties can be obtained. The polymer can be hard and crystalline or elastic and rubbery. The most widespread PHA is homopolymer of 3-hydroxybuytric acid, poly(3-hydroxybutyrate) (PHB). This material is highly crystalline, brittle and stiff with a melting temperature of 180° C. However, the introduction of different hydroxyacids monomers such as 3-hydroxyvalerate (3HV) or 3-hydroxyhexanoate (3HHx) into the PHB chain greatly improves the material properties. PHB is optically pure and possesses piezoelectricity which helps in osteoinduction, the process of inducing osteogenesis. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV), has a lower melting temperature and lower crystallinity than PHB. The mole percentage of 3HV in the polymer is important in determining the properties of the copolymer. P(3HB-*co*-3HV) containing more than 20 mol% of 3HV units can be used to make films and fibers with different elasticity by controlling the processing conditions (Flickinger and Drew, 1999; Sudech et al., 2000; Philip et al. 2007).



$R = CH_3\text{-}C_2H_5$ Short-chain-length PHAs
$R = C_3H_7\text{-}C_{14}H_{29}$ Medium-chain-length PHAs

Figure 7. General structure of PHA . Flickinger and Drew (1999). Reproduced with permission.

Besides the fact that PHA are considered as an alternative to traditional petrochemical plastics, they have also many medical applications. PHA are frequently used in tissue engineering as bone plates, osteosynthetic materials and surgical sutures. They are useful in the slow release of drugs and hormones. PHA fibers are especially sought after to make swabs

and dressing materials for surgery. PHA are produced by fermentations and, hence, do not contain the left-over undesirable metal catalysts used in chemical synthesis. The greatest contribution of PHA to medicine has been in the cardiovascular area. Tepha, Inc. specializes in manufacturing pericardial patches, artery augments, cardiological stents, vascular grafts, heart valves, implants and tablets, sutures, dressings, dusting powders, pro-drugs and micro particulate carriers using PHA (Williams and Martin, 2002; Philip et al., 2007).

Analysis and economic evaluation of the bacterial PHA productions suggested, that the cost of substrate (mainly carbon source) contributed the most significantly (up to 50%) to the overall production cost. Thus, cheap waste substrates attract attention of both scientific researches and industrial companies in order to reduce PHA production cost. Many agriculture and food industry by-products and wastes such as molasses (Page, 1992), waste frying plant oils (Obruca et al., 2010; Budde et al., 2011) or waste glycerol stemming from biodiesel production (Ashby et al., 2005) have been studied for PHA production. Among them, whey seems to be probably the most promising cheap substrate facilitating cheap PHA production. Thereby, there is high number of studies dealing with PHA production from whey.

PHA Production by Naturally Producing Bacteria

PHA production from cheese whey by naturally PHA producing and lactose utilizing strain was studied by Yellore and Desai (1998) who studied PHB production employing *Methylobacterium* sp. ZP24, isolated from a local pond. The isolate was able to utilize deproteinized whey and produce 1.1 g L^{-1} of polymer. Introduction of $(NH_4)_2SO_4$ enhanced the PHB yields 2.5-fold, so the bacterium produced 2.6 g L^{-1} of PHB which represented 44% of cell dry mass. Further, the production process was scaled-up by Nath et al. (2008). Since *Methylobacterium*sp. ZP24 showed growth associated PHB production, an intermittent feeding strategy having lactose and ammonium sulfate at varying concentration was used towards reaching higher yield of the polymer. About 1.5-fold increase in PHB production was obtained by this feeding strategy. Further increase in PHB production by 0.8-fold could be achieved by limiting the dissolved oxygen levels in the fermentor. The decreased oxygen level is thought to increase flux of acetyl-CoA towards PHB accumulation over TCA cycle. Maximal yields reached 3.91 g L^{-1} when *Methylobacterium* sp. ZP24 was able to convert 1 g lactose into 0.32 g of PHB.

Another bacterium which is capable of direct transformation of lactose into PHB is *Bacillus megaterium*. Obruca et al. (2011) employed *B. megaterium* CCM 2037. Optimization of medium composition in terms of whey dilution, Mg^{2+}, $(NH_4)_2SO_4$ and PO_4^{2-} supplementation improved PHB yields about 50-fold (biomass and PHB yields 2.82 and 1.05 g L^{-1}, respectively) as compared to whey alone. Furthermore, PHB yields were improved by about 40% by introducing 1% ethanol into the medium at the beginning of the stationary phase of growth (biomass 2.87 g L^{-1}, PHB 1.48 g L^{-1}). Pandian et al. (2010) converted whey into PHB using *Bacillus megaterium* isolated from brackish water. Medium was supplemented with rice bran and see water and also fed-batch fermentation strategy was developed so the maximum production of PHB (11.32 g L^{-1}) occurred at the 36th hour of cultivation.

Azotobacter vinelandii was used for PHB production on whey using bacterial peptone as nitrogen source. It was observed that by increasing the inoculum size the time of incubation can be reduced considerably (Dhanasekar et al., 2001). Further, *Pseudomonas hydrogenovora*

produced PHA on enzymatically cleaved lactose in whey permeate. The application of hydrolyzed whey permeate turned out to be advantageous as compared to the utilization of pure sugars. Sodium valerate was introduced into fermentation media to incorporate 3HV units into PHA structure and form P(3HB-*co*-3HV) copolymer. The process yielded 1.44 g L^{-1} of copolymer containing 21% of 3HV (Koller et al., 2008). Pantazaki et al. (2009) focused on mcl-PHA production from cheese whey employing *Thermus thermophilus* HB8 under nitrogen limitation. PHA was accumulated up to 35% of its biomass after 24 h of cultivation. Heteropolymer consisted of the short chain length 3-hydroxyvalerate and the medium chain length 3-hydroxyheptanoate, 3-hydroxynanoate and 3-hydroxyundecanoate. Futher, Povolo et al. (2013) has reported that *Hydrogenophaga psedoflava* is capable of accumulation of terpolymer consisting of 3-hydroxybutyrate, 3-hydroxyvalerate and 4-hydroxybutyrate from structurally unrelated carbon sources such as lactose and cheese whey. Terpolymer reveals superior mechanical properties as compared with PHB or P(3HB-*co*-3HV). On the other hand, PHA content was rather low (in range of 3 – 10 % of cell dry weight).

Very interesting fermentation strategy of PHA production from cheese whey was recently suggested by Koller et al. (2011). The authors of the study developed viable strategy to cope with potential contamination during PHA production from cheese whey employing *Hydrogenophaga psedoflava*. Using whey as substrate the risk of microbial contaminations rises as whey itself often (over 75% of the whey samples) contains infectants; e.g. the spore forming, gram positive bacterium *Bacillus cereus*. During PHA production, growth of *B. cereus* occurs at the expense of the production strain, resulting in the loss of the fermentation batch. Thermal processing, which is frequently used to prevent microbial contamination is expensive, time- and energy-consuming and does not necessarily have the desired effect on thermal resistant spore forming bacteria.The authors observed that application of vancomycin inhibited growth of contaminat *B. cereus* but not PHA producing strain. Moreover, the specific PHA production rates of *H. pseudoflava* decreased with increasing antibiotics in a minimal medium but in media supplemented with yeast extract the specific PHB production rates increased as the antibiotics concentrations increased.

Furthermore, cheese whey might also serve as a very promising nitrogen source for PHA producing bacteria. Protease hydrolyzed whole cheese whey applied as a complex nitrogen source improved PHA production by *Cupriavidus necator* on waste frying oils about 40 %, product yield coefficient reached 0.94 g PHA per g oil and final PHA content in cells exceeded 90 % of cell dry weight. Positive effects of whole whey hydrolyzate on PHA production were atrributed to amino acids composition. Whole whey hydrolyzate was rich in proline, leucine, isoleucine and methionine, which were obsrved to promote PHA accumulation in bacterial cells. Therefore, hydrolyzate of cheese whey seems to be superior to more expensive complex nitrogen sources such as soya peptone, yeast extract or casein hydrolyzate.

PHA Production by Genetically Engineered Bacteria

Apart from those microbes mentioned in the previous text, there is a great number of bacterial strain which are either not able to utilize lactose or they do not accumulate PHA. Therefore, a lot of efforts were targeted on genetic modification of particular bacterial strain in order to reach highly efficient production of PHA from cheese whey.

Cupriavidus necator is a well-known PHA producer, but unable to grow on lactose. Thus, Povolo at al. (2010) choose one of intracellular PHB depolymerases, enzyme degrading

intracellular stocks of PHA, to insert *lac*genes of *E. coli*. This would have the effect to allow polymer production on lactose and, in the same time, to remove part of the PHA intracellular degradation system. Disruption of PHA depolymerase was achieved by gene replacement after isolating a fragment of this gene and interrupting it with a cartridge containing the *lac* genes and a synthetic promoter. Growth and polymer production studies of the genetically modified strain mRePTin whey permeate and hydrolyzed whey permeate as carbon sources, were performed. Lower PHA degradation and higher yields were obtained as compared to the wild-type strain.

Finally, many researchers reported very efficient PHA production using *Escherichia coli* harboring PHA biosynthetic genes of various natural PHA producers. Fermentation strategies for the production of PHB from whey by recombinant *Escherichia coli* strain CGSC 4401 harboring the *Alcaligenes latus* PHA biosynthesis genes were developed by An et al. (2000). The pH-stat fed-batch cultures were carried out with a concentrated whey solution containing 280 g of lactose equivalent per liter. Cell and PHB concentrations of 119.5 and 96.2 g L^{-1}, respectively, were obtained at the 37.5th hour of cultivation. In the second study, An et al. (2001) even improved the fermentation strategy. The same *E. coli* strain was employed for PHB production using cell recycle membrane system. The final cell concentration, PHB concentration and PHB content of 194 g L^{-1}, 168 g L^{-1} and 87%, respectively, were obtained at the 36.5th h by the pH-stat cell recycle fed-batch culture using whey solution concentrated to contain 280 g lactose L^{-1} as a feeding solution.

Different recombinant *E. coli* strain was used for PHB accumulation in whey-based broth. The timing of PHB biosynthesis in recombinant *E. coli* was controlled using the agitation speed of a stirred tank fermentor. PHB content of 80% could be obtained with oxygen limitation by decreasing the agitation speed to only 500 rpm. Final yields reached were 31 g L^{-1} of biomass containing 80% of PHB (Kim, 2000). Nikel et al. (2005) aimed at statistical optimization of culture medium using powdered cheese whey by recombinant *E. coli* strain harboring biosynthetic geneses from a natural isolate of *Azotobacter* sp. The study demonstrated that the optimal concentrations of the three components, defined as those yielding maximal biomass and PHB production in shaken flasks, were 37.96 g L^{-1} of deproteinated milk whey powder, 29.39 g L^{-}1of corn steep liquor and 23.76 g L^{-1} phosphates. The cultivation in the optimized medium yielded 9.41 g L^{-1} biomass and 6.12 g L^{-1} of PHB in the culture broth.

In another study Nikel at al. (2006) employed the same recombinant strain for PHB production fed-batch mode using concentrated and deproteinated whey solution containing 25% lactose as a feeding solution.

The table 4 provides summary of PHA production processes from cheese whey reported in literature. Despite the legislative restrictions stemming from the application of genetically modified strains, it seems that recombinant *E. coli* is the most promising candidate for industrial production of PHA using whey. Employing these recombinant strains, it is possible to achieve very high cell densities in relatively short time. Because PHA production is, in these cases, growth associated PHA contents in cells reach up to 87% of cell dry weight that is furthermost of all the bacterial strains tested so far. This is very important factor influencing the economics of PHA production, because PHA content of the produced biomass strongly affects the efficiency and the price of the down-stream process. For example, Lee and Choi (1999) reported that a relatively low PHB content in cells – 50% results in a high recovery cost of 4.8 \$ kg^{-1} PHB. On the other hand, the recovery cost for a process with 88%

PHB cell content was only 0.92 \$ kg^{-1} PHB. A lower PHB content clearly resulted in a high recovery cost. This is mainly due to requirement of large amounts of digesting agents for breaking the cell walls and to the increased cost of waste disposal.

Table 4. Summary of PHB productions on cheese whey published in literature

Producent	Substrate	Fermentation mode	Biomass [g L^{-1}]	PHA [%]	PHA [g L^{-1}]	$Y_{p/s}$[1] [g g^{-1}]	Reference
Methylobacterium sp. ZP24	Deproteinized whey $(NH_4)_2SO_4$	Erlenmayer flask, Batch	5,9	44	2,6	*n.a.*[2]	Yellore and Desai (1998)
Methylobacterium sp. ZP24	Deproteinized whey $(NH_4)_2SO_4$	Fermentor, Fed-batch	*n.a.*	*n.a.*	3,9	0,32	Nath et al., 2008
Hydrogenophaga psedoflava	Cheese whey	Erlenmeyer flask	1,46	10.1	0.15	*n.a.*	Povolo et al. (2013)
Bacillus megaterium CCM 2037	Deproteinized whey $(NH_4)_2SO_4$, phosphate	Erlenmayer flask, Batch	2,87	52	1,5	0,08	Obruca et al. (2011)
Bacillus megaterium SRKP-3	Whey Rice Bran, Sea Water	Fermentor, Fed-batch	*n.a.*	*n.a.*	11,3	*n.a.*	Pandian et al. (2010)
Azotobacter vinelandii MTCC 124	Whey Bacterial peptone	Erlenmayer flask, Batch	*n.a.*	*n.a.*	1,4	*n.a.*	Dhanasekar et al.(2001)
Pseudomonas hydrogenovora	Hydrolyzed whey permeate	Fermentor, Fed-batch	*n.a.*	12	1,4	*n.a.*	Koller et al. (2008)
Thermus thermophilus HB8	Deproteinized whey	Erlenmayer flask, Batch	1,6	35	0,5	*n.a.*	Pantazaki et al. (2009)
Cupriavidus necator (*E. coli* lac genes)	Whey	Erlenmayer flask, Batch	8,8	23	2,0	*n.a.*	Povolo et al. (2010)
Escherichia coli (*A. latus* PHA biosynthetic genes)	Concetrated whey	Fermentor, Fed-batch	119,5	81	96,2	*n.a.*	Ahn et al. (2000)
Escherichia coli (*A. latus* PHA biosynthetic genes)	Concetrated whey	Fermentor, Fed-batch, Cell recycle system	194	87	168,0	*n.a.*	Ahn et al. (2001)
Escherichia coli (*C. necator*PHA biosynthetic genes)	Whey	Fermentor, Fed-batch, Oxygen limitation	31	80	25,0	*n.a.*	Kim (2000)
Escherichia coli (*Azotobacter* sp. biosynthetic genes)	Deproteinized whey powder Corn steep liquor	Erlenmayer flask, Batch	9,4	65	6,1	*n.a.*	Nikel et al. (2005)
Escherichia coli (*Azotobacter* sp. biosynthetic genes)	Deproteinized whey concetrate	Fermentor, Fed-batch	70	73	51,1	*n.a.*	Nikel et al. (2006)

Notes: [1] Yield in terms of g of PHA per g of substrate; [2] *n.a.* stands for: data not available.

7. ECONOMIC CONSIDERATION

In general, fermentative production of any substance or material can hardly compete with its chemical synthesis (if possible) in terms of productivity and economic parameters. A fermentation process requires relatively high capital and operating cost for the process of production as well as for the process of product separation and purification. Therefore, fermentative productions can be economically reasonable in those commodities which cannot be efficiently synthetized *via* chemical synthesis or the chemical synthesis yields product with undesired properties. Actually, this chapter focuses on this type of products.

The table 5 shows the market prize of some of the substances and materials include in this chapter. It is important to note that these prices are more or less orientational and the real price of high purity and highly defined commodity can be simply several times higher depending on its potential application.

Because the cost of fermentation medium is one of the most important operating costs for biotechnological production (representing up to 40 % of the process cost), there is always an effort to reduce it as much as possible. Among cheap substrates, whey is one of the most promising candidates. For instance Lee (1996) suggested cheese whey as the most promising substrate for PHB production (see table 6). Similarly, van Wegen et al. (1998) reported that the process of PHB production employing recombinant *E. coli* harboring PHA biosynthetic genes was profitable if 60 % of glucose substrate had been be substituted by evaporator-concentrated cheese whey. The cost of whey based medium is significantly lower than those of pure or waste substrates. Moreover, if the cheese whey can be used as the sole carbon source as has been described for instance in carotenoids, lactic acid or polyhydroxyalkanaotes, the utilization of whey not only decreases the price of final products, but it may also improve the process in terms of yields and also enhance quality of the final product.

Table 5. Market prices of some of the substances mentioned in this chapter

Substance	Price [$/kg]
Vitamin B12	10 - 30
β-carotene	10 - 200
Nisin	50 - 600
Xanthan (food grade)	1,8 - 3
Dextran	1 - 200
Pullulan	15 - 30
Hyaluronic acid	5000 - 7500
PHB	3 - 10

Gonzales et al. (2006) evaluated production of lactic acid from the economic point of view. The highest contribution to the total investment cost corresponds to the concentration step, representing 40% of the total cost, whereas the fermentation step requires the highest operating cost (47% of the total operating cost). Thanks to the fact that cheap ultrafiltred whey was used as a substrate, the proposed process was demonstrated to be economically viable. The cost resulted to be 1.25 $ kg^{-1} for 50% (w/w) lactic acid.

Table 6. Costs of substrates for PHB production (Lee, 1996)

Substrate	Substrate cost [$ kg^{-1} of PHB]
Glucose	1.35
Sucrose	0.72
Methanol	0.42
Acetic acid	1.56
Ethanol	1.00
Cane molasses	0.52
Hydrolyzed corn starch	0.58
Hemicelluloze hydrolyzate	0.34
Cheese whey	0.22

Wolf-Hall et al. (2009) focused on the development of a low-cost medium for production of nisin by *Lactococcus lactis* subsp. *lactis* using either condensed corn soluble or cheese whey as inexpensive growth media. The use of cheap substrates reduced the medium costs for nisin production from 600 $ kg^{-1} nisin (based on Laurel–Tryptose broth medium) to 35–40 $ kg^{-1}.This represents a 93% reduction in costs. With further development of pH control methods and carbohydrate supplementation, there is a perspective of the further increase of nisin yields to levels comparable to, or even exceeding those, of commercially used media, thereby further reducing nisin production costs.

As was menationed several times in this chapter, important motivation factor for cheese whey utilization as substrate for biotechnological production is elimination and valorization of the problematic waste. Therefofore, ecological aspect of the proposed processes are closely related with economic ones. Minimizing the environmental impact must be taken into account during the technological development phase to the same extent as to decreasing costs for achieving economic competitiveness. Such “double optimization” in terms of ecological and economic benefit is shaping technology and influencing important engineering decisions. Hence, Koller et al. (2013) performed life cycle assessment of PHA production from cheese whey. As the major outcome of the study, the resulting ecological footprint was comparable with that of competing fossil plastics. Additionally, optimization potentials to further increase the ecological competitiveness of PHA production such as energy requirement of the fermentation or logistical aspect of the process were identified. In addition, the developed PHA production process was compared with production of whey powder as the competing, conventional application of surplus whey. Also in this case, the novel PHA production process was superior according to the calculations.

To sum up, extensive research has been, and is continuing to be, conducted on profitable bio-products that can be obtained from cheese whey. The process of whey conversion into the high valuable products can not only be economically feasible but it would also provide very efficient solution of surplus whey disposal.

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Chapter 4

APPLE POMACE: SOURCE OF VALUE ADDED PRODUCTS

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ABSTRACT

The solid residue of apple juice extraction is the apple pomace. This processing waste represents up to 30% of the original fruit and consists of a complex mixture of peel, core, seed, calyx, stem, and soft tissue. It has high water content and is mainly composed of insoluble carbohydrates such as cellulose, hemicellulose, and lignin. Simple sugars, such as glucose, fructose, and sucrose, as well as small amounts of minerals and vitamins can also be found. This residual material is a poor animal feed supplement because of its extremely low protein content and high amount of sugar, and its production in large amounts has been reported world-wide. The direct disposal of agro-industrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabolites, with a higher commercial value. The application of agro-industrial by-products offers a wide range of alternative substrates, thus helping solve pollution problems related to their disposal. Attempts have been made to use apple pomace to generate several value-added products, such as pectin, polyphenols, edible fibers, enzymes, single cell protein, pigments, aroma compounds,

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alcohols, organic acids, polysaccharides, biohydrogen, antioxidant substances and mushrooms among others. This chapter reviews recent developments regarding process and products, as well as research works that employed apple pomace as a substrate for different industrial and biotechnological applications.

Keywords: Agro-industrial residues, solid-state fermentation, submerged fermentation, bio-products, apple pomace

INTRODUCTION

Growing international production and marketing of fruits has led to increasing accumulation of fruit wastes such as citrus pulp, seeds and peels, grape pomace among others (Volanis et al., 2006; Bampidis and Robinson, 2006). Over the past few decades, an increasing trend toward efficient utilization of natural resources has been observed around the world. The direct disposal of agro-industrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabolites, with a higher commercial value (Villas-Bôas and Esposito, 2000; Albuquerque et al., 2006; Vendruscolo et al., 2007; 2009a; 2009b).

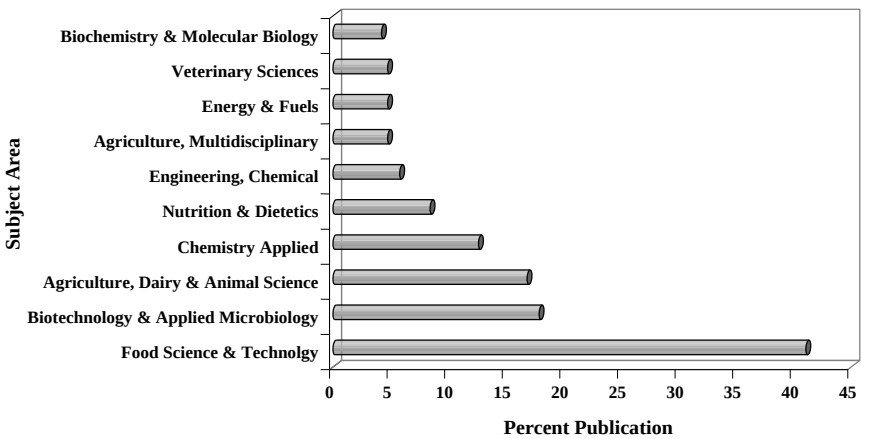
The industrial processing of apples is performed mainly for the production of juice, cider, sauce, jelly, and pulp. Fruits that are not suitable for consumption *in natura* are processed, generating large amounts of residues (Villas-Bôas and Esposito, 2000). Apple pomace is the primary by-product of apple processing and consists of the press cake from pressing apples for juice or cider and from pressing peel and core wastes in producing apple sauce and slices (Hang, 1987). The pomace is the solid residue from juice production and represents around 30% of the original fruit (Villas-Bôas and Esposito, 2000).

