WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 2, 199-224. Review Article ISSN 2277– 7105

MISCELLANEOUS USE OF ENZYMES

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Article Received on
15 Dec. 2019,

Revised on 05 Jan. 2020,
Accepted on 25 Jan. 2020,

DOI: 10.20959/wjpr20202-16783

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ABSTRACT

Enzymes are catalysts that act under gentle reaction conditions (room temperature and 4.0 < pH < 9.0). They present high specificity and enantiomer selectivity, enabling them to be largely used in industry (food, animal feeding, pharmaceutical, biotechnological, chemical, textile, laundry, waste treatments, leather, pulp and paper), clinical and chemical analyses, and therapy. Due to their specificity (hydrolysis of prostaglandin ethyl ester by esterase, for instance) and enantiomer selectivity (conversion of fumaric acid into malic acid or L-aspartic acid by fumarase), they have been used in the modification of complex and labile substances – largely used in immunology, endocrinology, among others – by introducing in the molecule structure one or two chiral carbon and/or a specific chemical group at a pre-defined position (conversion of progesterone into 11-hydroxyprogesterone, for example).

KEYWORDS: Enzymes, industrial enzymes.

INTRODUCTION

Enzymes are specialized high-molecular weight proteins composed of amino acid building blocks and are in general natural substances produced by all living organisms. There is a special group of intracellular enzymes – called ribozymes –, whose building blocks are ribonucleotides.[1] Enzymes act as catalysts and conduct about 95% of all physiological processes pivotal for the growth and life of all living matter. They can accelerate the processes of synthesis or decomposition of organic substances (prostaglandins, hormones, antibiotics, fats, carbohydrates, among others) under moderate conditions of pH, pressure and temperature.

When compared with another catalysts, organic or inorganic, the enzymes have uncommon characteristics regarding specificity (they act on a particular substance or insert a chemical group – such as the hydroxyl group – at a pre-selected position inside the substrate molecule, as in the conversion of progesterone into 11-hydroxyprogesterone) and enantiomer selectivity (they sort out only one of the two isomers present in a racemic mixture).

The initial use of enzymes was set when α-amylase from *Aspergillus oryzae* and invertase from *Saccharomyces cerevisiae* were produced at an industrial scale in the beginning of the twentieth century.[2] Since then, more and more enzymes were identified and produced in significant amounts that led to applications in industry (food, animal feeding, pharmaceutical, cosmetic, fragrance, chemical etc.), in medicine (as drugs), and in clinical and chemical analytical procedures (as reagents).

The aim of this review is to analyze the use of enzymes in detergents, effluent and waste treatments, flavor production, leather, textiles, pulp and paper, edible oils, animal feeding, analytical procedures, medicine, and organic synthesis (biotransformations).

ENZYMES IN DETERGENTS

Detergent is a generic term that encompasses all cleaning products available in the market. However, those containing enzymes (proteases, lipases, amylases, and cellulase) are used in households, industrial and hospital laundry, and household dishwashing since 1960.

Approximately 30% of the overall worldwide enzyme production is funneled to the detergent industry. Enzymes line with surfactants, bleaching compounds and builders as the main formulation ingredients of cleaning products.[3]

The most used are **proteases** due to their broad substrate specificities and capability of functioning to some extent some under extreme conditions found in domestic washing (temperatures of 20 to 70^{o}C, at a pH up to 11, and high concentrations of surfactants, polyphosphates and chelating agents).

Current trends on energy conservation promote low temperature fabric washing, requiring enzymes with a high catalytic activity at room temperature.

In laundry, proteases touch their substrates on stains glued on a fabric surface. Therefore, they must have the ability to be adsorbed by insoluble fragments, which occurs optimally when the pH value of the detergent solution is close to that of the enzyme pI (isolectric pH).

Lipases have been used in detergent formulations since 1988, aiming to wash out fatty stains deposited on the fabric surface. The lipase hydrolyzes the triglycerides present in fats, freeing hydrophilic compounds such as fatty acids, diglycerides, monoglycerides and glycerol.[4] All of them are removed by washing the fabric in an alkaline condition (pH: 8.0-11.0). In alkaline pH, the lipase maintains at least 60% of its overall activity as the temperature ranges from 20^{o}C to 55^{o}C.[3] Under these conditions, a complete wash out of a fatty stain from the fabric occurs after two or three washing cycles, shorter than the seven/eight washing cycles required for non-lipolytic washing.

