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Application of Biotechnology to the Production of Natural Flavor and Fragrance Chemicals.

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ABSTRACT

During the past years biocatalytic production of fine chemicals has been expanding rapidly. Flavors and fragrances belong to many different structural classes and therefore represent a challenging target for academic and industrial research. Here, we present a condensed overview of the potential offered by biocatalysts for the synthesis of natural and natural-identical odorants including flavors and fragrances, highlighting relevant biotransformations using microorganisms and isolated enzymes. The industrial processes based on biocatalytic methods are discussed in terms of their advantages over classical chemical synthesis and extraction from natural sources. Recent applications of the biocatalytic approach to the preparation of the most important fine odorants are comprehensively covered. Flavors and fragrances are extremely important for the food, feed, cosmetic, chemical and pharmaceutical industries. Most available flavor compounds are now produced via chemical synthesis or extraction. Drawbacks of such chemical processes are the formation of undesirable racemic mixtures and the growing aversion of the consumer towards chemicals added to his food, cosmetics and other household products. This has caused flavor companies to direct their attention towards flavor compounds of biological origin, so called natural or bio-flavors. Up to now, plants were also an important source of natural flavor and fragrances represented by their essential oils However, active components are often present in minor quantities or in bound form which make their isolation not economic or difficult leading to expensive flavor or fragrance compounds. Apart from plant cell and tissue culture techniques a directly viable alternative route for flavor synthesis is based on microbial processes, i.e. fermentation (= *de novo*) and bioconversion of appropriate precursor compounds. This review presents the current state of the art of bioflavor-synthesis, based on microorganisms (bacteria, fungi, yeasts) and their enzymes, with emphasis on currently commercialized processes. It also comments on regulatory aspects of biotechnological production of aroma-compounds such as the advances in solid state fermentation, the bioreactors used for the production. A comprehensive referenced literature survey of de novo fermentation and of bioconversion processes for flavor-compound synthesis and recovery of volatile compounds and the modeling approaches used for the theoretical study of transfer in these processes concludes this review.

Keywords: Microorganisms; Bio-flavors, Fragrances; Hydrolytic Enzymes; aroma compounds, biotransformation; plant cell cultures.

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INTRODUCTION

Flavors and fragrances are generally extracted from aromatic plants since times immemorial. The composition of flavor compounds from plant sources range from single to complex substances. Their chemical compounds are elucidated by the modern sophisticated techniques like mass spectroscopy and NMR and followed by production on industrial scale suing chemical synthesis. Flavors are generally used as food additives and they are mostly produced through artificial means i.e. chemical synthesis or by extraction from plant sources. Recently due to consumer's increased interest and health awareness in natural products, there has been more stress towards the use of flavors and fragrances obtained from natural sources. Research has shown that microbes can be used to produce aroma and fragrance compounds which can be labeled "natural". Man is using microbial systems from ages to develop aroma to fermented products like beer, wine, cheese etc. from ages. Vanillin (1874) and coumarin (1868) were the first synthetic flavor and fragrance compounds made available for use in the food industry. The worldwide demand of flavors and fragrances was estimated to be US$ 16 billion in the year 2013. Most of these flavoring and fragrance compounds are prepared by chemical synthesis and only a small fraction of the demand are met from plant or through microbial sources [1].

Flavors and fragrances have a wide application in the food, feed, cosmetic, chemical and pharmaceutical sectors [2]. These compounds greatly influence the flavor of food products and govern their acceptance by consumers and their market success. The increasing consumer preference for natural products has encouraged remarkable efforts towards the development of biotechnological processes for the production of natural flavor compounds [3].

These compounds represent a wide variety of chemical classes including hydrocarbons, alcohols, aldehydes, ketones, acids, esters or lactones, all of which are used as food flavoring additives [4].

The methods for obtaining aroma compounds include the direct extraction from aromatic plants, chemical transformations and biotechnological transformations (which include microbial and enzymatic biotransformations, *de novo* synthesis and the use of genetic engineering tools) [5].

There are some challenges that encounter the use of natural flavors from natural sources including (i) low concentrations of the product of interest (ii) seasonal variation of the quality of the extract and (iii) possible ecological problems involved with the extraction especially if it takes place using organic solvent for extraction as in case of essential oil from flowers [3].

Despite the great industrial application of aroma compounds produced *via* chemical synthesis (still responsible for a large portion of the market due to the satisfactory yields), this strategy is also associated with a number of environmental challenges and hardly presents adequate regio- and enantio-selectivity to the substrate, resulting in a mixture of molecules. In addition, the increasing interest for "natural" labeled products has led to intense research on the microbial production of the so-called 'bioflavors' [6-7].

This great interest for natural products has encouraged aroma industries to develop new biotechnological processes to obtain aroma compounds naturally. Although many biotechnological processes have been reported, most of them have not yet been applied to the industrial production of these compounds due to the low yields and therefore to the high costs of down-stream processing. This results in an increasing price of the naturally produced compounds, which is 10–100 times higher than that of the synthetic ones. For example, the γ-decalactone (flavor compound of peach) extracted from a natural source costs about US$6000/kg, while the synthetic one costs US$150/kg [8].

In this sense, there is a growing demand for biotechnology to provide alternatives for the production of natural flavorings and fragrances [9]. Thus, biotechnology offers the potential to produce natural aroma compounds in a commercial scale with many advantages over the chemical processes: the reactions occur at mild conditions, present high region- and enantio-selectivity and do not generate toxic wastes. Additionally, there are some compounds that may be produced exclusively *via* biotechnology [10].

Although microorganisms have been used to produce flavors for a long time, especially when considering the preparation of traditional fermented foods and beverages, only recently the relationship between microbial development and the typical desirable flavor of fermented foodstuff was recognized. Further analysis and optimization of such food fermentations led to the investigation of pure microbial strains and their capacity to produce specific single flavor molecules, either by *de novo* synthesis or by converting an

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added substrate/precursor molecule [2]. Thus, this review is intended to cover all aspects of flavor compounds formation from fungi and bacteria, focusing on recent progress and highlighting the most relevant developments in this area. This review is intended to demonstrate important advances with micro-organisms and plant cell that are able to transform flavor precursors directly into flavor molecules (biotransformation) or produce flavors along multi-step processes (bioconversion and *de novo* synthesis). Representative data are presented to demonstrate that a wide variety of compounds possessing unique and potent flavor properties have been isolated as naturally occurring constituents of biocatalysts, such as fungi and bacteria. The aim of this review is to view the current state of the art of bioprocesses used for the production of natural flavor compounds, with emphasis on the methodologies used to improve the production yields, and on the applications of *in situ* product removal techniques. Several approaches will be described with particular attention to microbial and enzymatic syntheses of natural aromatic com-pounds, engineering aspects of solid state fermentation, chiral separation and extraction, mathematical modeling and design of bioreactors.

DEFINITION OF BIOTECHNOLOGY

Biotechnology is defined as: the use of living systems for the production of useful products. These systems may be plants, animals, microbes or any other parts of their organisms. According to this definition, the biotechnological methods for production of flavor and fragrance compounds include:

1- whole microbial cell,
2- Utilization of microbial pure isolated enzymes (as inTable1)
3- Plant cell and tissue culture
4- Genetic engineering of an already existing aromatic plant.

At the moment, the whole cell microbial processes seem to be the most promising for the production of pure aroma and flavor compounds or complex mixtures of them.

In principle there are two kinds of biotechnological aroma production:

a- *de novo* synthesis and
b- Biotransformation/bioconversion.

De novo synthesis implies the production of aroma compounds using simple cultivation media without any special additions (Fig.1). On the other hand, biotransformation/bioconversion refers to the synthesis of one or several aroma substances by adding precursors of the products to the cultivation media. While *de novo* synthesis uses the metabolic spectrum of the microorganisms and there fore in general produces a mixture of several aroma compounds, the biotransformation/bioconversion leads to one major product, which is produced by one (biotransformation) or several (bioconversion) biochemical steps [11].

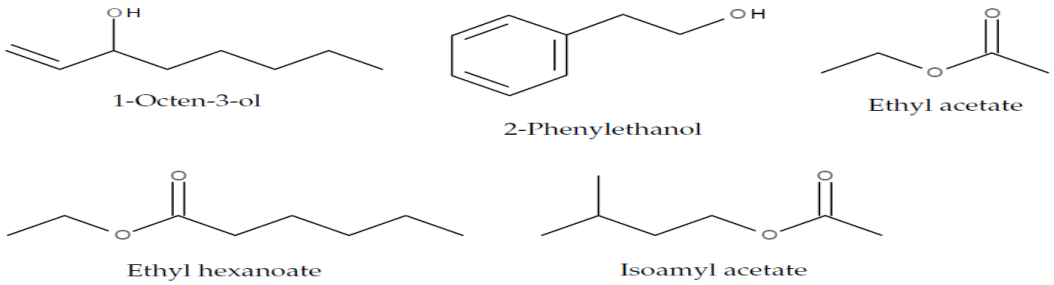

Fig.1. Structure of some biofl avors obtained by *de novo* synthesis.

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Table 1: Flavors and Fragrances Produced by some Microorganisms or using Enzymes Adopted from [6]

Flavour type	Flavour active component	Microorganism/enzyme involved in production
Almond	Benzaldehyde	*Ischnoderma benzoinum* (a bracket fungus)
Apple and pine apple	Butyric acid	*Clostridium butyricum*
Flavour component of dairy products	2,3-butanedione	*Lactic streptococci*
Rose-like odor	Citronellal	*Rhodotorula minuta* (yeast)
Flavour component of many essential oils	(+)-Curcumene	*Saccharomyces cerevisiae* (baker's yeast)
Peach	γ-Decalactone	*Yarrowia lipolytica* (yeast)
Coconut-peach	δ-Decalactone	Enzymatic reduction of the α, β-unsaturated compound (massoia lactone)
Flavour component of many essential oils	(+)-Dehydro-curcumene	*Saccharomyces cerevisiae* (yeast)
Citrus type fragrance	(-) Isopulegol	Lipase (*Pseudomonas* sp.)
Mint	(-)-Menthol	Lipase (*Candida rugosa*)
Expensive fragrance compound	Nor-patchoulenol	*Pithomyces* sp. (mould)
Flavour component of many essential oils	(+)-Nuciferal	*Saccharomyces cerevisiae* (yeast)
Rosary	Phenolethanol	*Kluyveromyces* sp. (yeast)
Spearmint flavour	β-pinene	Lipase
Raspberry	Raspberry Ketone	*Beauveria bassiana* (fungus)
Chocolate flavours	Thaumatin and monellin	*Kluyveromyces* sp. (yeast)
Flavour component of many essential oils	(+)-Turmerone	*Saccharomyces cerevisiae* (yeast)
Vanilla	Vanillin	*Pycnoporus cinnabarinus* (fungus)

Flavors and fragrances: types and synthesis

Flavors and fragrances are broadly divided into two categories: natural and nature-identical. Natural flavors are prepared by extraction from plants or by enzymatic or microbial processes, and nature-identical flavors and fragrances are synthesized chemically or by conversions of natural substrates. The natural flavors are produced either by de novo synthesis using microbes or plants or through single-step biotransformation of natural substrates by microbes or their enzymes or plant cells (e.g. synthesis of nootkatone using citrus cell cultures). In de novo synthesis, microbes transform carbon or nitrogen compounds into flavor molecules with the help of enzymes such as lipases, proteases, nucleases and some glycosidases. These enzymes are extensively used in industry for the synthesis of flavoring compounds as they catalyze single-step 2 transformations of substrates into natural flavor molecules. Furthermore, microbiological or enzyme based processes have also been developed where complex substrates such as lignin, phenyl-propanoids and phenolic stilbenes are converted to the desired flavors and fragrances [12].

Natural flavors

United States Code of Federal Regulations (1985) and European Communities (1988) legislations have meant that 'natural' flavor substances can only be prepared either by physical processes (extraction from natural sources) or by enzymatic or microbial processes, which involve precursors isolated from nature (Fig.2). This classification created a dichotomy in the market because compounds labeled 'natural'

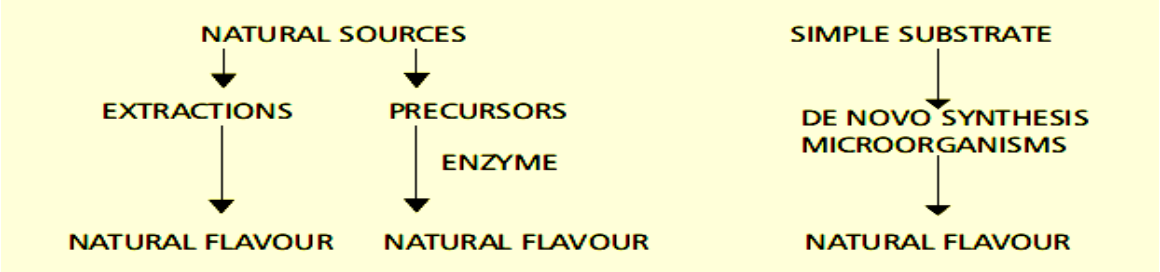

Fig.2. The three pathways for the preparation of 'natural' flavors. The first two involve the extraction of the flavor or precursors from natural sources.The last method is the de novo synthesis of the flavor by microorganisms growing on simple substrates such as glucose and sucrose.

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Become profitable products whereas other flavors that occur in nature but are produced by chemical methods must be called 'nature-identical' and are less appreciated by consumers. These differences have stimulated much research aimed at developing new biotechnological processes for these flavoring compounds. The 'natural' routes for flavor production are the bioconversions of natural precursors using biocatalysis, *de novo* synthesis (fermentation) and isolation from plants and animals. Although from the chemist's point of view there is no difference between a compound synthesized in nature and the identical molecule produced in the laboratory, the price of a flavor sold as natural is often significantly higher than a similar one prepared by chemical synthesis. For example, vanillin (Fig.3, compound 1) is the most important flavor in terms of consumption levels [13]. This compound occurs in the pods of tropical Vanilla orchids (mostly *Vanilla planifolia*) at levels of 2% by weight, but less than 1% of the global market is covered by the extracted compound. The value of vanillin extracted from pods is variously calculated as being between US$1200/kg and US$4000/kg, whereas the price of synthetic vanillin, that is vanillin prepared mainly from guaiacol, is less than US$15/kg. Therefore, several biotechnological processes for natural vanillin production have recently been developed including the bioconversion of lignin, phenylpropanoids (ferulic acid, eugenol, isoeugenol) and phenolic stilbenes (isorhapontin) in addition to the de novo biosynthesis [12].

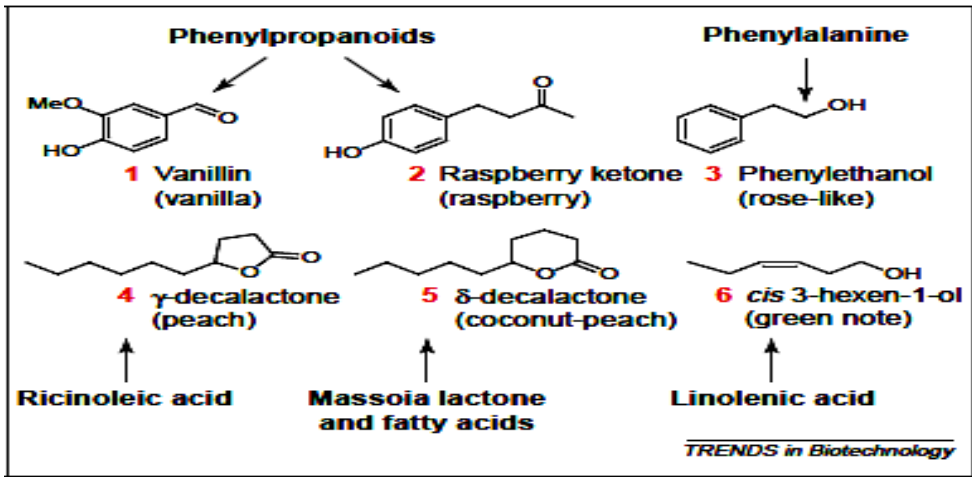

Fig.3. Examples of some relevant natural flavors prepared by biotransformation.

Similarly, raspberry ketone (Fig.3, compound 2) and 2-phenylethanol (Fig.3, compound 3) are phenylpropanoids used in industries as flavors and/or fragrance ingredients. Compound 2 is the key flavor molecule of raspberries in which it occurs in trace amounts (4 mg of ketone from 1 kg of berries). Compound 3 has a rose-like odor and occurs in fermented foodstuffs and in many essential oils. For both compounds extraction is unsuitable and their main mode of production is the bioconversion of some natural precursors [14]. 4-(4-hydroxyphenyl) butan-2-ol (betuligenol), its O-glucoside (betuloside) and 4-hydroxybenzalacetone are possible precursors for biotechnological production of raspberry ketone performed by oxidation of the secondary alcohol of the first two compounds and by double bond saturation of the third, using different microbial systems [15-16]. In the context of biogeneration of raspberry ketone in the fungus *Beauveria bassiana*, it emerged that odour inactivation of compound (2) occurs through Baeyer-Villiger oxidation to tyrosol [17]. Moreover, 2-phenylethanol (3) and its acetate are currently produced by yeast degradation of natural L-phenylalanine [18].

Lactones (Fig. 3, compounds 4, 5) and cis-3-hexenol (Fig. 2, compound 6) are also natural flavors produced at the industrial scale. Compounds (4-5) and analogues with up to twelve carbon atoms are widespread in fermented food, milk products and in a variety of fruits in minute amounts. Some of these materials are manufactured by degradation, via b-oxidation, of natural hydroxy-fatty acids [19-20]. Specifically, the γ-decalactone (compound 4) is obtained by chain shortening of C-18 ricinoleic acid (from castor oil) by different microorganisms. Improvement of the processes caused the selling price of compound (4) to decrease from US$ 12000/kg in 1986 to US$ 500/kg in 1998 [19]. Similarly, some precious γ-lactones containing an odd number of carbon atoms are accessible by degradation of natural hydroxy acids [21]. Interestingly, δ-decalactone (5) can be obtained by natural modification either by oxidation of hydroxy-fatty acids or by enzymic reduction of the α-β-unsaturated compound (massoia lactone) the main component of massoi bark oil

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[22]. Linolenic acid is the natural precursor of cis-3-hexen-1-ol (leaf alcohol) (Fig. 3, compound 6). This compound has an odour of freshly cut grass and is essential for obtaining the 'green' organoleptic note in many formulations. The 'green notes' obtainable by distilling plant oil are expensive and different biotransformations were developed. The lipoxygenase- and hydroperoxide lyase-mediated oxidation of linolenic and linoleic acid produce cis-3-hexen-1-al and hexen-1-al, which can be reduced by yeast to the corresponding alcohols [23]. Additionally, n-hexanol is easily accessible by microbial reduction of the carboxylic group of extractive C-6 caproic acid [24].

Many biocatalytic processes for other attractive flavors have recently been described. In spite of this the number of industrial applications is limited and the cases illustrated above are the more promising ones. Moreover, an additional problem in this area is the occurrence of adulterations with readily available 'nature-identical' products. The achievement of new analytical methods for discriminating between natural and nature-identical flavors has become essential [25]. Different studies based on stable isotope characterization of aroma have showed promising results and are now applied by specialized laboratories to prove authenticity [26-27].