Large amounts of apple pomace are produced world-wide (Table 1), and, being highly biodegradable, its disposal represents a serious environmental problem. In Brazil, about 800,000 tons of apple pomace are produced per year (Vendruscolo et al., 2008), and it is mostly used as organic fertilizer and animal feed. The use of apple pomace as animal feed has been widely reported. Diets using this residue have been tested for fish (Vendruscolo et al., 2009a; 2009b), poultry (Ayhan et al., 2009; Kang et al., 2010), cattle (Manterola et al., 1993a, 1993b; Ghoreishi et al., 2007; Abdollahzadeh et al., 2010a, 2010b), lamb (Sirhan et al., 1994; Szucsne, 2000), swine (Gutzwiller et al., 2005; Fujitani et al., 2008) and ewes (Szucsne, 2000; Volanis et al., 2006). This utilization is, however, limited due to a low protein and vitamin content of the residue, which means a low nutritional value. Therefore, alternative approaches to current disposal methods for pomace are necessary, focusing on the maximum production of pomace by-products.

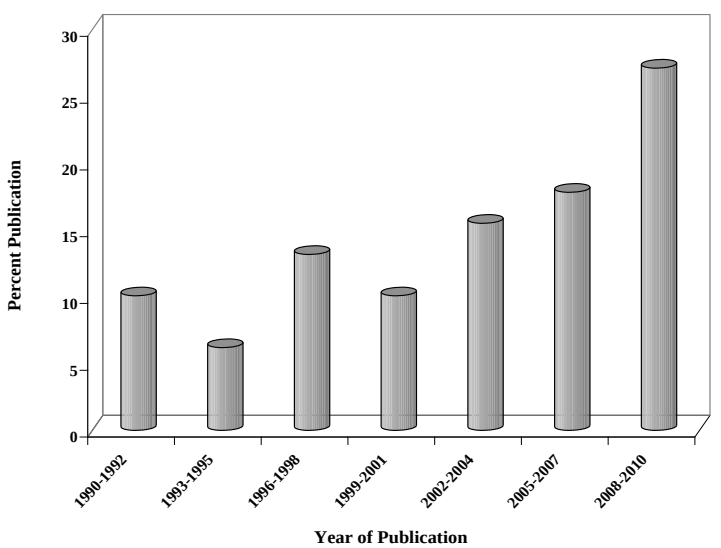
Since 1990, a continuous increase was observed in the number of publications in peer reviewed journals per year dealing with apple pomace utilization (Figure 1a). In this period, R&D initiatives were more focused on apple pomace conversion into value added product development (Sudha et al., 2007; Bhushan et al., 2008; Vendruscolo et al., 2008), or on extracting the bioactive constituents for food fortification or food enrichment (Figure 1b). Approximately 45% of all apple pomace papers were published from 2005 to 2010.

Table 1. World-wide apple pomace generation

Country	Thousands of tonnes	Reference
New Zealand	20	Lu and Foo (1998)
Spain	20	Garcia et al. (2009)
United States	27	Roberts et al. (2004)
Iran	97	Pirmohammadi et al. (2006)
Japan	160	Takahashi and Mori (2006)
Germany	250	Endreβ (2000)
Brazil	800	Vendruscolo et al. (2008)
China	1000	Wang et al. (2010)
India	1000	Manimehalai (2007)



(a)



(b)

Figure 1. R&D publications on apple pomace: concerning (a) subject-wise percentage and (b) year-wise (Source: Web of Science, 2011).

Many researchers, looking for value-added products, have proposed the use of apple pomace for the production of enzymes (Berovic and Ostroversnik, 1997; Zheng and Shetty, 2000a; Favela-Torres et al., 2006; Josh et al., 2008; Liu et al., 2008; Tao et al., 2009), organic acids (Hang and Woodams, 1984; Shojaosadati and Babaeipour, 2002; Gullón et al., 2007; 2008; Kumar et al., 2010; Dhillon et al., 2011a, 2011b, 2011c), pectin (Hunt, 1918; Hwang et al., 1998; Kumar and Chauhan, 2010; Min et al., 2010; Rha et al., 2011), protein-enriched feeds (Hours et al., 1985; Bhalla and Joshi, 1994; Devrajan et al., 2004; Albuquerque et al., 2004; 2006; Vendruscolo et al., 2007; 2009a; 2009b; Tao et al., 2009) baker's yeast (Bhushan and Joshi, 2006), edible mushrooms (Worral and Yang, 1992; Zheng and Shetty, 2000b), ethanol (Ngadi and Correia, 1992a, 1992b; Paganini et al., 2005; Chatanta et al., 2008), butanol (Voget et al., 1985), hydrogen (Doi et al., 2010; Feng et al., 2010, Wang et al., 2010), aroma compounds (Bramorski et al., 1998; Medeiros et al., 2000; 2006; Tsurumi et al., 2001), natural antioxidants (Foo and Lu, 1999; Lu and Foo, 2000; Sudha et al., 2007; Garcia et al., 2009; Kosmala et al., 2010; Suárez et al., 2010; Ajila et al., 2011; Oszmiański et al., 2011; Sudha, 2011), edible fibers (Grigelmo-Miguel and Martín-Belloso, 1999; Masoodi et al., 2002; Paganini et al., 2005; Sudha et al., 2007), energy (Xu et al., 2011), phytochemicals (Lavelli and Corti, 2011), biofilms (Sablani et al., 2009), among others.

Thus, value addition of such residues will not only provide the means to industrial economic stability but will also help in reduction of environmental pollution, judicious management of natural bioresources and availability of nutritionally enriched food products, as well as industrially important biomolecules (Bhushan et al., 2008).

APPLE POMACE COMPOSITION

The apple pomace is a heterogeneous mixture consisting of peel, core, seed, calyx, stem, and soft tissue (Grigelmo-Miguel and Martín-Belloso, 1999). According to Carson et al. (1994), the evaluation of pomace of three apple cultivars indicated that it has 2.2-3.3% seed, 0.4-0.9% stem and 70.0-75.7% apple flesh. The pomace has high water content and is mainly composed of insoluble carbohydrates such as cellulose, hemicellulose, and lignin. Simple sugars, such as glucose, fructose, and sucrose, as well as small amounts of minerals, proteins, and vitamins, are part of apple pomace composition (Villas-Bôas and Esposito, 2000; Zheng and Shetty, 2000a; Jin et al., 2002; Queji et al., 2010). This composition varies according to the apple variety used and the type of processing applied for juice extraction, especially regarding how many times the fruits are pressed (Paganini et al., 2005; Queji et al., 2010). Table 2 shows the chemical composition of apple pomace and Figure 2 shows different purposes for apple pomace applications.

As shown in Table 2, apple pomace contains high amounts of sugar and appears to be an excellent substrate for bioprocesses, being rich in different carbon sources. The decrease in the reducing sugar content, in addition to being an indicator of cell growth, is an important aspect concerning the reduction in the organic matter, since when this material is used for fish feeding the content of sugars is directly related to the added organic load (Vendruscolo et al., 2009a; 2009b). The pomace is very inexpensive and is abundantly available during the harvesting season, and several microorganisms can use this apple residue as a substrate.

Table 2. Examples of physical-chemical composition of apple pomace

Composition	Hang and Woodams (1987)	Joshi and Shandu (1996)	Villas-Bôas and Esposito (2000)	Albuquerque (2003)
Moisture (%)	75.6	3.97	80	79.2
Carbohydrates (%)	9.5-22	48	nd	59.8
Reducing sugars (%)	5.7	nd	15	10.8
Fiber (%)	4.3-10.5	14.7	40.3	38.2
Protein (%)	5.1	5.8	4.1	3.7
Lipids (%)	4.2	3.9	nd	nd
Ash (%)	2.8	1.82	2	3.5
Pectin (%)	1.5-2.5	nd	5.5	7.7
pH	nd	4.2	nd	4.0
Titrable acidity (%)	nd	2.6	nd	0.13
Water activity	nd	nd	nd	0.97

nd – not determined.

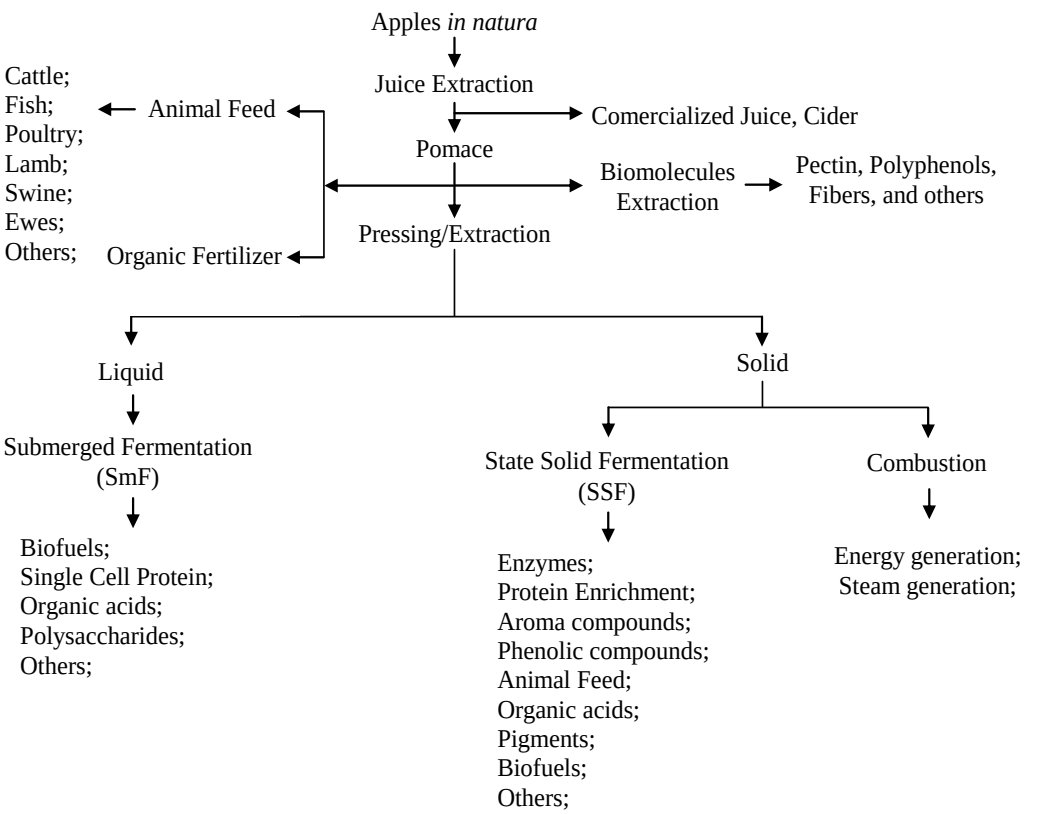


Figure 2. Different purposes for apple pomace applications.

Pectin

Production of pectin is considered the most reasonable way of utilizing apple pomace both from an economical and from an ecological point of view. Apple pomace is a natural source of pectic substances, being an important raw material for pectin production throughout the world. In comparison to citrus pectins, apple pectins are characterized by superior gelling properties. Pectin is slightly soluble in water and therefore, the pulp or pomace resulting from the pressing of ripe fruit contains practically all of the pectin. Hot water will slowly extract the pectin and for this reason fruits are cooked to a pulp with water before extracting the juice for jelly-making (Hunt, 1918). Several attempts have been made to efficiently extract pectin from the apple pomace (Hwang et al., 1998; Canteri-Schemin et al., 2005; Marcon et al., 2005; Shin et al., 2005; Wang et al., 2007, Kumar and Chauhan, 2010; Rha et al., 2011).

Hwang et al. (1998) used twin-screw extruder to disintegrate apple pomace to facilitate production of apple pectins by hot water extraction. The yield of water soluble polysaccharides increased with increasing specific mechanical energy, but intrinsic viscosity decreased and concluded that extrusion could be applied on apple pomace to produce high yield and high quality pectins, replacing conventional acid extraction. Canteri-Schemin et al. (2005) studied the effects of particle size, apple variety, and type of acid on the extraction of pectin from apple pomace. The authors found that higher extraction yields around 14% when pomace particles larger than 106 μm and smaller than 250 nm were used. Marcon et al. (2005), using an experimental design, found that the best yield of pectin from 168 mg g^{-1} of dried apple pomace was obtained at l00°C and 80 min. Significant differences in pectin yield were observed when the pomace from different apple varieties was used. Citric acid and nitric acid showed the highest yields on pectin extraction, among other organic and mineral acids tested for acid extraction. Wang et al. (2007) studied the applicability of microwave-assisted extraction to obtain pectin from apple pomace. They studied the effect of four different factors on the pectin yield. An extraction time of 20.8 min, pH 1.01, solid:liquid ratio of 0.069, and a microwave power of 499.4 W produced the highest extraction yield (157.5 mg g^{-1} of dried apple pomace). According to the authors, these process conditions allowed an important reduction in the time required for pectin extraction. Rha et al. (2011) used the apple pomace to extract pectin of which structure was modified by hydroxamation to improve its antioxidant effect. As the pectin obtained from apple pomace was treated with alkaline hydroxylamine, the hydroxamic acid content of the pectin derivative increased from 2.68 to 10.43%. The pectin derivatives were also shown to have enhanced antiradical activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) in a dose-dependent way. Moreover, the hydroxamation improved the scavenging effect of the pectin which was even 3-fold higher than that of the native one. Thus, the introduction of hydroxamic acid into the apple pomace pectin structure seemed to be a useful tool for improving biological activities of pectins.

Fibers

Diets rich in fibers have been associated with the prevention, reduction, and treatment of several diseases, including pulmonary disease, asthma, diabetes, diverticulitis, coronary heart disease, colon cancer, and obesity (Boyer and Liu, 2004). Fibers obtained with different

extraction methods and from different sources behave differently during their transit through the gastrointestinal tract (Grigelmo-Miguel and Martín-Belloso, 1999; Masoodi et al., 2002; Canteri-Schemin et al., 2005). Apple pomace is characterized by a high content of fibers (cellulose, hemicellulose, pectin, β-glucans, gums, and lignin). It is widely known that dietary fibers play an important role in human health, and the availability of high-quality foods with high dietary fiber content is of great importance for human fiber intake (Boyer and Liu, 2004; Figuerola et al., 2005, Sudha et al., 2007).

Leontowicz et al. (2001) evaluated the effect of diets supplemented either with 10% wt/wt sugar beet pulp fiber or apple pomace fiber on lipids and lipid peroxide production in 60 male Wistar rats fed with cholesterol. Diet supplementation with the sugar beet pulp, as well as with the apple pomace fiber, significantly hindered the increase in plasma lipids and decreased the levels of HDL and total phospholipids. These results demonstrated that fibers mixture have hypolipidemic properties, which can be attributed to their water-soluble components. Sudha et al. (2007) incorporated finely ground apple pomace in wheat flour and studied its rheological characteristics. The authors verified that apple pomace like any other fiber source increases the water absorption capacity of the flour. In general apple pomace affected the elastic properties of the wheat flour dough as well as the pasting properties. Apple pomace having high amount of total dietary fiber can function as a valuable source of dietary fiber in cake making. Cakes prepared from 25% wt/wt of apple pomace-wheat flour blend had 14.2% total dietary fiber with high acceptable quality. Addition of apple pomace in cake making can avoid the addition of other flavoring ingredients as the cakes prepared with apple pomace had pleasant fruity flavor.

The use of apple pomace fibers as metal adsorbents has also been reported. Borycka (2002) used black currant pomace and apple pomace as fiber preparations to determine the dependence between the processes of cadmium sorption and magnesium desorption, as well as to assess the suitability of fiber compositions as selective metal sorbents (Cd, Mg) in selected foodstuffs. The author found that the fiber preparations tested possess a high cadmium binding capacity (about 35% on the average) and that these materials have the ability to desorb magnesium. According to Ahmaruzzaman (2011), phosphate apple waste can be considered as an outstanding adsorbent for the removal of Cd (II) metals. It presented a high adsorption capacity of 36.2 mg g^{-1}. In a previous work, Lee and Yang (1997) investigated the copper removal from a solution to evaluate the cation-exchange capacities of apple residues. The optimal pH range for copper removal by apple residues was shown to be from 5.5 to 7.0, and the maximum percentage of copper removal was 91.2%. Column experiments showed that the dynamic capacity of chemically modified apple residues was four to five times higher than that of raw residues which contained acidic groups such as carboxylic and phenolic functional groups. Thus, the study has shown that modified apple residues could be applied successfully for metal removal from wastewater.

Polyphenols

Apple pomace has also been reported as a source of polyphenols, such as flavonols, isoflavones, flavanones, monomeric and oligomeric flavonols, dihydrochalcones, anthocyanins, anthocyanidins, as well as others. As a result of its abundance, and owing to the increasing interests in new natural sources of antioxidant products, apple pomace has been

investigated as a potential source of bioactive polyphenols during recent years (Escarpa and Gonzalez, 1998; Lu and Foo, 1997; 2000; Zheng and Shetty, 2000b; Schieber et al., 2003; Manach et al., 2004; Carle and Schieber, 2006; Adil et al., 2007; Sudha et al., 2007; Ćetković et al., 2008; Garcia et al., 2009; Sudha, 2011). Antioxidants are substances that are able to prevent or retard the oxidation of lipids, proteins and DNA; and to protect the compounds or tissues from damage caused by oxygen or free radicals. Therefore, their health promoting effects reduce the risk of various diseases (Manach et al., 2004).

In view of the fact that most of the phenolic compounds remained in the apple pomace, the interest is focused on the apple pomace as a potential source of bioactive phenolics, which can be used for various purposes in the food, pharmaceutical and cosmetic industry (Ćetković et al., 2008). Lu and Foo (2000) indicated that the polyphenols, which are mainly responsible for the antioxidant activity, are present in apple pomace and hence could be a cheap and readily available source of dietary antioxidants.

Will et al. (2006) obtained apple pomace extracts containing 31 to 51% polyphenols, rich in cinnamate esters, dihydrochalcones, and flavonols. Savatovic et al. (2005) found high contents of phenolic acids (6.38 mg g^{-1}), flavonoids (1.01 mg g^{-1}) and flavan-3-ols (0.70 mg g^{-1}) in 80% methanol extract of Induna apple pomace. These compounds clearly contributed to the high antioxidant activity observed for the Induna apple pomace extracts. The same research group identified and quantified by HPLC phenolic acids, flavan-3-ols, flavonoids, and dihydrochalcons in Granny Smith apple pomace. The total content of phenolic acids, flavonoids and flavan-3-ols in apple pomace determined spectrophotometrically, was 7.02 mg g^{-1}, 0.51 mg g^{-1} and 8.80 mg g^{-1}, respectively. These contents show that apple pomace represent a valuable source of antioxidant and anticancer phytochemicals, considering its antioxidant and antiproliferative activities (Savatovic et al., 2008). In the same way, Lu and Foo (1997) observed the presence of some phenolic constituents, as procyanidins and quercetin glycosides, which have been shown to exert strong antioxidant activity *in vitro*. Therefore, polyphenols from apple pomace are considered as high value-added compounds. They may be used as functional food ingredients and as natural antioxidants, being able to replace their synthetic equivalents, which may cause some type of rejection (Schieber et al., 2003). Sudha et al. (2007) demonstrated that apple pomace also has the potential for use in cake making as a good source of polyphenols which has antioxidant properties. The total phenol content in wheat flour and apple pomace was 1.19 and 7.16 mg g^{-1} respectively, where cakes prepared from 0 and 25% of apple pomace blends had total phenol content of 2.07 and 3.15 mg g^{-1} indicating that apple pomace can serve as a good source of both polyphenols. Garcia et al. (2009) analyzed eleven different cider apple pomaces for low molecular phenolic profiles and antioxidant capacity. Major phenols were flavanols, dihydrochalcones (phloridzin and phloretin-20-xyloglucoside), flavonols and cinnamic acids (chlorogenic and caffeic acids). The group of single-cultivar pomaces had higher contents of chlorogenic acid, (-)-epicatechin, procyanidin B2 and dihydrochalcones, whereas the industrial samples presented higher amounts of up to four unknown compounds, with absorption maxima between 256 and 284 nm. The antioxidant capacity of apple pomace, as determined by the DPPH and ferric reducing antioxidant power (FRAP) assays, was between 4.4 and 16.0 g ascorbic acid kg^{-1} of dry matter, thus confirming that apple pomace is a valuable source of antioxidants.

Organic food is nowadays perceived by the consumers as a high quality food and with guaranteed production safety. Health consciousnesses of modern consumer have been

increasing and market development gives new possibilities to organic food producers. Unfortunately food processing causes the reduction of bioactive substances in final product as fruit pomace, juice or jam. Rembialkowska et al. (2006) produced apple pomace of five apple cultivars: Lobo, Jonagold, Cortland, Boskoop and Idared. The fruits were collected from one organic and one conventional orchard. The authors evaluated the content of flavones, total polyphenols, vitamin C and antioxidant activity in the fresh and pasteurized apple pomace. Results indicated that organic apple pomace contained more total polyphenols including flavones, more vitamin C and higher antioxidant activity than conventional ones. It was also verified that the pasteurization process influenced negatively upon the bioactive substances content and on antioxidant properties of the apple pomace. Thus, apple pomace obtained from organic cultivars appears to be a better source for antioxidant compounds.

Emerging technologies such as subcritical extraction and microwaves have been used from polyphenols extraction from apple pomace. Adil et al. (2007) used the ethanol and carbon dioxide on polyphenols extraction from apple pomace. Total phenolic content and antiradical efficiencies of the extracts from apple pomace obtained by subcritical (CO_2 + ethanol) was higher than the extracts obtained by ethanol extraction, indicating that less but more active polyphenols were selectively extracted by subcritical (CO_2 + ethanol) extraction compared to ethanol extraction. The optimum ethanol concentration and extraction time were 20% ethanol and 40 min. Total phenolic content and antiradical efficiencies were 0.47 mg gallic acid g^{-1} of dried apple pomace and 3.30 mg DPPH mg^{-1} of dried apple pomace, respectively. Bai et al. (2010) used a simple and efficient microwave-assisted extraction of polyphenols from industrial apple pomace by the maximization of the yield using response surface methodology and HPLC analysis. The results demonstrated that industrial apple pomace contains chlorogenic acid, caffeic acid, syrigin, procyanidin B2, (-)-epicatechin, cinnamic acid, coumaric acid, phlorizin and quercetin, thus confirming that apple pomace are a valuable source of polyphenols. This study can be useful for the development of industrial microwave-assisted extraction of polyphenols from apple pomace.