Amylases are enzymes that catalyze the hydrolysis of starch in low MW sugars and oligosaccharides. The starch adheres to the fabric surface aggregating other dirty on stains. The most used are heat stable microbial α-amylases. Depending on the origin of the α-amylase, its optimal activity pH can vary from 6.5 to 9.0 (*Bacillus licheniformis* α-amylase) or from 4.5 to 7.0 (*Bacillus amyloliquefaciens* α-amylase).

Cellulases have been used in detergent formulations since the 1980s. They catalyze the hydrolysis of β-1,4 glycosidic bonds in cellulose, freeing short chain oligosaccharides consisting of glucose units. Unlike other detergent enzymes, cellulases do not act on stains, but on cellulose, reconstituting the microfibers and damaged fibers which appear on the tissue during wash and wear of a garment. Till now, no damage was observed to the strength of textiles despite the cellulose being the main constituent of fabrics. The macroscopic effects resulting from the cellulase action on the garment are color intensification, enhancing the softness of the fabric surface, and improving the removal of particles of grime entrapped in the twill of the fabric. The intensity of the effects on garments depends on the dose, the washing conditions, and the detergent formulation.

Detergents containing enzymes are commercialized as either powder or liquid. Currently, the enzyme itself is presented as granules as a preventive measure against allergenic problems which can affect either domestic users or workers at detergent factories.[5]

Enzyme compatibility with detergents involves stability during storage and washing. During the use, enzymes are exposed to **denaturation** (unfolding of the molecule structure due to high temperature and/or harsh environment; the addition of Ca^{2+} at 500ppm can stabilize alkaline and super-alkaline proteases), **undesired chemical reactions** (an oxidizing agent modifying a critical amino acid residue located at the active site, for example), and **proteolysis** (which can be minimized by adding to the formulation propylene glycol and/or reversible protease inhibitors such as glycine and borates). Storage stability depends on whether the product is a liquid or a powder detergent.

In a liquid detergent, all ingredients can have a direct impact on the enzyme. Thereby, the addition of an adequate surfactant, mainly non-ionic, can reduce the rate of denaturation. This is not the case for a granulated enzyme in powder detergents. The formulation and the type of granulation have a major influence on the stability. Granulation introduces a barrier between the harmful surrounding and the enzyme inside granules. The major parameters for stabilizing enzymes in powder detergents are storage at low temperature and humidity. However, if the formulation has a bleaching system (perborate plus tetra acetyl ethylene diamine, for instance), the enzyme activity can decrease due to the oxidative action of oxygen free radicals – generated during the reaction promoted by bleaches – on amino acids that are constituent of the enzyme. In this case, an oxygen free radical sequester, such as ascorbic acid, must be added.[6]

During washing, enzyme stability depends on factors such as detergent composition and dosage, pH of the detergent solution, ionic strength of the detergent solution, washing temperature, washing time, mechanical handling, water hardness, level of soiling, and type of textile.

Finally, enzyme detergents can also be used in cleaning machinery parts, ultrafiltration and reverse osmosis membranes and gadgets such as lens, endoscopes, dentures and electrodes.[7]

EFFLUENT AND WASTE TREATMENTS

Currently, one of the greater aims is to minimize damages inflicted to the environment by any form of residue (gaseous, liquid or solid) generated by the industrial activity.

The best situation would be that where no waste is discharged into the environment, i.e., the plant is structured for recovering and reworking all residues generated. Unfortunately, such

situation is almost never achieved. Thereby, the only way to avoid damages to the environment is treating the waste.

Waste treatment can result in a marketable byproduct – a situation in which financial benefit can be reverted to the company – or in an effluent inoffensive to the environment, resulting in expenditure to the company. However, this expense can be reverted into benefits to the company since the plant is operated using good environmental practices. Thereby, the company could ask for an environment certification (based on the ISO 14001), which could aggregate value to the company assets.

The ways of treating wastes and pollution clean-up can be carried out by chemical and/or physical processes or biological agents (microorganisms and/or enzymes).

The use of microorganisms in industrial waste streams can be by activated sludge with or without anaerobic digestion. Moreover, the application of microorganisms to waste treatment and pollution clean-up can involve the disposal of municipal waste into methanogenic landfills and the treatment of land which has already been polluted with undesirable and noxious compounds.

Enzymes acting on wastes generated by food industry – still rich in sugars, proteins and fats – can result in valuable byproducts. However, if the waste effluent comes from another type of industry – normally rich in noxious chemicals –, the direct treatment of enzymes aims to reduce its pollution power before throwing it to the environment.