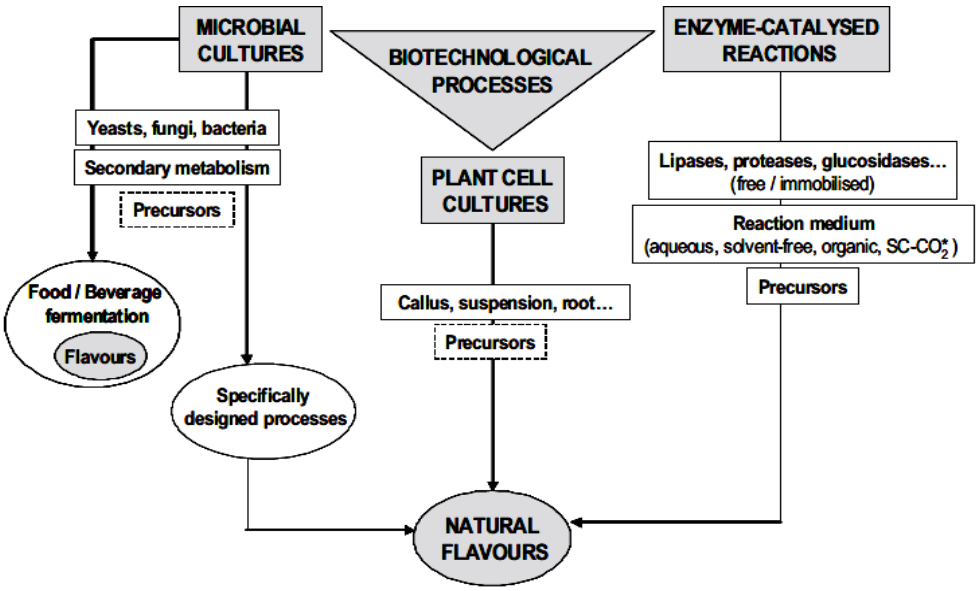


Fig.4. Biotechnological processes for the production of flavor compounds
***SC-CO2: supercritical carbon dioxide**

Nature-identical flavors

The method of production of the nature-identical flavors and fragrances is determined by stringent economic considerations. Although the biocatalytic approaches to these compounds are often expensive, different applications have been described. Environmentally friendly conditions and high chemical selectivity make biocatalytic approaches attractive. Two separate fields should be examined: (i) industrial production and (ii) academic synthesis (synthesis not used for industrial production but mainly for scientific interest) of fine flavors. Few applications are related to the first case in which isolated enzymes were mainly used. Lipases are the favorite catalyst because they show remarkable chemoselectivity, regioselectivity and enantioselectivity. Moreover, they are easily available on a large scale and remain active in organic solvents [28-29].

Generation of natural aroma compounds by the different biotechnological a approaches

The conventional routes of chemical synthesis or isolation of aroma compounds from plants are still viable, but the biotechnological generation is becoming increasingly attractive because of the increasing demand for natural flavors compounds produced *via* biotechnological ways. A market price of a bioflavor is actually of 100–500$/kg. More than100 flavors compounds (single constituents and complex flavor mixtures) are commercialized. An alternative route for bioflavor-synthesis is based on microbial biosynthesis or bio-conversion. These bioprocesses based on microorganisms (bacteria, fungi, yeasts) and their enzymes are part

of white biotechnology. The principle of this technology consisted of the use of renewable resources, clean production, less pollution and less energy intensive processes in biological systems, such as whole cells or enzymes, used as reagents or catalysts.

Flavors and Fragrance chemicals biogeneration using whole microbial cell

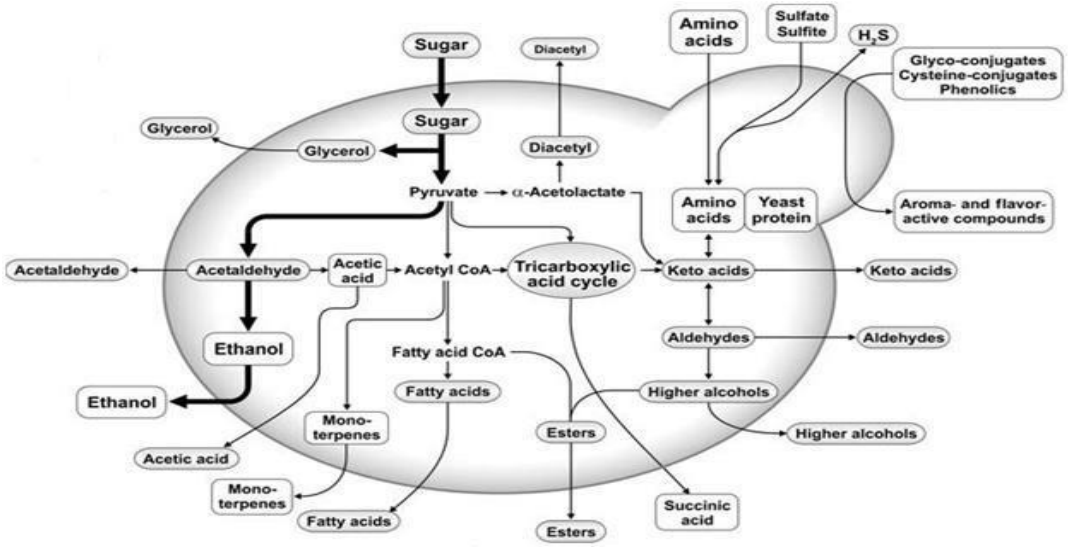
Several microorganisms have been selected in recent years for the development of flavors *via* biotechnological processes. Biotechnological approaches can be divided into two classes: microbiological and enzymatic methods.

Microbiological methods used for the synthesis of natural molecules and especially flavors can be subdivided into *de novo* synthesis and biotransformation (Fig. 5). The first consisted of the production of aroma compounds after metabolizing cells by using simple cultivation media, whereas biotransformation refers to the use of microbial cells to perform specific transformation of precursor into desired product (Table 4). *De novo* synthesis should be therefore used for complex targets or product mixtures, whereas biotransformations are able to carry out single-step processes.

Flavors and Fragrance compounds can be classified in families based on their chemical compounds, physicochemical or sensorial properties (Table 2). Alternative classifications could be established as a function of the chemical family of the precursor used for their production by bioconversion. Detailed information on the production of some commonly used food aroma compounds by microorganisms is presented below.

Alcohols:

Alcohols are produced as a result of bacterial fermentations, various yeast also produces long-chain complex alcohols that possess unique organoleptic properties. In a study, *Saccharomyces cerevisiae* was immobilized on delignified cellulosic material and gluten pellets. The delignified cellulosic material resulted in higher amounts of ester production while gluten pellets produced higher amounts of alcohol [30]. An important aroma-related alcohol is 2-phenylethanol which possesses a rose-like smell. It is usually chemically synthesized from substrates such as toluene, benzene, styrene, or methylphenylacetate [31].). The natural 2-phenylethanol is mainly extracted from rose petals that involve a high-cost process. Specific strains of yeast like *Kluyveromyces marxianus, Saccharomyces cerevisiae, Hansenula anomala* are also capable of producing aroma compounds by bioconversion of 2-phenylalanine into 2-phenylethanol [32]. However, there is a limitation of synergistic inhibition due to the presence of ethanol and 2-phenyl ethanol that reduces the tolerance of *Saccharomyces cerevisiae* in conversion to 2-phenylethanol [33].



The metabolic role of yeast in the development of flavor compounds

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Table 2. Classification of food aroma compounds based on their chemical compound

Alcohols	Aldehydes	Esters	Fatty acids	Ketones
1,2-butanediol	acetaldehyde	methyl acetate	acetate	acetophenone
2-butanol	decanal	ethyl acetate	butyrate	acetone
2-3-butanediol	heptanal	ethyl butyrate	caproate	2,3-butanedione
ethanol	(Z)-4-heptenal	ethyl hexanoate	decanoate	2,3-pentandione
2-ethylbutanol	hexanal	ethyl isobutanoate	isobutyrate	2-butanone
2-ethylhexanol	2-hexenal	ethyl octanoate	2-methylbutyric acid	3-hydroxy-2-butanone
2-heptanol	isohexanal	ethyl butanoate	3-methylbutyric acid	2-heptanone
hexanol	2-methylbutanal	isobutyl butanoate	octanoate	2-hexanone
isobutanol	3-methylbutanal	2-methyl-1-butyl acetate	phenylacetate	3-methyl-2-butanone
2-methylbutanol	2-methylpropanal	3-methyl-1-butyl acetate	propionate	4-methyl-2-pentanone
3-methylbutanol	nonanal	3-octyl acetate	valerate	2-nonanone
2-methylpropanol	(E,E)-2,4-nonadienal	pentyl acetate	**Lactones**	2-octanone
2-nonanol	(Z)-2-nonenal	phenethyl acetate	δ-decalactone	1-octen-3-one
(Z)-1,5-octadien-3-ol	(E)-2-nonenal	ethyl butyrate	γ-decalactone	2-pentanone
2-octanol	octanal	propyl butyrate	γ-butyrolactone	3-pentanone
1-octen-3-ol	butanal	2-hidroxyethyl	δ-dodecalactone	2-tridecanone
1-pentanol	pentanal	propionate	δ-octalactone	2-undecanone
phenylethanol	propanal	2-methyl-2-ethyl-3-	(Z)-6-dodecen-δ-lactone	
2-phenylethanol	propenal	-hydroxyhexyl propionate		
1-nonanol	thiophen-2-aldehyde	ethyl 2-methylbutanoate		
		ethyl 3-methylbutanoate		

Aromatic compounds	Pyrazines
vanillin	2,3-diethyl-5-methylpyrazine
benzaldehyde	2-ethyl-3,5-dimethylpyrazine
β-phenethyl alcohol	2-methoxy-3-isopropylpyrazine
trimethylbenzene	

Methyl ketones:

Methyl ketones contribute to cheese flavors and hence are in great demand. The presence of methyl ketones ($RCOCH_3$) was first observed in the spores of mold ripened cheese *Penicillium roqueforti* [34]. The characteristic odor and taste of ripened cheese is due to the presence of methyl ketones, particularly methyl n-pentyl ketone as well as other short chain that contribute to fruity-spicy notes to fragrances. The mechanism of action of *P. roqueforti* is that the fungal mycelium converts fatty acid chains with less than 14 carbon atoms ($RCH_2CH_2CO_2H$) into methyl ketones. In each reaction, the acids are oxidized to methyl ketones with one less carbon atom than the original acid. Similarly it is also possible to convert vegetable oil and triglycerides to methyl ketones [34].

Diacetyl:

Diacetyl ($CH_3COCOCH_3$) is a naturally occurring flavor chemical that possesses a strong typical 'butter' odor and flavor on dilution. Therefore it is widely used to mimic the buttery taste to the dairy and other milk products. It was assumed that diacetyl was produced from acetoin by microbiological oxidation. The major diacetyl producing bacteria are *Lactococcus lactis, Lactobacillus* sp*, Streptococcus thermophilus,* and *Leuconostoc mesenteroides*. A method for increasing the diacetyl production from bacteria such as *S. diactilactis*, *S. cremoris* and *S. lactis* has been patented. The use of humectants such as glycerol or sucrose lowers the water activity of the medium and results in greater diacetyl production. The production of diacetyl is further enhanced by a low pH (less than 5.5), low temperature and aeration. A pH below 5.5 enhances the activity of citric acid permease and reduces the activity of diacetyl reductase [34].

Lactones:

Lactones are chemically defined as cyclic esters of γ-and δ-hydroxy acids. Lactones are responsible for various taste and odor like oily-peachy, creamy, fruity, nutty, coconut, etc. Various fungi are capable of producing lactones from substrates like triolein, sebum, lecithin, oleic acid and Tween 80. A coconut aroma is very popular as a food flavour and chemically they include γ-octalactone and γ-nonalactone. Another lactone having a coconut odour is 6-pentyl-2-pyrone that is produced by fungus *Trichoderma viridae*. Other fungi involved include *Tyromyces sambuceus* and *Cladosporium suaveolens* that efficiently generate the coconut-flavored lactones γ-decalactone and δ-dodecalactone from substrates ricinoleic acid and linoleic acid, respectively. The milky, buttery and coconut-like flavor provided by these lactones are desirable in dairy and milk products. However, lactones are also responsible for stale flavor of heated milk if used in excess amounts. Some yeasts such as *Candida tropicali*s or *Yarrowia lipolytica* possess the ability to degrade ricinoleic acid to C16, C14 and C12 acids and accumulate δ-decalactone which contributes to fruity and oily flavor that are commonly used in peach, apricot and strawberry aromas [34].

Butyric Acid:

Butyric acid ($CH_3CH_2CH_2CO_2H$) is mainly produced by obligate anaerobic bacteria belonging to the genera *Clostridium, Butyri-vibrio, Eubacterium* and *Fusarium.* They are generally used in low concentration to supply butter-like flavor to different food products like natural cheese flavors. Its derivative pentyl butyrate provides a strong, ethereal, fruity, pungent odor resembling that of pear, pineapple and banana. Butyric acid is naturally present in ester form in butter ranging from 2-4% but its isolation process is very difficult and expensive. Thus microbial production through fermentation process is an attractive and cost effective alternate. The clostridia, particularly *C. acetylbutyricum* possess the ability to produce organic solvents such as acetone and butanol which can be modified and adapted to produce butyric acid. The optimum culture conditions include maintaining the pH of the medium above 5.0 in order to direct the fermentation away from solvent so that formation of butyric acid is favored [34].

Esters:

Esters are generally added in food products like beverages, candies, jellies, jams, baked goods, wines, and dairy products such as cultured butter, sour cream, yogurt, and cheese. They contribute to a fruity aroma to the food. The derivatives of esters like acetate esters, such as ethyl acetate, hexyl acetate, isoamyl acetate and 2-phenylethyl acetate provide typical flavor in wine and other grape-derived alcoholic beverages. Several strains of lactic acid bacteria synthesize ethyl esters and thioesters. *Lactococcus lactis* possess unique esterase enzyme that is responsible for the formation of these aroma ester compounds [35]. Several non-*Saccharomyces* wine yeasts are also the producers of acetate ester [36]. The yeasts *Hanseniaspora guilliermondii* and *Pichia anomala* are potent 2-phenylethyl acetate and isoamyl acetate producers, respectively. In cheese production, ethyl or methyl esters of short-chain fatty acids contribute to the fruity flavour while thioesters derived from thiols are associated with cabbage or sulphur aromas [37].

Pyrazines:

Pyrazines are chemically heterocyclic, nitrogen-containing compounds that are normally produced during conventional cooking or roasting of food through the Maillard reaction. This class of flavor compounds is responsible for a nutty and roasted flavour in food. However, the microwave cooking does not favor pyrazine formation and so the natural pyrazines are externally added as food additives to provide a roasty flavor. There are few microorganisms that synthesize pyrazines, e.g. bacteria such as *Corynebacterium glutamicum* produce important quantities of tetramethylpyrazine from amino acids [34].

Vanillin:

Vanillin is a unique flavor chemical that occurs in *Vanilla planifolia* beans. Its chemical formula is 4-hydroxy- 3-methoxybenzaldehyde. This flavor is widely used in foods, beverages, perfumes, pharmaceuticals and in various medical industries [12]. Although vanillin can be chemically synthesized, but there is an increasing demand for natural vanillin [38]. The direct extraction of vanillin from vanilla beans is expensive and limited which makes this compound a promising target for biotechnological flavor production. Vanillin is also

produced as an intermediate compound in the microbial degradation of several substrates such as ferulic acid, phenolic stilbenes, lignin, eugenol and isoeugenol. Several bacterial and fungal strains of *Pseudomonas putida, Aspergillus niger, Corynebacterium glutamicum, Corynebacterium* sp*., Arthrobacter globiformis* and *Serratia marcescens* are capable of conversion of natural eugenol and isoeugenol from essential oils into vanillin [12-39].

Benzaldehyde:

Benzaldehyde is naturally extracted from fruit kernels such as apricots and imparts a flavor similar to cherry. This extraction also leads to a formation of toxic hydrocyanic acid that is undesirable. An alternative method of extraction is microbial fermentation without producing any harmful by-products. However, benzaldehyde is toxic towards microbial metabolism and its accumulation in the culture medium inhibits cell growth. Thus, only a few microorganisms such as *Pseudomonas putida* and the white rot fungi *Trametes suaveolens*, *Polyporus tuberaster, Bjerkandera adusta* and *Phanerochaete chrysosporium* can be used as benzaldehyde producers. They are involved in the biosynthesis of benzaldehyde from phenylalanine [34].

Terpenes:

Terpenes are widespread in nature and mainly found in plants as constituents of essential oils. The common ones are linalool, nerol, geraniol and citronellol and they are most flavor-active due to their low sensory threshold. Research has shown that aromas and fragrances can also be produced through microbial oxidation of monoterpenes. Terpenes are usually produced by fungi belonging to group ascomycetes and basidiomycetes. The fungus *Ceratocystis moniliformis* produces several aroma products such as ethyl acetate, propyl acetate, isobutyl acetate, isoamyl acetate, citronellol and geraniol. Many microorganisms have an ability to break down terpenes and to carry out specific conversions producing value added products. Some of the enzymes involved in terpene biosynthesis have been sequenced, e.g., monoterpene synthase (isolated from sweet basil) is a key enzyme involved in the production of geraniol and has been sequenced to produce recombinant geraniol synthase [40]. In addition, another geraniol synthase was cloned from the camphor tree *Cinnamomum tenuipilum* and was expressed in *E. coli* [41]. Functional genomics was used to identify the genes for monoterpene synthases from *Vitis vinifera* grapes in order to characterize the enzymes by expression in *E. coli* and subsequent analysis [42].

Flavor and fragrance biogeneration using *de novo* synthesis

Microbial processes, *i.e.* fermentation (*de novo*) constitute a viable and economical alternative route for flavor synthesis. In contrast to biotransformation, which leads to one major product produced by specific conversion (s), *de novo* synthesis produces a mixture of several aroma compounds by using the whole metabolic system of the microorganism. Microorganisms can synthesize flavors as secondary metabolites during fermentation on nutriments (Table 3). Whole cells act on carbohydrates, fats and proteins, and further convert the breakdown products to flavor components. The primary metabolites are produced in considerable amounts with starter cultures, but only traces of more complex aroma chemicals are produced by the same sources. The flavor pro-files produced by the fermentation of by-products of the food industry with traditional starter cultures are well described. This process is however not very promising because of the low concentrations of produced flavors [43]. More attention should be paid to starter cultures with enhanced flavor potential. Moreover, an immediate improvement is often prevented by a lack of metabolic knowledge. The use of genetic engineering techniques may have potential by inserting genes from other microorganisms, and for the expression, in microorganisms, of genes of vegetable and animal origins that encode useful flavor molecules. Although the development of genetic engineering techniques, the application of these approaches remains sensitive due to the complexity of cellular potential and of regulation in the host microorganism. Also, the public perception of “natural aroma” produced through genetically modified organisms is not always good and feasible for commercial production.

By developing microbiological processes for flavor synthesis, it is important to identify strains that produce significant amounts of the desired components. It is generally preferred to use strains GRAS (generally recognized as safe) obtained from traditional fermentations. The quality as well as the amounts of the produced aroma is mainly dependent on the composition of the culture medium especially the carbon and

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nitrogen sources. For the mycologist, taxonomic classification is also based on odors liberated by the fungi [44].

Flavor synthesis by filamentous fungi represents an area of development in the next few years. Filamentous fungi constitute an important source for flavoring compounds (Bigelis, 1992). The volatile spectra of fungi, especially of basidiomycetes, are closer to those of plant (Table 3). *De novo*-produced flavor compounds of these fungi are chemically identical to the volatiles of higher [6]. These fungi possess a unique extracellular enzyme sys-tem (the so-called secretome) have already been shown to produce two kinds of bioflavors by using two pathways: *de novo* synthesis biotransformation [46-47].

Various flavor molecules have been identified from mycelial cultures of higher fungi such as: benzene derivatives, lactones, terpenes, aldehydes, alcohols, esters and ketones [48]. The production yields were generally low. Exceptionally, the use of *Polyporus durus* has allowed to pro-duce 100 mg/L of 4-octanolide [49]. Similarly, high levels of 4-methoxybenzaldehyde and benzaldehyde have been obtained by the use of *Ischnoderma benzoinum* culture.