Recently, Lavelli and Corti (2011) studied the effect of drying and long-term storage on phytochemical contents of apple pomace. Air-drying at 60°C was better than vacuum-drying at 40°C in terms of anthocyanin and flavanol retention; no adverse effect of drying was observed for flavonols, dihydrochalcones and hydroxycinnamic acids. The maximum stability occurred for all apple phytochemicals at the lowest a_w. At a_w 0.75 degradation of all phytochemicals occurred with the following stability ranking: phloridzin > chlorogenic acid > quercetin 3-*O*-galactoside > epicatechin > procyanidin B2 and cyanidin 3-*O*-galactoside. A promising feature of pomace is its exclusive high content of phloridzin, which showed relatively high stability during long-term storage. Apple pomace showed high antioxidant content and consequently high radical-scavenging activity and antiglycation activity.

Energy Generation

The utilization of biomass as a renewable energy resource is of great importance in responding to concerns over the protection of the environment and the security of energy supply. A major issue with any biomass conversion process is the cost of transporting the biomass to a central processing plant. The following survey therefore concentrates on biomass residues that are generated in processing plants and are thus already available in

large quantities at one location. In addition, such processing plants require energy and process heat and could easily use the energy produced by the conversion of the residues (Xu et al., 2011).

Dried apple pomace can be utilized as fuel for steam generation in processing plants which will help to make a significant contribution to the energy budget (Fischer, 1984). Sargent et al. (1986) studied the economic feasibility of in-plant combustion of apple processing wastes. They suggested that reductions in fossil fuel and waste disposal costs could be achieved by apple processors through in-plant combustion of apple pomace. Xu et al. (2011) studied the pyrolysis of apple pomace which has been carried out in a pilot bubbling fluidized bed pyrolyzer, operating under a range of temperature from 300 to 600°C and two vapor residence times, with the aim of determining their pyrolysis behavior including products yield and heat balance. The thermal sustainability of the pyrolysis process was estimated by considering the energy contribution of the produced gases and of the liquid bio-oil in relation to the pyrolysis heat requirements. Takahashi and Mori (2006) studied the hydrogen production using apple pomace at high temperatures (>973 K). The reaction was carried over a commercial steam reforming Ni catalyst using a fluid bed reactor. The Ni catalyst could mediate the reaction between apple pomace and H_2O to produce hydrogen. The balance between the reactant apple pomace and the produced gases, together with thermogravimetric data, suggested that apple pomace was decomposed to H_2, CO, CH_4, CO_2 and carbon deposits prior to steam reforming, and the resultant deposited carbon reacted with H_2O to produce H_2, CO, and CO_2. This conclusion was supported by the observation that potassium and calcium compounds added to the Ni catalyst considerably increased the extent of gasification, probably by promotion of the reaction between deposited carbon and H_2O. Enhancement of the reaction between deposited carbon and H_2O is a possible way to develop a high performance system for H_2 production from steam reforming of biomass.

MICROBIAL STRAINS CULTIVATED ON APPLE POMACE

Bacteria, levеduriform and philamentous fungi have been cultivated on apple pomace for different purposes. Philamentous fungi, especially basidiomycetes, are the most suitable microorganisms for growing on fruit processing residues. Their ability to grow on the surface and even inside the medium explains why these organisms are often used for residue bioconversion. Table 3 summarizes a list of different biotechnological applications of apple pomace, with the respective microorganism and the fermentation process applied: solid-state fermentation (SSF) or submerged fermentation (SmF).

Selection of a suitable strain is one of the most important criteria in state solid fermentation. The vast majority of wild type microorganisms are incapable of producing commercially acceptable yields of the desired strains product. The selection of a substrate for SSF process depends upon several factors, mainly related with the cost and availability and, thus, may involve their screening. Agro-industrial residues, particularly of tropical origin offer potential advantages for their application as substrates. In SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the micro-organisms growing in it should be considered as the ideal substrate. However, some of the

nutrients may be available in sub-optimal concentrations, or even not present in the substrates. In such cases, it would be necessary to supplement them externally. It has also been a practice to pre-treat (chemically, or mechanically) some substrates before use in SSF processes (e.g. lignocellulosics), which makes them more easily accessible for microbial growth (Pandey et al., 2008). Tools as radial growth rate can be used to the screening of microorganisms for different substrates (Gabiatti et al., 2006).

From Table 3, it is possible to observe that SSF is the most commonly used process for the valorization of apple pomace through microorganism cultivation. SSF can be affected by different factors. Among them, the selection of a suitable strain and the setting of process parameters (physical, chemical, and biochemical) are crucial (Durand, 2003; Mitchell et al., 2003; Raghavarao et al., 2003).

Considering productivity aspects of fermentation processes, the presence of large amounts of water in SmF may be a disadvantage when the desired product needs further purification because water needs to be separated from the product by energy-consuming techniques such as centrifugation, evaporation, or filtration. From this point of view, SSF might have an economic advantage over SmF. A certain amount of water will always remain necessary in SSF for the microorganisms to remain alive, but restricted water availability can prevent the outgrowth of undesired microorganisms in non-sterile cultivations, especially in combination with extreme pH, thus reducing the need for sterilization. However, the number of species that can be applied in low water content SSF is restricted. Thus, because of the low water content, SSF can have advantages over SmF, such as superior productivity, low waste water output, and improved product recovery (Lonsane et al., 1985).

Biomass is a fundamental parameter in the characterization of microbial growth. Its measurement is essential for kinetic studies on SSF. Direct determination of biomass in SSF is very difficult due to the problem of separating the microbial biomass from the substrate (Pandey et al., 2000a). This is especially true for SSF processes involving fungi because the fungal hyphae penetrate into and bind tightly to the substrate. However, for the calculation of growth rates and yields, it is the absolute amount of biomass that is important. Glucosamine is a useful compound for the estimation of fungal biomass, taking advantage of the presence of chitin, poly-N-acetylglucosamine, in the cell walls of many fungi (Raimbault, 1998).

BIOPROCESSES INVOLVING APPLE POMACE

Production of Enzymes

The most important area of apple pomace utilization is the production of enzymes, especially pectic ones. For the production of enzymes, several process parameters, such as temperature, moisture content, carbon dioxide partial pressure, and periodic mixing of the solid substrate mash, are of fundamental importance. The advantage of solid-state fermentation for pectolytic enzyme production compared to conventional submerged fermentation can be pointed out. SSF uses less water in the process, reducing the production of liquid wastes and their associated disposal problems. In this case, the final enzymatic product could be use as an enzyme complex, immobilized in the dry biomass. Therefore, only drying and pulverization would be needed. Using SmF, the enzymes have to be extracted

from biomass, and much more solvent is required, generating large amounts of effluent. Another advantage of using SSF is that low moisture content leads to fewer contamination problems, and thus, simpler operations with sterile conditions can be used.

Table 3. Bioprocesses using apple pomace as substrate

Application	Organism	Process	Reference
Antioxidant Compounds			
Phenolic	*Trichoderma viride, T. harzianum, T. pseudokoningii*	SSF	Zheng and Shetty (2000a)
Phenolic	*Phanerocheate chrysosporium*	SSF	Ajila et al. (2011)
Aroma Compound Production			
Aroma compounds	*Rhizopus* sp., *Rhizopus oryzae*	SSF	Christen et al. (2000)
Aroma compounds	*Kluyveromyces marxianus*	SSF	Medeiros et al. (2000)
Aroma compounds	*Saccharomyces cerevisiae*	SmF	Muranaka et al. (2006)
Fruity aroma	*Ceratocystis fimbriata*	SSF	Bramorski et al. (1998)
Enzyme Production			
β-glucosidase	*Aspergillus foetidus*	SSF	Hang and Woodams (1994b)
Cellulase	*Aspergillus niger*	SSF	Tao et al. (2009)
Cellulase	*Trichoderma* sp.	SSF	Sun et al. (2011)
Ligninolytic enzymes	*Phanerocheate chrysosporium*	SSF	Gassara et al. (2010; 2011)
Lignocellulolytic enzymes	*Candida utilis*	SSF	Villas-Bôas et al. (2002)
Pectin methylesterase	*Aspergillus niger*	SSF/SmF	Joshi et al. (2006)
Pectinase	*Aspergillus niger*	SSF	Tao et al. (2009)
Pectinase	*Aspergillus niger*	SSF	Josh et al. (2008)
Pectinase	*Polyporus squamosus*	SmF	Pericin et al. (1999)
Pectolytic enzymes	*Aspergillus niger*	SSF	Berovic and Ostroversnick (1997)
Polygalacturonase	*Aspergillus niger*	SSF	Hang and Woodams (1994a)
Polygalacturonase	*Lentinus edodes*	SSF	Zheng and Shetty (2000a)
Proteinase	*Aspergillus niger*	SSF	Tao et al. (2009)
Xylanase	*Aspergillus niger*	SSF	Liu et al. (2008)
Biofuels			

Application	Organism	Process	Reference
Butanol	*Clostridium acetobutylicum, C. butylicum*	SmF	Voget et al. (1985)
Ethanol	*Saccharomyces cerevisiae*	SSF	Ngadi and Correa (1992a; 1992b)
Ethanol	*Saccharomyces cerevisiae*	SSF	Chatanta et al. (2008)
Hydrogen	*Clostridium* spp.	SmF	Doi et al. (2010)
Hydrogen	Anaerobic activated sludge	SmF	Feng et al. (2010)
Hydrogen	Anaerobic activated sludge	SmF	Wang et al. (2010)
Heteropolysaccharide Production			
Chitosan	*Gongronella butleri*	SSF/SmF	Streit (2004)
Chitosan	*Gongronella butleri*	SmF	Vendruscolo (2005)
Chitosan	*Gongronella butleri*	SmF	Streit et al. (2009)
Heteropoly-saccharide	*Beijerinckia indica*	SmF	Jin et al. (2002)
Xanthan	*Xanthomonas campestris*	SSF	Stredansky and Conti (1999)
Nutritional Enrichment			
Animal feed	*Gongronella butleri*	SSF	Vendruscolo (2005)
Animal feed	*Gongronella butleri*	SSF	Vendruscolo et al. (2007; 2009a; 2009b)
Animal feed	*Candida utilis, Torula utilis, Saccharomyces cerevisiae*	SSF	Joshi and Shandu (1996)
Nutritional enrichment	*Candida utilis, Kloeckera* sp.	SSF	Devrajan et al. (2004)
Nutritional enrichment	*Candida utilis, Pleurotus ostreatus*	SmF	Villas-Bôas and Esposito (2000)
Nutritional enrichment	*Rhizopus oligosporus*	SSF	Albuquerque et al. (2004; 2006)
Single cell protein	*Saccharomycopsis lipolytica, Trichoderma reesei*	SmF	Hours et al. (1985)
Single cell protein	*Candida utilis*	SmF	Albuquerque (2003)
Organic Acids			
Citric acid	*Aspergillus niger*	SSF	Shojaosadati and Babaeipour (2002)
Citric acid	*Aspergillus niger*	SSF	Kumar et al. (2010)

Table 3. (Continued)

Application	*Organism*	Process	Reference
Citric acid	*Aspergillus niger*	SSF	Hang and Woodams (1984)
Citric acid	*Aspergillus niger*	SmF/SSF	Dhillon et al. (2011a; 2011b; 2011c)
Lactic acid	*Lactobacillus rhamnosus*	SmF	Gullón et al. (2007; 2008)
Vinegar	-	SmF	Takahashi et al. (2011)
Other Products			
γ-Linolenic acid	*Thamnidium elegans, Mortierella isabelina, Cunninghamella elegans, C. echinulata*	SSF	Stredansky et al. (2000)
Bioinoculant	*Trichoderma viride, T. harzianum, T. pseudokoningii, Penicillium* sp., *Rhizopus oligosporus*	SSF	Zheng and Shetty (1998)
Edible mushrooms	*Lentinus edodes, Pleurotus ostreatus, P. sajor-caju*	SSF	Worrall and Yang (1992)

Polygalacturonases or hydrolytic depolymerases are enzymes involved in the degradation of pectic substances. They have a wide range of applications in food and textile processing, degumming of plant rough fibers, and treatment of pectic wastewaters. Bacteria, yeasts, and fungi under both SmF and SSF conditions are able to produce these enzymes (Favela-Torres et al., 2006). Hang and Woodams (1994a) compared five strains of *Aspergillus niger* cultivated on apple pomace for the production of polygalacturonase. The highest enzyme activity of 25,000 U kg^{-1} of apple pomace was obtained after 72 hours of cultivation at 30°C. The polygalacturonase showed an optimum activity at 40°C and pH 4.5. Zheng and Shetty (2000b) compared the production of polygalacturonase by *Lentinus edodes* in SSF from apple pomace, cranberry pomace, and strawberry pomace as substrate. Strawberry pomace was the best substrate for polygalacturonase production with 29.4 U g^{-1} of dried strawberry pomace, followed by apple pomace with 20.1 U g^{-1} of dried pomace. An increase in polygalacturonase production was obtained with the supplementation of the apple pomace with 20% of polygalacturonic acid, obtaining 26.8 U g^{-1} of dried apple pomace. Hang and Woodams (1994b) studied the production of β-glucosidase using apple pomace as substrate for three *Aspergillus* species. *A. foetidus* NRRL 337 produced a β-glucosidase with higher activity than the enzyme from *Aspergillus fumigatus* and *A. niger* under similar cultivation conditions.

Berovic and Ostroversnik (1997) studied the production of a pectolytic enzyme complex by *Aspergillus niger* from solid-state fermentation. It was based on the utilization of an

inexpensive substrate: the apple pomace, combined with soya flour, wheat bran, and mineral salts. Carbon dioxide partial pressure should be kept low, and moisture content has to be less than 50%. The best result was obtained with a moisture content of 38% and temperature equal to 35°C. Hang and Woodams (1995) evaluated the potential of using apple pomace as a substrate for the production of β-fructofuranosidase by three *Aspergillus* species. *A. foetidus* NRRL 337 was found to produce the higher β-fructofuranosidase activity with 2,700 U kg^{-1} of dried apple pomace. *A. oryzae* NRRL 1988 and *A. niger* NRRL 2270 produced enzymes with 100 and 1,660 U kg^{-1} of dried apple pomace, respectively. The β-fructofuranosidase from *A. foetidus* was stable over a large pH range (3.4-6.0), and at 50°C, but lost about 50% of its activity after 20 min at 60°C.

Pericin et al. (1999) used *Polyporus squamosus* for the bioconversion of apple pomace and the production of pectinases in liquid medium, simultaneously. The microbial converted apple pomace which had its protein content increased (about six times), with a very high (75%) protein digestibility. The amino acid profile was appropriate for young animal feed formulations, due to its high histidine and lysine content. Crude enzyme preparations, obtained by ultrafiltration of the liquid culture, were used for apple juice clarification. Recently, Joshi et al. (2006) reported the production of pectin methylesterase by *A. niger* using apple pomace as culture medium comparing the SmF and SSF. The pectin methylesterase activity was 2.3 times higher when produced by SSF than by SmF. This study corroborates the fact that SSF is the more adequate process for apple pomace bioconversion.

Seyis and Aksoz (2005) investigated the use of apple pomace, orange pomace, orange peel, lemon pomace, lemon peel, pear peel, banana peel, melon peel, and hazelnut shell as substrate for xylanase production using *Trichoderma harzianum*. The maximum enzyme activity was observed when melon peel was used as the substrate for SSF, followed by the apple pomace and hazelnut shell. Liu et al. (2008) optimized the state solid fermentation on production of extracellular xylanase by *Aspergillus niger* using apple pomace and cotton seed meal as substrate. The results from this study would be of significance for the agriculture and enzyme industries to develop innovative techniques to utilize these inexpensive agro-industrial wastes for enzyme production using SSF process. Tao et al. (2009) produced multienzyme bio-feed, with the biodegration of anti-nutritional factors such as pectin and tannins in apple pomace, and obtained the nutritional enrichment of the fermented substrate. The mixture of apple pomace and cotton seed powder, supplemented with ammonium sulfate and monopotassium phosphate, was proved to be the optimum medium for the mixed strains of *Aspergillus niger* M2 and M3. The activities of pectinase, proteinase and cellulase achieved were 21,168; 3,585 and 1,208 U g^{-1}, respectively.

Villas-Bôas et al. (2002) found a novel lignocellulolytic activity of *Candida utilis* during SSF on apple pomace. Hydrolytic and oxidative enzymes of *C. utilis,* excreted to the culture medium during solid state fermentation were identified, evaluated, and quantified. The soluble lignin fraction of the apple pomace was consumed at very significant levels of 76%, when compared to the unfermented apple pomace. The enzyme produced by *C. utilis* with the highest activity was a pectinase of 239 U mL^{-1}. The yeast showed a significant manganese-dependent peroxidase activity of 19.1 U mL^{-1} and low cellulase (3.0 U mL^{-1}) and xylanase (1.2 U mL^{-1}) activities, suggesting that *C. utilis* has the ability to use lignocellulose as a substrate. Recently, Sun et al. (2011) studied the feasibility of using apple pomace for cellulase production by *Trichoderma* sp. by solid state fermentation. The results indicated that

initial moisture level of the medium, incubation temperature and inoculum size influenced the cellulase production greatly. The supplementation of lactose and corn-steep solid to the apple pomace favored the enzyme formation markedly.

Production of Aroma and Phenolic Compounds

A chemical compound has a smell or odor when two conditions are met: the compounds needs to be volatile, so it can be transported to the olfactory system in the upper part of the nose, and it needs to be in a sufficiently high concentration to be able to interact with one or more of the olfactory receptors (Kohl et al., 2001).

Today, the consumer has a preference for natural food additives. The use of biotechnology for the production of natural aroma compounds by fermentation or bioconversion using microorganisms is an economic alternative to the difficult and expensive extraction from raw materials such as plants (Daigle et al., 1999). Currently, it is estimated that around 100 different aroma compounds are produced commercially by fermentation (Medeiros et al., 2006).

Bramorski et al. (1998) analyzed the production of aroma compounds by *Ceratocystis fimbriata* under seven different medium compositions (prepared by mixing cassava bagasse, apple pomace, amaranth, and soya bean). The aroma production was growth dependent, and the maximum aroma intensity was detected in a few hours around the maximum respirometric activity. The medium containing apple pomace produced a strong fruity aroma after 21 hours of cultivation. This same medium was used by Christen et al. (2000) for the production of volatile compounds by *Rhizopus* strains. Authors found that the production of volatile compounds was related mainly to the used medium, and no difference was observed among the studied strains. The odors detected have a slight alcoholic note, and the apple pomace produced intermediate results, compared with the amaranth grain supplied with mineral salt solution.

Almosnino et al. (1996) evaluated the production of volatile compounds hexanal and 2,4-decadienal obtained from polyunsaturated fatty acids through the action of a specific apple pomace enzymatic system. The production of these compounds was quantitatively improved by increasing the fatty acids and the enzyme complex concentrations in the reaction medium. The importance of an exogenous oxygen supply during bioconversion was also shown. Some physiochemical factors involved in the expression of the pomace enzymatic system were screened, and a temperature of 25°C was set as favorable for the bioconversion. The authors found that at basic conditions, hexanal production was 2.1 times higher and the production of 2,4-decadienal was 3.3 times lower, when compared to aldehyde production in the control reaction medium.

Muranaka et al. (2006) cultivated a high-ethyl caproate-producing strain, *Saccharomyces cerevisiae* 3703-7, isolated from cerulenin-resistant mutants of sake yeast in apple pomace extracts. The yeast produced ethyl caproate, ethyl acetate, and isoamyl acetate from the apple pomace extract rich in sugars, such as glucose, fructose, and sucrose. After 8 days of the strain 3703-7 incubation in the pomace extract containing 10.8% sugars, 5.23% ethanol was produced, and the production amounts of ethyl caproate, ethyl acetate and isoamyl acetate were 3.12, 7.13, and 0.45 ppm, respectively. The authors obtained distilled liquor which had

the flavor of alcohol containing 32.3% ethanol, possessing a sweet aroma typical of ethyl caproate in addition to an apple flavor.

Ajila et al. (2011) showed that the enrichment of apple pomace with phenolic antioxidants can be carried out by SSF using the white rot fungus, *Phanerocheate chrysosporium*. The phenolic content was higher in fermented apple pomace than the apple pomace and the antioxidant activity was correlated with the increase in polyphenolic content. The extraction of polyphenol content varied depending on the type of solvent, temperature of extraction, extraction time and the method used. SSF thus, not only resulted in value addition to the apple pomace, but also improved the antioxidant activity.

The malic polyphenol production from apple peel waste was invented by Yu (2002) (Patent CN 1335311-A). The malic polyphenol mixture consists of catechin, epicatechin, phloretin and proanthocyanin. These substances present several physiological activity and may be used in food as antioxidant, as well as in the pharmaceutical industry as antimutagenic and antianomalism agents. Apple peel pomace was used as a low cost raw material for extracting the malic polyphenol mixture. The process includes the following steps: extraction, adsorption, separation and spray drying or decompression drying. Tomochika et al. (2003) invented a bacteriostatic agent suitable for beverages using malic polyphenol. The product containing the mixture of malic polyphenol effectively inhibited the growth of bacteria and molds, and was appropriate to be added into beverages, commercialized by Asahi Soft Drinks Co. (Patent JP 3423664).

Nitrogen-Enriched Pomace

Cells of algae, fungi, yeasts, and bacteria are composed of up to 60% high quality protein. These organisms multiply quickly under different conditions, being able to consume diverse types of industrial residues (Anupama and Ravindra, 2000). Considering that traditional animal protein sources, such as meat and milk, have a higher cost and, as such, are not accessible to a large part of the global population, the production of alternative protein sources, such as those originated by microorganisms, appears to be an attractive solution for raising protein intake (Albuquerque et al., 2004; 2006). Furthermore, the use of agro-industrial residues for growing microbial cells as a suitable protein source for human consumption is an interesting approach for adding value to industrial by-products (Anupama and Ravindra, 2001).

The use of biotechnological manipulated ingredients for the production of animal feed has been growing each year. During microbial processing, along with the conversion of lignocellulosic waste into foods, an increase in protein content and an improvement in the digestibility of the substrate are observed (Villas-Bôas et al., 2003). The presence of a large quantity of organic material such as reducing sugar can lead to the complete extinction of the oxygen in the water, causing the disappearance of fish and other forms of aquatic life, increasing costs with the oxygenation system of lakes (Vendruscolo et al., 2009a; 2009b).