Evaluation of biological waste upgrade viability is based on **a)** the knowledge about the chemical and physical characteristics of the waste, **b)** the waste nature – if it is a genuine one or an underutilized byproduct, **c)** the commercial novelty of the byproduct obtained, **d)** the waste abundance, **e)** the economic viability of waste treatment considering legal requirements, cost and logistic for collecting, and long-term policy on waste source processing.

Some aspects regarding the enzyme-assisted processing of waste and byproducts are **a)** the economic recovery and reuse (sugars recycling in confectionary after α-amylase hydrolysis of starch present in the waste), **b)** the energy conservation and material economy (cellulases in the extraction of flavors and colors, byproducts with high market values), **c)** process

economy and upgraded byproducts (for example, use of proteases in the reduction of viscosity and "stickiness" of concentrated wash water from fish meal industry used as supplement of culture media for fermentative processes), **d)** alternative higher value derivatives (when the raw waste is a complex mixture of substances, polymers included, and the separation of one of them is not economically viable, a pool of enzymes – proteases, amylases and pectinases – can be used to hydrolyze the polymers resulting in a medium valuable as a fermentation feedstock, and **e)** the attainment of new source materials (for example, using low active proteases in cheese whey concentrate leads to obtaining a mixture of proteins with changed foaming and gelling capabilities, enabling whey to be used in food formulations).[8]

The direct use of enzymes in effluent treatment originated from industries other than the food industry is still incipient. This is due to the harshness of the chemical composition of wastes against the delicate enzyme structure. The peroxidase/hydrogen peroxide combination (PHPC) is the best studied system for treating wastes rich in phenols and aromatic amines. These compounds, in presence of free hydroxyl radicals (generated by PHPC), are converted into insoluble polymers, which are separated by filtration or decantation. After drying, they are incinerated.[9]

Growing public awareness about environmental problems in recent years will push the academic and industrial researches to developing new enzyme means for waste disposal.

FLAVOR PRODUCTION WITH ENZYMES

Practically all types of natural and industrialized foods have characteristic flavors and aromas. For example, citrus juices have a little bitter taste due to the presence of naringin and limonin, two compounds located at the white layer of citrus fruits.

Thousands of volatile chemicals belonging to different classes of organic compounds (esters, amines, alcohols, alkenes, terpenes, aldehydes and ketones) have been identified in food. The "food aroma" is a mixture of hundreds of compounds. For example, the apple aroma/flavor is composed by at least fifteen different chemicals (1-butanol, 2-methyl-1-butanol, hexanal, 1-hexanol, furfural, among others).

Market surveys on flavoring compounds have demonstrated that consumers prefer foodstuffs that can be labeled "natural." The perception of "natural" as better has led to an increased

demand for flavor and fragrance chemicals that may be considered "natural." Plants are the best natural source of flavor chemicals. However, there are setbacks regarding plant source, such as few suppliers, expense of isolation, variability in the amount, and quality of final product from different geographical sources. These concerns have resulted in a high price for natural source chemicals and a search for alternate supplies of the desired compounds. One way of alleviating this problem has been the development of biotechnological processes – either via microbial fermentation or enzymatic reaction – for the production of specific flavor and fragrance chemicals and complex mixtures with a cheese, fish and meat aroma, among others.

Food aroma/flavor can be produced by a variety of processes including enzymatic and microbial action, food processing, cooking, and chemical interactions.[10] A convenient way to consider these chemicals is to divide them into three broad groups: **a)** the heat-derived or Maillard browning aroma chemicals, which are formed when food is cooked or heat-processed, such as the aroma of meat and coffee, **b)** chemicals formed during heat-processing (via Maillard reaction) from a nonvolatile chemical precursor formed in the course of a fermentation step, such as observed in cocoa and bread, **c)** biologically-derived aroma chemicals, often referred to as secondary metabolites, arise by microbial fermentation, action of endogenous enzymes, end-products of plant metabolism or enzymes added during processing.

Regarding aroma/flavor production by enzymatic ways, there are three main approaches, i.e., ***in situ*** – for example, the taste of beer is formed by cooking the mashed cereal must with hops before the fermentation of the broth by the beer-yeast –, **enzyme catalyzed reaction** for the production of specific flavor chemicals (for instance, the sweetener aspartame is synthesized by thermolysin from aspartic acid and phenylalanine methyl ester;), and **enzyme-modified foods** for the production of savory flavors such as meat, cheese, and fish.[11]

The cheese manufacture is an example of ***in situ*** and **enzyme-modified-food** approaches.