Ceratocystis fimbriata and *Ceratocystis moniliformis* are known to produce a wide range of complex aroma such as: peach, banana, pear, rose or citrus, depending on the strain and environmental conditions [50-51]. The advantages of using these fungi consist of their relatively rapid growth and the variety of complex aroma mixtures synthesized [50]. Bluemke and Schrader [52] have developed an integrated bioprocess for the production and recovery of *de novo* synthesized aroma compounds by using *C. moniliformis*. The main aroma products of the fungus *C. moniliformis* were citronellol, geraniol, ethyl acetate, propylacetate, isobutyl acetate and isoamyl acetate.

Yeasts are considered to be a source of volatile terpenes [53]. Some terpenes produced by yeasts are shown in Table 3. Low amounts of limonene, linalool, terpineol, myrcene may be obtained by cultivating *Kloeckera Torulopsis* strains. Drawert Barton [54] have demonstrated that the biosynthesis of citronellol by *Kluyveromyces lactis* did not depend on special precursors, but was stimulated by the addition of L-asparagine.

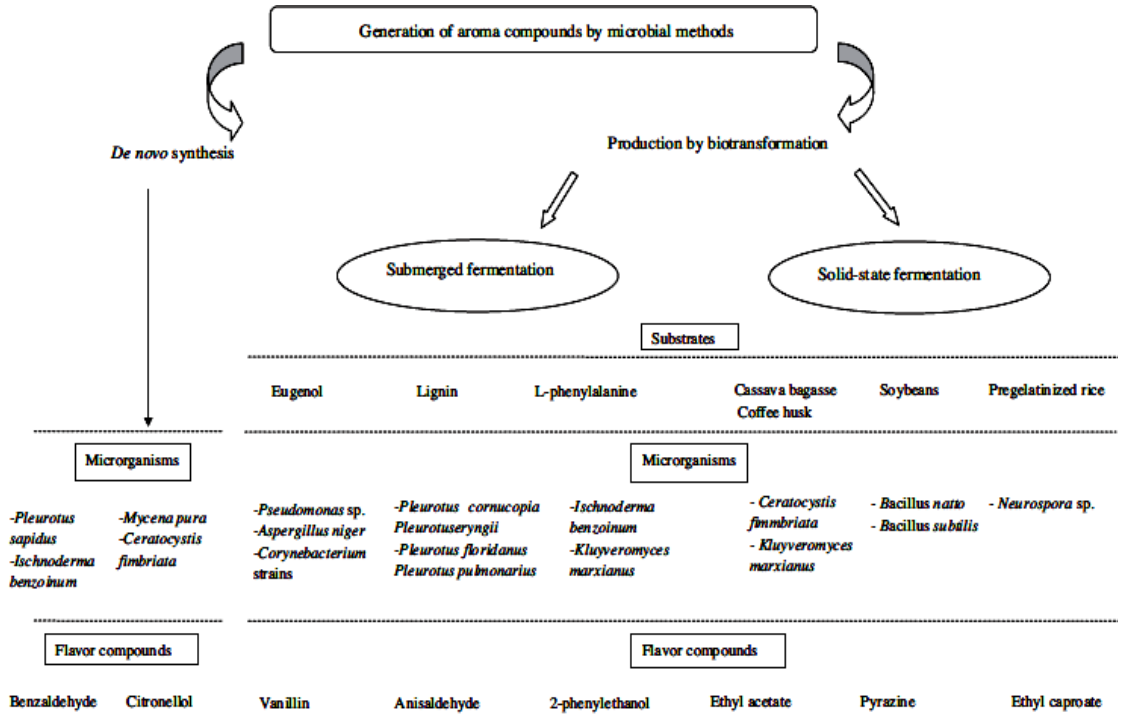

Fig. 5. Examples of natural flavors prepared by microbiological methods.

Flavor and fragrance biogeneration using microbial transformation

Microbes may be used for large-scale biotransformation of pre-cursors to natural flavors (Fig. 5). The selected microbe should be grown under conditions that favor the synthesis of the desired flavor components.

Cells possess generally the redox system required for the synthesis of flavors and the necessary system required for the regeneration of cofactor. Thus, the use of whole cell in microbial transformation allows overcoming the problems associated with the use of enzyme-based redox system. In fact, by regenerating the enzyme chains and the required cofactors and transporters, the continuous flow of metabolites is maintained. However, in microbial transformation, all steps have to proceed under sterile conditions in order to avoid accumulation of unwanted metabolites and therefore to minimize the inhibition of producer cell by substrate or byproduct. So, all steps from the preparation of nutrients to the final down-streaming of product have to be controlled to have an efficient production.

Synthesis of 'bioflavors' by microbial transformation rep-resents a field of investigation that gains a growing interest. Two approaches may be used to produce flavor molecules by bioconversion: submerged fermentation and solid state fermentation (Fig. 5).

Flavor and fragrance biogeneration using submerged fermentation.

Several microorganisms are currently known for their ability to synthesize different aroma compounds. These components are produced as secondary metabolites during fermentation on nutrients. Several researchers have studied the production of aroma compounds by submerged fermentation SmF of several microorganisms [43-55-56]. Attempts to use SmF for flavor production resulted generally in low productivity, which hampered their industrial application [57].

Bensoussan *et al.,* [55] have demonstrated that the aroma compounds produced by morel mushroom mycelia are dependent on the used strains on the culture conditions. The formulation of the culture media represents one of the most important parameters influencing not only the growth state but also the quality the amounts of flavors generated by microbes. In order to reduce the production-costs, various synthetic substrates have been used for the formulation of the culture media in several fermentation processes. The substrates used in submerged fermentations processes may have multiple sources such as: by-products of the food industry or residues of the paper industry. The production of an aromatic mycelium extract allowed the valorization of various by-products. Basidiomycetes [58] ascomycetes [55] were shown to transform successfully the by-products of the food industry to complex highly interesting natural flavor mixtures. Bosse *et al.,* [58] have used the agricultural by-products as substrates for the synthesis of complex flavor mixtures by basidiomycetes. By using apple pomace, these authors have demonstrated that the most potent biotransformation products generated by *Tyromyces chioneus* were: 3-phenylpropanal, 3-phenyl-1-propanol benzyl alcohol [58].

Bensoussan *et al.,* [55] have produced flavor compounds with fruity notes after using malt extract as substrate in the submerged fermentation process. The culture of *Morchella* in submerged conditions allowed the obtention of aroma with amounts in the same order with the quantities extracted from fruiting bodies (70–170 ppm). The malt extract has been also used as a substrate by Bensoussan *et al.,* [55] for the production of 1-octen-3-ol by *Morchella* in submerged fermentation process. In comparison to fruiting bodies fresh or dehydrated, the authors have demonstrated that the mycelium contained a greater quantity of l-octen-3-o1. This aliphatic alcohol, produced by microorganisms, is of considerable importance in fragrance applications. It was produced by the mycelia homogenate of the edible mushroom *Pleurotus pulmonarius*grown in submerged culture through oxidative breakdown of linoleic acid [59]. Assaf *et al.,* [59] have cultivated fungus in submerged culture with a medium based on a mixture of soybean meal soybean oil supplemented with mineral solution.

Monoterpenes such as α-pinene and limonene are also inexpensive starting materials for microbial transformations. The fungal biotransformation of these natural precursors to more valuable aroma compounds offers a very interesting alternative source of natural flavors. Terpenes are usually isolated from the essential oil of many plants and are relatively cheap. Monoterpenes used as substrates have led to a great variety of oxyfunctionalized components [60].

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Table 3 – Microbial processes used for the production and recovery of several flavor compounds.

Flavors	Flavoring types	Microbial preparation	Recovery	Yields/ productivities	References
Caroxilic acids					
Propionic acid	Raspberry, cognac, butter	*Propionibacterium* from lactose	Hollow-fiber membrane extractor	1 g/L h	Jin and Yang (1998)
		Propionibacterium acidipropionici from waste activated sludge		68.4%	Chen et al. (2013)
		Batch fermentation *Propionibacterium freudenreichii*, co-fermentation of glycerol and glucose		0.54–0.65 g/g 0.18–0.23 g/L h	Wang et al. (2013)
Butyric acid	Butter, cheese, nut, fruit, others	Batch fermentation *Clostridium tyrobutyricum* on glucose		0.46 g/g 73 g/L	Song et al. (2010)
		Batch culture *Clostridium tyrobutyricum* on glucose and xylose in a fibrous-bed bioreactor		0.45–0.54 g/g sugar 0.48–0.60 g/L h	Wei et al. (2013)
		Fed-batch culture *Clostridium tyrobutyricum* on glucose and xylose		0.48 g/g sugar 0.51 g/L h	Wei et al. (2013)
		Kluyveromyces lactis *De novo* synthesis		0.37–0.83%	Jiang (1995)
Hexanoic acid		Bach culture *Clostridium acetobutyricum*	*In situ* extractive fermentation	0.34 g/L h	Jeon et al. (2013)
ESTERS					
Ethyl acetate	Fruity smell	*Ceratocystis moniliformis* *De novo* synthesis	An integrated bioprocess by interlinking a pervapor-ation membrane module		Bluemke and Schrader (2001)
		Solid state fermentation on coffee husk *Ceratocystis fimbriata* bioreactors: columns (laboratory scale) and horizontal drum (semi-pilot scale)		28.55 μmol/L g of initial dry matter	Medeiros et al. (2006)
		Solid state fermentation on cassava bagasse and giant palm bran *Kluyveromyces marxianus*		418 and 1395 μmol/L g substrate	Medeiros et al. (2000)
2-Phenyl ethanol	Rose-like aroma	A continuous reactor *Saccharomyces cerevisiae* sp. strain R-UV3	Use of macroporous resin as *in situ* adsorbent	0.90 g L/h	Wang et al. (2011)
		Aerobic fed-batch hybrid bioreactor *Saccharomyces cerevisiae*	Membrane separation techniques: microfiltra-tion	7.5 g/L	Mihal et al. (2012a)
		Integrated fermentation/membrane extraction process *Kluyveromyces marxianus* CBS 600	Membrane extraction by using a hollow fiber module	4.0 g/L	Adler et al. (2011)

Allylic hydroxylations of monoterpene hydro-carbons are interesting reactions because of the multiple bioactivities of many of resulting aroma compounds [61]. The allylic oxidation of limonene (obtained from orange peel oil) yields to cis: trans-carveol carvone: important aroma compounds in foods beverages

[61]. The cis: trans-carveol carvone are generally obtained with amounts (a few milligrams per liter range) which are insufficient for industrial applications. In fact, terpene transformations suffer generally from the volatility of the substrate from the toxicity of terpenes toward microorganisms. In order to improve the production yields of the desired products (cis: trans-carveol carvone) by *Pleu-rotus sapidus*, Onken Berger [61] have conducted the precultures in the presence of limonene. An adaptation of the precultures with small amounts of limonene has allowed to double the concentration of carveol to increase the concentration of carvone by a factor of 3–4.

Table 4 – Examples of flavor compounds obtained from microbial *de novo* synthesis.

Flavors	Sensorial description	Species	
		Fungi	Yeast
2-Phenyl ethanol	Rose-like aroma	*Pleurotus sapidus* *Polyporus* sp.	*Saccharomyces cerevisiae* *Kluyveromyces marxianus*
Citronellol	Fresh, rose-like	*Mycena pura* *Ceratocysis moniliformis* *Ceratocystis fimbriata*	*Kluyveromyces lactis*
Geraniol	Sweet, rose-like, fruity	*Ceratocysis moniliformis* *Ceratocystis variospora*	*Kluyveromyces lactis*
γ decalactone	Peach aroma	*Sporobolomyces* sp.	*Rhodotorula glutinis*
Pyrazines	Roasted, nutty flavor	*Aspergillus* sp.	
1-Octen-3-ol, 1-octen-3-one	Green	*Lentinus edodes* *Grifola frondosa* *Pleurotus pulmonarius* *Penicillium camemberti*	
Benzaldehyde	Bitter almond	*Ischnoderma benzoinum* *Agaricus subrefecens* *Agaricus bisporus* *Armillaria mellea* *Pleurotus sapidus* *Polyporus* sp. *Tyromyces sambuceus*	
Anisaldehyde	Vanilla-like, anise-like	*Pleurotus sapidus* *Polyporus benzoinus* *Bjerkandera adusta* *Trametes suaveolens*	
Methyl benzoate Ethyl benzoate	Fruity	*Agaricus subrefecens* *Mycena pura* *Phellinus* sp. *Polyporus tuberaster*	

Monoterpene alcohols (geraniol and nerol) were also used as substrates in submerged fermentation processes of *Aspergillus niger* [56]. The main bio-conversion products obtained from geraniol and nerol were linalool, α-terpineol, 2,6,6-trimethyl-2-vinylte trahydropyran and an unidentified compound.

The production of aromatic aldehydes such as vanillin benzaldehyde by biotransformation using microorganisms has been also extensively investigated [62]. The bioconversion routes as well as the enzymes implied in the synthesis of vanillin banzaldehyde are shown in (Table 5). These flavors are marketed on a scale of several thous tons per year. Benzaldehyde was the first aromatic aldehyde identifed by Liebig Wöhler [63]. The identification the synthesis of vanillin marked the beginning of the modern flavor industry [64]. Vanillin appears as an intermediate in the microbial degradation of several substrates including: ferulic acid, phenolic stilbenes, eugenol isoeugenol [65-66-62]. Eugenol isoeugenol are essential oil components that can be used as inexpensive substrates for the production of vanillin by biotransformation (Table 5). Shimoni *et al.,* [67] have isolated microbial strains which were able to produce vanillin from isoeugenol. They have produced high amounts of vanillin (0.61 g/L vanillin: molar yield of 12.4%) when *Bacillus subtilis* was grown in the presence of isoeugenol as the sole carbon source.

Stentelaire *et al.,* [62] have developed a biotechnological process for the production of vanillin from vanillic acid by*Pycnoporus cinnabarinus*. These authors have demonstrated that over a concentration of 1000 mg/L, vanillin was shown to be highly toxic to the growth of *P. cinnabarinus*. To over-come this toxicity, they

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have used a selective XAD-2 resin for the reduction of vanillin concentration in the medium for minimizing the formation of unwanted by-products. In this case, the production of vanillin has reached 1575 mg/L [62].

Table 5 – Routes for the bioproduction of some relevant flavors by microbial transformations.

Flavours	Metabolic intermediates	Biocatalysts	Microorganisms	References
Vanillin	Eugenol			
	↓	Eugenol hydroxylase	*Pseudomonas* sp. HR199	Overhage et al. (1999)
			Aspergillus niger	Priefert et al. (2001)
	Coniferyl alcohol		*Corynebacterium* strains	Priefert et al. (2001)
	↓	Coniferyl alcohol dehydrogenase	*Arthrobacter globiformis*	Priefert et al. (2001)
	Coniferyl aldehyde			
	↓	Coniferyl aldehyde dehydrogenase		
	Ferulic acid			
	↓	Feruloyl-CoA synthetase /and Enoyl-CoA-hydratase/lyase	*Pseudomonas putida*	Berger (1995)
			Streptomyces setonii	Muheim and Lerch (1999)
	Vanillic acid			
	↕	Vanillin dehydrogenase	*Pycnoporous cinnabarinus*	Falconnier et al. (1994)
			Phanerochaete chrysosporium	Lesage-Meessen et al. (1996)
	Vanillin			
γ –decalactone	12-hydroxy-9-octadecanoic acid			
	↓	β-oxidation	*Aspergillus niger*	Cardillo et al. (1989)
	10-hydroxy-7-hexadecanoic acid		*Pichia etchellsii*	Cardillo et al. (1989)
			Cladosporium suaveolens	Cardillo et al. (1989)
	↓	3 β-oxidations	*Sporidiobolus salmonicolor*	Lee et al. (1995)
			Yarrowia lipolytica	Rabenhorst and Gatfield (2002)
	4-hydroxy-7-decenoic acid		*Rhodotorula glutinis*	Cheetham et al.(1988)
	↓	cyclisation		
	γ –decalactone			
Benzaldehyde or 2phenylethanol	L-Phenylalanine			
	↓	Transaminase	*Polyporus tuberaster*	Kawabe and Morita (1994)
	Phenylpyruvic acid		*Ischnoderma benzoinum*	Krings et al. (1996)
	↓	Decarboxylase	*Bjerkandera adusta*	Lapadatescu et al. (1999)
	Phenylacetaldehyde			
	→ Benzaldehyde			
	↓		*Ischnoderma benzoinum*	Krings et al. (1996)
		Dehydrogenase	*Kluyveromyces marxianus*	Fabre et al. (1998)
	2-phenylethanol		*Saccharomyces cerevisiae*	Stark et al. (2002)

Filamentous fungi especially basidiomycetes, have been largely reported to metabolize ferulic acid to vanillic acid vanillin, but always with low yields of vanillin [68]. Lesage-Meessen *et al.* [69] have developed a two-step process to produce vanillin: (i) by transforming ferulic acid into vanillic acid with the ascomycete, *Aspergiflus niger*, (ii) by transforming vanillic acid into vanillin with basidiomycetes: *P. cinnabarinus* or *Phanerochaete chrysosporium*. The vanillin was produced with a concentration of 237 mg/L [69].

In addition to the interest accorded to vanillin benzaldehyde, aromatic alcohol such as: 2-phenylethanolbecomes also one of the most useful fragrance chemicals in perfume cosmetic industry. The

biotransformation route used for the production of this compound by microorganisms is showed by Table 5. The 2-phenylethanol produced naturally is preferred especially when it is used as a food additive. Wang *et al.* [70] have used yeasts to convert l-phenylalanine to 2-phenylethanol in submerged fermentation processes. Due to the inhibition toxicity of obtaining product toward microorganisms, these authors have developed a continuous approach using macroporous resin as an *in situ* adsorbent in order to minimize the inhibition by product therefore to improve the cells viability. In fact, it is often necessary to control the concentration of the end product in the fermentation broth because above a certain value, the flavor molecules negatively influence the cell physiology by affecting the membrane fluidity, consequently, the loss of cell viability.

Flavor and fragrance biogeneration using solid state fermentation.

There have been significant developments in solid state fermentation (SSF) as potential technology for the production of microbial products over the past few years. SSF is the state-of-the-art technology that is used in many biotechnological applications for the production of high value-added products. The SSF is used to produce various products such as: biologically active secondary metabolites [71-72], biopesticides [73], enzymes [74-75-76], biosurfactants [77], bio-fuel [78-79], bioremediation [80] natural flavors [81-50-82-83]. The production of desired compounds is generally coupled with the stationary growth phase of the used microorganism.

Solid-state fermentation denotes the cultivation of microorganisms on solid material in the absence of a free aqueous phase. The solid material is non-soluble acts both as physical support as source of nutrients [84]. The substrate must possess enough moisture to support growth metabolism of microorganism [85]. The selection of a proper substrate is a key parameter in SSF applications. The choice depends upon several factors related to the cost availability. The choice of available substrate is also mainly related to the goal of producing a specific product. The substrate used in SSF technology could be a naturally occurring solid substrate such as agricultural crops, agro-industrial residues or inert support [86-87-85]. Agro-industrial residues have been used as efficient substrates in several bioprocesses (Fig. 5). The application of these sources presents several advantages such as: solid waste management [88-89-90], biomass energy conservation, production of high value products little risk of bacterial contamination [91].

In addition to substrate, there are several important factors, which affect SSF processes. These include selection of suitable microorganism, optimization of process parameters (physical, chemical biochemical) isolation of the product. Water activity (^{a}w) of substrate in SSF should be also evaluated because of its determinant influence on microbial activity. Based on minimal water activity requirement, the fungi yeast are the only suitable micro-organisms used for SSF. Several works have managed bacterial cultures by using SSF processes [87]. The first fungus used for solid-state biotechnology was *A. niger* [88-90-92]. It has been used for the production of citric acid in surface culture on a solid support [92].