Rahmat et al. (1995), working with the yeasts *Kloeckera apiculara* and *Candida utilis* Y15 grown on apple pomace, observed a total crude protein content of 7.5% after 72 hours of growth at 30°C for both microorganisms. This apple pomace-based biotechnological product was shown to be potentially interesting as a stock-feed supplement, with a high nutritional quality (concerning the concentration of essential amino acids) that was two times higher than

the non-fermented pomace. Joshi and Sandhu (1996) studied the protein enrichment of dried apple pomace by SSF using three different yeasts *Saccharomyces cerevisiae*, *Candida utilis*, and *Torula utilis*. After the removal of ethanol, it was verified that the pomace was enriched in crude protein (3-fold), fat (1.5- to 2-fold), and vitamin C (2-fold). However, soluble protein content presented a slight increase, while the sugar content was reduced. There was an enhanced Zn (1- to 5-fold), Mn (3- to 5-fold), and Fe content (more than 2-fold) in the fermented pomace compared to its counterpart. Copper concentration also increased. An increase in microelements (Fe, Cu, Zn, and Mn) by fermentation and drying improved the nutritive value of the apple pomace. The yeast *C. utilis* gave the highest crude protein, while *S. cerevisiae* gave the best soluble protein content and *T. utilis* the lowest fat concentration. A slight reduction in the energy value (caloric values) of the fermented apple pomace compared to the unfermented pomace was observed due to the decrease in the sugar content.

Aiming at the production of single cell protein (SCP), Albuquerque (2003) cultivated *Candida utilis* CCT 3469 cells in SmF using the watery extract of apple pomace as substrate. The yeast produced cells containing up to 48% crude protein, 3.7% lipids, and 8.2% ash, representing a proteinous biomass with great potential for use as a supplement for animal feed.

Zheng and Shetty (1998) compared *Trichoderma*, *Penicillium*, and *Rhizopus* species in SSF for the production of beneficial fungi using glucosamine and soluble protein as the indicator of growth. The optimal water activity was found to be 0.98 and moisture content of 81% at 25°C. However, if the medium contains significant amounts of soluble proteins or peptides, only the glucosamine content can be used as the growth indicator.

To increase the apple pomace protein content, Albuquerque et al. (2006) cultured the fungus *Rhizopus oligosporus* CCT 4134 in SSF, using aerated column reactors. The influence of adding nitrogen sources and buffer solutions on soluble protein production, as well as on substrate pH value during growth was investigated. The solid cultivation lead to an increase of more than five times over the initial protein content, at a pH range considered optimum for growing *R. oligosporus*, which produced around 30% soluble protein. The fungi colonized the substrate very quickly, showing a great potential for being used in apple pomace bioconversion.

Song et al. (2005a) developed a method for producing SCP from apple pomace by dual SSF. This method comprises four different steps: (1) preparation of an SSF medium with pulverized apple pomace, (2) inoculating cultured mixed mature strains for SSF, (3) rapidly drying the resulting fermentation product at low temperature, and (4) subjecting the dried product to solid fermentation in another SSF medium to obtain the final product (Patent CN 1673343). The same group of researchers (Song et al., 2005b) developed another method for producing feed protein by liquid-solid fermentation of apple pomace (Patent CN 1663421). As compared with solid fermentation, this method has the advantage of reduced consumption of medium for seed culture, reduced cost, and applicability to large-scale production.

Animal Feed

Vendruscolo et al. (2009a) used the filamentous fungi *Gongronella butleri* for nutritional enrichment of the apple pomace in SSF. After cultivation for 7 days, the biologically treated material was added at 30% to the feed of conventional meal and used as feed for Nile Tilapia

over a period of 30 days. Growth characteristics of fishes fed with enriched pomace were compared to the control. Height, length, and mass of the fishes were analyzed, and, after 30 days, the fish fed with the biologically treated apple pomace presented an increase of 13% in length, 11.5% in height, and 44% in body mass. These results indicate that *G. butleri* protein-enriched apple pomace can be used as a supplement in fish diets, which represents a value-added application for apple pomace. Abdollahzadeh et al. (2010a; 2010b) evaluated the effects of replacing alfalfa hay with ensiled mixed tomato and apple pomace on ruminal fermentation, blood metabolites, chewing activity and apparent digestibility of diets in six multiparous mid-lactating Holstein dairy cows. The substitution of ensiled mixed tomato and apple pomace in dairy cow's diet was associated with a reduction of chewing activity, better digestibility and higher blood metabolites concentration. Feeding ensiled mixed tomato and apple pomace resulted in a significant higher concentration of acetic, propionic acids, and total volatile fatty acids in rumen and lower rumen pH. It was concluded that, ensiled mixed tomato and apple pomace might efficiently comprise up to 30% of the diets. The nutritive value of tomato and apple pomace could be improved when they are used together (50:50) in dairy cows diet.

Kang et al. (2010) conducted an experiment to investigate the effects of dietary supplementation of fermented apple pomace on laying performance and egg quality of laying hens. The chickens that were fed with diets containing 1.0% of fermented apple pomace showed higher egg production than the animals fed with other diets, suggesting the possibility that fermented apple pomace could be used as an alternative to antibiotic growth promoters.

Apple powder producing process and apple leftover feed compounding process were developed by Cui (2001; Patent CN 1307830-A). This invention is related to an apple powder, which can be used as a food additive because it possesses a certain sugar content and nutritional value. Song et al. (2005b) developed a system for the production of feed albumen by SSF of apple dregs liquid (Patent CN 1663421-A).

Production of Alcohols

Bioethanol is considered one of the most important renewable fuels due to the economic and environmental benefits of its use. Lignocellulosic biomass is the most promising feed-stock for producing bioethanol considering its global availability. The energy gain that can be obtained when non-fermentable materials from biomass are used for cogeneration of heat and power makes bioethanol a very interesting product. The so-called lignocellulosic biomass includes agricultural residues, forestry wastes, municipal solid waste, agroindustrial wastes, food processing, and other industrial wastes. The lignocellulosic complex is the most abundant carbohydrate in the world and is present in sugarcane bagasse, wood chips, saw dust, paper residues, and grass, among many others (Alzate and Toro, 2006).

Hang et al. (1981) used apple pomace as a substrate for ethanol production in SSF. At the end of the cultivation, the alcohol content of the fermented apple pomace was determined by gas chromatography, and the sugar was analyzed as glucose by the phenol-sulfuric method. The sugar was reduced from an initial concentration of 10.2% to less than 0.4%, and the final concentration of ethyl alcohol was greater than 4.3%, representing a fermentation efficiency of approximately 89%. Alcohols produced from apple pomace via SSF were methyl, ethyl, propyl, butyl, and amyl alcohols. Ethyl alcohol was produced at the highest levels, while

propyl, butyl, and amyl alcohols were found in much lower concentrations. Methyl alcohol was not formed by the yeast *S. cerevisiae*. However, this alcohol was a hydrolytic product formed by the action of pectinesterases present in apples.

A solid-state fermentation process for the production of ethanol from apple pomace by *S. cerevisiae* was described by Khosravi and Shojaosadati (2003). A moisture content of 75% (wt/wt), an initial sugar concentration of 26% (wt/wt), and a nitrogen content of 1% (wt/wt) were the conditions used to obtain 2.5% (wt/wt) ethanol without saccharification and 8% (wt/wt) with saccharification. The results indicate that the alcohol fermentation from apple pomace is an efficient method to reduce waste disposal, with the concomitant production of ethanol. Nogueira et al. (2005) evaluated the alcoholic fermentation of the aqueous extract of apple pomace. Apple juice, pomace extract, and pomace extract added with sucrose provided after fermentation 6.90, 4.30, and 7.30% ethanol, respectively. A fermentation yield of 60% was obtained when pomace extract was used, showing that it is a suitable substrate for alcohol production. Chatanta et al. (2008) used different microbial strains *Saccharomyces cerevisiae* MTCC 173 (ethanol production), *Aspergillus foetidus* MTCC 151 (pectinase) and *Fusarium oxysporum* MTCC 1755 (cellulase) individually as well as in consortia for ethanol production from apple pomace in solid state fermentation. With *S. cerevisiae* MTCC 173 ethanol was recovered after 72 hours of incubation at 30°C with Bucchi rotary vacuum evaporator and sugar concentration decreased to 0.25% and on the other hand, with co-cultures, i.e. *S. cerevisiae* MTCC 173, *A. Foetidus* MTCC 151, *F. oxysporum* MTCC 1755, the ethanol increased to 16.09% (v/wt) and sugar concentration further decreased to 0.15% after 72 hours of growth. Voget et al. (1985) used apple pomace for butanol production by employing strains of *Clostridium acetobutylicum* and *C. butylicum*. Yields of butanol between 1.9 and 2.2% using fresh apple pomace were reported. The high percentage of consumed sugars and the yield of butanol show that apple pomace is a potential useful substrate for butanol production by fermentation.

Production of Hydrogen

The need for alternative energy sources has increased in recent years, due to rapid depletion of fossil fuels. Direct utilization of fossil fuels results in considerable environmental problems due to CO_2, SO_X, NO_X emissions causing air pollution and global warming (Argun et al., 2008). Environmental pollution due to the use of fossil fuels and their shortage requires the search for alternative energy sources that are environmental friendly and renewable (Wang et al., 2008). Hydrogen is considered as the most promising alternative energy, since it produces only water when combusted with oxygen and has a 2.75 times higher energy yield (122 kJ g^{-1}) than hydrocarbon fuels (Kim et al., 2009).

Hydrogen can be produced via several methods, including electrolysis of water, thermo-catalytic reformation of hydrogen and biological processes. Biological process mainly comprises the photosynthetic hydrogen production and fermentative hydrogen production or dark fermentation. One novel method to produce biohydrogen is H_2 fermentation using H_2 producing bacteria such as anaerobic and/or photosynthetic bacteria (Bartacek et al., 2007; Kalinci et al., 2009; Mathewsa and Wang, 2009). The biological H_2 production by algae, phototropic bacteria or anaerobic fermentation bacteria is not only an environmental friendly but also energy saving process (Das and Veziroglu, 2001). Biomasses such as crops,

agricultural and industrial wastes and cellulosic municipal solid wastes can be used to produce H_2 by fermentation after being hydrolyzed to sugars (Wang et al., 2010). Moreover, organic wastes can be used as fermentation substrates for biohydrogen production, which facilitates both waste treatment and energy recovery.

Potential of H_2 production from highly concentrated, carbohydrate-rich wastewaters was reviewed by Van Ginkel et al. (2005). The biogas produced using wastewater from apple processing and potato processing industries contained 60% hydrogen with no methane generation. Doi et al. (2010) used anaerobic hydrogen fermentation of apple pomace wastes mediated by rhizosphere bacterial microflora of rice. In the vial test, the optimal condition for hydrogen fermentation was initial pH 6.0, 35°C, and 73.4 g pomace per liter of medium, equivalent to 10 g hexose L^{-1}. In the batch experiment the hydrogen yield reached 2.3 mol H_2 mol^{-1} hexose. The time course of biogas production and PCR-DGGE analysis suggest that *Clostridium* spp. decomposed degradable carbohydrates rapidly and a part of the refractory carbohydrate (e.g. pectin) gradually in the apple pomace slurry. These results demonstrated that paddy fields are not only the site for rice production but can be valuable mine of industrial microbe due to their biodiversity. Higher efficiency of biohydrogen production can be expected using microflora that actively decomposes plant fibers (e.g. pectin, cellulose) as well as soluble carbohydrates in the apple pomace. In organic farming of rice, substantial amounts of fibrous organic materials such as rice straw compost are incorporated into soil and are digested in anaerobic conditions. Further exploration of paddy fields for effective microorganisms might contribute greatly to biological production of hydrogen and methane from biomass waste materials. Feng et al. (2010) studied the biohydrogen production from apple pomace by anaerobic fermentation with river sludge. The results show the potential of apple pomace as carbon source for the fermentative H_2 production. An optimal fermentation condition in this work was suggested using the apple pomace soaked in 6% ammonia liquor at room temperature for 24 hours, being fermented at 37°C, with initial pH 7.0, and fermentation substrate concentration of 15 g L^{-1}. Those conditions would produce a maximum cumulative H_2 yield of 101.08 mL g^{-1} total solid. Wang et al. (2010) performed a series of batch experiments to investigate the effects of the HCl-pretreated concentrations, enzyme hydrolysis time and temperature, the cellulase dosage, the ultrasonic time and the fermentation substrate concentration on hydrogen production from the anaerobic fermentation of apple pomace. A maximum cumulative H_2 of 134.04 mL g^{-1} total solid and an average H_2 production rate of 12 mL h^{-1} g^{-1} total solid were obtained from the fermentation of the enzyme-hydrolyzed apple pomace.

Production of Organic Acids

Among the various products obtained through microbial cultivation on apple pomace, organic acids are particularly important. The ratio of carboxylic acids manufactured microbiologically in the bulk of biotechnological products is very high (Finogenova et al., 2005; Warnecke and Gill, 2005; Vendruscolo et al., 2008). These compounds are valuable building blocks for chemical obtention, which can be used in several applications. Owing to the high carbohydrate content, other vital nutrients, high-moisture content, and abundant

availability, apple pomace can be utilized as an ideal substrate to cultivate different microorganisms for the production of organic acids such as citric, lactic, and acetic acids.

Among organic acids, citric acid production has been well studied and reported. Citric acid is widely used in several industrial processes, such as in the food and pharmaceutical industries. In recent times, much attention has been paid to the production of citric acid from diverse agro-industrial residues through SSF, such as those involving apple pomace (Pandey et al., 2000b). Citric acid production by *Aspergillus niger* is one of the finest and most commercially utilized examples of higher accumulation of intermediate products during fungus metabolism. The amount of citric acid manufactured annually exceeds 1.6 million tons, and its production is increasing at 5% a year (Finogenova et al., 2005; Sauer et al., 2008).

Hang and Woodams (1984) used apple pomace as a substrate for citric acid production by five strains of *Aspergillus niger*. *A. niger* NRRL 567 produced the greatest amount of citric acid from apple pomace in the presence of 4% methanol. The production of citric acid by fermentation of apple pomace by *A. niger* NRRL 567 was patented by Hang (1988, U.S. Patent 4767705-A). The yield was 88% based on the amount of sugar consumed. Shojaosadati and Babaeipour (2002) used apple pomace as substrate for the production of citric acid using *A. niger* in SSF. They evaluated several cultivations parameters, such as aeration rate, bed height, particle size, and moisture content. For citric acid yield, the aeration rate and particle size were the most important parameters. Neither the bed height nor the moisture content was found to significantly affect citric acid production. The operating conditions that maximized citric acid production consisted of a low aeration rate of 0.8 L min^{-1}, a high bed height of 10 cm, a large particle size ranging from 1.70 to 2.36 mm, and an elevated moisture content of 78%. Kumar et al. (2010) used SSF process for the production of citric acid from apple pomace left after juice extraction using *A. niger*. The yield of citric acid was optimized by varying the amount of methanol (1-5% v/w), temperature (25-35°C) and time of incubation (1-7 days) for fermentation process. Optimum yield of citric acid (46 mg g^{-1} of pomace) was recorded with 4% (v/wt) methanol after 5 days of incubation at 30°C. Use of apple pomace as a substrate might have economic value in the production of commercially valuable citric acid and in handling waste disposal problem. Dhillon et al. (2011a; 2011b; 2011c) used the apple pomace as an inexpensive substrate for citric acid production from *A. niger* NRRL 567 by submerged and solid state fermentation. The crucial parameters, such as total suspended solids and inducer concentration, were optimized by response surface methodology for higher production. Results indicated that total solids concentration, and methanol as an inducer, were effective with respect to higher citric acid yield and also indicated the possibility of using apple pomace as a potential substrate for economical production of citric acid and environment sequestration of waste carbon leading to mitigation of climate change.

Apple pomace has also been used for fatty acid production. Stredansky et al. (2000) evaluated the γ-linolenic acid (GLA) production in *Thamnidium elegans* by SSF. Apple pomace and spent malt grain were used as the major substrate components for the production of high-value fungal oil containing up to 11.43% biologically active GLA. *T. elegans* was grown for 8 days on a substrate consisting of a mixture of apple pomace and spent malt grain impregnated with peanut oil and nutrient solution. The solid-state fermentation process developed for GLA production was affected by diverse process variables, including substrate

composition and moisture, agitation, and aeration. Under optimized conditions, GLA yields up to 3.50 g kg^{-1} of moist substrate.

Lactic acid has a number of applications in pharmaceuticals, chemicals and food technology as acidulant, flavor, and preservative (Hofvendalh and Hahn-Hägerdal, 2000). Is mainly produced by fermentation of enzymatic hydrolyzates from starch extracted from grain. Cost reduction in lactic acid manufacture can be achieved, for example, by using cheaper raw materials, decreasing enzyme loadings, reducing the nutrient supplementation of fermentation media, and facilitating purification stages (Gullón et al., 2008). The same authors evaluated the potential of apple pomace (a solid waste from ciderand apple juice-making factories) for obtaining sugars and other compounds suitable as carbon sources for lactic acid fermentation. The effects of the cellulose to solid ratio and the liquor-to-solid ratio on the kinetics of glucose and total monosaccharide generation were assessed. When samples of apple pomace were subjected to enzymatic hydrolysis, the glucose and fructose present as free monosaccharides in the raw material were dissolved at the beginning of the process. A quantitative retention of lignin in the residual solid was observed, and 93% of uronic acids were dissolved during the enzymatic hydrolysis. These enzymatic hydrolyzates were used for lactic acid production by fermentation with *Lactobacillus rhamnosus*, leading to fermentation media containing 32.5 g L^{-1} of lactic acid. Mass balances showed that 46.5 kg of lactic acid can be produced from 100 kg of dry apple pomace by sequential hydrolysis and fermentation. Moreover, 13.4 kg of oligosaccharides (which can be used as ingredients for functional foods) and 8.2 kg of microbial biomass (which can be used as probiotic) were produced simultaneously.

Recently, Takahashi et al. (2011) investigated the production of brewed vinegar from apple pomace and its physiological function. The authors prepared a saccharified solution from apple pomace by cellulose and pectinase treatment. The solution contained 4.4 times more oligosaccharides and 2.5 times more polyphenols than the untreated sample. Moreover, the DPPH radical scavenging activities of the vinegars produced from the saccharified sample were higher than the vinegars produced without enzymatic saccharification. The results of a blind sensory evaluation of these vinegars indicated that the vinegar produced from apple pomace and commercial apple vinegar was equally accepted. As expected, an increase in the functional composition of the vinegar produced from apple pomace was observed. In addition, the vinegar had good flavor and taste.

Production of Heteropolysaccharides

Long-chain, high-molecular-mass polymers that dissolve or dispense in water to give thickening or gelling properties are indispensable tools in food product formulation. Such food polymers are also used for secondary effects, which include emulsification, stabilization and suspension of particulates, control of crystallization, inhibition of syneresis, encapsulation, and film formation (Vuyst and Degeest, 1999). The utilization of agro-industrial by-products for the production of polysaccharides by microorganisms has many advantages, such as reducing production cost and recycling natural resources (Jin et al., 2006). Factors limiting the use of microbial exopolysaccharides include their economical production, which requires a thorough knowledge of their biosynthesis and an adapted

bioprocess technology, the high cost of their recovery, and the nonfood bacterial origin of most of them (Vuyst and Degeest, 1999).

The production of xanthan through SSF of apple pomace-based substrates was studied by Stredansky and Conti (1999). The authors mixed the apple pomace with spent malt grains, which acted as an inert support to increase the medium porosity. The highest xanthan yield of 325 g kg^{-1} of dry apple pomace was obtained using a ratio of apple pomace to inert support of 2:3. Jin et al. (2002) examined the potential of three agro-industrial by-products to be used as substrate for the production of heteropolysaccharide-7 (PS-7) by *Beijerinckia indica* in submerged fermentation under the same cultivations conditions. By-products from apple juice production, soy sauce production, and the manufacturing processes of Sikhye (fermented rice punch), a traditional Korean food, were tested. The apple pomace was found to be the best carbon source for PS-7 production compared to the other by-products, giving a production or 4.09 g L^{-1} after 48 h or cultivation. When Sikhye by-product was used as substrate, 3.00 g L^{-1} of PS-7 was formed, and using the soy sauce residue, 0.96 g L^{-1} of PS-7 was observed.

Production of Biopolymers

Biopolymers are a special class of polymers produced by living organisms. Starch, proteins and peptides, DNA, and RNA are all examples of biopolymers, in which the monomer units, respectively, are sugars, amino acids, and nucleic acids. Polymers from plant, animal, and microbial origin play an important role in food formulations. Food polymers are long-chain, high-molecular-mass molecules that dissolve or dispense in water to give texturizing properties (Vuyst and Degeest, 1999).

Streit (2004) and Streit et al. (2009) studied the production of fungal chitosan in submerged and state solid fermentation using the water extract of apple pomace and the pressed apple pomace as substrate, respectively. Among the microorganisms studied, the fungus *Gongronella butleri* yielded the best results for the production of chitosan in SmF and SSF. Growth on the watery extract of apple pomace, *G. butleri* presented the highest productivity of 0.091 g L^{-1} h^{-1} and chitosan content in the biomass of 178.3 mg g^{-1} of dried apple pomace for a medium with 40 g L^{-1} of reducing sugars and supplemented with 2.5 g L^{-1} of sodium nitrate. Analyzing the chitosan production curve, it was possible to conclude that the chitosan extraction from the biomass should be carried out at the end of the exponential phase and during the beginning of the stationary phase of fungus growth, where the biopolymer represents 21% of the cell dry weight. Vendruscolo (2005) used an external loop airlift bioreactor for chitosan production by *Gongronella butleri* CCT 4274 on the watery extract of apple pomace. The experiments using higher levels of aeration provided grater concentrations of biomass and chitosan, attaining 8.06 g L^{-1} and 1,062 mg L^{-1}, respectively.

Production of Edible Mushrooms

Mushroom production is one of the few large-scale commercial applications of microbial technology for bioconversion of agricultural and forestry waste materials to valuable foods. The annual world production of cultivated mushrooms has been increasing significantly, and many methods for production and processing are being developed (Ohga and Kitamoto,

1997). Lignocellulosic materials are the main substrates for mushroom production because agricultural residues are very attractive for that purpose.

Apple pomace has been used for mushroom cultivation in SSF. Worrall and Yang (1992) compared the cultivation of shiitake (*Lentinula edodes*) and oyster mushrooms (*Pleurotus ostreatus* and *Pleurotus sajor-caju*) on apple pomace and sawdust, individually or used as a mixture. Yield on sawdust alone was generally lower than on apple pomace, which provided a faster mycelia growth. Five shiitake isolates and two *Pleurotus* spp. produced higher fresh weights on a mixture of equal parts (by weight) of apple pomace and sawdust than on either substrate alone. Highest yields were generally obtained using apple pomace mixed with ash.