There are several advantages of SSF when compared with submerged fermentation. The SSF technology is more effective in several aspects including higher fermentation productivity, extended stability of products, low production costs, lower energy low water dem . Because SSF processes are performed at lower water activities, the growth of contaminating bacteria yeasts can be minimized. Also, microorganisms involved in SSF have a higher metabolic potential since they proliferate in an almost natural environment. Studies the production of fungal enzymes in SSF have repeatedly shown that SSF, in comparison with SmF, provides higher volumetric productivities [93]. This makes SSF the preferred technique for many applications such as the production of enzymes flavors.

Today with a better underst ing of biochemical engineering aspects particularly the mathematical modeling the design of bioreactors, it is possible to scale up SSF processes. In recent years, there has been a significant improvement in underst ing of how to design scale up SSF bioreactors. The key to these advances has been the application of mathematical modeling to describe various physicochemical biochemical phenomena within the system [94-95-96]. Modeling could be a good tool for scale-up studies but such results need to be validated by experimental findings. However, due to the heterogeneous nature of the substrates, which are structurally nutritionally complex, there are some difficulties on the determination of substrate consumption on the determination of the kinetics of reactions in SSF systems.

Micro-organisms play an important role in the generation of natural compounds, particularly in the field of food aroma (Table 6). It is estimated that around 100 aroma compounds are produced industrially by microbial fermentations [97]. A few species of yeasts fungi have generally been preferred, to be used in industrial applications due to their GRAS (generally regarded as safe) status.

The production of flavors presents another aspect of SSF application. Many works have reported the production of aroma in SSF by microorganisms including bacteria fungi (Table 6). The use of these micro-organisms in SmF resulted in low productivity of aroma compounds [57], which reduced the operation of this process in industrial application. SSF could be of high potential for this purpose [7]. Several researchers have studied the production of aroma compounds by SSF from several microorganisms such as *Neurospora* sp. [98], *Zygosaccharomyces rouxii* [99], *Aspergillus* sp. [100].

Fungus from the genus *Ceratocystis* such as *C. fimbriata* has a great potential to produce a wide variety of fruit-like or flower-like aromas (peach, pineapple, banana, citrus rose) (Table 6). This fungus has a great potential for ester synthesis because it grows rapidly it has a good ability to sporulate. *Bacillus natto B. subtilis* have been also cultivated on soybeans in SSF for the respective production of 2,5-dimethylpyrazine tetramethylprazine [101-102]. Pyrazines, especially alkylpyrazines are heterocyclic compounds which have a nutty roasty flavor. These compounds are used as additives in a wide variety of foods [103]. Besson *et al.* [101] Larroche *et al.* [102] have demonstrated the suitability of SSF for the production of these compounds.

A mixed-culture of fungus yeast, which cannot be established in SmF, allows the producing of various aroma components [104]. Fu *et al.* [82] have identified 29 volatile aroma compounds when bamboo shoots were fermented in SSF. It was not possible for these authors to produce this flavor combination when fermentation was realized in SmF.

Several substrates have been used for aroma production in SSF such as: cassava bagasse, sugar cane bagasse, apple pomace, giant palm bran, coffee husk [105-106]. Christen *et al.* [50] have employed different substrates (wheat bran, cassava bagasse sugar cane bagasse complemented with a synthetic medium) for the growth for the aroma production by the mold *C. fimbriata*. They have demonstrated that the substrate had a direct influence on the nature of the volatile compounds produced by the fungus. By using sugar cane bagasse complemented with a synthetic medium containing glucose, a fruity aroma has been produced, while the use of leucine or valine containing medium gave a banana aroma. Soares *et al.* [107] have demonstrated that the odor produced by the culture depended on the amount of added glucose. The addition of glucose decreased the aroma intensity (Table 6). These authors have also reported the production of strong pineapple aroma when SSF was carried out using coffee husk as a substrate for *C.fimbriata*. This fungus was also cultivated by Bramorski *et al.* [108] in order to produce fruity aroma. They have evaluated the potential of several agro-industrial residues such as cassava bagasse, apple pomace, amaranth soybean. While amaranth medium produced a pineapple aroma, media with other substrates produced a strong fruity aroma.

Medeiros *et al.* [109] have cultivated a strain of *Kluyveromyces marxianus* in SSF using five agro-industrial residues. With palm bran, ethanol was produced with the highest concentration, but with cassava bagasse, the major component was ethyl acetate.

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Table 6 – Some examples of aroma produced by microorganisms in solid-state fermentation.

Microorganisms	Substrates	Aroma	Intensities	References
Ceratocystis fimmbriata	Cassava bagasse with valine Cassava bagasse with leucine	Banana aroma Banana aroma	478 μmol eq. ethanol $^{-1}$ 414 μmol eq. ethanol $^{-1}$	Christen et al. (1997)
Neurospora sp.	Pregelatinized rice	Ethyl caproate, Isoamyl alcohol 3-Methyl-1-butanol 1-Octen-3-ol Ethyl acetate		Yamauchi et al. (1989) Pastore et al. (1994)
Trichoderma viride	Agar	Coconut aroma		Gervais and Sarrette (1990)
Aspergillus strain	Cellulose fibers	Methyl ketones		Humphrey et al. (1990)
Zygosaccharomyces rouxii	Miso (soyfoods)	4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone		Sugawara et al. (1994)
Ceratocystis fimbriata	Coffee husk	Ethyl acetate 28.55 μmol/L/g of initial dry matter		Medeiros et al. (2006)
Kluyveromyces marxianus	Cassava bagasse Palm bran	11 fruity aroma: ethyl acetate Ethanol 09 fruity aroma: ethanol Ethyl acetate	1395 μmol/L/g 1163 μmol/L/g 418 μmol/L/g 251 μmol/L/g	Medeiros et al. (2000)
Rhizopus oryzae	Amaranth grain	Ethanol	More than 80%	Bramorski et al. (1998)
Ceratocystis fimbriata	Cassava bagasse Apple pomace Soybean Amaranth Coffee husk	Fruity aroma Pineapple aroma		Bramorski et al. (1998) Soares et al. (2000)
Ceratocystis fimbriata	Coffee husk supplemented with 20% glucose Coffee husk supplemented with 30% glucose	Pineapple aroma Pineapple aroma	6.58 mmol/L/g total volatiles 5.24 mmol/L/g total volatiles	Soares et al. (2000)
Kluyveromyces marxianus	Cassava bagasse Palm bran	Fruity aroma		Medeiros et al. (1999)
Bacillus natto	Soybeans	2,5-Dimethylpyrazine		Besson et al. (1997)
Bacillus subtilis	Soybeans	Tetramethylprazine		Larroche et al. (1999)

Flavor and fragrance biogeneration using pure isolated enzymes.

There are about 4000 known enzymes about 200 have been mainly commercialized for stereo selective organic synthesis also for the biotechnological production of flavor com-pounds. The use of enzymes presents a set of advantages like high selectivity, high efficiency, and high reaction speed catalytic activity in both reaction directions. The use of enzymes in flavor synthesis allows the obtaining of compounds which could be labeled as “natural” when substrates from natural origins are used (Fig.6). It has been reported that these compounds show better odor color [110].The enzymatic conversion allows also obtain of high productivities of aroma compounds if compared to the direct extraction from plants.

There are however some drawbacks by using enzymes in bioprocesses such as the need of long complicated steps for enzyme isolation purification (Fig. 6). Another problem deals with the difference of solubility between the reagents. Bioconversion is usually performed in an aqueous environment; however, many flavor precursors flavor products are not well-soluble in water. Biphasic systems (Aqueous/organic; aqueous/gas) were shown to overcome the solubility problems [111].

The worldwide market of enzymes is more than US $1billion per year. Approximately 60% of enzymes are produced in Europe [112]. Microbial enzymes play the greatest role in production of flavor compounds (Table 5).The most popular classes of enzymes used in biotechnology processes are hydrolytic enzymes, transferases, oxireductases lyases (Table 7). The oxidoreductases used for flavor generation are: alcohol

dehydrogenases, peroxidases including chloroperoxidase, lipoxygenase, and amine oxidase vanillyl alcohol oxidase [113]. These enzymes have been mentioned for their potential application in the production of flavor compounds are involved in the enhancement of the organoleptic characteristics [114]. Many different plant volatiles are synthesized *via* a few basic pathways. Once they are produced *via* these pathways, their diversity is achieved *via* additional modification reactions such as acylation, methylation oxidation/reduction [115].

Table 7 – Enzymatic processes used for the production of several flavor compounds.

Substrates	Flavoring types	Enzyme-catalysed reaction	Yields/productivities	References
Esters				
Ethyl butyrate	Pineapple flavor	Lipase from *Rhizopus oryzae* immobilized in a hydrophilic polyurethane foam Molar ratio ethanol/butyric acid (0.257–2.443)	47% 2.21 μmole/mL h	Grosso et al. (2013)
		Fusarium solani pisi cutinase in an organic solvent media (iso-octane)	84% 1.15 μmol min^{-1} mg^{-1}	De Barros et al. (2009)
Ethyl valerate	Green apple flavor	*Fusarium solani pisi* cutinase in an organic solvent media (iso-octane)	96% 1.06 μmol min^{-1} mg^{-1}	De Barros et al. (2009)
		Lipase from *Staphylococcus simulans* immobilized on $CaCO_3$ support Molar ratio ethanol/valeric acid of 1 and 20% (w/w) of water was added to the reaction mixture	51%	Karra-Chaabouni et al. (2006)
Butyl butyrate	Fruity flavor	Lipase from *Candida cylindracea* Molar ratio butanol/butyric acid of 8 and lipase concentration of 75 U/mg	63.33%	Salihu et al. (2013)
Butyl acetate	Pineapple flavor	Lipase from *Rhizopus oligosporus* NRRL 5905 immobilized onto cross-linked silica gel Transesterification of vinyl acetate with n-butanol	50%	Mahapatra et al. (2009)
		Immobilized lipase from *Rhizopus oryzae* in a solvent-free system Molar ratio butanol/acetic acid of 1	60%	Ben Salah et al. (2007)
Propyl acetate	Pear flavor	Lipase from *Rhizopus oligosporus* NRRL 5905 immobilized onto cross-linked silica gel Transesterification of vinyl acetate with n-propanol	56%	Mahapatra et al. (2009)
Isoamyl acetate	Banana and pear flavors	Immobilized *Candida antarctica* lipase B in ionic liquid solvent The reaction medium was a biphasic mixture of excess of isoamyl alcohol and ionic liquid	Nearly 100%	Feher et al. (2008)
Hexyl acetate	Fruity odor	Lipase from *Staphylococcus simulans* immobilized on $CaCO_3$ support Molar ratio hexanol/acetic acid of 1 and 10% (w/w) of water was added to the reaction mixture	41%	Karra-Chaabouni et al. (2006)
Aldehydes				
Hexanal	Grass like	Bioconversion of linoleic acid by sequential action of lipoxygenase and hyroperoxide lyase from radish	1.7 mg/kg plant material	Brunerie (1989)
		Hyroperoxide lyase from guava by using sunflower oil as precursor	5 g/kg of reaction medium	Muller et al. (1995)
		Bioconversion of hydrolyzed sunflower oil by sequential action of lipoxygenase and hyroperoxide lyase from spinach	54%	Marczy et al. (2002)
3Z-hexenal	Green odor of freshly cut leaves	Bioconversion of linolenic acid by sequential action of lipoxygenase and hyroperoxide lyase from radish	57.7 mg/kg plant material	Brunerie (1989)
		Bioconversion of hydrolyzed linseed oil in enzymatic liquid/gas reactor by sequential action of soybean lipoxygenase and hydroperoxide lyase from olive leaves	50% 0.36 g/kg of reaction medium	Ben Akacha and Gargouri (2009)

Table 7 – (*Continued*)

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Substrates	Flavoring types	Enzyme-catalysed reaction	Yields/productivities	References
2E,6Z-nonadienal	Cucumber or watermelon	Bioconversion of mixture of polyunsaturated fatty acids by sequential action of lipoxygenase and hyroperoxide lyase from viola leaves	600 mg/kg plant material	Hausler et al. (2000)
Alcohols				
2E-hexenol		Bioconversion of linolenic acid in 1 L reactor by sequential action of lipoxygenase, hyroperoxide lyase from guava and alcohol dehydrogenase from baker's yeast	63% 3.79 g/kg of reaction medium	Muller et al. (1995)
3Z-hexenol	Grassy, green odor	Bioconversion of linolenic acid by sequential action of lipoxygenase, hyroperoxide lyase from watermelon and alcohol dehydrogenase from baker's yeast	990 µg/g of plant material	Holtz et al. (2001)
		Bioconversion of hydrolyzed linseed oil in enzymatic liquid/gas reactor by sequential action of soybean lipoxygenase, hydroperoxide lyase from olive leaves and alcohol dehydrogenase from baker's yeast	3.54 g/kg of olive leaves	Ben Akacha and Gargouri (2009)

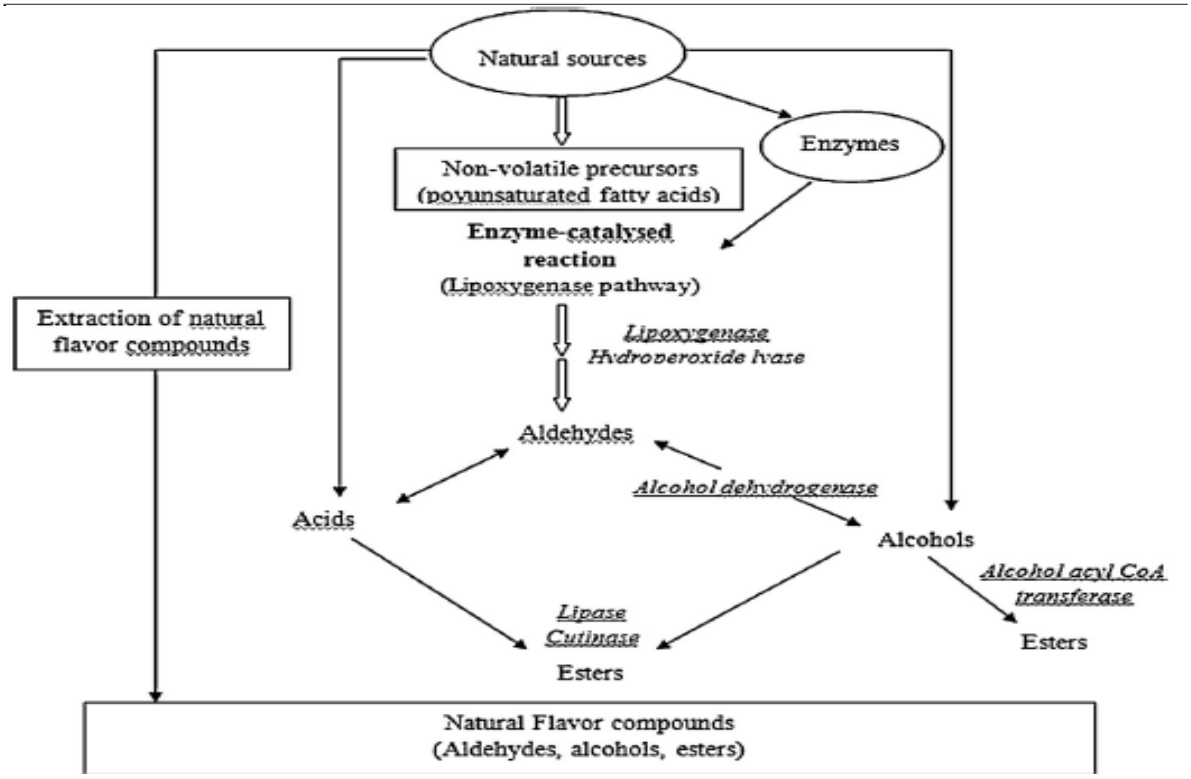

Fig. 6. The pathways for the preparation of some natural aroma compounds: by extraction from natural sources and *via* the biocatalytic routes.

Aliphatic esters production by enzymes

Aliphatic esters are generated by hydrolytic enzymes (Table 7).These enzymes are the most employed in organic chemistry because of their provide advantages such as: low cost, wide availability, substrate specificity and activity without the need for cofactors. The use of hydrolytic enzymes as industrial

catalysts for ester synthesis is generally conducted in organic media (Table 7). Terpenic esters synthesis through direct esterification and transesterification in low water content media was described in many works [116-117-118].

The use of organic solvent media for enzymatic ester synthesis presents several advantages among them: the increased solubility of non-polar substrates products the shifting of the thermodynamic equilibrium of the reaction to favor ester synthesis over hydrolysis. The water activity of the reaction medium is largely responsible for the determination of the predominance of each type of reaction [119-120]. Log P (the partition coefficient between water organic solvent) is generally used to describe the solvent hydrophobicity. It has been reported that solvents with intermediate log P (around 4) are suitable for esters synthesis [121].

Use of lipases (esterase)

The use of lipases for a variety of biotechnological applications is rapidly increasing. Lipase has proved to be of great interest in the industrial flavor ester synthesis. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are known as efficient biocatalysts that catalyze the hydrolysis of esters of glycerol with prefer-ably long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. Lipases have been employed for direct esterification transesterification reactions to produce esters of glycerol [122], aliphatic alcohols [123] terpenic alcohols [116]. Such esters maybe considered as naturals when produced by lipase-mediated syntheses from substrates having natural origins.

Lipases are nowadays widely used in industrial scale with applications in food, detergent, cosmetic pharmaceutical industries [124-125-126-127]. The most prominent applications of lipolysis which have a good potential are flavor formation from fat the esterification reaction of alcohol acid to carboxylic esters with fruity notes (Table 8).

The biotechnological synthesis of flavor compounds by lipase concerns esters lactones. In cosmetic perfumery industries, the lipases are used in the synthesis of flavors by transesterification reactions or by direct esterification (Table 8). Esters of acids from acetic acid to hexanoic acid alcohols from methanol to hexanol, geraniol citronellol have been synthesized using several lipases. Lipases from *Mucor miehei* *C ida rugasa* are the most widely lipases used in industrial applications for the production of a number of commercially important esters [128-129].

The behavior of lipase toward substrates is different depends especially on the number of carbon atom chain length of fatty acids. The esterification of short-chain fatty acids alcohols has not received much attention because substrates with low molecular weight have less affinity for these lipases than long-chain substrates [118]. These results agree with those found by Abbas Comeau [130]. These authors have demonstrated after the use of immobilized lipase of *Mucor* sp. that butyl caproate was more readily synthesized than butyl butyrate, which was readily synthesized than butyl propionate. Ethyl propionate ethyl butyrate synthesis had lower yields for the same reaction time than methyl caproate ethyl caproate which were produced with conversion yields of 92 98%, respectively. Moreover, the odor intensity of esters decreases with the increase of molecular weight [131]. Also, esters with low molecular weight such as ethyl butyrate play an important role in the food industry as aroma constituents [132]. Ethyl butyrate is an important component of many fruit flavors such as pineapple passion fruit. This compound was synthesized by using lipases from different sources: *Rhizopus oryzae* lipase [133], *Cida rugosa*, *Cida antarctica* [133], *C ida cylindracea* [134].