Patulin is an important mycotoxin in apples and apple products, and it is also a marker of quality in the apple and apple juice industry (Morales et al., 2007; González-Osnaya et al., 2007; Katerere et al., 2008). A maximum of 50 μg L^{-1} for patulin in apple juice was considered acceptable by the Codex Alimentarius Commission. Concerned about deleterious effects of patulin in food, some investigators are trying to find chemical, physical, or biological agents able to decrease the quantity of patulin or its fungal producer (Iha and Sabino, 2008). Thus, it is extremely important to know the quality of this product and especially the content of possible toxic substances.

Production of Baker's Yeast

Baker's yeast (*Saccharomyces cerevisiae*) is part of our everyday nutrition because it is widely used in baking, beverage technology, starter cultures of various types of foods, SCP production, and food supplements. Fermentation raw materials are major contributors to the cost of low-value products such as baker's yeast (Ferrari et al., 2001). The production of baker's yeast using agro-industrial residues as a substrate has become an interesting alternative for reducing production costs.

Hours et al. (1985) used apple pomace for single cell protein from *Saccharomycopsis lipolytica* and *Trichoderma reesei* in batch and fed batch cultures. The results obtained indicate the technical feasibility of production of single cell protein from apple pomace by using unsterilized medium. The composition of the product obtained compared with alfalfa, suggests that it will only require adjustment of fiber content. It is important to bear in mind that these results were obtained with a partially altered substrate. By using fresh apple pomace, the protein values attained can be substantially increased, as was shown in small scale experiments. Values between 19 and 22% of protein on dry basis in the final product were achieved. Bhushan and Joshi (2006) used apple pomace extract as a carbon source in an aerobic-fed batch culture for the production of baker's yeast. The fermentable sugar concentration in the bioreactor was regulated at 1 to 2%, and a biomass yield of 0.48 g g^{-1} of sugar was obtained. Interestingly, the dough-raising capacity of the baker's yeast grown on the apple pomace extract was apparently the same as that of commercial yeast. The use of apple pomace extract as substrate is an interesting alternative to molasses traditionally used as a carbon source for baker's yeast production.

Production of Pigments

Color is an important attribute related to the visual appeal and the quality of food products. Due to increasing attention about the safety of synthetic colorants, the use of natural sources of colorants has been widely considered (Reyes and Cisneros-Zevallos, 2007). The pigment production by microorganisms that are capable of producing natural dyes, among them, seaweed, algae, yeast and filamentous fungi are alternatives to increase production of natural compounds against the synthetic ones. The use of fungi for the production of commercially important products has increased rapidly over the past half century. Pigments producing microorganisms and microalgae are quite common in Nature. Among the molecules produced are carotenoids, melanins, flavins, quinones and more specifically monascins, violacein, phycocyanin or indigo (Dufossé et al., 2005; Dufossé, 2006).

Attri and Joshi (2005) determined the effect of carbon and nitrogen sources on carotenoid production by *Micrococcus* sp. from apple pomace. Maximum biomass and pigment concentration were 4.13 g L^{-1} and 9.97 mg per 100 g of medium, respectively. Optimal conditions for carotenoid production were 35°C, pH 6.0, and cultivation time of 96 hours. In similar work, the same authors Attri and Joshi (2006) studied carotenoid production by *Chromobacter* sp. Using the same basic medium, they found a high production of biomass (6.6 g L^{-1}) and carotenoids (46.6 mg per l00 g of medium) and with a shorter incubation period of 48 hours. Powder apple pomace was used as a less expensive medium than Wickerham's synthetic medium for pigment production by *Rhodotorula*. The use of 50 g L^{-1} of apple pomace in the medium produced the highest yield of biomass and carotenoids. Addition of 0.3% (v/wt) ferrous ammonium sulfate gave the highest pigmentation (Sandhu and Joshi, 1997; Attri and Joshi, 2006).

CONCLUSION

This chapter has attempted to demonstrate the industrial processes applications of apple pomace. This residue, as much as its aqueous extract present a great potential for use as substrates in biotechnological processes, as well as raw material for pharmaceutical and food industries. Several studies and patents have been described regarding the employment of this residue for the production of value-added compounds, such as enzyme, SCP, biopolymers, fatty acids, polysaccharides, biofuels, and organic acids, among many others. Industrial applications of the apple pomace are interesting not only from the point of view of low-cost substrate, but also in solving problems related to the disposal of the pomace, a pollution source that has been gaining a lot of attention on apple-producing areas around the world. Several operational variables must be considered and optimized in order to effectively use the apple pomace in bioprocesses; strain type, reactor design, aeration, pH, moisture, and nutrient supplementation are only a few examples of these fundamental process variables that are crucial for the economic viability of using the apple pomace as a substrate for biotechnological applications. Nevertheless, some of the issues described here are still under investigation, in a laboratory scale, but the potential of this solid residue to be employed into different kinds of industrial processes is evident.

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Chapter 5

TRENDS IN PRECISION AGRICULTURE FOR SUGARCANE BIOREFINERIES COMPETITIVENESS

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ABSTRACT

The world sugarcane agroindustry participates with 70% of the sucrose production and integrates agricultural activities of growing, harvesting and transportation of sugarcane with production in sugar mills. However, it has challenges related to low productivity and socio-economic aspects that are a risk to food security and the agro industrial conversion to biorefineries. Regarding sugarcane productivity, Remote sensing (RS) is a vital component of a wide variety of applications across disciplines as a decision support system that combines multi-source information and could be very useful for agricultural applications and has been used to detect or assess a variety of agricultural variables: crop discrimination, and inventory, growth stage, health, nutrient requirement, spatial variations in productivity, biomass estimation and area estimation and soil physical and chemical properties sampling with complex biophysical environments by satellite imagery. It can provide farm managers data that can allow them to make quick decisions concerning their operations at various spatial scales. However, remote sensing data has not been used to its fullest potential for management of sugarcane crops largely because these data are not readily available to researchers and farmers, to understanding of effects of sensor characteristics such as spatial, spectral and radiometric resolution and scene characteristics such as acquisition date etc. and as well as integration of conventional field survey information with remote sensing data. Potentially, one of the most powerful tools, processes and techniques in precision agriculture (PA) is the use of remote sensing, Geographic Information System (GIS) and Global Positioning System (GPS), which has the ability to rapidly provide a description of canopy crops, mapping spatial variability, quality and Modeling to predict the yields for reduction of costs of production and competitiveness. The objective of this paper is to review the status and

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development of the documented applications of the role of remote sensing technology to optimize sugarcane production and improve productivity and diversification projects. We discuss advantages, limitations, strategies and future perspective with special emphasis on farm sugarcane, contribution of vegetation components to reflectance signals, as well as mechanistic relationships between biochemical processes and spectral indexes, which are compared and summarized in detail.

SUGARCANE

Sugarcane is a member of the plant family *Graminae* of which only the species *Saccharum officinarum* L. is grown commercially. It is from the Andropogoneae tribe in the grass family Poaceae, and is a tall growing cash crop cultivated in the tropical and subtropical regions of the world located with uncertain and variable climate from sea level to 1,500 m at latitudes between 36.7°N and 31.0°S because of its ability to store high concentrations of sucrose in the internodes of its stalk.

Sugarcane is widespread in tropical areas worldwide with a total of 23.8 million ha as a single crop; it has a sucrose content of 10–18% and a fiber content of 10–15% at harvest. The plant is harvested and processed to extract the final product sucrose. (Approximately 79% of total sugar production and currently, 71% of the world's sugar is consumed in the countries of origin). The average yield of sugarcane in the world is around 65 metric tons per hectare; the sugar recovery is 8.5 %.

Sugarcane is commonly propagated asexually whereby the mature cane is cut, producing new shoots and root systems from the underground part of the stalk. Its growth rate slows down with its sucrose content increasing about 120 days following planting. Normally sugarcane will be ready for harvest 12 to 18 months after planting, the harvested part being the sugarcane stalks. The new plant emerging after the first cut is known as the first ratoon, and successive generations are named accordingly.

The sucrose content of the sugarcane plant decreases with increasing number of ratoons, and depends on regionally varying environmental conditions and the variety of sugarcane grown. Different regions, mainly developing countries, display different accuracy responses to the climate outlook and several suggestions for future research are made, such as incorporating different climate forecasts and creating consortiums between climate forecasters, crop modelers and decision makers.

Traditionally, sugarcane is harvested manually; the senescent leaves (trash) and stalks, after burning, are removed by people using big knives. In favorable years there is more sugar production than needed. In adverse years it falls short due to the spatial, temporal, and ecological complexity of these processes. Some sugarcane varieties exhibit multiple modes of growth and senescence within a single annual cycle (Aguilar-Rivera, 2012).

By 2012 World sugarcane acreage was 26,088,636 ha with 1,832,541,194 t. of produced raw material. Brazil is the world's largest sugarcane grower with a 721,077,287 t. concentrated in 9,705,388 ha (39.34 % world sugar cane acreage and 37.2% of the world production). Those are mostly driven to ethanol (55%) sugar and others derivatives (45%). India is the world's second largest sugarcane producer (19 %). The four other major world producers are China, Thailand, Pakistan and Mexico.The five largest exporters since 2009/10,

Brazil, Thailand, Australia, EU and SADC, are expected to supply approximately 93% of all world free market exports (FAOSTAT, 2014; De Figueiredo et al., 2010)

Cultivation of sugarcane for bioethanol is increasing and the area under sugarcane is expanding. Much of the sugar for bioethanol comes from large plantations where it is grown with relatively high inputs and machinery. There is also risk that the sugar industry is expanding on marginal lands where the costs of preventing or repairing environmental damage may be high. With regards to the sugar outlook in developing countries, more than 80% of the ethanol produced in 2020 is expected to be based on sugar cane, which results from the dominance of Brazilian ethanol production. Brazil's sugar production, as one of the lowest cost sugar producers with considerable capacity to expand sugar cane area on a large scale, along with the projected growth in ethanol production, will be key determinants of global sugar production, which is projected to reach over 209 Mt in 2020-21 (OECD/FAO (2011).

This way, certain properties of the sugarcane plant to bioethanol with regard to commercial sugarcane farming are of major importance from an economical and social point of view. Geographic information about the sugarcane harvest development is an essential tool for crop estimation systems for processing. First of all, sugarcane is a perennial grass, and hence, is normally not integrated into a rotation system; it is commonly carried out as a monoculture and requires inputs of freshwater, pesticides and fertilizers. Furthermore, the fact that sugarcane is harvested by cutting the stalk and removing the leaves and tops which, if harvesting is not mechanized, is very labor-intensive. High sucrose content levels, which are attained at full maturity, only last for a relatively short period; therefore cane must be harvested within a limited period of time, making harvest labor-intensive and seasonal. Sugarcane agriculture, as with most other crops, is controlled by a complex system of environmental determinants such as climate, landform and soil.

Climate is a key driver that affects sugarcane productivity levels. The ideal climate is a long, warm growing season with a fairly dry, sunny, cool but frost-free ripening and harvest season free from hurricanes and typhoons.

Firstly, climate directly determines the processes of yield accumulation and the amount of sugar produced. Secondly, climatic conditions influence the development and spread of fungal diseases, insects, pests and weeds, which can restrict crop growth. Thirdly, climate, and rainfall in particular, sets the potential for runoff and deep drainage with possible environmental impact associated with the movement of nutrients and pesticides. If seasonal climate forecasts can be applied and integrated into the farm management system, forecasting offers the potential to improve farm management systems by improving yields, planting opportunities, application of fertilizers, herbicides, pesticides and irrigation, all of which influence the ecological sustainability of sugarcane production (Everingham et al. 2002)

Climate change impact assessment studies have been generally been carried out on individual crops. However, the performance of individual crops will vary significantly, when they are evaluated as part of a system. Hence, for understanding the impact of climate change on sugarcane agriculture, cropping systems need to be assessed. In this direction, simulation models have been developed and evaluated to understand the behavior of cropping systems under different climate change scenarios. Apart from the impact of climate change, the long-term sustainability of many cropping systems needs to be evaluated but it is difficult to justify using a climate forecast system to enhance decision-making capability if the climate forecast is unable to add value to current decision making approaches (Adiku, 2006).

Sugarcane puts high demands on the soil because of the use of heavy machinery and because large amounts of nutrients are removed with the harvest; biocides and inorganic fertilizers introduce risks of groundwater contamination, eutrophication of surface waters, soil pollution, and acidification. Farmers experience severe environmental constraints with regard to natural hazards that cause damage to the plant and often result in heavy yield losses. These characteristics, and the prevailing economic conditions under which sugarcane agriculture is carried out, make sustainable and optimal farming practices a particular necessity because the sugarcane is produced on small farms around the world (Mazumdar, 2007)

Sugarcane cultivation can substantially contribute to the supply of renewable energy, but improved crop husbandry and precision farming principles are needed to sustain and improve the resource base on which production depends (Van Antwerpen et al., 2013).

COMPETITIVENESS OF SUGARCANE AGROINDUSTRY

Sugar is a significant commodity contributing an annual gross value of production to the world economy. Growing sugarcane and the associated processing into raw sugar and several coproducts and byproducts such as ethanol, molasses, vinases, filter mud, bagasse and others are some of the largest and most important rural industries and underpin the economic stability of many developing countries characterized by deep complexity.

All sugar industries are being exposed to the low world price for sugar or are otherwise facing reduced income from sugar exports and hence are feeling the pressure of reduced viability.

Sugar is exported in several countries; industry success has been built on, and continues to require, the world's best practice in production, handling and marketing, as well as a benchmark for quality, supply reliability and service. At the farm gate, the sugar industry must maintain export competitiveness by innovation, particularly through mechanization, new farming practices and diversification.

The long-term sustainability of the sugarcane industry depends on continued high-quality raw sugar production, and capitalizing on energy potential and bioproducts. This will involve a range of products utilizing sugarcane biomass, and the capacity to maintain sustainable production with limited impact on the environment (Dias et al., 2013).

In addition to innovative improvements in sugarcane growing, harvesting, milling and sugar manufacturing, industry stakeholders must support initiatives that identify and foster innovation and diversification opportunities, which provide for a forward-looking and ‘broader’ sugarcane industry (Arjchariyaartong, 2006).

While fuel ethanol, electricity co-generation and other products (such as furfural) are presently a small part of industry production, these present alternatives could contribute to industry diversification and profitability while attracting positive environmental outcomes. An opportunity for the sugarcane industry lies with diversification into biofuels, other products from sugarcane, and complementary uses of sugarcane land. Diversification brings with it opportunities for exploring new markets and new products (Barnejee, 2004).

The industry is faced with several challenges and opportunities, which will incorporate both research and development and other solutions. Some these issues are:

- Restructuring of mills and mill operations
- Closure of mills putting pressure on transport systems
- Opportunities for diversification with co-generation and ethanol
- Development of genetically modified (GM) sugarcane varieties
- Declining terms of trade and the increasingly volatile sugar prices, in combination with other factors external to the industry, continue to affect farm viability
- Margin pressure is likely to lead to larger farms, corporate farming and increased sugarcane farming by mill owners (vertical integration) to manage costs
- Need for productivity increase with new technologies to improve cost competitiveness with most major producing countries and to maintain viability
- Environmental pressures and climate change will further impact input costs and farm availability (DAFF, 2001)

Sugar production activities provide significant full time and temporary employment in sugar factories, sugar transformation, transportation and exports. The sugar industry has high crushing capacities, but it could suffer from increasing wages in the future because of low labor productivity worldwide mainly in developing countries. Therefore, the study of sugar cane and the sugar industry's competitiveness (productivity, diversification and profitability) with new approaches are important, especially with the increasing liberalization of the world market and agricultural trade. Agro industry has grown into one of the world's main exporters, despite the handicap of low agricultural yields, poor cane quality, mediocre factory recoveries and a short average milling season. This serves to demonstrate that some low technology/low input/low output industries (at least on the agricultural side) can be economically competitive.

There are several reasons for this paradox. Probably the most convincing is that low yielding cane land is typically unattractive for most other crops as well. Therefore, the 'opportunity cost' of that land is low, and the price at which farmers are willing to grow cane is correspondingly low. At the other extreme, very fertile, highly productive land acquires a market value that is eventually too high to sustain cane agriculture. Research, development and extension (RD&E) is critical for increasing productivity, efficiency and profitability and ensuring sustainability and diversification capacities in sugarcane industries, where sugar industries will be relatively more efficient in terms of sugar yields and the costs of raw material low (the expenses of the processing industry on cane purchases necessary to produce one ton of sugar) (Fry, 1998).

However, notwithstanding these advantages, the sugar industry has slipped in the international competitiveness league. The conventional wisdom is that the world sugar market is a residual one, which is unrepresentative of the bulk of the trade in sugar, since only a minority of sugar is actually bought or sold at world prices. As a result, a relatively small wave or exchange in the world supply/demand balance has a large impact upon the world sugar price.

The world sugar price is more volatile than any other major commodity; and the world market has often experienced periods in the past when world sugar prices were either ridiculously low or ridiculously high in relation to local conditions for cane farming (Arjchariyaartong, 2006).

World sugar prices are being used more and more as a basis for decisions on whether to support new investments in sugar production and derivatives such as bioethanol. For both these reasons, it seems logical to suppose that sugar production costs and world prices will be related to one another (Aguilar et al., 2010).

The cost competitiveness of sugarcane producers depends on three main factors: the ability of the industry to boost its technical efficiency and take measures to reduce its production costs; the climate; and input prices. Input prices in turn depend on two main influences: the state of the local economy, and the exchange rate and competitiveness in terms of technical efficiency; which has long achieved the highest levels of productivity in terms of yields of sugar per hectare. (Plant productivity is a result of complex interactions among climate, soil, and leaf area displayed). However, many of the determinants of cost competitiveness, notably exchange rates, are not under the control of sugar producers (Arjchariyaartong, 2006; Banerjee, 2004; Zimmermann, 2002).

One of the few aspects of production which is fully under the control of sugar producers is the technology that is applied. The constraints that are imposed by these technical factors are higher fiber content and higher sugar losses, factory utilization rates, expressed in tons of sugar produced per ton of daily crushing capacity. Determining the competiveness is necessary to study the structure of sugarcane and sugar production, to analyze costs and returns of sugarcane and sugar production from the domestic and international sugar market, to examine the competitiveness of the sugar industry and identify indicators which are divided into technology, input costs, production economies, product quality and enterprise differentiation, diversification, advertising and promotion, and external factors to describe strategies of sugarcane growers and sugar factories for improving competitiveness.

In the same way, competitiveness of sugarcane production is influenced by the following location factors (comparative advantage)**:** temperature, rainfall, topography, location of farm, farm size, land tenure, need for irrigation/draining, varieties, sugarcane diseases, possibility of machinery use, sugar yields, distance to the sugar mill or distillery (transportation), duration of crushing season (zafra), cane payment system and crop diversification have all become significant factors affecting the production costs and productivity (comparative advantages). It has become increasingly important to develop tools that will enhance sugarcane productivity (TSH, TCH) and economic profits while simultaneously conserving the environment (Rivera, 2014; Banerjee, 2004; Chavarría et al., 2002).

Precision agriculture (PA) is one of many potential solutions to overcome these problems and has grown and evolved to incorporate the best of multidisciplinary science and technology to solve the riddle posed by spatial and temporal variability. In sugarcane, production is rapidly increasing, markets are forced to become more globally competitive and the production system is expected to become more environmentally friendly. In response to these pressures, precision agriculture PA promises a framework to address the economics associated with an increase in production while conserving the environmental resource base on which agriculture depends (Zamykal, 2009).

There is evidently a huge increase in the demand for feedstock worldwide and in many minds sugarcane will be a critical component for the anticipated growth. Leaders, senior managers and researchers, management, policy makers of sugarcane growing and processing enterprises and others stakeholders share in the development and advancements of sugarcane industries across the globe. It is therefore not surprising to see large investments by several multinational businesses in sugarcane R&D. The additional requirement for feedstock will

bring huge new demands on higher production per area land, production under more marginal conditions and effective utilization of existing knowledge.

However, even with a full adoption of existing technology we will not be able to adequately meet the additional market requirements. Innovative research solutions and effective technology transfer will be critical to ensure the relevance of current sugarcane R, D & E and several critical traits needed to be reexamined.

While social, political and economic changes are occurring in a developing agricultural sugarcane country, farmland is affected by both urbanization and industrialization. This process also gives rise to certain changes in the human environment and leads to changes in land use patterns that can result in overuse and natural environmental degradation.

The sugar industries play a key role in the transition from a developing country to a newly industrialized country. Developing countries sugar producers need to anticipate the influence that changes in land use will have on both the human and natural environment, keeping in mind that careful consideration of the land use policies relating to rural industry is a long-term undertaking (Sentíes-Herrera et al.,2014).

SUGARCANE DIVERSIFICATION

With changing sugar markets in the U.S.A., E.U. and around the world, innovation and environmental protection through value-addition and diversification will be crucial for the sustainability of the sugarcane industry.

Commercial sucrose has very high purity (>99.9%) making it the purest organic substance produced on an industrial scale. Several value-added commodity products are currently produced from sucrose by chemical or biotechnological derivatization. These products include oligo- and polysaccharides for specialty markets, surfactants and citric, gluconic, and lactic acids for the food sector. Increasing use of biotechnological methods to derivatize the sucrose molecule is envisaged, to add special functionalities to the sucrose products like biodegradability, biocompatibility, and non-toxicity, which are becoming important in the emerging bio-economy (Rivera, 2014).

The sugarcane industry is currently faced with the reality that sugar, molasses, and bagasse can no longer be regarded as the final products of a sugar mill or distillery. Instead, the sugar industry should be regarded as a biomass-based industry that is not only equipped to manufacture products for the food sector, but also value-added biofuels, energy, and chemicals for the non-food sector as a biorefinery (Ghatak, 2011). Sugarcane fits well into the emerging concept of a renewable carbohydrate feedstock because of its availability, and because it is amongst the plants giving the highest yields of carbohydrates per hectare. Commercialization of value-added products of sugarcane, such as fuel ethanol will depend mostly on economic factors, such as government subsidies. Vibrant global market in biofuels based on sugarcane-based ethanol is economically feasible. Cane sugar production by-products can be considered either as waste, affecting the environment, or as a resource when an appropriate valorization technology is implemented (Eggleston et al., 2007.)

Rudorff et al., (2010) concluded that sugarcane residues represent 11% of worldwide agricultural residues. It has a negative impact on air quality due to the land use, especially considering the burning practice.

There is already international trading in biofuels, but it is highly limited compared with its potential. In the current climate of accelerating fossil fuel prices, biofuels represent an increasingly attractive displacement for some of our fossil fuel addiction. Not only do they substitute for oil, but they also produce lower emissions. Sugarcane ethanol could be an important substitute for a portion of fossil fuels and also offer potential benefits for international development (Gómez-Merino et al., 2014; Rivera, 2014; Aguilar-Rivera, 2012).