Use of cutinases

Cutinases, also known as cutin hydrolases (EC 3.1.1.74) are enzymes of phytopathogenic fungi that grow on cutin as the sole carbon source. Cutin is a biopolyester that constitutes the structural component of higher plant cuticle [135]. This enzyme displays hydrolytic activity on cutin polymers efficiently hydrolyzes soluble esters emulsified triacylglycerols [136]. Cutinases belong mostly to the group of serine hydrolase family, which contains serine group in its active site. This is a group which shares catalytic properties with lipases esterases. Cutinase is therefore considered as an enzyme intermediate between these enzymes. According to Carvalho *et al.* [137], cutinase is an esterase that hydrolyzes cutin, but it is also classified as a lipase. However, cutinases present an interesting option, since they do not require interfacial activation, unlike

classical lipases [137]. This feature makes them able to hydrolyze soluble esters as well as emulsified triacylglycerols [138]. They are therefore interesting in several industrial processes involving hydrolysis, esterification, trans-esterification reactions.

Table 8– Applications of immobilized lipases for the synthesis of several natural aroma compounds.

Sources of lipases	Immobilization support	Volatile esters	Sensory description	References
Candida antarctica	Immobilization on acrylic resin	(Z)-3-hexenyl acetate	Green note flavor	Bourg-Garros et al. (1998a); Bourg-Garros et al. (1998b)
Candida rugosa	Immobilization onto amine functionalized polypropylene membrane	Ethyl valerate	Green apple flavor	Bayramoglu et al. (2011)
Candida rugosa	Immobilization into calcium alginate gel	Isoamyl acetate Ethylvalerate Butyl acetate	Banana flavor Green apple flavor Pineapple flavor	Ozyilmaz and Gezer (2010)
Candida rugosa	Immobilization in hydrophilic polyurethane foam	Ethyl butyrate	Pineapple aroma	Pires-Cabrala et al. (2007)
Mucor miehei	Immobilized lipase	(Z)-3-hexenyl acetate	Green note flavor	Shieh and Chang (2001)
Rhizopus oryzae	Immobilized in three different kinds of supports: EP100, Eupergit®CM and octadecyl-sepabeads	Ethyl butyrate	Pineapple aroma	Guillen et al. (2012)
Rhizopus oryzae	Immobilization in hydrophilic polyurethane foam	Ethyl butyrate	Pineapple, passion fruit and strawberry	Rodriguez-Nogales et al. (2005); Grosso et al. (2013)
Rhizopus oryzae	Covalent immobilization on activated silica	Methyl butyrate Octyl acetate	Fruity odor of pineapple, apple, and strawberry Fruity orange flavor	Garlapati and Banerjee (2013)
Rhizopus oligosporus	Immobilization onto cross-linked silica gel	n-Butyl acetate n-Propyl acetate	Pineapple flavor Pear aroma	Mahapatra et al. (2009)
Burkholderia cepacia	Immobilization on sodium alginate	Ethyl valerate	Green apple flavor	Padilha et al. (2013)
Staphylococcus simulans	Immobilization onto $CaCO_3$	Isoamyl acetate	Banana flavor	Ghamgui et al. (2006)
Thermomyces lanuginosus	Immobilization on styrene divinylbenzene beads	Butyl butyrate	Fruity flavor	Martins et al. (2013)
Rhizomucor miehei	Immobilization on anion-exchange resin	Isoamyl isovalerate	Apple flavor	Chowdary et al. (2000)

Lipases are the most commonly used enzymes for the synthesis of different aroma. Until now, lipases esterases are used for the production of a wide range of ester products in non-conventional media. But, because most of lipases have higher affinity for long-chain length substrates [139] low-molecular weight substrates have generally an inhibitory effect on these enzymes [130], cutinase has shown a great potential for the esterification trans-esterification reactions since it showed selectivity toward the production of short-chain carboxylic acid esters. For this feature also because of the low stability of lipases under industrial process conditions [140], the use of cutinases was effective in several areas such as: fine chemicals pharmaceuticals, detergents, food industry polymer chemistry [141].

Cutinases act on a wide range of substrates. The catalytic potential of cutinases is mainly related to alkyl esters synthesis, which are involved in many applications as flavor compounds (Table 7). Short chain alkyl

esters present fruity flavors which are very appreciated in many applications especially in fruit-flavored products like jam, beverages, wine dairy. Ethyl acetate has many uses, such as artificial fruit essences aroma enhancers. De Barros *et al.* [142] have worked on the optimization of the alkyl ester synthesis by using lyophilized *Fusarium solani pisi* cutinase. The esterification was achieved in organic medium (isooctane) by using ethanol short-chain fatty acids (C2–C6). The authors have demonstrated that cutinase was more effective for the synthesis of an alkyl ester with a chain length of substrates in the range of C4–C6 with esterification yields higher than 95%against an esterification yield of 90% when an alcohol chain length longer than C6 has been used. Dutta Dasu [143] have also demonstrated the effectiveness of cutinase from *Burkholderia cepacia* for the synthesis of alkyl ester with short-chain length. The use of butyric acid valeric acid with butanol gave the best yields of esterification, which reflects the specificity of the enzyme for short-chain length fatty acids.

De Barros *et al.* [142] have worked on the synthesis of hexyl octanoate which is a flavor compound incorporated in a wide range of aroma such as apple, banana, cider, grape melon. The synthesis was catalyzed by cutinase from *F. solanipisi* in a mini emulsion system. These authors have demonstrated that the variation in molar ratio of substrates has a significant influence on enzyme activity reaction yield due to inhibitory effects on cutinase activity. In the used micellar system, a maximum yield of 86% was obtained for hexyloctanoate by using an alcohol: acid molar ratio R = 1. Above this ratio, a strong inhibition effect of cutinase activity was observed.

Use of acyltransferases

Alcohol acyltransferase (AAT) plays a major role in the biosynthesis of volatile esters, which constitute a major class of compounds contributing to the aroma of many fruits. Esters are associated with 'fruity' attributes of fruit flavor typically increase to high levels in the ripening process [144-145].

The volatile esters are formed by esterification of alcohols carboxylic acids: CoA ester as the acyl donor (Fig. 6). Acetyltransferase (EC 2.3.1.84) transfer an acyl group from a donor (often CoA) to the hydroxyl, amino, or thiol group of an acceptor molecule to yield an acyl ester derivative. This enzyme has been detected in many fruits including banana [146], apple [147-148-149], melon fruit [150-151] strawberry [152-153].

The AAT presents various properties as a function of source [154]. The molecular weight is going from 50 kDa (apricot) to 400 KDa (melon). D'Auria *et al.* [115] have characterized an acyltransferase which catalyzes the formation of (Z)-3-hexenyl acetate from acetyl-CoA 3Z-hexenol. They have proved that the encoded acetyl acetyltransferase has the ability to catalyze several medium-chain-length aliphatic benzyl-derived alcohols, but has the highest catalytic efficiency with 3-Z-hexenol.

The volatile ester composition of fruits (produce a wide range of short- long-chain acyl esters) is mainly dependent on the availability of the substrates acyl-CoAs alcohols the properties of the AAT enzyme. Shalit *et al.* [155] have tested AAT by using various acyl-CoA alcohols. These authors have demonstrated that the maximum activity of strawberry AAT was obtained when acetyl-CoA hexyl alcohol were used as substrates. The same result has been shown by Pérez *et al.* [152].

Ueda Ogata [156] found out that the formation of volatile esters in banana fruit was a coenzyme A dependent reaction. According to Shalit *et al.* [155], the acetyl-CoA butyl alcohol are the preferred substrates for the banana AAT. Previous works performed using bananas strawberries have demonstrated that volatile esters present in each fruit's aroma are mainly dependent on the substrate specificity of AAT. The AAT has a determinant role in flavor biogenesis in these species [157-153].

Alcohol acyltransferase plays also a major role in the process of fermentation by microorganisms [158]. The mechanism of ester synthesis in microorganisms involves two enzymes: alcohol acyltransferase (AAT) esterase [159-158].

Use of Glucosidases

Glucosidases (EC 3.2.1.21) widely disturbed through plant kingdom; catalyze the hydrolysis of D-glycoside bond to release non-reducing D-glucose residue and terminal aglycone. Properties of glycosidases mainly in relation to their role in flavor release are now considered.

It is well known that aroma compounds can be present in fruit in a free state /or in the form of glycosides. In a great number of fruits, a significant part of important flavor compounds is accumulated as a non-volatile fraction, which is known as glycosidic aroma precursor. Glycosidic flavor precursors were detected in different parts of the plant as in green parts, roots, rhizomes seeds [160]. In these tissues, any amounts of flavor compounds are bound as non-volatile sugar conjugates. The sugar moiety includes generally glucose (*O*-β-D-glucosides), but there are other glycones like pentoses, hexoses, dis-accharides trisaccharides [161]. The aglycone part of glycosides is often represented by monoterpenes, C13-norisoprenoids, benzene derivatives long-chain aliphatic alcohols.

The widespread occurrence of glycosylated secondary metabolites such as flavonols, anthocyanins, monoterpenes, norisoprenoidic compounds in plant tissues, confirms the central role of glycoside hydrolase on metabolism there-for its involvement in a large number of major biological processes. In enzymatic release of aroma volatiles, a sequential hydrolysis is involved: (i) the hydrolysis of diglycosides, involving several exoglycosidases depending on the sugar moieties of the substrates. (ii) The cleavage is then made in the inter sugar linkage by an endoglycosidase to release the aglycone glucose [162]. The reaction rate of glycosidase differs as function as the substrate used. Park Noble [163] has demonstrated that glycosides of tertiary alcohols such as linalool, linalool oxides a-terpineol are more readily hydrolyzed than those of primary alcohols such as geraniol nerol. These authors found that after 2 years of storage at 10°C, more than half of the glycosides of geraniol were still present in a muscat wine, while glycosides of linalool were totally hydrolyzed.

The effect of glycosidases on flavor recovery from glycosides in fruit juice production in winemaking has attracted much attention has been reported in many works [164-165]. The use of β-glucosidases in the wine industry is interesting because they can promote the liberation of aromatic compounds from monoterpene glycoside precursors present in young wines [166]. Su *et al.* [167] have also approved the important role of glycosidase in determining the beverage quality of tea. The aroma compounds of tea such as: monoterpene alcohols (such as linalool geraniol) aromatic alcohols (such as benzyl alcohol 2 phenylethanol) are present as monosaccharide or disaccharide glycoside precursors in fresh leaves of tea plants. Su *et al.* [167] have demonstrated that after treatment with immobilized glucosidase, the essential oil of tea beverage was improved obviously the total amount in green tea black tea was increased by 20.69 6.79%, respectively. These authors concluded that immobilized glucosidase could be used to treat tea beverage successfully by hydrolyzing the glycosidic aroma precursors effectively. Nevertheless, the stability of immobilizedglucosidase exhibited remarkable improvement may be a useful biocatalyst for aroma-increasing in the tea beverage industry. González-Pombo *et al.* [168] have also worked on the aroma enhancement of wine by using co-immobilized *A. niger* glycosidase. These authors have demonstrated the excellent stability of immobilized enzyme in wine conditions they highlighted the importance of exogenous glycosidases for the enhancement of wine flavor. The treatment of muscat wine with the biocatalyst for 20 days has significantly increased the amounts of free monoterpenes (from 1119 to 2132 g/L).

Use of Multi-enzymatic system

Aliphatic aldehydes alcohols (C6 C9 compounds) are considered as attractive flavors in the aroma industry conferring a fresh green character authenticity to goods [169]. The multi-enzymatic system "lipoxygenase pathway" presents the most widely accepted enzymatic pathway for conversion of polyunsaturated fatty acids to these compounds. Green leaf volatiles are represented by C6- orC9-aldehydes alcohols which are formed through the lipoxygenase pathway. In many higher plants, this enzymatic pathway is activated following a chemical or mechanical aggression of plant tissues. The formation of aldehydes alcohols in the plant is therefore related to the cell destruction.

The lipoxygenase pathway is a multi-enzymatic system [180] in which polyunsaturated fatty acids (linolenic or linoleic acid) are converted by the sequential action of lipoxygenase, hydroperoxidelyase alcohol-

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dehydrogenase into aldehydes alcohols (Fig. 6).The initial step in the lipoxygenase pathway should be the liberation of free fatty acids by lipolytic enzymes [171-94]. Once liberated, these polyunsaturated fatty acids are oxygenated by the action of lipoxygenase which catalyzes the di-oxygenation of linoleic linolenic acids to form both 13- or 9-hydroperoxides depending on the enzyme selectivity. The formed hydroperoxy-fatty acids may be further metabolized by several metabolic pathways [172]. One of them is mediated by hydroperoxide lyase alcohol-dehydrogenase involving conversion into aldehydes alcohols. In this case, the hydroperoxide lyase specifically cleaves the hydroperoxy linoleic hydroperoxy-linolenicacids (on C13 or C9 position) to form aldehydes (C6 or C9), as well as oxo-acids (C12 or C9). The final enzyme involved in the lipoxygenase pathway is alcohol dehydrogenase. In the presence of cofactor NADH/NAD, this enzyme catalyzes the interconversion of aldehydes (C6 or C9) alcohols (C6 orC9) (Fig. 7).

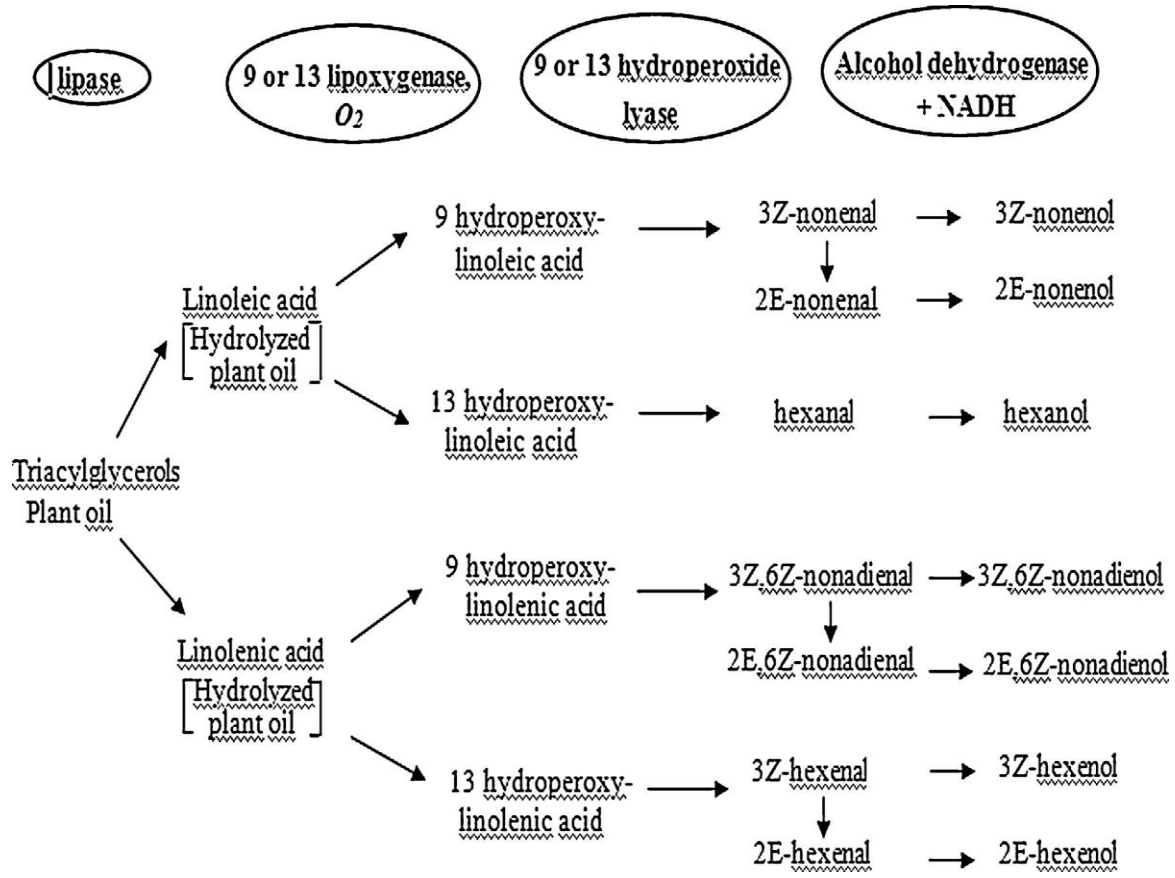

Fig.7. Major products, flavor precursors and enzymes involved in the lipoxygenase pathway.

Use of Lipoxygenase

Lipoxygenases (EC1.13.11.12) are non-heme iron-containing fatty acid dioxygenases, which are widely distributed in the plant kingdom animals possess diverse functions [172]. Specific lipoxygenase (LOX) genes have been identified in several fruit species such as: apple [173-147], strawberry [174], pear [175] kiwi fruit [176]. Lipoxygenase catalyzes the regio- stereo-specific dioxygenation of polyunsaturated fatty acids (C18:2 C18:3) containing a (1Z, 4Z)-pentadiene system to give the optically active monohydroperoxides [187].

Polyunsaturated fatty acids are oxygenated either at carbon atom 9 (9-LOX) or at C-13 (13-LOX) of the fatty acid leading to two groups of compounds: 9-hydroperoxy- 13-hydroperoxy fatty acids (Fig. 7). The specificity of lipoxygenase depends on the source. 13-Hydroperoxy fatty acid is the major product of the soybean lipoxygenase [178-179], melon [180] cucumber [181]. In tomatoes, lipoxyge-nase produces mainly 9-hydroperoxy fatty acid [182]. The same specificity was demonstrated in potato [183-184]. For Galliard Phillips [178], the relative amounts of the 13 9-hydroperoxyfatty acids in soybean depend on the pH of the

surrounding medium. Two lipoxygenase activities exist in plants: one is specific to the position 9 is active at acidic pH, the other is specific to the position 13 is active at basic pH [185].

There are differences between the lipoxygenases from different sources, however, common structural features were observed. A more comprehensive classification of plant lipoxygenases based on comparison of their primary compound, has been proposed. In soybeans, four isoenzymes have been distinguished (LOX-1, LOX-2, LOX-3a LOX-3b). Each isoenzyme has a molecular weight of 100 kDa [179]. Lipoxygenase activity varies according to the stage of development. For leaves of watermelon, the maximal activity was observed on the sixth day of germination [180]. In alfalfa, the most important activity was observed in the first second day of germination [181]. The activity is also influenced by the seasons, it is maximal in summer may disappear in winter [186].

The LOX pathway generates two kinds of aldehydes by using hydroperoxy-fatty acids as precursors: C6 or C9 aldehydes. The nature of aldehyde depends on the position of hydroperoxide function on the specificity of HPLS (Fig. 7). C6 aldehydesare volatile compounds that show a significant green note flavor [187]. These molecules result from the cleavage of the 13-hydroperoxy fatty acids in the presence of 13HPLS [188]. The hexanal is produced from 13-hydroperoxy linoleic acid is characterized by green odor, while the 3Z-hexenal has a scent called green or herbaceous occurs from 13-hydroperoxy linolenic acid. The3Z-hexenal is an unstable molecule that isomerizes to 2E-hexenal “leaf aldehyde” which is characterized by note of cutgrass [185].

C9 aldehydes are responsible for the fresh note [189]. They are produced from 9-hydroperoxy fatty acid isomers in the presence of 9HPLS. The 3Z-nonenal is produced from 9-hydroperoxy linoleic acid is characterized by the smell of melon. It is the major product resulting from the cleavage with pear HPLS extract. This compound isomerizes to 2E-nonenal [189]. The 3Z, 6Z-nonadienalis produced through the action of 9HPLS on 9-hydroperoxylinolenic acid. This compound isomerizes to 2E, 6Z-nonadienalwhich is characterized by a slightly green note fat naturally encountered in cucumber.