AGRICULTURAL MODELS FOR SUGARCANE

While the first green revolution brought increases in productivity, these carried environmental costs and the next generation of farmers will be in the vanguard to reduce those impacts. Now, major components of the agroecological sensor web and information infrastructure such as individual sensors including remote (e.g. satellite, aircraft, unmanned drone), automated *in situ* and direct human observations collect data at multiple spatial scales are used as data-inputs to agronomic, agroecological and integrated models for key industry decisions. The output of the models can be used by producers for real-time management and long-term planning, consumers to discriminate products based on their environmental footprints, scientists to study the dynamics of agroecosystems and policy makers to guide policies to further reduce environmental impacts of agricultural production (Zhao et al., 2010; Inoue, 2008; Lisson et al., 2005; Colomboa et al., 2003).

However, the increasing complexity of data assimilation methods and of models describing agroecosystem functioning has significantly increased computational demands that maximize (crop) production while minimizing the environmental impacts. Current agroecosystem models, also known as Soil–Vegetation–Atmosphere (SVAT) models, not only incorporate biological and physiological knowledge of plants, but also model the interactions between plants and their environment to driving variables like weather conditions, nutrient availability and management variables. Output of the models usually is final yield or accumulated biomass. However, specialized SVAT models require such a large amount of input that it is virtually impossible to gather all of them with a sufficient degree of confidence. Remote Sensing (RS) data can provide information on meteorological, vegetation, and soil conditions over large areas (Dorigo et al., 2007)

Traditional methods of assessing spatial variability over large fields, such as tissue analysis and soil testing are both labor and cost intensive and require many point samples. In a detailed sampling they are generally expensive and time demanding. Other methodologies have not been rigorously explored. The agronomic diagnosis method was developed by agronomists to understand the origin of crop yield variability, identify important limiting factors, and define new cropping systems with a sound knowledge of the local conditions with access to regional accounting statistics; but unfortunately, there is still great difficulty in estimating the accuracy and anticipation necessary to supply all the information desired and necessary for a reliable statement on the situation of the whole sugar industry and to monitoring and managing the sugarcane crop (completing the field survey) which justifies the study and development of methods employing remote sensing (RS) (Bongiovanni, 2008; Benvenuti, 2005).

The theoretically-based alliance between remote sensing and conventional sugarcane statistics, in a way that is adequate to estimate crop areas, results in a step ahead in the efficient use of data from orbital sensors for agriculture aims on a regional and national level. Because of the spatial nature of these applications, efforts to parameterize a cropping systems model for spatial simulations may be limited by a lack of information to describe the required cultivar, management, soil, and meteorological inputs for unique spatial units (Aguilar-Rivera et al., 2013).

Nevertheless, the quantity of information extracted from time series of optical images is often restricted by several factors: acquisition gaps, atmospheric conditions, imperfect radiometric normalization, radiometric confusion, etc. Therefore, to make credible decisions, this information needs to be supplemented with data from other sources (complementary sources). Such information increases the effectiveness in fields and factories (El Hajj, 2009).

Thorp (2010) have shown that uncertainty in the model input parameters may degrade the predictive performance of the model, which data assimilation procedures aim to correct by periodically readjusting the simulation using spatial information from remote sensing images. Although this approach may sound reasonable for addressing the issue of limited model input data for spatial simulations, further evaluation efforts using thorough measured datasets are warranted to understand the feasibility of using remote sensing data to reliably update cropping system model simulations.

Monitoring of vegetation on a global, regional or local scale is essential for understanding dynamic biosphere processes related to food security, poverty alleviation and sustainable development. Several airborne or space-borne multispectral or hyperspectral sensors are currently able to measure the electromagnetic radiation reflected or emitted from vegetation and perennial crops and sugarcane in particular.

The acquired data are used to deduce the large-scale spatio-temporal distribution of vegetation, by using field boundaries assessment and estimate its biochemical (e.g. content of chlorophyll or water) and biophysical (e.g. leaf area index or foliage clumping) properties, disease, pest and weed infestations. Remote sensing technologies and vegetation properties are essential for ecological modeling of carbon and nutrient cycles, and estimating vegetation production on local, regional, national and global scales, natural resources management, ecosystem change detection, environment preservation and in many other world significant problems. Vegetation monitoring is among the priorities of remote sensing being associated with plant growth assessment, stress detection, yield forecasting (Kumar, 2013; Majeke, 2008; Hatfield et al., 2008; Curran, 2001; Fourty, 1996; Baret et al., 1994).

However, crop models are increasingly used to assist agronomic research and management of sugarcane production. Examples are management of irrigation and stress factors, yield benchmarking, biomass accumulation, and partitioning of the different stalk and non-stalk components. At present, sugarcane crop models that range in scale and complexity are designed to study the interactions of ecosystems with environmental drivers of change such as APSIM-Sugarcane, (*Agricultural Production System Simulator*)- *sugarcane* (Keating, 2003), CANEGRO (*sugarCANE Growth model*) (Inman-Bamber, 2005, 1993), AUSCANE, CENTURY, Canesim, MOSICAS, SUCRETTE, SUCROS, PREDPOL and QCANE (Galdos, 2010 and O'Leary, 2000,1999).

Sugarcane simulation models generally have acceptable accuracy for simulating total biomass and even stalk biomass, but current models and conceptual basis cannot adequately simulate variation and spatial and temporal distribution. Most properties in an agricultural

field show spatial dependence at many scales; therefore geostatistics is usually preferred to describe spatial variation. This is not the case for sucrose yield.

Given this variety of tools, there are many opportunities to improve the data flow between agroecological sensors, models, and end-users. Tighter integration between models and decision support systems can provide added value to decision-making processes by providing timely, relevant information to the hands of decision makers and at a small scale, to aggregate data from several monitors within a field. However, an integrated information infrastructure foundation will be needed to transform raw data into useful products, and thereby ensure effectiveness (Zaks, 2011).

Lisson et al. (2005) presented a summary of the principle physiological findings that have contributed to the key processes simulated in sugarcane simulation growth models, the integration between basic field research, model development and application and on-ground impact but they do not include a spatial component appropriate to Precision Agriculture (PA) and they are too complex to be used by a farmer or his consultants.

PRECISION AGRICULTURE PA

Precision agriculture, geostatistical tools or precision farming or site-specific management is defined as the art and science of utilizing advanced technologies for enhancing crop production while minimizing potential environmental pollution (Khosla, 2003, 2001). This technology recognizes the inherent spatial variability that is associated with most fields under crop production. PA is an innovative, integrated and internationally standardized approach aimed at increasing the efficiency of resource use and reducing the uncertainty of decisions required to manage variability on farms. PA has emerged as a valuable component of the framework to achieve this goal.

The concept of Precision Agriculture was formulated in 1986. It is based on the fact that variability of soil fertility, soil depth, micro-relief, microclimate, weed species, etc. are natural and site-dependent and therefore have a direct bearing on crop production. By this method, systematic surveys of the vegetative cover and crop yield can be better controlled and low-yielding areas can be better managed resulting in increased productivity. Other names used in Precision Agriculture are: Precision Farming, Prescriptive Farming, Variable Rate Farming, Site-Specific Management, Soil Specific Crop Management, Farming by Computer, Farming by Satellite, Computer-assisted Agriculture, Automated Agriculture, Farming by Foot, Cyberfarm, etc. (Liaghat, 2010; Daughtry et al., 2005; Bappel, 2005; Bastiaanssen, 2000).

Precision Agriculture has already been applied in the sugar industry for competitiveness, helping to provide answers to such questions as:

- Can spatial variability be managed to allow variable rate applications?
- What are the interacting factors that most affect yields in small areas?
- How can precise variable rate inputs be applied to the desired area?
- As a result of the effective management of variable rate inputs, can there be a reduction of costs of inputs?
- What are the costs involved and how cost-effective can Precision Agriculture be?

- Will it involve other changes in management of fields?
- Will this method of farming be possible for the small planters' community?
- In spite of success, what will be its rate of acceptance by the planting community? (Jhoty, 1998)

The importance of PA has been widely recognized in numerous papers (Amaral et al., 2013; Hamzeh et al., 2013; Demattê et al., 2013; Aguilar et al., 2012; Mutanga et al., 2009; Campos et al. 2009; Nascimento et al., 2009; Schaepman, 2009, 2005; Guimarães Ferreira, 2008; Paruelo, 2008, Knop, 2007, Dorigo et al., 2007, Lu, 2007; Bongiovanni et al., 2006; Sivakumar et al., 2004) as a key contributor in crop production technology around the globe, but so far, this technology is only becoming practicable on large farms.

A great source of motivation for this sugarcane PA development comes from the fact that the sugar industry relies strongly on agricultural inputs to increase yield and instead of treating specific high-infested or nutrient deficient areas, large areas are often uniformly treated with herbicides and fertilizers.

PA is based on innovative systems approach and these new systems of approach depend on a combination of fundamental technologies such as Geographic Information System (GIS), Global Positioning System (GPS), computer modeling, ground based/airborne/satellite remote sensing (RS), variable rate technology and advanced information processing for timely in-season and between season crop management. Protocols for PA implementation can be encapsulated in three general steps: (1) Gathering information about variability, (2) Processing and analyzing information to assess the significance of variability and (3) Implementing change in the management of inputs. The importance of PA evolved during the era of agricultural mechanization in the 21th century, where there is strong economic pressure to treat large fields with uniform agronomic practices (Rizzi, 2005). PA provides a means (Thematic maps) to automate Site-Specific Management (SSM), Variable-rate technology (VRT) and 'precision crop management' (PCM) using information technology, thereby making SSM practical in commercial agriculture:

- Crop information such as growth stage, health, and nutrient requirements advise markets on crop production and progress as well as for food security related questions, input to models, and on-farm management.
- Detection of plant stress in order to minimize the adverse effects on plant growth.
- Management of newly emerging pests and diseases.
- Soil physical and chemical properties, depth, texture, nutrient status, salinity and toxicity, soil temperature, and productivity potential based on topography for determining management zones to improve fertilizer management to mitigate waste.
- Microclimatic data (seasonal and daily) such as canopy temperature, wind direction and speed, and humidity as an important source of agrometeorological data in models that simulate the interactions of complex sugarcane systems.
- Surface and sub surface drainage conditions to improve Irrigation management to mitigate salinization, irrigation facilities, water availability and planning of other inputs.

- Yield maps are the key for managing crops in terms of spatial variation from several seasons and provide valuable information for defining and evaluating public policies designed to foster the use of new technologies in the sugar and ethanol industry.
- Delineating management zones (MZs) or Agro-ecological Zoning to optimize crop land use by increasing production, while decreasing agricultural inputs which consider environmental, economic, and social aspects to guide both sustainable expansion of sugarcane production and investments mainly in the biofuel sector.
- Data, remotely sensed imagery, can provide within-season monitoring to aid reliable and feasible management of the growing crop.
- Optimization of harvest operation scheduling, with reliable estimates of productivity, characterizing management alternatives, creating more realistic scenarios for decision analysis simulations and optimization in sugar mills, increasing the efficiency of management and strategic decision making during the cropping season.
- The maps generated and field data are used for monitoring the cane area, delivering inputs and planning locations for new potential planting areas and provide information on the life-cycle environmental impacts of agricultural production (Song et al. 2009, Machado, 2007; Almeida et al. 2006; Gilbert, 2006).

Precision farming technologies today are being studied and adopted for varied cropping systems. Besides the traditional crops, i.e., corn (*Zea mays* L.), soybean (*Glycine max* L.), wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.), precision farming practices are now being implemented in potato (*Solanum tuberosum* L.), onion (*Allium cepa*), tomato (*Lycopersicon lycopersicum*), sugar beet (*Beta vulgaris* L.), forages, citrus, grape (*Vitis* spp.), and sugarcane (*Saccharum* spp.) (Koch, 2003).

Many authors explored the use of PA and ground-based techniques in sugarcane for crop monitoring, especially for mapping cropping operations (sowing, harvest, irrigation, etc.), for detecting growth anomalies and changes, analyzing trends and for predicting yield. This is being done by survey of resources at different scales using traditional and remote sensing techniques, collection of collateral information like slope, topography etc, preparation of a set of resource maps (hydrogeomorphology, soils, land use/cover, surface water/drainage/watershed etc.) and generation of action plan maps giving site specific recommendations for development and management of agriculture, ground water recharge, fuel and fodder as well as for sugarcane soil conservation (Misra et al., 2013; Salgado, 2010; De Lira et al., 2009; Begue, 2008; Xavier, 2006, 2003; Ueno, 2005; Pontes et al., 2005, Simoes et al., 2005, 2003).

Zamykal (2009) describes three criteria that are required for the implementation of precision agriculture in sugarcane. First, there must be significant spatial and/or temporal variability in the soil and crop conditions within a field and among fields within a region. Second, the sugar industry must be able to identify and quantify the observed field variability. Describing variability depends on uniting the best of farmer's knowledge on field variability, positioning and information technology and quantitative expertise. Third, it is the ability and willingness to reallocate inputs and change management practices to improve productivity and profitability while improving environmental stewardship. These three criteria provide a foundation for the implementation of precision agriculture and are essential for any industry to address when embarking on the process of managing variability at any scale.

The principles of precision agriculture PA can be extended across a range of scales in sugarcane-growing communities to include the region, mill, farm, and paddock and sub-paddock level. Indeed, precision agriculture is about putting the right input, in the right amount, at the right place, in the right manner at the right time.

For precision agriculture to be successful, industry must collect, analyze, process and synthesize large amounts of information from a range of integrated enabling technologies. However, quantifying variability alone will not constitute successful precision agriculture in sugarcane production. Success will be measured by the extent to which these technologies are adopted by industry (Liaghat, 2010; Song et al., 2009).

The implementation of a PA and the requisite data collection are subject to various economic and physical constraints and the extent and style of PA implementation should be tailored to the specific geographic context. Bramley (2009) reviewed PA research and adoption around the world and considered it to be ideally suited to sugarcane production, although sugarcane producers on a large scale have not yet adopted it. It will depend on simpler and more efficient technologies and increased perception of profitability as a result of using PA along with the training of skilled labor.

REMOTE SENSING ON SUGARCANE

Remote sensing (Satellite imagery) is a new and developing discipline that incorporates advanced technologies (multi-temporal coarse resolution data based analysis) to enhance the efficiency of farm inputs with econometric and weather-based techniques in a profitable and environmentally sensible way. It is a response to the recognition that land is indeed variable. Numerous papers have the objective of retrieving quantitative information using vegetation reflective and emissive spectra to detect changes in ecosystem processes, such as carbon fixation, nutrient cycling, net primary production and litter decomposition which are all important in defining global biogeochemical cycles and identifying changes in climate (Murillo et al. 2010; Govender 2009, 2007).

The use of remote sensing technology in crop condition monitoring begins in developed countries in the West. After more than 30-years of research around the world following crop condition monitoring models, a *conceptual framework* has been developed to study the relationships between production efficiency and climatic variables. They have quantified and indicated that solar radiation, annual rainfall, water deficiency, and maximum air temperature are the main factors affecting the sugarcane production efficiency. They are: Direct monitoring models, Classification model, Same-period comparing model, Crop growing process monitoring model, Crop Growing model and Diagnosis model; this can help more countries, departments, organizations and individuals to have the opportunity to use crop condition information (Ji-hua, 2008; De Souza, 2008) .

To model a sugarcane farm in remote sensing; the sugarcane leaves represent the main surfaces of plant canopies where energy and gases are exchanged. Their optical properties are essential to understanding the transport of photons within vegetation. Most papers (Thorp, 2010; Adam et al., 2009; Schaepman, 2009, 2005; Jacquemoud, 2009, 2001; Hatfield et al., 2008; Nagendra, 2008, Kumar, 2001, 1973; Moran, 1997) have focused on the leaf spectral properties (hemispherical reflectance and transmittance) in connection with their biochemical

content (chlorophyll, water, dry matter, etc.) and their anatomical structure establishing empirical relationships between the variables of interest and the leaf reflectance or transmittance, or better still, by directly using a physical model.

The spectral variation of canopy reflectance or Canopy Reflectance Sensitivity to Leaf Reflectance is mostly governed by the optical properties of the elements such as the leaves: lignin, protein, cellulose, hemicellulose, sugar, starch, chlorophyll and water that are materials directly or indirectly involved in bio-geochemical cycles and productivity (Soares et al., 2008; Wah Liew, 2008; Merzlyak, 2008; Brizuela et al., 2007; Senay et al., 2000; Monteith, 1976).

Since leaf intrinsic scattering properties show very little spectral variation, leaf optical properties are related to their absorption properties. Relationships between anatomical and biochemical canopy leave parameters and assessment of physiological plant activity have been documented at different levels by Ustin (2013); Feng *etal* (2008); Curran (2001, 1990, 1989); Blackburn, (2008). These papers identified that the driving forces behind remote sensing for farm management are the need for information on foliar chemistry and increased understanding and ability to measure foliar spectra.

The optical properties of leaves with their biochemical process is a priority to connect the method of conventional statistical analyses (soil, foliar, productivity etc.) with the data that is acquired from the remote sensed image to estimate the biochemical productivity of leaves. Remote sensing techniques provide a very convenient way of data acquisition capable of covering a large area several times during one season, so it can play a unique and essential role in providing remote sensing measurements to the biochemical characteristics of crops such as sugarcane. It collects data in the fields to define standards for the interpretation and identification the sugar cane stages, satellite imagery interpretation for mapping sugar cane areas; going to the field to check inconsistencies in the interpretation, reinterpreting the satellite images for generation of thematic maps; generation of thematic maps containing only the areas planted with the sugar cane in different dates; generation of spreadsheets and graphs with the quantitative data of the areas, green phytomass estimation, plant growth evaluation and yield prediction and analysis of the sugar cane plantation changes in the study area (Aguilar et al., 2010; Rodrigues et al., 2007; Gitelson et al., 2003; Daamen, 2002; Asner, 1998; Daughtry et al., 1982).

Ground-based VIS and NIR spectral measurements have been carried out along with phenological and biometrical observations in order to establish empirical relationships between plant reflectance features, growth variables, productivity and treatments applied (Rudoff, et al., 2010, Borisova, 2009; Huber, 2008; Shengyan, 2002).

The revision of the state of the art of sugarcane remote sensing by Abdel-Rahman (2010, 2009 and 2008) concluded that when light interacts with sugarcane canopies, as with any land surface, it is partly reflected, absorbed and/or transmitted. The reflectance spectra of crop canopies are a combination of the reflectance spectra of the plants and the underlying soil; the spectral response of the sugarcane plant depends on four factors: canopy architecture, foliar chemistry, agronomic parameters and the geometry of data acquisition and atmospheric conditions. However, the most important factor affecting the optical properties of the sugarcane canopy is its geometrical structure; the amount of light intensity penetrating the sugarcane canopy and interacting with the lower leaves. This is related to the canopy architecture that is also influenced by the pigments in the leaves, such as chlorophyll a and b, carotene, xanthophyll and anthocyanins.

Other foliar nutrients can affect sugarcane spectral behavior either directly by absorption or indirectly through their influence on plant physiological processes, that affect the vegetative development and interfere with sugarcane spectral response (Zhao et al. 2010; Delegido *etal* 2009; Adam et al. 2009; Majeke, 2008; Zarco, 2008; Wulder, 1998; Datt, 1998).

Jacquemoud et al., (2009); Farias, (2008) and Terashima (1995) concluded that radiactive transfer models have helped in the understanding of light interception by plant canopies and the interpretation of vegetation reflectance in terms of biophysical characteristics. Since they attempt to describe absorption and scattering, the two main physical processes involved in such a medium, canopy radiactive transfer models are useful in designing vegetation indexes, performing sensitivity analyses, and developing inversion procedures to accurately retrieve vegetation properties from remotely sensed data.

Measurement of spectral reflectance or spectral reflectance characteristics of sugarcane leaves (Spectral Signature and chlorophyll content) (Knipling, 1970) is non-destructive, rapid and relatively cheap, and can be applied across spatial scales; so remote sensing has attracted a great deal of attention in terms of application for crop monitoring so far (Figure 1).

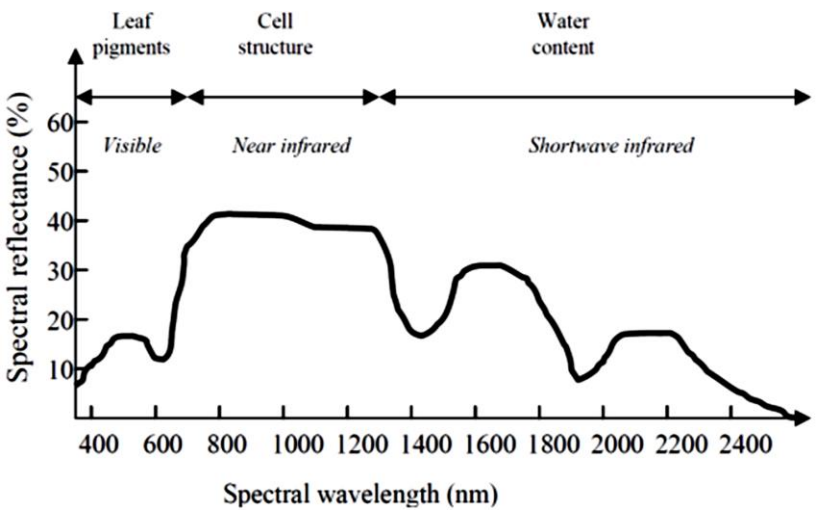

Figure 1. Typical reflectance sensitivities from vegetation (spectral signature) as controlled by leaf pigments, cell structure and water content (Govender, 2009).

Sugarcane spectral behavior is also affected by different agronomic parameters. The most important of these factors is the leaf area index (LAI). For the potential use of remote sensing for making day-to-day sugarcane management decisions, there is overall importance to the cane growers of timely, up-to-date and accurate data. Despite the recent development of new sensors and processing techniques, very few cane farmers presently have access to regular images of their farms, and even when they do, slow turnaround of the processed product continues to be a problem (Xie et al. 2008; Elwadie, 2005; Schaepman, 2005; Koetz, 2005; Daamen, 2002; Pellegrino, 2001; Schmidt, et al. 2000; Hadsarang, 2000; Narciso, 1999; Lee-Lovick, 1991).

This approach could also be applied to elucidate the biophysical factors that affect sugarcane crop yields at a regional and local scale: physical processes, including regional

pedoclimatic conditions; structural components, corresponding to the agricultural systems and management practices adopted; institutional effects, involving governmental actions affecting price, credit, commercialization, and incentives; and research and development, related to innovations to increase yield and solve problems that restrict agricultural-related activities (Marin et al. 2008).

The application of remote sensing and GIS techniques in the management of agricultural resources are increasing rapidly due to improvement in space borne remote sensing satellites in terms of spatial, spectral, temporal and radiometric resolutions. Many conventional approaches of handling multi-thematic information to arrive at optimal solutions are being computerized using GIS utilities. Keeping in mind the development in satellite, computer and communications technologies, the following opportunities and challenges exist in the application of remote sensing GIS and precision sugarcane farming technologies worldwide (Straschnoy, 2006; Patil, 2002).

However, Aguilar (2010) concluded that as the variability in the growing conditions become more complex, the multidisciplinary partnership between farmers, scientists and information technologists becomes integral for actuating the precision agricultural process.

Remote sensing data and techniques have a large potential for use in the prediction of sugarcane yield [tons of stalks per hectare (t ha^{-1})] and has been studied with satellite data and agrometeorological models for:

- Monitoring sugarcane health, condition and nutritional status in maintaining the competitive advantages of the sugar industry.
- Detection of nutrient and water deficiencies
- Use of remote sensing and precision farming technologies in intercropping/multiple cropping situations.
- Identifying ways and means of reducing the cost of RS, GIS and PA technologies and time gaps in collection, interpretation and dissemination of data to enable their usage on a large scale (Murillo et al., 2010; Da Silva, 2005, 2001; Zhou, 2003; Graeff 2003; Lumsden, 1998).

The use of remote sensing for mapping sugarcane crops has already been attempted by several researchers (Aguilar-Rivera et al., 2013, 2012; Gonçalves, 2010, 2008; El Hajj, 2009; Soares et al., 2008; Fortes, 2006; Singels, 2005).

Vikesh et al., 2010; Auynirundronkool et al. 2008; Johnson et al. 2008; Govender et al. 2009, 2007, Senay et al., 2000, and Bowker, 1985 determined the correlation analysis between the difference reflectance index (Spectral Signature) and chlorophyll content. However, only a few investigations using hyperspectral or Multispectral sensor (separability or discriminating features of different varieties of sugarcane) data in agriculture are related to sugarcane (Apan et al., 2004; Gers, 2003) and its cultivar discrimination (Galvao et al., 2006, 2005) (Figure 2).

Schmidt et al., (2001) investigated the use of a multispectral sensor to identify sugarcane varieties and to determine various crop conditions, including moisture stress, crop age, nutritional status, ripener response, and yield potential.

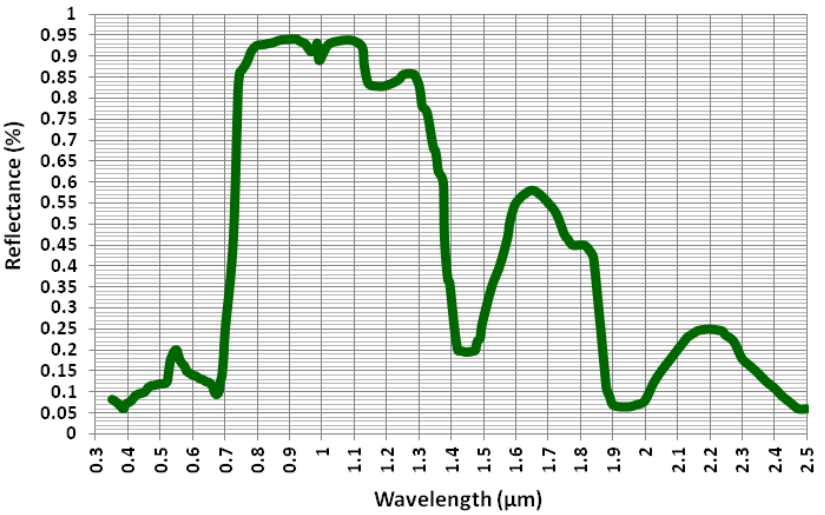

Figure 2. Sugarcane spectral signature.

Gers (2003, 2001) attempted to utilize Landsat 7 Enhanced Thematic Mapper Plus (ETM+) and SPOT imagery to determine sugarcane phenology and to discriminate between different varieties.

Johnson et al., (2008) reported the use of hyperspectral remote sensing techniques, with high spectral resolutions, in combination with plant pigment analysis may significantly improve the ability to discriminate between sugarcane varieties. Wavelengths ranging from 560 - 720 nm could be used to discriminate between varieties.

Remote sensing (RS) techniques have been widely considered to be a promising source of information for land management decisions, Remotely sensed images acquired by aircraft and satellite-based sensors: IKONOS, Quick bird Advanced Very High Resolution Radiometer (AVHRR), Advanced Space Borne Thermal Emission and Reflection Radiometer (ASTER), Satellite Pour l' Observation de la Terre (SPOT), Landsat Thematic Mapper (Landsat TM), and Moderate Resolution Imaging Spectrometer (MODIS). Each satellite sensor has different spatial, temporal and spectral characteristics (Brito Silva, 2011; Yang et al., 2011; Malenovsky, 2009; Xie et al., 2008; Carbonell, 2007; Daamen, 2002). Satellite sensors that have a higher spatial resolution include IKONOS and Quick bird, while Landsat TM and SPOT can be viewed as medium spatial resolution sensors and MODIS, as lower resolution (Figure 3). SPOT and Landsat are the most commonly used satellite sensors for sugarcane plantations (Rudoff et al., 2010; Upadhyay, 2008).

They have the potential to provide a synoptic view of an entire field, a continuous description of growth and development and not just the sample sites, within the time and space requirements of PCM applications for several agricultural products, such as herbicides, fertilizers, insecticides and seeds to a smaller coefficient of variation (CV) for conventional management (Xie et al., 2008; Machado, 2007). Remote sensing (RS) provides a primary source of input data for GIS and many PA applications. A GIS can provide farm managers an effective method to visualize, manipulate, analyze and display spatial data, providing the backbone of a PA system (Narciso, 1999).

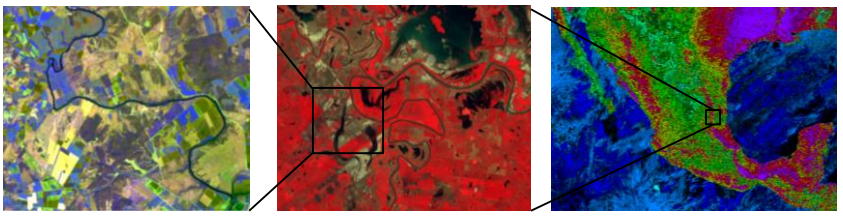

Figure 3. SPOT 5 HVR, Landsat ETM+ and NOAA-AHVRR imagery for sugarcane land at Huasteca, Mexico (Aguilar et al., 2010).

The amount of detail contained in the imagery is dependent on the attributes of the satellite (spatial and spectral resolution). Embedded within the spectral signature is detailed quantitative and qualitative information about the medium being examined e.g. crop vegetation; to fully exploit the information captured by the modern satellite apparatus. Statistical analysis methods are needed to extract relevant information embedded within the more voluminous image data, so that agricultural sugarcane managers can capitalize on this knowledge to improve sustainable agronomic practices (Everingham et al., 2007).

It is interesting to note that sugarcane cultivation presents favorable characteristics to be monitored to quantitatively evaluate several aspects related to the sugarcane cultivation dynamic by using remote sensing satellite images multispectral and multi-temporal such as Landsat MSS, TM, ETM + or SPOT 5 HVR:

1) To map the recent expansion of sugarcane cultivation and the spatial dynamics land cover changes
2) To monitor the sugarcane harvest for the classification of fields with and without straw burning practices prior to harvesting
3) To identify the land use prior to the additional sugarcane expansion of crop year (Aguilar-Rivera, et al., 2012)

Rudoff et al., (2010, 2004, 1985), Schmidt et al., (2000), mentioned numerous applications of remote sensing and key aspects in the agribusiness of sugar cane:

- Sugarcane characteristics of cnterest for remote sensing: A key aspect that promotes the use of remote sensing imagery for applications in sugarcane is the fact that it is a semi-perennial crop that reaches its maximal growth in summer. Therefore, the long permanence of the crop in the field increases the chance of acquiring cloud free images, which is fundamental for mapping the cultivated areas, monitoring the harvest and evaluating the land use change dynamic.
- Growth Cycle: An understanding of the sugarcane (Saccharum spp. L.) growth cycle is important for the correct identification and mapping of the different crop classes in remote sensing images. The sugarcane cycle is semi-perennial and begins with the planting of a stem cutting that grows for about 12 months (year sugarcane) or 18 months (year-and-half sugarcane). After the first harvest, the ratoons are harvested annually for a period of about 5 to 7 years or more. Successive harvests lead to a gradual yield loss until the crop is no longer economically profitable. At this point, the cycle is interrupted, and the area is renovated with the planting of new stem

cuttings. Should the ripe sugarcane not be harvested, it will keep on growing to be harvested in the following year.

- Harvest: Sugarcane harvesting extends from April to December (southern Hemisphere) or November to May (northern Hemisphere) and is performed in two ways: either manually or mechanically. For manual harvesting, the sugarcane crop is burned prior to cutting the stems in order to eliminate the straw thus making the harvest easier. For mechanical harvesting, burning the straw is unnecessary since the machine cuts off stems and straw leaving the latter scattered on the ground. Interestingly, with mechanical harvesting, the straw residues that remain on the field after the stems are removed look very bright in satellite images, whereas burned areas, where the straw was converted to ashes, appear dark and more similar to bare soil.
- Expansion of Cultivation: The direct land use change caused by the recent and significant expansion of sugarcane can be evaluated from remote sensing images previously recorded for sugarcane plantations. This evaluation allows locating and quantifying the actual amount of pasture land, food production areas, natural vegetation and reforestation, which were converted to sugarcane plantations, and provides relevant information that would otherwise be difficult to obtain.

In the established scenarios based on the current agricultural management systems and the climate patterns that affect development of the sugarcane crop, a new source of uncertainty must be taken into account–the temperature and water availability scenarios resulting from climate changes and/or global warming; especially how sugarcane crops respond to a new thermal regime, the physiological and metabolic adaptations, and the consequent modifications to which crops will be subject (Brunini, 2010).

VEGETATION INDEX

Vegetation Indexes are mathematical transformations, usually ratios or linear combinations of reflectance measurements in different spectral bands, especially the visible and near−infrared bands. They are widely used in remote sensing practice to obtain information about surface characteristics from multispectral measurements, taking advantage of differences in the reflectance patterns between green vegetation and other surfaces (Ustin, 2013; España-Boquera, 2006; Elwadie, 2005; Payero et al., 2004; Imanishi, 2004; Brog; 2002). Through the years, a great number of vegetation indices have been proposed, ranging from very simple to very complex band combinations as:

- Modified Chlorophyll Absorption in Reflectance Index (MCARI):
  - $MCARI=[(R_{700}-R_{670})– 0,2(R_{700}-R_{550})] R_{700}/R_{670}$
  - $R\lambda$ reflectance (nm)
- Transformed CARI (TCARI):
  - $TCARI=3[(R_{700}-R_{670})– 0,2(R_{700}-R_{550})R_{700}/R_{670}]$
- MERIS Terrestrial Chlorophyll Index (MTCI):
  - $MTCI = [(R_{750}-R_{710})/(R_{710}-R_{680})]$

- Triangular Chlorophyll Index (TCI):
  - $TCI=1,2(R_{700}-R_{550})–1,5(R_{670}-R_{550})(R_{700}/R_{670})^{1/2}$
- Red – edge model (R-M):
  - $R–M = R_{750}/R_{720} – 1$
- Normalized Difference Vegetation Index (NDVI):
  - $NDVI = (R_{800}-R_{670}) / (R_{800}+R_{670})$
- "Green" NDVI:
  - $NDVI_{Green} = (R_{750}-R_{550})/(R_{750}+R_{550})$
- Normalized Area Over reflectance Curve (NAOC):

$$NAOC = 1 - \frac{\int_{643}^{795} R \, d\lambda}{152 \, R_{795}}$$

A number of studies have shown that the normalized difference vegetation index (NDVI) derived by dividing the difference between infrared and red reflectance measurements, by their sum, provides effective measure of photosynthetically active sugarcane biomass.

Tucker (1979) studied different linear combinations of RED, GREEN, and NIR bands for monitoring vegetation properties such as biomass, leaf water content, and chlorophyll content. He found that the ratio and related NIR and RED linear combinations were superior to the GREEN and RED linear combinations for monitoring vegetation.

NDVI = (NIR-RED)/(NIR+RED)

The value of this index ranges from-1 to 1. The common range for sugarcane green vegetation is 0.2 to 0.8 (Figure 4).

Several studies also discussed the suitability of temporal NDVI profiles for studying vegetation phenologies, especially those of crops such as sugarcane to identify (i) areas having different vegetation cover types and (ii) agricultural areas following different crop-calendars (Aguilar et al., 2010; Abdel-Rahman, 2008).

Several authors have sought to map land-cover phenology, dynamics and degradation through multi-temporal NDVI data obtained through AVHRR / NOAA images (Murillo et al., 2010; Nascimento et al., 2009; Heuminski de Ávila, 2009, De Bie et al., 2008; Lucas; 2007).

The value of information is in the improved decisions it enables. The typical image of precision agriculture is of an intensive crop management system, served by high technology. This contrasts strongly with the image of developing agriculture as a low- or no-technology activity, undertaken by subsistence farmers with minimal resources or inputs.

Expanding the current agroecological monitoring and analysis systems will not only require new technologies, but cooperation between governments, academia, private industries and farmers as well (intersection of science, technology, agriculture, and policy in decision-making processes). However, in an innovative multidisciplinary approach, this will require a more robust system to collect, analyze and disseminate data on the functioning of the agricultural sugarcane system. There are few published studies that provide data on the adoption of PA in sugarcane countries and producers to obtain higher yields, lower costs, minimization of environmental impacts and improvements in sugarcane quality and the

impacts of PA technologies, information sources used for implementing these technologies, synergies, problems and obstacles that affect their adoption.

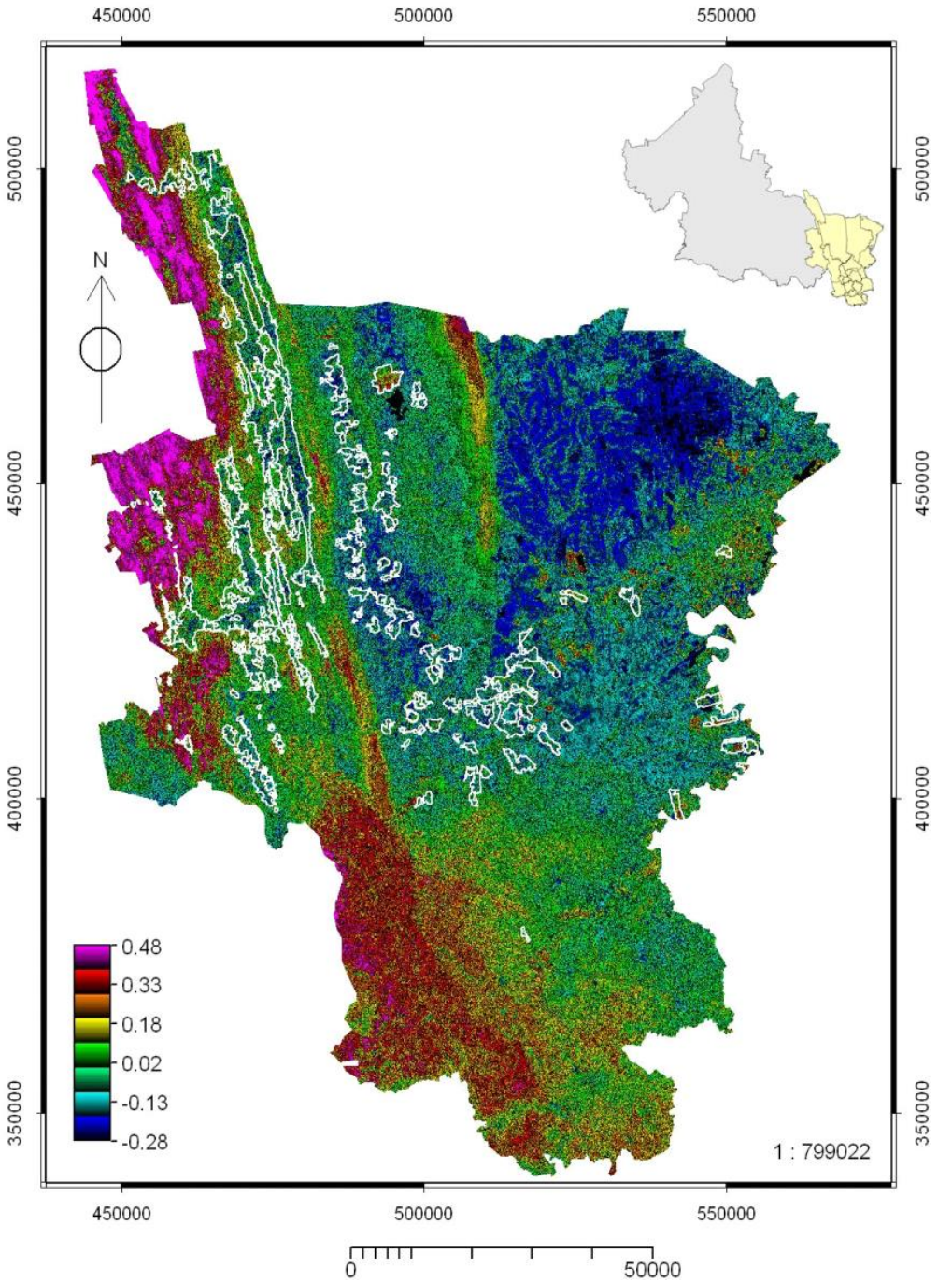

Figure 4. Sugarcane NDVI from Huasteca Mexico (Aguilar et al., 2010).

Understanding the complex interactions among the multiple factors affecting crop growth is the foundation of any attempt to improve management systems.

What makes PA different is its ability to capture data on the production practices actually applied in the field and on the results achieved.

A basic premise of PA is that more and better information can reduce the uncertainty of the producer's place in decision-making.

A unique combination of remote sensing data and detailed (stratified) ground survey is necessary to generate a spatial database of different cropping systems to characterize the cropping systems with respect to their diversity and land utilization capability. The cropping system information must be integrated with information related to the soil nutrient level and water quality in order to understand the sustainability and ecological impact of different cropping systems such as sugarcane.

Extending our understanding of the State-of-the-art optical remote sensing of vegetation canopies, and the methods used to retrieve remotely quantitative biophysical and biochemical characteristics of vegetation canopies, is required for unambiguous interpretation of remotely sensed data (geostatistical techniques) on competiveness.

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Chapter 6

FRUIT BASED FUNCTIONAL BEVERAGES: PROPERTIES AND HEALTH BENEFITS

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ABSTRACT

Fruit juice has been considered as one of the staple beverages from many decades. Fruit juice and juice-based beverages have increasingly been promoted on a health platform and has been consumed worldwide due to the natural abundance of carbohydrate, essential vitamins, minerals and other nutrients in them.Fruit juices also possess several reported health benefits, such as aid in digestion, high antioxidant properties, anti-inflammatory properties, and combating several diseases. A functional beverage is a non-alcoholic drink product that is formulated with ingredients, such as raw fruits, herbs, vitamins, minerals, amino acids and other bioactive compounds that provide specific health benefits. In the recent years, functional beverages have become increasingly popular due to its several specific health benefits. The present chapter focus on various fruit based functional beverages, their nutritional benefits, physiological benefits and functional attributes to human health.

Keywords: Fruit juice, functional beverage, non-alcoholic beverage, bioactive compounds

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LIST OF ABBREVIATION

CVD	Cardiovascular disease
DPPH	1,1-diphenyl-2-picrylhydrazyl radicle
CHD	Coronary heart disease
DNA	Deoxyribonucleic acid
UTIs	Urinary tract infections
LDL-C	Lipoprotein cholesterol
5-HT	5-hydroxytryptamine
DHA	Docosahexeanoic acid
EPA	Eicosapentaenoic acid

1. INTRODUCTION

Various functional foods and non-alcoholic beverages with nutraceutical value and positive health benefits are now available in the market.A nutraceutical is any substance considered as a food, or part of food which in addition to its normal nutritional value, provides health benefits including disease prevention and health promotion (Hegde et al., 2014). Likewise, a functional beverage is a non-alcoholic drink which consists of ingredients, such as herbs, vitamins, minerals, amino acids or additional raw fruit or vegetables in its formulation.Fruits and fruit-based products, such as fruit juice, fruit juice drinks, and fruit nectarsarehave been gaining popularity as a functional drink due to their reported health benefits, such as aid in the prevention of cancer, aid in digestion, anti-inflammatory properties, increased bone strength and treatment of cardiovascular disease (CVD), among others (Jandric et al., 2014). The demand for fruit-based product in the juice market is substantially growing over recent years due to its wide range of health benefits owing to the presence of large amounts of various phytochemicals in it (Hassan et al., 2010). Recent research indicates that the market for fruit juice and juice drinks has been increased by 43% from 1999 to 2009(Caswell, 2009).

Currently, there are several fruit-based ready-to-drink beveragesrich in different classes of biologically active anti-oxidants, polyphenolics, isoprenoids, essential vitamin and mineral contents are available in the market (Mintel, 2008). There are also reports on development of functional beverages by combining various fruits and other plant extracts to formulate drinks with particular health benefits, such as treatment of CVD (Gunathilake et al., 2013). Various fruits, such as orange, cranberry, blueberry, grape, apple among other fruits with high nutrient value and excellent antioxidant propertiesare being used in formulation of functional beverage are being explored by nutritional researchers (Morton et al., 2008; Gunathilake et al., 2013). Recently, beverage consisting of extracts of fruits, such as apple, cranberry and blueberry rich in different classes of phenolics together with ginger extract and cardio-protective minerals, amino acids and vitamins has been investigated for ability to reduce the risk of CVD (Gunathilake et al., 2013). The present chapter gives an insight into fruits and fruit-based juices, their nutraceutical importance, functionality, and health benefits in treatment or prevention of various health problems.

2. AN OVERVIEW OF FRUIT JUICE AND HEALTH BENEFITS

It is generally believed that fruit and vegetable consumption is associated with good health. The health benefits of fruit and fruit-based drinks stem from their micronutrients, antioxidants, various bioactive compounds and phytochemicals present in them (MargettsandButtriss, 2003; Gryet al., 2007). Several investigations have been carried out in the past decade on various health benefits of the fruit and fruit based functional drinks. This section deals with some of the health benefits of consuming fruit juice in combating various diseases (Caswell, 2009).