The biosynthesis of aldehydes at large scale was conducted by mixing polyunsaturated fatty acids with plant material. This later should contain the enzyme activities of lipoxygenase pathway [190-191-192]. Many publications patents have used reactors processes for the preparation of green aroma compounds or for the production of intermediate products of lipoxygenase pathway (Table 7). The synthesis can be carried out either by using polyunsaturated fatty acid or by using a precursor introduced as oil hydrolyzate**.** Kerler *et al.* [193] have produced aldehyde by using hydroperoxide lyase as catalyst hydrolyzed linseed sunflower oils as precursors. The sequential catalysis of linseed oil has allowed to produce mainly the 2E-hexenal. Its isomer: 3Z-hexenal (less stable) was produced in smaller amounts. The highest yield of hex-anal production was obtained when the sunflower oil was used as a substrate. The authors have also produced the C9 aldehydes. The major component was the 2E, 6Z-nonadienal. Hausler *et al.* [194] have used HPLS from viola leaves to produce C9 aldehydes. They have demonstrated that the addition of a mixture of precursors of polyunsaturated fatty acid as function of the reaction has a beneficial effect on the enzymatic reaction. This process enabled them to produce 600 mg of 2E, 6Z-nonadienal/kg of plant material.

Németh *et al.* [195] have produced 37% of hexenal relative to the 13-hydroperoxy-linolenic acid concentration by using HPLS from green bell pepper. By using HPLS activity from guava, Muller *et al.* [192] have produced hexenal with an overall yield of 36%. 54% of hexanal relative to the 13-hydroperoxy-linoleic acid concentration was produced by Marczy *et al.* [196] by using spinach leaves HPLS.

The enzymes implied in lipoxygenase pathaway especially HPLS are known by their low stability under experimental conditions, which limits their industrial applications. Immobilization is generally used to enhance the stability of these enzymes. Schade *et al.* [197] have used immobilized enzymes extracted from different plant tissues, such as leaves of tomato strawberry to produce hexanal. The biocatalysts were immobilized in alginate matrix in a fixed bed reactor. The use of optimal conditions allowed them to produce 80.2 µg of hexanal/g fresh weight of tomato leaves 22.4 µg/g fresh weights of strawberry leave. This production was 112 times higher than the amount produced endogenously by the same amount of plant tissue used. The biosynthesis allows not only to enhance the production yields of aroma compounds but also offers the possibility of using enzyme activities extracted from different sources to make successive reactions [170-187-198]. Ben Akacha *et al.* [199] have worked on the production of hexenol from hydroperoxy linolenic acid

in one step by a coupled enzymatic reaction involving mint HPLS yeast ADH. The authors have proposed a mathematical model to predict the performance of the system to optimize the productivity.

Use of hydroperoxide-lyase

Hydroperoxide lyase (HPLS) is a suitable enzyme for the biocatalytic production of aldehydes (C6 C9), which are responsible for the "green note" in many fruits vegetables. The hydroperoxide lyase (HPLS; EC 4.1.2.92) is a member of the cytochrome P450-family, including CYP74. In lipoxygenase pathway, this enzyme catalyzes the cleavage of 13- 9-hydroperoxides of linoleic linolenic acid into volatile C6- or C9-aldehydes C12- or C9-oxoacids, respectively [200] (Fig. 6). HPLS is widely distributed in the plant kingdom is expressed at different compartments of plant: leaves, cotyledons, seedlings seeds. This enzyme is presented in the green leaves of higher plants with significant amounts, in opposite to the leaves with low chlorophyll content which contain low HPLS activity [185].

HPLS was first cloned from green bell peppers is designated as CYP74B [201]. HPLS has been identified in several plants, including tomato fruit [202], guava fruit (*Psidium guajava* L.) [203], cucumber (*Cucumis sativus* L.) [204], melon [169], watermelon seedlings [180], tealeaves [205], alfalfa seedlings [206], soya leaf [179], spinach leaves [207], olive fruits [208], olive leaves [170] mint leaves [209-210]. Each plant may contain two forms of HPLS (13HPLS: with specific cleavage position on carbon13, 9HPLS: with specific cleavage position on carbon 9) but with different levels of expression. For guava fruit cucumber, 13HPLS is the dominant form, which leads to the major production of C6-aldehyde: 3Z-hexenal in guava fruit. The 3Z-nonenal was preferentially produced in melon.

Use of alcohol dehydrogenase

Alcohol dehydrogenase (EC 1.1.1.1) is an oxidoreductase that contributes to flavor development by interconverting aldehyde alcohol, depending on the presence of cofactor other effectors in the medium [211-212]. Alcohol dehydrogenase (ADH) is the last enzyme involved in LOX pathway, which acts by reducing C6 or C9 aldehydes to their corresponding alcohols (Fig. 7). This enzyme was identified in microorganisms, animals plants. It was purified from different plant tissues such as soybean seeds [213], barley wheat seeds [214] cucumber fruit [215]. In fruits, ADH has been related to the interconversion of aldehyde alcohol, to their accumulation in the fruit during ripening [216]. ADH is also involved in a wide range of responses to other stresses, elicitors to abscisic acid [217]. However, the ADH gene expression has been shown to be tissue-specific [147]. In tomato, Longhurst *et al.* [218] have identified two ADH genes. ADH1 is found in pollen, seeds young seedlings, ADH2 is contained in tomato fruit during ripening when there is an increase in the synthesis of flavors. Therefore, these authors have suggested that ADH2 may play an important role in flavor development.

In LOX pathway, the use alcohol-dehydrogenase extracted from baker's yeast has allowed to produce alcohols from their corresponding aldehydes [210]. Aldehydes alcohols are high value molecules widely used in flavors to impart both the green character the impression of freshness. The practical application of this dehydrogenase can be, however, quite challenging remains limited by the high cost of cofactor. This owes in part to the difficulty of regeneration of the cofactor (NAD/NADH or NADP/NADPH) of crucial role for the reaction. So, an efficient economical cofactor regeneration system is necessary [219]. This approach has found novel applications in biocatalysis is critical to the economical viability of industrial scale biotransformation using oxidoreductases. Many chemical, enzymatic electrochemical methods have been investigated to regenerate various cofactors [220-221]. These methods include the addition of second enzyme to the system to catalyze the regeneration reaction, which is known as enzyme-coupled process, or the addition of substrate for the second reaction catalyzed by the same enzyme used in the main reaction, which is known as a substrate-coupled process. Several NADH regeneration systems have been performed with living cells [222]. Living cell metabolism offers not only the internal supply of reduction equivalents, but also prevents costly enzyme isolation steps provides stability for the biocatalyst [223]. Ben Akacha Gargouri [170] have worked on the enzymatic production of C6-aldehydes alcohols by using LOX from soja HPLS extract from olive leaves. The whole cells of yeast containing ADH activity has been used by these authors in order to avoid the expensive additional use of cofactor (NADH) required to ADH activity. They have demonstrated that the hexenals were successfully reduced into their corresponding alcohols by adding yeast cells *Saccharomyces*

cerevisiae. Significant amounts of 3Z-hexenol (up to 3.54 g/kg of olive leaves) were produced extracted with a yield of 47.7% with high purity when permeabilized yeast cells were used.

Several studies have focused on the *in situ* conversion of aldehydes into their corresponding alcohols by using alcohol-dehydrogenase (Table 7). Baker's yeast "*S. cerevisiae*" has been used in several studies as a source of this activity to produce hexenol from hexenal [170-224-213]. The 3Z-hexenol called "leaf alcohol" can be produced by lipoxygenase pathway from linolenic acid (Fig. 6). The organoleptic properties of this compound make it of special importance in the field of aroma chemicals.

In the process of production of alcohol from hydroperoxy-fatty acid, when the reaction of cleavage by HPLS the reaction of reduction by ADH are conducted separately, the cleavage product (3Z-hexenal) isomerizes to form 2E-isomerbefore the start of the second reaction. This results in the predominant production of the 2E-hexenol, which is of less importance than its isomer 3Z-hexenal. Moreover, the ADH reaction can be simultaneously conducted with HPLS reaction to convert 3Z-hexenal into its corresponding alcohol, which avoids isomerization of the double bond [224-192].

Muller *et al.* [192] have mixed guava homogenate (containing HPLS activity) and baker's yeast (containing ADH activity) to produce C6 aldehydes and their corresponding alcohols. These authors have demonstrated that the temperature is a determining factor in the isomerization process. By using low temperature (20◦C), 2E-hexenol was produced with minimal yield (10%) compared to 3Z-hexenol. However at 50◦C, they have mainly produced 2E-hexenol (63%).

Ben Akacha Gargouri [170] have proposed an enzymatic liquid/gas reactor, where the synthesis of C6-compounds was coupled to their extraction. Hexenals were produced in two steps: (1) 13-hydroperoxy-linolenic acid was produced from hydrolyzed linseed oil in presence of soybean lipoxygenase. (2) 3Z- 2E-hexenals (up to 0.36 g/kg of reaction medium) were produced from 13-hydroperoxy linolenicacid in presence of olive hydroperoxide-lyase. The authors have also used ADH activity containing within whole cells of yeast. Whole cells of yeast were used especially to avoid the expensive additional use of cofactor (NADH) required for ADH activity. In order to reduce the number of steps needed to produce to extract the hexenol, the reduction reaction catalyzed by ADH subsequent product extraction were carried out in the reaction medium where hexenal was produced by using LOX HPLS. Ben Akacha Gargouri [170] have demonstrated that the coupled enzyme system allowed increasing the conversion rate. They have produced extracted 3Z-hexenol at a yield of 47.7% with high purity.

Bioprocesses for the production and recovery of aroma compounds

Bioreactor designs

The selection of a proper reactor configuration is an important aspect in designing industrial enzymatic synthesis of natural aroma compounds. There are various bioreactor configurations used, depending not only on the nature of reactants and catalysts but also on the bioprocess costs. Several economic requirements have to be considered. The most important are: cheap, robust and simple mechanical design, easy to scale-up and low power consumption. Two types of bioreactors are generally used for microorganisms and enzymatic syntheses of aroma compounds: the packed-bed reactor and the fluidized bed reactor.

In addition to bioreactor configurations, it is very important to choose the operation mode. Bioreactors can be operated indifferent modes, such as: batch, fed-batch and continuous. (i) In batch operation; no fresh material is introduced or removed from the bioreactor during processing, meaning all nutrients are presented from the start. (ii) In a fed-batch culture, the feed is added continuously to the bioreactor in order to keep nutrient levels and the growth rate at a constant predefined value. (iii) In continuous operation; fresh material is introduced and the product is removed continuously during processing.

The use of soluble enzymes for the industrial-scale synthesis of flavor compounds is economically unprofitable. The enzymes lack generally reusability in continuous syntheses due to their low stability complex separation techniques [225]. The immobilization rep-resents a suitable alternative to accomplish the enzymatic syntheses of aroma compounds. Immobilization of enzymes results in several advantages such as:

the easier recovery reuse of the enzyme, it may simplify the use of enzymes in continuous-flow reactors immobilization of enzymes results often in an improved stability. Packed-bed bioreactors are therefore the most frequently used for aroma synthesis. The ratio between substrate enzyme is much lower in a packed bed reactor than in conventional batch reactors, these results in shorter reaction times [226]. Commercially, bed reactors are best used in continuous mode. This operational mode has many advantages over a batch packed bed reactor: facilitates the automatic control operation, minimizes the labor overhead costs allows the stabilization of operating conditions.

Use of packed-bed reactors for flavor biosynthesis

Packed-bed reactor in continuous mode constitutes one of the mostly used reactors for the synthesis of esters. These reactors have been reported as being the most suitable for industrial scale applications because of their high efficiency, low cost ease of construction, operation maintenance. This configuration has been used in many studies for lipase-catalyzed ester synthesis of butyl oleate [227], ethyl oleate [228], and erythrityl laurate [229] citronellyl butyrate [230]. Ju *et al.* [231] have used the lipozyme (*Rhizomucor miehei*) for the synthesis of hexyl laurate in a solvent-free system by using a continuous packed-bed reactor. These authors have evaluated the effects of synthesis parameters by using response surface methodology three levels have been optimized: the temperature of reaction, the mixture flow rate the concentration of lauric acid. They have demonstrated that the production rate was significantly affected by the mixture flow rate by lauric acid concentration. The use of optimized conditions has allowed to reach 81.58 ± 1.76 µmol min^{-1} of hexyl laurate [231].

Carta *et al.* [232] have used a nylon-immobilized lipase from *C. cylindracea* in batch continuous-flow reactors for the synthesis of ethylpropionate, isoamylpropionate isoamylbutyrate. They have demonstrated that the packed-bed reactor was operated successfully when the synthesis of these esters was achieved in continuous mode. Covalent immobilization has allowed forming a very stable lipase by maintaining the active conformation of this enzyme by reducing the denaturing effects of environmental factors.

Use of fluidized bed reactors for flavor biosynthesis

Fluidized bed reactors are widely used in many industrial applications including biocatalytic reactions and aerobic fermentation processes. In this reactor, the bed materials are homogeneously suspended in a fluid stream, which presents the advantage of proper mixing over other contacting methods. Also, pressure drop across the bed appears to be much lower than that in the corresponding packed bed reactor. This configuration allows also to work in a continuous operational mode and to improve the heat and mass transfers.

Jakovetic *et al.* [233] have used a fluidized bed reactor for the synthesis of ethyl cinnamate by immobilized lipase (*C. antractica*). These authors have compared the time course for the enzymatic synthesis of ethyl cinnamate, over a 24h period, by using two reactor modes: batch fluidized bed bioreactors. They have demonstrated that the reaction proceeded more quickly in the fluidized bed bioreactor (6 h) than in the batch one (23h) with respective ester productivity of 0.361 0.089 $mmolh^{-1g-1}$of lipase. Jakovetic *et al.* [233] have demonstrated, after the calculation of mass transfer limitations in both bioreactors, that external internal mass transfer limitations were negligible that the esterification was a kinetically controlled reaction in both bioreactors.

Fluidized bed reactor has been also used by Saponjic *et al.* [234] for the synthesis of amyl caprylate by *C. rugosa*lipase (immobilized on Sepabeads). High production yields of amyl caprylate have been reached by using both batch continuous syntheses. Continuous batch esterification allowed to obtain yields of 90.2 100% within 14 24h, respectively [234]. The immobilized lipase from *C. rugosa* has been also used by Damnjanovic *et al*. [235] for the synthesis of geranyl butyrate in a fluidized bed reactor. These authors have worked on the improvement of the process performance by studying the bioreactor hydrodynamic characteristics the reaction conditions. Damnjanovic *et al.* [235] have demonstrated that the flow rate strongly influences ester production by immobilized *C. rugosa* lipase in fluidized bed reactor. Thus, the productivity reached 6.1 $mmolL^{-1h-1}$ if a flow rate of 2.67 mL min^{-1} was used against a productivity of 5.8 $mmolL^{-1h-1}$ obtained at a flow rate of 3.70 mL min^{-1}. Flow rate was proved to be significant for reactions catalyzed by immobilized enzymes, since it has a reciprocal relationship with residence time. Usually, longer

residence time enhances the reaction by enabling longer enzyme–substrate contact, which can enhance productivity. Many studies have suggested that lower flow rates longer residence times are more productive due to pro-longed contact between enzyme substrates [236-237]. Using increased flow rate led to a reduction of contact time between the enzyme surface substrates, which cause the reduction of enzymatic conversion [234]. It appears however that at high flow rates, mass transfer is enhanced diffusional limitations are reduced. A higher value of the solid–liquid mass transfer coefficient was found by Jakovetic *et al.* [233] in a fluidized bed bioreactor by using an intense fluid flow.

Methods used for the extraction of aroma compounds

Interest in mild separation techniques for the isolation of aroma components has increased during the past decade. The extraction method used should provide an extract with sensory characteristics as close as possible to the complete product. It is therefore important to maintain ingredient functionality of aroma during processing by the development of non-thermal and nondestructive technologies that can replace conventional processes of separation.

Commercial production of aroma compounds by bio-technological ways often lacks economic profitability. The microbial biosynthesis is generally limited by the low productivity or poor concentrations of target compounds in the fermentation broth. The main reason of low levels of productivity is due to the accumulation of hydrophobic aroma compounds having an inhibitory effect in the fermentation medium [238-239]. Once produced, these inhibitory compounds have to be removed from the vicinity of the cells [240]. *In situ* product removal is a powerful tool to overcome these limitations [240]. As well as allowing the recovery of aroma compounds by the fast removal of the products from the producing cells, this concept (i) enables detoxification of the culture medium, resulting in an improvement in the efficiency of biosynthesis, (ii) minimizes product losses (*e.g.* by degradation, evaporation), (iii) reduces the number of subsequent downstream processing steps [240]. In this process, upstream downstream processes are coordinated (Fig.8).

Aroma recovery both from fermentation media other biological media is not an easy task since aroma is usually diluted in a complex matrix. Additionally, aroma fractionation may be needed when aiming at recovering separately a particular aroma or a group of aroma compounds. Various bioprocesses, aiming at defining suitable strategies for the synthesis /or recovery of aroma have been studied on a laboratory scale [241-170]**.** Continuous removal was achieved using techniques based on extraction, permeation, adsorption evaporation (Figs. 8 9).

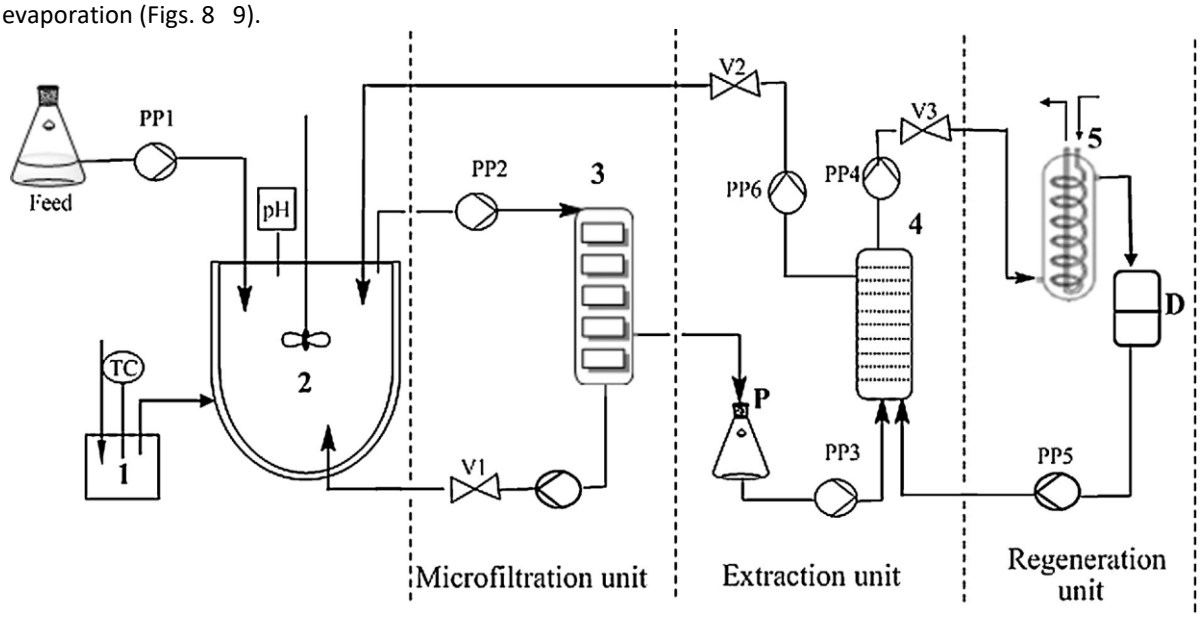

Fig.8. Experimental setup used for production of natural aroma compounds by a hybrid system consisting on microfiltration and membrane extraction interconnected with the bioreactor. (1) Thermostat; (2) bioreactor; (3) microfiltrationmembrane module; (4) hollow fiber membrane module; (5) condenser. D: distillate; P:permeate; PP(1-6):peristaltic pumps; V(1-3):needle valves.

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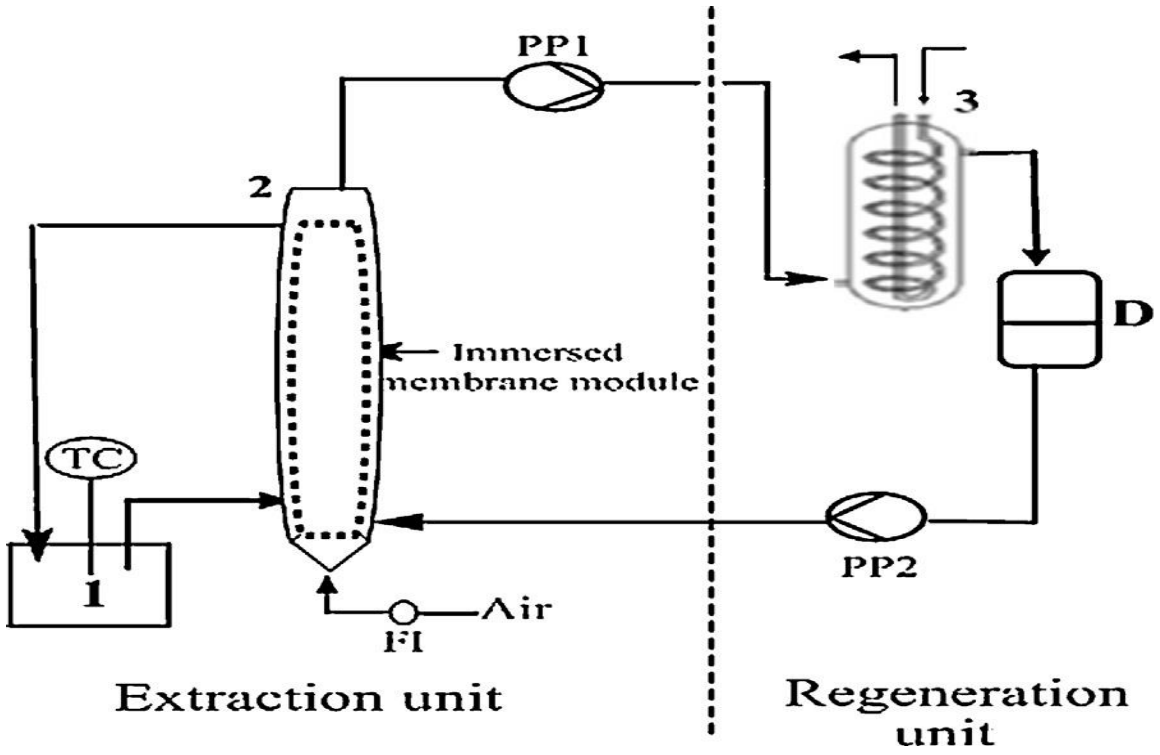

Fig.9. Schematic set-up of bioreactor system for aroma production by hybrid system consisting of an air lift reactor and immersed extraction hollow fiber membrane module.(1) Thermostat; (2) air lift reactor; (3) condenser. D: distillate; PPi: peristaltic pumps; FI: flow indicator.

Chiral separation of racemic mixtures of aroma compounds:

Many components of natural products can be chiral, which leads to the formation of one pair of enantiomers. Enantiomers are stereo isomers, with a molecular asymmetry, that exhibit non super imposable mirror images. These compounds have identical physical chemical properties, except that they rotate polarized light in opposite directions. So, the two enantiomers are classified as dextro (+) or levo (−). There are remarkable examples of enantioselectivity for flavor compounds. Most of them are chiral show different odor properties according to their absolute configuration [242-243]. For example, carvone or linalol are two enantiomers that exhibit different odor properties. Chiral discrimination represents one of the important principles of odor perception [244]. The difference in specific olfactive profile makes generally the preference for one of the enantiomers, which is often of main importance [245].

The final olfactive properties of natural product depend on the enantiomeric ratio, which represents a valuable parameter for differentiating natural flavor compounds from those of synthetic origin [246]. Zawirska-Wojtasiak [245] has studied the characteristic enantiomeric ratio of 11-octen-3-ol in various species of edible mushrooms. Eight carbon atom compounds particularly 1-octen-3-ol represents the main odorant of the mushroom which is formed by the enzymatic oxidative breakdown of linoleic acid. This compound may exist in two optically active forms: (R)-(−)-octen-3-ol having a fruity mushroom-like characteristic, (S)-(+)-octen-3-ol with a moldy grassy note [247]. Zawirska-Wojtasiak [245] has shown, after determining the ratio of 1-octen-3-ol optical isomers in several species of edible mushrooms, that the optical purity of (R)-(−)-octen-3-ol was very high in all mushroom species. The highest yield was obtained in *Agaricus bisporus* (98.5%) the lowest in*Xerocomus badius* (82.1%).

Applications of chiral active compounds concern mainly the synthesis of pharmaceuticals, but enantiomerically pure compounds are increasingly needed for the production of flavors and agrochemicals. In flavor industry, the production of optically enriched or optically pure compounds is of crucial importance. These individual species may have interesting odor properties, which differ from those of a corresponding racemic mixture. Menthol is an outstanding example. (–)-L-menthol represents the desired enantiomer used in many applications with an estimated global market of 5000 tons per year [248].

The production of many types of useful optically active compounds, whose demand is estimated to be growing, has attracted much attention. The synthesis of enantiomerically pure compounds by using processes which are more economical and more environmentally are being developed. There are many enzymatic and microbial resolution methods used to separate enantiomers in racemic mixtures.

Enantiomerically pure primary secondary alcohols are important as intermediates in aroma flavor enhancing compounds. These chiral compounds can be obtained by processes based on the microbial or enzymatic reduction of the corresponding ketones. The active enantiomer can be also obtained by the kinetic resolution of the racemic alcohols *via* hydrolysis of the appropriate ester with lipase. Lipase-catalyzed reactions have been applied in the kinetic resolution of enantiomeric mixtures of primary secondary alcohols [249]. By ensuring the region selective enantioselective biotransformations, this enzyme is widely used for the efficient syntheses of chiral drugs, fragrances pheromones [126].

Immobilized *C. antarctica* lipase B has been used by Franssen *et al.* [250] for the kinetic resolution of branched-chain fatty acids. By esterifying racemic 4-methyloctanoic acid with ethanol, only the ester R was obtained, which proved that(S)-4-methylctanoic acid was not converted [250].

Chojnacka *et al.* [251] have studied the kinetic resolution of racemic mixtures of several secondary aliphatic allylic alcohols in lipase-catalyzed stereoselective transesterification with various vinyl esters as acyl donors. After testing different lipases, these authors have demonstrated that Novozym435 (*C. antarctica*) proceeded with good enantioselectivity was very effective in the acylation of (±) hepten-3-ol (±)-5-methylhexen-3-ol.

The optical resolution of racemic secondary alcohols by enzymatic or microbial esterification transesterificationis much more applied than primary alcohols. The optical resolution of primary alcohols is more difficult [252]. Oda *et al.* [253] have developed a double coupling system for the optical resolution of racemic citronellol. They have used acetyl coenzyme A (obtained from the metabolism of glucose), the subsequent acetylation of the primary alcohol by acetyl coenzyme A was achieved with alcohol acetyltrans-ferase. Oda *et al.* [253] have demonstrated that *Pichia kluyveri* IF0 1165 was effective for the optical resolution of racemic citronellol provided S-citronellyl acetate with high enantioselectivity yield.

(−)-Menthol is one of the most important terpenoids flavoring agents in natural peppermint oil. After vanillin, it is the most widely used aroma chemical worldwide. The characteristic odor of peppermint oil the typical refreshing effect resulted from the (−)-menthol. This later finds applications in cosmetic, pharmaceutical, flavor, pharmaceutical, tobacco toothpastes. The racemic mixture of menthol D-/L-menthol can be resolved enzymatically. Many lipases (from *Penicillium*, *Rhizopus*, *Trichoderma* from various bacteria) are generally used for such application. These enzymes preferentially hydrolyze the (−)-menthyl esters enantiomer, whereas (+)-menthyl esters are not hydrolyzed. The use of lipase from recombinant *C. rugosa* by Vorlova *et al.* [254] for enantioselective hydrolysis of racemic menthyl benzoate has allowed to produce optically pure L-(−)-menthol with yield higher than 99%.

Some examples of bioprocesses elaborated for coupled synthesis/extraction of aroma compounds

The elaboration of complete bioprocesses for the synthesis of aromatic compounds is of crucial importance for economical production of molecules with natural properties. The dem for natural aroma compounds, from microbial fermentation or biotransformation, is important due to the strong preference by consumers the need of bio-based natural ingredients in foods, cosmetics pharmaceuticals. Jin *et al.* [255] have scaled up the synthesis of short-chain flavor esters in a 5 L batch stirred reactor after determining the optimal conditions for esters synthesis. The synthesis was catalyzed by *C. antarctica* lipase B-displaying *Pichia pastoris* whole cells which is characterized by its low preparation cost simple recycling procedure. Jin *et al.* [255] have demonstrated that the used biocatalyst showed good tolerance for high substrate concentrations excellent operational stability. A yield of 95% has been reached for several esters. Ethyl acetate is an important volatile compound manufactured on a large scale for use not only as a solvent but also as aroma enhancer. The annual production of this compound is of 1.5 million tons [256]. Ethyl acetate can be produced naturally by using *K. marxianus* which is able to convert lactose into ethyl acetate. Löser *et al.* [256] have studied the effect of Fe on growth of *K. marxianus* the efficiency of production of ethyl acetate by this yeast on a pilot scale

since some Fe is required for the growth of yeasts containing the biocatalyst needed for ester synthesis. *K. marxianus* DSM 5422has been cultivated aerobically in whey-borne medium using1 L or 70 L stirred reactor. The mass of produced ethyl acetate related to the sugar consumed was amounted to 0.239 g/g when 10 mg/L of Fe were supplemented to the pre-culture [256]. Butyric acid is also used in many industrial applications. It can be used as the pure acid to enhance butter-like notes in food flavors or in the form of esters used as additives for increasing fruit fragrance. The biotechnological production useful for the economical butyric acid production on industrial scale is of great interest. Jiang *et al.* [257] have used fibrous bed bioreactor with *Clostridium tyrobutyricum* cells immobilized in the fibrous matrix packed in the reactor for producing butyric acid from cane molasses. The fermentation was achieved in batch, repeated-batch fed-batch reactors. Several cane molasses pretreatment techniques have been investigated sulfuric acid treatment gave the best results regarding butyrate concentration. The fed-batch fermentation from cane molasses pretreated with sulfuric acid has increased the concentration of butyrate which was obtained with a concentration of 55.2 g/L [257]. In order to get high yields productivities, it is important to choose the convenient reactor design the convenient system for the removal of volatile compounds because some of these compounds are strong inhibitor of the biomass growth or biotransformation. To overcome the product inhibition, continuous product removal from the fermentation medium is necessary. There are several possible ways for simultaneous removal of volatile products from the bioreactor such as: the two-phase extraction [241-258]; the adsorption [70] the pervaporation [259].

The membrane based solvent extraction represents a special type of two-phase extraction. Membrane extraction is commonly used for applications where product removal from the reactor medium is required [260]. In this case, the organic solvent is physically separated from the aqueous phase with a membrane in a membrane module the contact between phases is mediated only by the pores of the membrane. During the bioprocess, the membrane separation technique such as microfiltration membrane extraction interconnected with the bioreactor in one hybrid system, can be done by (*i*) an external module for membrane extraction: in this case the fermentation medium with biomass can be led directly to the extraction membrane module then back to the bioreactor [261] or the medium is first treated by microfiltration (prevent fouling of the extraction module) before passing through extraction module. The permeate is then led to the extraction module where the product is extracted to the organic phase, fermentation medium without product is returned back to the bioreactor (Fig. 8) [262]. (ii) the extraction membrane module can be also immersed directly in the bioreactor medium (Fig. 6). In this case, the use of microfiltration mem-brane a pump for microfiltration or extraction circuit is not necessary [263-264]. The membrane separation technique such as microfiltration membrane extraction interconnected with the bioreactor in one hybrid system is one among the membrane reactors mainly used in recent years [265-266]. This process has been commonly used for the separation of the rose-like aroma: 2-phenylethanol [262-267-264]. 2-Phenylethanol (with toxic concentration of around 4 g/L for *S. cerevisiae* (Stark *et al.,* [249] should be removed from the fermentation medium to overcome the inhibition of biotransformation. Mihal *et al.* [261] have produced 2-phenylethanol by using a hybrid system coupling an aerobic fed-batch bioreactor to the membrane separation techniques such as cross-flow microfiltration membrane based solvent extraction (Fig. 8). The feed from the bioreactor was led to the microfiltration unit to obtain a fermentation medium without yeast cells the permeate was continuously led to the extraction module, where 2-phenylethanol was extracted to the organic solvent. Solvent can be regenerated from the extract in a regeneration unit by distillation. Raffinate (fermentation medium without product) was then returned back to the bioreactor (Figs. 8, 9). Mihal *et al.* [264] have also developed a system in which the extraction membrane module was immersed directly in the medium in the bioreactor. The hybrid system used for the biotransformation of l-phenylalanine to 2-phenylethanol by *S. cerevisiae*, consisted of an air lift reactor an immersed extraction hollow fiber membrane module. 2-phenylethanolwas produced with high volumetric production (up to 18.6 g/L) the conversion yield was about 100%.

Genetic Engineering for the Production of Natural Flavor Compounds: General Aspects and Process Improvement

Genetic engineering provides tools such as simple gene deletions or amplifications, DNA rearrangements in a species, trans-species gene transfer bioanalytical screening monitoring techniques, others that could benefit the biocatalysts the biotechnological production of natural flavors [268]. From the technical point of view, genetic engineering applied to food products has reached an interesting status, however, legal public concern are the major drawbacks for its application. Furthermore, there are few, but

important reports on genetic engineering to produce flavor compounds, specially reporting the over expression of some genes in *E. coli* [269].

Some researchers have described *Escherichia coli* utilization to obtain vanillin by bioconversion [270-271]. *Escherichia coli* JM109/pBB1 has been studied to produce vanillin from hydrolyzed corn cob. This by-product of corn industry was hydrolyzed for 6 h with 0.5N NaOH at solid/liquid ratio of 0.084 $g.g^{-1}$ allowed obtaining a hydrolyzate containing 1171±34 $mg.L^{-1}$ ferulic acid 2156±63 $mg.L^{-1}$ *p*-coumaric acid that was used as a medium for vanillin bioproduction. Biomass pre-cultivated once in unsterilized hydrolyzate was able to effectively convert ferulic p-coumaric acids to a mixture of vanillin, vanillic acid vanillyl alcohol provided with the typical vanilla flavor. At initial biomass concentration of 0.5 gL^{-1} (dry matter), a maximum value of vanillin concentration was 239±15 $mg.L^{-1}$. The authors suggested that the by-product of corn can be used as an interesting raw material for vanillin bioproduction in bench-scale bioreactor [271].

Additionally, some other studies reported the production of modified crops with improved flavor, color resistance to pests. Some examples are 'golden rice' (rich in carotenoids) transgenic tomatoes with improved concentrations of (*Z*)-3-hexenal (*Z*)-3-hexen-1-ol, characteristic compounds of tomato flavor, which are derivatives from lipid metabolism [272-273].

Gounaris *et al.* [274] presented a very detailed review reporting the recent advances on the field. Improvements of flavor production after over expressed enzymes involved on the biochemical routes were already reported. Large yields were also reported, making it possible to get commercial scale processes.

Classical reports, like the production of flavor compounds by genetic modified microorganisms, the expression of the enoyl-SCoA hydratase/lyase enzyme from *P. fl uorescens* in *E. coli* should be cited. The microorganism proved to be capable of converting ferulic acid into vanillin [275]. A Research group from Washington State University has isolated cDNAs from mint species coding for limonene hydroxylases [276-277]. The cDNAs were overexpressed in *E. coli S. cerevisiae* resulted in the production of (–)-*trans*-carveol (-)-*trans*-isopiperitenol. The authors confirmed that the enzyme sequences were very similar to the ones from the original plant. Other reports about the over expression of genes involved on the flavor biotransformation are already described [278-228].

There are few, but important reports about the genetic engineering applied to biotransformation processes to obtain flavor compounds. It has been shown to be a promising topic to be addressed, as after the advent of DNA recombinant techniques, direct genetic approaches for increasing biotransformation rates and simplifying the process have been driving studies in this area.

Plant Cell Cultures for the Production of Natural Flavor Compounds

Plant cell cultures appear as a viable method to produce a wide range of flavors aromas characteristic of their plant origin (Table 9), [34]. This approach is based on the unique biochemical genetic capacity, the totipotency of plant cells [279-280]. Every cell of a plant culture contains the genetic information necessary to produce numerous chemical components that constitute natural flavor (Fig.10). Feeding intermediates of the biosynthetic pathway can enhance the production of flavor metabolites by precursor biotransformation. Some authors [281-282] summarised the advantages of plant cell culture technology over conventional agricultural production, pointing out the following aspects:

- It is independent of geographical and seasonal variations, political interference and other environmental factors. It also offers a defined production system, which ensures a continuous product supply, as well as uniform quality and yield,
- It is possible to produce novel compound that are not normally found in parent plants, either directly or through stereo- and regiospecific biotransformations of cheap precursors,
- High-productivity species can be selected.
- Costs can be decreased and productivity increased by automatization of cell growth control and regulation of metabolic processes.
- Efficient downstream recovery.
- Rapid production.

Table 9. Flavors from plant cell cultures [34]

Products	Plant species
2,3-butanedione, (E,Z)-2,6-nonadienal and (E,Z)-2,6-nonadien-1-ol	*Agastache rugosa*
Apple aroma	*Malus silvestris*
Cinnamic acid	*Nicotiana tabacum*
Caryophyllen	*Lindera strychnifolia*
Basmati flavour	*Oryza sativa*
Cocoa flavour	*Theobromo cacao*
Flavanol	*Polygonum hydropiper*
Garlic	*Allium sativum*
Monoterpenes	*Perilla frutescens*
Onion	*Allium cepa*
Triterpenoid	*Glycyrrhiza glabra glandulifera*
Vanillin	*Vanilla planifolia*

Nevertheless, some problems need to be solved before plant cell cultures are extensively used for plant metabolite production. The technology for large scale suspension cultures should be further developed, since it differs from that commonly employed for microbial systems. Sensitivity to shear stress, relatively long growth cycles, low yields, progressive loss of biosynthetic activity and rare product secretion are some of the features of plant cell cultures that need special attention. However, some strategies have been developed to stimulate biosynthetic activities of cultured plant cells by optimization of culture conditions, selection of high-producing strains, precursor feeding and elicitation. Also, cell immobilization techniques could help to prolong viability periods, maintain high cell density in the bioreactors and reduce shear stress, amongst other advantages. Besides, regulation of plant secondary metabolism at the biochemical and genetic levels could lead to improved production systems [282-283].