The beneficial effects of fruit and vegetables on CVD and cancer risk are well documented (MargettsandButtriss, 2003; Stanneret al., 2004). Various studies showed that fruit-based drinks offer remarkable benefits in combating CVD and cancer. For instance, Bazzanoet al. (2002) reported that consuming more than three servings of fruit and vegetables per day had a 42% reduction in stroke mortality and a 27% reduction in risk of CVD mortality compared to those who consumed less than one serving per day. Epidemiologic and clinical trials suggest that fruit consumption may lower the risk of CVD through blood pressure lowering effects. A meta-analysis study reported by Dauchetet al. (2006) showed that there is a 4% reduction in the risk of coronary heart disease (CHD) for each additional serving of fruit and vegetable and 7% for intake of fruit per day. Several studies also been conducted to show significant protective effect of fruits and vegetables against a variety of forms of cancer (Block et al., 1992). Studies conducted by 'American Institute for Cancer Research' on diet and cancer concluded that fruits and vegetables may have protective effects against a range of cancers including mouth, stomach and bowel cancer (WCRF/AICR, 2007). Variousstudies showed the positive impact of fruit juices on reducing breast cancer risk, risk of renal cell carcinoma, oxidative DNA damage, reduction in oxidative cell damage (involved in the pathogenesis of atherosclerosis, cancer and diabetes) and an increase in blood glutathione levels (an antioxidant) (Ruxtonet etal., 2006;Weiselet etal., 2006; Caswell, 2009). Citrus fruit juice consumption has been associated with protection against ischemic stroke (Joshipuraet al., 1999). Cranberry juice has long been associated with the treatment of urinary tract infections (UTIs) (Jepson and Craig, 2008). It was reported that one of the influencing mechanisms in the development of atherosclerosis is oxidation of the cholesterol-rich low density lipoprotein cholesterol (LDL-C) particles (Steinberg et al., 1989; Witztumand Steinberg, 1991; Aviram, 1993). The oxidation of LDL-C increases the atherogenicity and enables diffusion of lipids into the arterial wall, causing the occlusion of coronary arteries (Dembitsky et al., 2011). Frequent consumption of grape juice which is rich in variety of flavanoids has been associated with improved endothelial function within the lining of blood vessels, flow mediated vasodilation and reduced LDL-susceptibility to oxidation (Stein et al. 1999). Moreover, the dietary fiber and polyphenols of fruits improve lipid metabolism and prevent the oxidation of LDL-C, which helps in lowering the development of atherosclerosis (Gorinstein et al., 1998). The grape juice consumption reduces platelet aggregation associated with atherosclerotic disease development (Freedman et al., 2001; Keevil et al., 2000). Consumption of two to three Kiwifruits a day has shown 15% reduction in blood triglyceride levels and platelet aggregation response to collagen (Duttaroyand Jorgensen, 2004). It has also been demonstrated that consumption of berries are associated with increased plasma hydrophilic or lipophilic antioxidant capacity (Chidambara et al., 2010). There are also some

suggestions in the literature that daily consumption of citrus juices and those from berries (at doses of at least 200 mg of vitamin C per day or more) help in combating cold and flu symptoms (Dembitsky et al., 2011).

3. FRUIT BASED BEVERAGE SOURCES AND NUTRITIONAL BENEFITS

Several fruits and fruit juices consumed in our day to day diet contain various bioactive compounds, such as flavonoids, vitamins, minerals, carotenoids and many other important phytochemicals with various medicinal and functional properties which prevent several chronicle diseases associated with heart, liver and kidney. Table 1 presents some of the important bioactive compounds found in fruits commonly used for preparation of juices.

Table 1. Principal bioactive active compounds present in some fruits commonly used for preparation of fruit based juices

Fruit type	Bioactive compounds	Reference
Apple (*Malus domestica*)	Gallic acid, chlorogenic acid, catechine, rutine, quercetin, epicatechin	Lee et al., 2003; Dhillon et al., 2013
Avocado (*P. americana Mill.*)	Serotonin, Monounsaturated fatty acids and sterols	Feldman and Lee, 1985; Plaza et al., 2009
Banana (*Musa paradisiaca*)	Serotonin, Flavonoids Lignans	Someya et al., 2003
Berry (family *Rosaceous*, genus *Rubus*)	Ellagitannins, Anthocyanins, Proanthocyanidins, Stilbenes, Cyanidin-3-O-g lucoside, Pelargonidin-3 -O-glucoside, Quercetin-3-glucoside, Myricetin-3-glucoside	Määttä-Rįįhįnen et al. (2004)
Dragon fruit (*Hylocereussp*)	Tannins, DPPH (1,1-diphenyl-2-picrylhydrazyl), Vitamin C	Rebecca et al., 2010
Grapes (*Vitisvinifera L.*)	Unsaturated fatty acids, Phenolic acids Flavonoids	Maier et al., 2009
Guava (*Psidiumguajava*)	Vitamin C, β-caryophyllene, Aromadendrene and O-containing sesquiterpenes	Ekundayo et al., 1991; Chen et al., 2008
Litchi (*Litchi chinensisSonn.*)	Epicatechin, Procyanidin A2, β-citronellol, Geraniol	Sun et al., 2010; Sun et al., 2010
Mango (*M. indica L.*)	β-carotene, Mangiferin, Catechins, Quercetin, Kaempferol and llagic acids	González-Aguilar et al., 2008; Gil et al., 2006
Orange (*Citrus sinensis*)	Gallic acid, Poncirin, Didymin, vitamin C	Ramful et al., 2010
Persimmon (*Diospyros kaki L.*)	7-methyl-1,4,5-trihydroxynaphthalene 4-O-(6′-O-β-xylopyranosyl)-β-glucopyranoside, p-Coumaric acid, Catechin, Epicatechin and Epigallocatechin	Giordani et al., 2011; Gondo et al., 1999
Pineapple (*AnanascomosusMerr.*)	Gallic acid, Catechin, Epicatechin, Serotonin and Ferulic acid	Yi et al., 2006
Pomegranate (*Punicagranatum L.*)	Gallic acid, Caffeic acid, Catechin, Quercetin and Anthocyanins	Poyrazoglu et al., 2002

Fruit type	Bioactive compounds	Reference
Rambutan (*Nepheliumlappaceum L.*)	Ellagic acid (EA), Corilagin, and Geraniin.	Thitileadecha et al., 2008
Star fruit or Carambola (*Averrhoacarambola*)	Proanthocyanidins, Catechin or Epicatechin	Shui and Leong, 2004a, 2004b

The following section discusses some of the fruits, fruit juices and their nutraceutical importance.

3.1. Avocado (*Perseus americana*)

Avocado belongs to the Lauraceae family, which is a native plant of Mexico and Central America. There are about 500 varieties of avocados all over the world, which differ in fruit shape and color (Dembitsky et al., 2011). Non-processed avocado does not have any specific taste, however, its pulp is widely consumed as a fruit juice. Typically, the avocado pulp contains fat, protein, mineral salts like, manganese, phosphorous, iron and potassium, fiber, folic acid and vitamins (Vitamin E, B1, B2, and D).Avocado is also rich in serotonin 5-hydroxytryptamine (5-HT) which is animportant class of neurotransmitter and a signaling molecule that plays central role in the development of neural circuits and plasticity (Serfaty et al., 2008). Avocado is also rich in several bioactive compounds such as sterols (β-sitosterol, stigmasterol, and campesterol) and monounsaturated fatty acids (oleic acid, linoleic acid, palmitoleic acid, cis-vaccenic acid, and γ-linolenic acid) (Plaza et al., 2009).

Research has shown that avocado has a positive influence on short-term memory and reduces the risk of cardiovascular disease. High level of omega-6 fatty acids and mono-saturated fatty acids present in the avocado helps control cholesterol levels and improve cardiovascular health (Dembitsky et al., 2011). The high levels of antioxidants and vitamin E in the avocado help prevent skin aging and skin damage. Avocado also contains carotenoid, lutein which helps in keeping eyes safe from oxidative stress, macular degeneration, and cataracts. Several studies had also shown that avocado can inhibit the growth of cancerous cells without damaging the healthy cells.

3.2. Apple (*Malus domestica*)

The apple of rose family (*Rosaceae*) is the most widely cultivated deciduous tree fruit that originated in Central Asia and spread worldwide with more than 7500 cultivars like Gala, Red delicious, Fuji, Pacific Rose, Golden delicious, McIntosh, Lobo, Ananasrenette, Yellow transparent, Pink lady etc. Originating from its wild ancestor, *Malussieversii*different cultivars had been raised for diverse uses including fresh eating, cooking and production of cider, syrup, jelly, juices and various alcoholic beverages (Setorki et al., 2009). Apples are used to control diarrhea or constipation and softening and passing of gallstones. Research suggests that daily consumption of apple can modestly help reduce body weight. Apple also have a role in prevention of cancer (especially lung, colon and prostate cancer), diabetes, dysentery, fever, heart problems, warts, and a vitamin C deficiency condition called scurvy (Setorki et al., 2009). Apple has high content of dietary fiber having antioxidant properties

and are associated with prevention of diverse diseases, such as diverticular and coronary heart diseases. High content of polyphenols, phenolic acids as well as hypolipidemic and antioxidant properties of apples makes this fruit desirable as a dietary supplement (Larrauri et al., 1996; Belitz and Grosch, 1999). The phenolic acids found in apple are caffeic, *p*-coumaric and ferulic acids which are acknowledged to have high antioxidant activity (Nardini et al., 1995). Another compound, malic acid that is known to help the peristaltic movements in the human intestine has been reported in apple pomace (Halbware-Preisnotierung, 2007). Some of the phenolic compounds, such as gallic acid, chlorogenic acid, catechine, rutine or quercetine and triterpenes like betulinic acid, oleanolic acid or ursolic acid have also been reported (Grigoraş et al., 2012; Dhillon et al., 2013). Other predominant phenolic phytochemicals in apples are quercetin, epicatechin, and procyanidin B2 with considerable antioxidant activity (Lee et al., 2003; Dhillon et al., 2013).

3.3. Berries

Edible berries have been a part of human diet for centuries. The most commonly consumed berries are strawberries, raspberries, blackberries, blueberries, black currants and red currants. There are also a number of crosses between raspberries and blackberries available, such as the loganberry (Beattie et al., 2005). Berries contain several bioactive compounds which are essential for health. High amount of ascorbic acid (vitamin C), vitamin B, folic acid, anthocyanins and flavonols are present in berries. However, their concentrations are influenced by many factors including environmental conditions, degree of ripeness, cultivar, cultivation site, processing and storage of the fruit (Boyles and Wrolstad, 1999; Hakkinen and Torronen, 2000). Some of the health benefits of berries include enhancement of collagen synthesis, iron absorption, platelet aggregation, thrombus formation and may have a role in preventing heart disease, osteoporosis and a range of cancers, catalyzing nitric oxide formation and maintaining DNA stability (Byers and Guerrero, 1995; Davey et al., 2000; Khaw et al., 2001; Verhaar et al., 2002).

3.4. Citrus Fruits

Citrus is a genus of flowering plant in family Rutaceae consisting of fruits like orange, lemon, Tangor, grapefruit, Calamondin, Pamplemousses, Satsumah, Clementine, Mandarin etc. The genus is mostly cultivated for commercially important fruits which are consumed fresh, processed as juices and preserved as pickles (Nicolosi et al., 2000). The juice consists of high quantities of citric acid which is responsible for its representative flavor and taste. Citrus fruits are rich in vitamins, minerals, dietary fiber and also in biologically active compounds that help reduce risk of many chronic diseases. Adequate folate and vitamin C intake through citrus fruits help decrease the risk of cardiovascular disease, cancer, neural tube defects, anaemia, cataracts, osteoporosis and kidney stone disease (Cleveland et al., 1996; Fleming et al., 1998). Consumption of citrus fruits supplements plenty amount of vitamin C, folic acid, potassium and pectin along with phytophenolics, such as phenolic acids, flavonoids, stilbenes, tannins and lignans that scavenge free radicals to prevent several diseases listed above (Gorinstein et al., 2004; Proteggente et al., 2003). The flavonoid profile

of several citrus fruits consists of poncirin, dydimin, naringin, hesperidin, neohesperidin, neoeriocitrin and narirutin. Other than this rutin (flavonol glycoside) and rhoifolin, diosmin and isorhoifolin (flavone glycosides) were also identified in the extracts (Ramful et al., 2010). β-Cryptoxanthin was also reported as the chief carotenoid followed by β-carotene in several citrus fruits (Wang et al., 2007).

3.5. Guava (*Psidium guajava*)

Guava is an important fruit, which originated in the tropics of America. The fruit can be consumed as such or can be processed as juice. Consumption of ripen fruit with the skin improves digestion. The daily consumption of guava normalizes blood pressure and improves digestion (Dembitsky et al., 2011). The typical fruit composition consists of proteins, fats, calcium, phosphorus, iron, fruit sugar, vitamin A, vitamin C, and fiber. The vitamin C content of guava is almost 5 times more than oranges (Dembitsky et al., 2011). Guava also has a very high concentration polyphenols which act as an antioxidant (Isabelle et al., 2010; Kongkachuichai et al., 2010).

3.6. Grape (*Vitis vinifera*)

Grape fruit and juice is rich source of polyphenols, catechin, anthocyanidins and resveratrol. Several biological activities and health benefits have been reported for grape and grape juice. Grape is also an excellent source of bioactive polyphenols, such as anthocyanins, flavonols, dimeric, trimeric and polymeric procyanidins, and phenolic acids (gallic acid and ellagic acid) which make it an excellent health drink (Vislocky et al., 2010). Grape and grape related products are being investigated for health benefits against liver disease, cardiovascular disease, cancer development and progression, alzheimer's disease and other neurodegenerative disorders, aging and alterations in cognitive and motor function, antiviral activity, oral health, immune function and diabetes (Vislocky et al., 2010). Several studies have also been carried out to show the beneficial effects of grape products on liver (Bian et al., 1998; Bouhamidi et al., 1998).

3.7. Litchi (*Litchi chinensis)*

Litchi originated in the Kwantung province in southern China. The aril part of the fruit has a juicy, translucent texture and tastes sweet and slightly sours (Dembitsky et al., 2011). It is typically used in processed form as canned litchijuice/syrup. A large number of polysaccharides (mannose, galactose, and arabinose), antioxidants and polyphenols (epicatechin and procyanidin) have been identified in the pericarp tissues of harvested litchi fruits (Dembitsky et al., 2011). Health benefits of litchi include effective regulation of blood glucose levels, efficient free radicle scavenging activities of 1,1-diphenyl-2-picrylhydrazyl radicle (DPPH), hydroxyl radical and superoxide radical(Edel et al., 2006).

3.8. Mango (*Mangifera indica*)

Mango is one of the abundantly available fruit in the tropical region with a global production exceeding 27 million tons, and considered to be second largest tropical crop next to banana(FAOSTAT, 2008). Mango can be consumed as such or processed to juice and jam. Mango is rich in ascorbic acid,β-carotene, and phenolic contents (Gil et al., 2006;Gonzálezet al., 2008). Mango is abundant in several important bioactive compounds and antioxidants, such as polyphenols, flavonoids, flavanols and tannins (Liu, 2003). Some of the potent antioxidative polyphenols in mango are mangiferin, catechins, quercetin, kaempferol, rhamnetin, anthocyanins, gallic and ellagic acids, propyl and methyl gallate, benzoic acid, and protocatechuic acid. The amounts of various polyphenolic compounds in the mango differ from part to part, such as pulp, peel, seed, bark, leaf, and flower with most polyphenols being found in all the parts (Dembitsky et al., 2011). Several carotenoids, such as β-carotene, luteoxanthin, neochrome, 9-cisviolaxanthin, and unusual carotenoid ester violaxanthindibutyrate have also been identified in mango. (Dembitsky et al., 2011). The nutraceutical and pharmaceutical significance of mangiferin, a unique polyphenol in mango, have been demonstrated to combat degenerative diseases, such as heart diseases and cancer (Dembitsky et al., 2011). The polyphenols work mainly as antioxidants and help to protect human cells against oxidative stress damage leading to lipid peroxidation, DNA damage, and several degenerative diseases (Masiboand He, 2008).

3.9. Pineapple*(Ananas comosus*)

Pineapple is a juicy and fibrousfruitwith sweet and sour taste, native to South America and has now spread throughout the tropical world (Dembitsky et al., 2011). The fruit is used as fresh and processed to juice, jam, and glaze.Phenolic compounds such as, gallic acid, catechin, epicatechin and ferulicacidwere found in peel of pineapple, while pulp and core of the pineapplecontainsepicatechin and ferulic acid (Dembitsky et al., 2011; Yi et al., 2006). Some of the sterol components reported in the pineapple include, β-sitosterol, stigmasterol, citrostadienol, campesterol, isofucosterol, stigmast-4-en-3-one, valencene, nootkatone, ergostanol, ergostanol and stigmastanol (Dembitsky et al., 2011). Several biologically active phytochemicals, such as flavonoids, saponins, tannins, cardenolides and bufadienolideshave been reported in exocarp of pineapple (Dembitsky et al., 2011). A pharmacologically active compound called Bromelain is present in stems and fruits of pineapple, which has anti-inflammatory, anti-invasive and anti-metastatic properties. Recently, there are also reports on antitumor-initiating effects of bromelain in mouse model (Bhui et al., 2009).

3.10. Pomegranate (*Punica granatum*)

Pomegranate is a fruit predominantly cultivated in India, Spain, Israel and USA (Hegde et al., 2014). The pulp of pomegranate can be consumed fresh or processed to obtain juice. Some of the important bioactive compounds found in pomegranate include phenolic acids, such as ellagitannins, flavonoids, and tannins. It has also been reported that the juice, seed and peel of pomegranate contains steroid hormones, estrone and fatty acids such as punicic

acid, palmitic acid, stearic acid, oleic acid and linoleic acid (Viuda-Martos et al., 2010). One of the abundant polyphenols in pomegranate juice is a hydrolysable tannins called ellagitannins (also known as punicalagins), which is formed by binding of ellagic acid with a carbohydrate. Punicalagins has an excellent free-radical scavenging properties (Viuda-Martos et al., 2010).Some of the medicinal and functional properties of pomegranate incudeantioxidant, antitumoral or antihepatotoxic, and helps to improve cardiovascular health (Viuda-Martos et al., 2010). Pomegranate also exhibits antimicrobial, anti-inflammatory, antiviral and anti-diabetic properties (Viuda-Martos et al., 2010). Hepato-protection properties of pomegranate and its derivatives against hepatic oxidative stress have also been well studied in different animal models (Mena et al., 2011).

4. CURRENT TREND OF FRUIT BASED FUNCTIONAL BEVERAGES

Due to the increasing health consciousness, obesity concerns and life style choices the market trend for functional beverages is souring at 14% per year since 2002 (Mintel, 2008).Fruit-based functional beverages are the largest segment of sales of functional beverages as they account for 58% of the functional beverages sold in America (Mintel, 2008). Currently, there are number of commercial ready-to-drink beverages which claim to have high antioxidant potencies due to their perceived high content of polyphenolic antioxidants are available in market (Mintel, 2008) (Table 2).

Table 2. Annual sales of some fruit based juices in USA

Category	Sales (million $)
Apple Juice	11.6
Blended Fruit Juice	315.2
Cider	44.5
Cocktail Mixes	0.3
Cranberry Cocktail/Drink	2.5
Cranberry Juice/ Blend	0.7
Fruit Drink	684.4
Fruit Nectar	23.4
Grape Juice	0.4
Grapefruit Cocktail/Drink	0.5
Grapefruit Juice	130.1
Lemon/lime Juice	3.1
Lemonade	73.8
Orange Juice	3,078.9
Pineapple Juice	8.9
All Other Fruit Juice	8.6

Adopted from Thor and Savitry, 2012.

Minimally processed fruit juices are one of the major growing segments in the food market. Retention of most of the bioactive compounds of the fruit in the process of juice making is very important and challenging task in the commercial marketing. One of the effective approaches to fulfill this need is membrane processing technology. Membrane

processing provides a means of concentrating, fractionating and purifying fluids, generating two streams that are different in their compositional characteristics. Reverse osmosis, ultrafiltration and microfiltrationare the most common types of membrane filtration used in concentrating fruit juices (Gunathilake, 2012). Ultrafiltration and microfiltration are associated with the separation of larger macromolecules, using a selective filtration process, whereas reverse osmosis is used for concentration of juices (Girard and Fukumoto, 1999). Membrane processing techniques are relatively efficient and beneficial in terms of energy because no dehydration occurs during the process, which avoid the use of intensive heat transfer equipment and the ambient processes temperatures in the range of 4 to 50°C minimize the thermal damage of products rich in antioxidants (Girard and Fukumoto, 2000; Gunathilake, 2012).

The trend of blending of various fruit juices to improve its bioactive profile is a new area of research in the human nutritional research (Gunathilake, 2012). For instance,González-Molina et al. (2009)developed a health drink with improved organoleptic properties and enhanced bioactive content and antioxidant properties by combining lemon juice and pomegranate juice. The same research group has also reported that enrichment of 5% chokeberry with lemon juice substantially increase the antioxidative property and certain organoleptic properties of the drink (Gonzalez-Molina et al., 2008). Recently,Gunathilake et al., (2013) reported a formulation of functional beverage designed to be cardio-procreative by blending the juices of cranberry (50% v/v), blueberry (12.5% v/v), apple (37.5% v/v), ginger (2% v/v) and selected cardio-protective ingredients. Some other examples of blended functional juice include, 100% orange juice fortified cholesterol-lowering sterols from plant sources (Caswell et al., 2008),orange juice enriched with longer-chain omega-3 fatty acids, docosahexeanoic acid (DHA) and eicosapentaenoic acid (EPA) (Caswell, 2009).Various other combinations of fruits, such as orange, cranberry, blueberry, grape, and apple are also being explored in formulation of functional beverage to treat various diseases by human nutritional researchers (Gunathilake et al., 2013, Morton et al., 2008).

CONCLUSION

Extensive research in the past decade demonstrated that several types of micronutrients, antioxidants, phytochemicals and other nutraceuticals present in the fruit and fruit based juice benefit health in many ways. This fact is also evident from the trend of increased consumption of fruit juice and the considerable growth in the fruit juice market over the past 20-30 years. Though traditional fruit juices, such as orange and apple juice still dominate the market (over 70%), today new flavors, such as cranberry juice are constantly being introduced. Apart from targeting the consumer satisfaction, the commercial juice manufacturers are also focusingupon health benefits. New trend of juice enrichment with vitamins, minerals, as well as various different functional ingredients to enhance the nutritional properties has also been initiated to make the fruit juice healthier. Due to the fact that proper dietary supplements reign as the first line of defense to prevent chronic diseases and even to reverse it in its earliest stages, many people are turning into fruit based functional drinks. As several diseases like obesity, CVD, cancer, and liver disease are becoming widespread health concerns today, by adding fruit based functional juice to the regular diet,

people can add some extra assurance to their quest for preventing the accumulation of such diseases.

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