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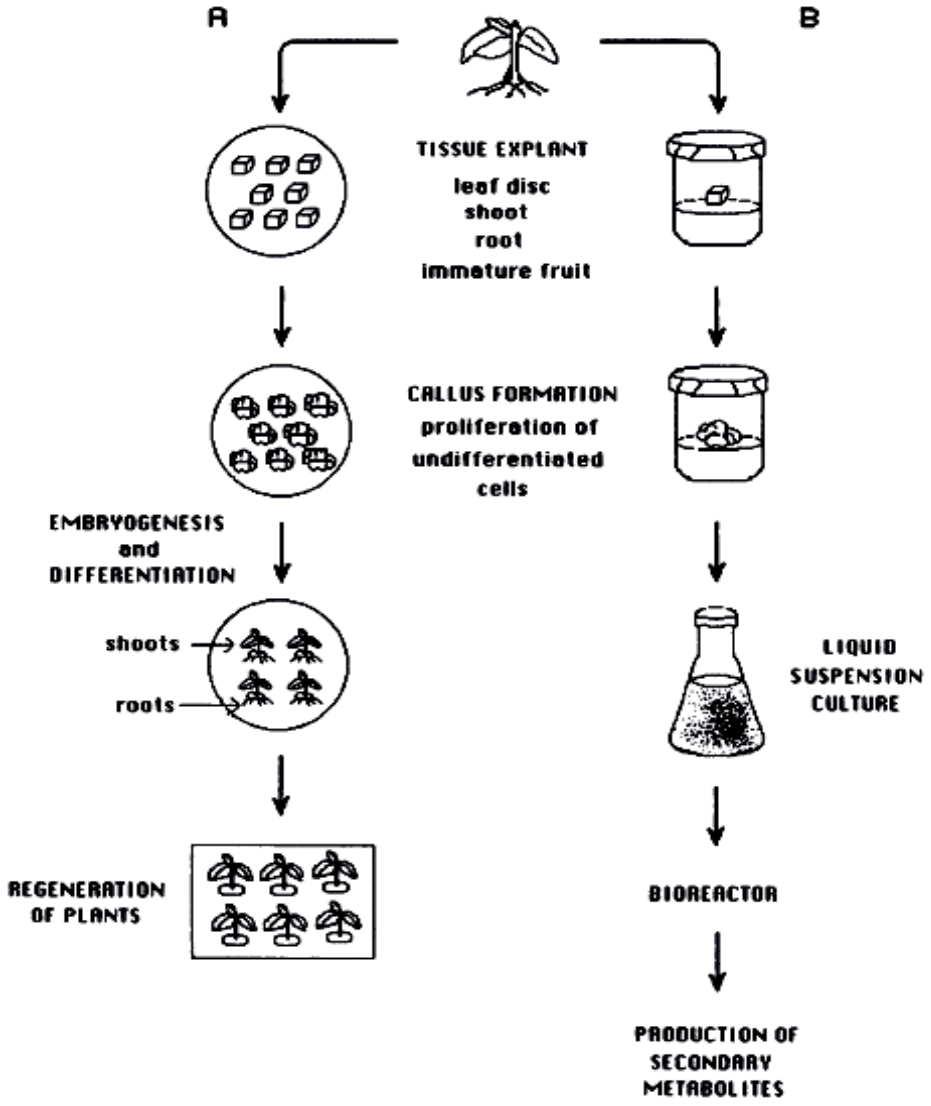

Fig.10. Plant tissue culture technology (A) micropropagation and (B) liquid suspension culture.

As for specific efforts related to flavor production by plant cell cultures, several researchers have investigated the synthesis of vanillin, a much sought-after flavor compound [281]. Plant cell cultures of *Vanilla planifolia* have been initiated from various plant cells tissues Fig.11 [284], the convenience of using elicitors to induce vanillic acid synthesis assessed [285]. Also, feeding of the precursor ferulic acid resulted in increase in vanillin accumulation [286-287]. Furthermore, the production of vanillin from ferulic acid with vanilla aerial roots on charcoal as a product reservoir has been described [288]. *Capsicum frutescens* root cultures have also been used for the bioconversion of ferulic acid to vanillin [289].

Some other works involve the production of monoterpenes (*i.e.* limonene, linalool, *etc*.) in callus tissues cell suspensions of *Perilla frutescens* [290], basmati rice volatile flavor components in callus cultures of *Oryza sativa* [291]. In some cases, the flavor profiles obtained in plant cell cultures differ from those encountered in the parent plants. Such was the case in suspension cultures of *Agastache rugosa* Kuntze (Korean mint), which had a marked cucumber/wine-like aroma, produced some interesting flavor-related alcohols (*i.e.* 2-phenylethanol) [292]. This alteration of the original flavor profiles can be deliberately induced by the addition of precursors, as demonstrated in root cultures of *Allium cepa* L. (onion) [293].

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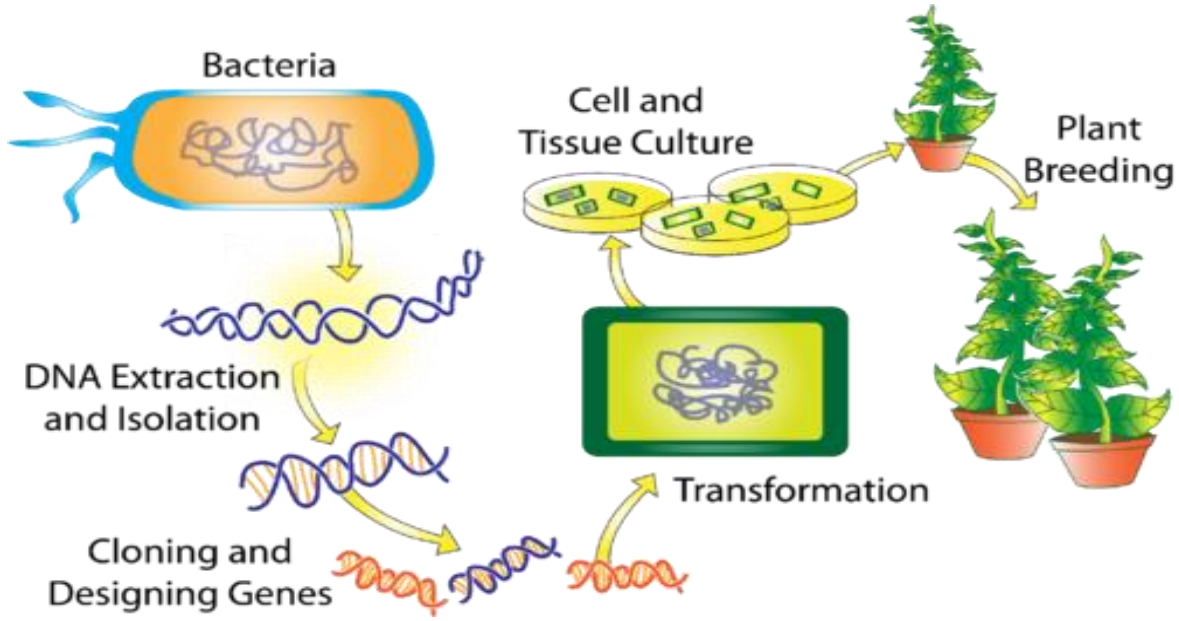

Fig. 11. The various of Plant cell cultures

Analyses and sampling techniques

A requirement in any process is to quantify the product formed. In the case of bioflavor production, this requires some specific consideration. Analytical difficulties may arise for the following reasons [294-295]:

1. 1-Low concentration levels.
2. Matrix effect of the sample.
3. Complexities of aroma compounds.
4. Variation of volatility.
5. Instability.
6. Lower sensitivity of laboratory instrumentation than the human olfactory system.

Isolation of aroma compounds from a matrix before identification is a very important step. Isolation methods of aromatics can be classified as:

1-Headspace sampling:
   a. Static headspace.
   b. Dynamic headspace.
   c. Purge and trap.
2. Solvent extraction and distillation.
3. Solid phase microextraction.
4. Direct thermal desorption.
5. Headspace sorptive and stir bar sorptive extraction.

Headspace sampling techniques

This can be divided into three categories: static headspace, dynamic headspace purge trap [296]. The basic principle of all headspace techniques is based on an investigation of volatile compounds in the headspace vapors above a solid or liquid sample. In the static headspace technique, a small amount of the atmosphere above the sample is injected to the GC column directly. However, in the dynamic headspace technique, analytes from a large amount of sample are first concentrated, then transferred to the GC. Dynamic headspace is usually used for analysis of solid samples, while purge trap refers to the analysis of volatiles from liquids by bubbling the purge gas through the samples. This technique has been used to isolate identify volatiles in different varieties of food samples [297-298-299-300].

Solvent extraction and distillation

Most of the volatile compounds are soluble in organic solvents, including diethyl ether, dichloromethane pentane [295]. Solvent extraction procedures can be applied to isolate volatile flavor compounds from non-fat-containing matrices aqueous distillates. However, a fat containing matrix may be further treated by steam distillation, molecular distillation or dialysis to isolate volatiles. Solvent selection is an important parameter for extraction. It is suggested that non-polar solvents, freons hydrocarbons, should be used for samples containing alcohol [294]. Diethyl ether methylene chloride are good solvents for general purposes. With respect to distillation techniques, efficiencies of separation depend upon the physical properties of the components the type design of distillation procedures. Steam distillation is the most common technique. This technique has some advantages, including simplicity of operation, no need for complex apparatus, reproducibility, rapidity range of samples h led for isolation of volatiles from non-volatiles [294]. If a sample has a large amount of lipid, high-vacuum distillation is recommended for the isolation of volatiles. High-boiling heat-sensitive compounds cannot be successfully separated at atmospheric pressure because of difficulties in heating the distillation equipment to very high temperatures, because of thermolability of certain compounds [301]. Application of higher temperatures during distillation may generate the formation of artifacts or undesirable volatile compounds. Specifically, samples rich in free amino acids sugars can interact during Maillard reaction Streaked degradation to form additional compounds [302]. High-vacuum transfer (HVT; molecular distillation) is commonly used for the isolation of volatile compounds from lipid-containing foods. HVT includes the direct transfer of volatiles from a matrix to a cold condenser. This technique requires a short distance between the condenser the sample the use of high-vacuum systems ($<10^{-3}$Torr). HVT decreases the formation of artifacts provides good recovery of volatiles [295].

A fast careful isolation of volatiles from complex matrices was developed by Engel *et al.* [303]; this is called solvent-assisted flavor evaporation (SAFE). The system is connected to a high vacuum pump. It allows isolation of volatiles from solvent extracts, aqueous foods, food suspensions matrices with high oil content.

Solid phase microextraction (SPME)

This technique is a simple solvent-free sample preparation technique [304]. It is based on the adsorption of volatile compounds to the polymeric stationary-phase coating of a fused silica fibre. The volatile compounds are then thermally desorbed in the injection port of a gas chromatograph. This technique is successfully applied for the characterization of flavor compounds found in different varieties of foods, including cheese, honey, wine, fruits vegetables, etc. [305-306-307-308-309-310].

Direct thermal desorption

This is a simple rapid sample-preparation technique. It does not require any solvent use. It is based on sparging volatiles from sample matrix transferring them onto the chromatographic column. Heat treatment is usually applied to a matrix to extract volatile compounds from samples. A cryofocusing unit or cold trap can be used to focus the volatiles at the head of the column [311].

Headspace sorptive (HSSE) and stir bar sorptive extraction (SBSE)

Both methods are solventless enrichment techniques. Volatile compounds are absorbed on a thick film of poly (dimethylsiloxane) (PDMS) coated onto a magnet incorporated in a glass jacket. Gas phase extraction is called headspace sorptive extraction. Sampling of liquid samples was done by immersing a stir bar in the liquid (SBSE). Then the stir bar is thermally desorbed on-line with capillary gas chromatography (GC) or GC–mass spectrometry (MS) [312-313].

SENSORY EVALUATION

The ultimate purpose of an industrially produced flavor compound is to induce a sensory effect, most often in humans. The human nose is a remarkably sensitive analysis instrument, the perceived response is affected by not only one compound but the complete composition, in a highly non-linear way. For this reason, merely instrumental analyses are not sufficient, but in addition it is necessary to assess the perceived intensity of a volatile compound by using human senses quantitative sensory evaluation methods. Humans perceive

smells using chemical sense, focusing on odor compounds. Volatile compounds become aroma compounds or odorants when they activate the sense of smell via olfactory or smell receptors in the nasal cavity , moreover, in the olfactory epithelium [314-315]. Aroma compounds may be either orthonasal, when odorants are sniffed or retronasal when odorants are semi volatile transferred via mouth to nose. The odor perception process is complicated , despite having been studied extensively in recent decades, it is still not fully understood. Odorants may be also flavor components, but actual flavor is a combination of retronasal smell, taste chemaesthesis (such as chemical irritation or pungency) [316-317-318]. In general, odorants are volatile compounds taste molecules are non-volatile compounds activating the sense of taste via taste receptors.

When volatile compounds are analyzed using GC coupled to any headspace-sampling or other sample preparation techniques, it is not certain whether detected compounds are actually contributing to odor or to flavor. Moreover, the quantity of volatiles in the original sample is not directly related to the intensity of the smell. One solution is to divide the GC eluent before the instrumental detector steer it partly to the human nose [299]. Using GC–olfactometry (GC–O), it is possible to find both odor volatile compounds [319]. For the human nose this task may be laborious, as well as for researchers involved in data processing. An example of specific methods used to identify the key odorants is aroma extract dilution analysis (AEDA) [320-321-322-323]. Instrumentally-analyzed volatile profiles may also be combined with sensory profiles. Usually, a trained sensory panel evaluates the most important sensory properties of a product in sensory laboratory (ISO 8589) conditions [324-325], e.g. following a general sensory profiling protocol. When the sensory profile is connected to instrumental analyses, it is important to keep the sample preparation method as similar as possible in both methods. Different data matrices are relatively easy to combine, using multiregression statistical methods to identify the key volatile compounds contributing to smell or flavor [326]. However, it is necessary to determine the target, such as orthonasal odorants, retronasal odorants or flavor compounds, when selecting the correct method of analysis for instrumental measurement human sensory evaluation.

CURRENT STATE AND FUTURE

Extensive reviews dealing with biotechnological production of flavor compounds have been published during the past few years. Research in the last decades has led to relevant achievements related to the enzymatic and microbial preparation of the most important fine odors. Nowadays, more than 100molecules are marketed.

Recent applications of the biotechnological approaches are comprehensively covered since the improvement of knowledge in analytical and synthetic methods and the replacement of classical organic methods by the emerging techniques of analysis and separation. These developments have allowed the preparation and separation of a considerable number of volatile compounds at industrial scale.

The production of natural aroma compounds by microbial fermentation leads to low amounts of desired products because the metabolic potential for *de novo* flavor biosynthesis is immense and a wide variety of products can be detected in culture media. An improvement is often prevented by a lack of metabolic knowledge. Precursor approach holds more promise. Several starting materials have been already used including lignin, eugenol, ferulic acid. Inexpensive, available and renewable natural precursors such as terpene hydrocarbons, can be converted to more highly valued flavors. Terpenoid flavor compounds, of high commercial interest, are in principle accessible by biotransformation of abundant natural terpene hydrocarbons used as precursors.

Moreover, the attempts to use submerged fermentations for flavor production resulted in low productivity, which hampered their industrial application. Solid state fermentation is a technology lesser explored than submerged fermentation, but that has been proven to give higher productivities of flavor compounds. Industrial wastes may be used in these processes as solid support and as a source of nutrients, which allows avoiding the use of expensive chemical components in the media formulation and makes possible the achievement of more economical fermentation processes.

Many biotechnological routes for the synthesis of attractive flavors have been recently described. However, the described processes have more academic than practical value. The number of industrial applications is therefore limited to some aroma components, such as vanillin, benzaldehyde, phenyl-alanine, decalactone. The industrial production often lacks economic profitability, mostly because microbial flavors are

often present only in low concentrations in fermentation broths, resulting in high costs for down-stream processing. There are other problems hampering the large scale production such as: (i) the toxicity of the hydrophobic aroma compounds, which leads to strong inhibitory effects toward microbial metabolism even at low concentrations, and there-for the cell growth is disturbed; (ii) the volatility and the low solubility in water of many flavors makes often their recovery difficult to perform.

In order to overcome these limitations, the processes for *in situ* product removal; by coupling bioreactor to the down-stream unit, have been developed in many works. These systems allow the fast removal of the products from the fermentation broths. The selective product recovery during downstream processing becomes a major issue for the bio-process development. Although the *in situ* product removal has been intensively studied, the applications remain limited to the laboratory scale. The developed systems are generally of high costs for a production on a large scale and often lack economic profitability. In the future, other combined approaches aiming at reducing the energy-intensity of the process, while keeping or improving its selectivity, should be considered.

In addition to microbial fermentations, recent developments of biocatalysis have made possible to obtain valuable aroma compounds. Biocatalysis becomes a useful tool for the specific catalysis of different enantiomers or regioisomers, which is not easily achieved by the less selective classical synthetic methods. Enantiomers or regioisomers show different sensorial properties; their specific enzymatic synthesis becomes therefore beneficial.

A great interest has been accorded especially to the use of non-conventional media for enzymatic syntheses. The non-conventional media have made possible the use of hydrolytic enzymes for the specific synthesis of a number of valuable hydrophobic aroma compounds, which allows overcoming several drawbacks, such as the low water-solubility of the synthesized products. The non-conventional media were generally applied for the production of food aromas, especially for the synthesis of esters by using lipases in low water-content media. Lipase represents one of the most enzymes commonly used for the production of aroma compounds. It is considered as the favorite catalyst because it is characterized by its remarkable chemoselectivity, regioselectivity and enantioselectivity. Also, lipases are easily available on a large scale and remain active in organic solvents.

The biotechnological approaches using enzymes have been proved to be efficient for the stereoselective organic synthesis and also for the biotechnological production of flavor com-pounds. However, the applications using isolated enzymes for the industrial production of natural flavors remain reduced. This is mainly due to the high cost of isolated enzymes and to their low stability under operational conditions. The application of enzyme technology in the field of aroma production would be easier, if less complicated steps for enzyme isolation and purification would be available. The hydrolytic character of enzymes makes also complicated the synthesis of valuable hydrophobic aroma compounds in conventional media. Lipases represent the most promising biocatalysts because of their versatility and selectivity. More attention should be paid to improve the catalytic properties and the stability of other interesting enzymes that can be used in production of aroma compounds. Molecular biological methodologies are necessary to optimize biocatalyst properties, *e.g.* by conferring higher stereoselectivity and thermostability.

As for every bioprocess, the screening of suitable biocatalyst, the adjustment of a full set of chemical and physical parameters and the design of the reactor must be considered for the efficient synthesis of flavor compounds. More attention should be also paid for the *in situ* recovery of volatile components. This will also contribute to make production profitable.

The field of production of natural aroma compounds is still in development and requires highly innovative processes. More academic studies have to be undertaken in order to find novel biocatalytic routes for flavor synthesis. This will depend on genetically improved biocatalysts and on process engineering features. Biotechnological advances, including genetic engineering techniques and modern techniques of molecular biology and process engineering, are now increasingly applied to enhance the efficiency of the biocatalysts. Several technologies can be used for this feature including; heterologous expression of genes, use of non-conventional media, whole-cell biocatalysis, immobilization of biocatalysts in large systems and cofactor regeneration. Many improvements can be also achieved through studies into reactors technology and through understanding of the interactions and of the transfers within these systems.

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CONCLUSION

The development of methods for the production of natural flavor and fragrance chemicals through biotechnological routes has evolved rapidly in recent years. The use of microbial cultures or enzyme preparations offers several advantages over traditional methodologies. The biotechnological approaches are applied to produce natural flavors and thus making them more suitable for consumption. This review describes several methodologies applied for the production and recovery of natural aroma compounds on an industrial scale.

In order to increase the diversity of produced compounds and to decrease the costs of production, the field of bio-flavors requires highly innovative processes such as exploiting the enantioselectivity of the enzymatic reactions involved in the biosynthetic pathways. A judicious choice of reactor type and operating conditions are also of great interest. Several parameters need to be optimized for both economic and environmental performance. The most important are: the reactor design, the operation mode, the biocatalyst stabilization, the recovery mode and the mass transfer, which is necessary for the reaction advancement. It is also important to minimize capital and operating costs in many bioproductions of chemicals for future scale-up. The use of highly efficient continuous bioprocess for the synthesis/extraction of natural aroma chemicals is economically effective and promising for further large-scale production of bio-flavors.

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