The flavor generation *in situ* is promoted by leaving the whey-free p-k-casein coagulum in a room for a long time, during which the characteristic flavor and aroma appear. This is mainly due to the action of selected or wild strains of microorganisms (*Lactobacillus sp.*) associated with the proteolytic and lipolytic activities of residual protease and lipase added in the first step of cheese making (enzymatic p-k-casein coagulation). The enzyme-modified cheese

production can be summarized as follows: off-cuts of cheese (with about 63% of dry solids) are mixed with water and emulsifiers; the cheese slurry (with about 45% of dry solids) is pasteurized (72°C for ten minutes) and cooled at 50°C. Enzymes (porcine pancreatic lipase – PPL – or microbial lipase – ML – and fungal protease) are added to the paste, which is left for at least 8 h at 50°C. Then, the paste is pasteurized (72°C for 25-35 min) and, finally, spray-dried. Normally, ML is preferred over PPL because **a)** the PPL is always contaminated by trypsin, whose proteolytic activity causes the appearance of bitter peptides in the final product, **b)** the PPL cannot be used for vegetarian and kosher products, and **c)** the fear of virus or prion presence in products of animal origin. Fungal proteases are the enzymes of choice because they do not produce a high level of bitter peptides and some of them contain very high levels of both carboxy- and amino-peptidases, which hydrolyze bitter peptides, a well prized side effect.[11]

The attainment of meat flavor is an example of **enzyme-catalyzed reaction**, although it can be obtained by acid hydrolysis (6M HCl) of soya protein at 180°C and pressure of 6 atm. The meaty flavor of soya hydrolyzed protein is due to the Maillard reaction involving sugars (from hydrolyzed carbohydrates) and amino acids (from hydrolyzed protein). The acidic soya hydrolysate is used in formulations of soups, sauces, snacks, pies etc., in spite of presenting the following as disadvantages: **a)** many countries consider it an artificial instead of natural flavoring, **b)** containing sodium chloride over 40% (residue of hydrochloric acid neutralization), **c)** high glutamate content, which is reproved by several countries (USA, included), and **d)** containing a low amount of mono- and dichloropropanol, potential carcinogens. The resilience on HCl substitution for proteases is due to its low cost. However, the substitution of acid hydrolysis for enzyme hydrolysis is possible when high-cost foods are produced. Moreover, as the ecological conscience of the community increases, entrepreneurs of this kind of industry will be pressured to take measures to reduce the probability of environment damages, leading necessarily to a full substitution of the acid for enzymatic hydrolysis. In this point of view, an alternative to the enzyme soya hydrolysate could be the use of yeast extract – previously treated with ribonuclease and deaminase – as a meat savory enhancer.[11]

Finally, flavor enhancer compounds of microbial origin such as monosodium glutamate, inosine monophosphate and guanosine monophosphate deserve to be remembered because

they help to obtain a certain type of flavor (meat, for instance) even in the presence of low concentrations of natural flavoring substances.[11]

LEATHER

Leather processing consists of six clearly defined steps, i.e., **curing** (the fresh skin is placed into a salt concentrated solution, then draining, addition of antimicrobials – non-ionic or anionic surfactant – and drying the flesh under the sun), **soaking** (consists on rehydration, washing away fat and dirt, swelling and cleansing the cured skin), **dehairing** (consists on removal of hairs from cured and soaked skin by using an alkaline mixture of hydrated lime, inorganic sulfides and amines (pH=11.0). Sulfides break the bonding protein fibrils within the hair and dissolve the proteins of the hair root. The skin is left in contact with this solution under agitation for several days), **dewooling** (consists on painting the skin with an aqueous solution constituted by hydrated lime, sodium chlorite and protease. The painted skin is left overnight at 30^{o}C, resulting a bright and clean skin), **bating** (consists on deliming, deswelling the collagen of the skin, degrading the protein fibers partially so that they become soft and able to accept an even dye), and **tanning** (consists on treating the bated skin with acid solutions in order to produce a further deliming without reswelling the collagen fibers. Staining chemicals are used obtaining the final leather with chromatic nuances).

Enzymes are useful catalysts in some stages of leathering (soaking, dehairing, dewooling and bating). The most valuable in leather industry are proteases – mainly the neutral and alkaline – and a raw extract of bovine and porcine pancreas called “pancreatin.” Indeed, pancreatin has amylolytic, lipolytic and proteolytic activities. Its use becomes more advantageous than the protease alone.

The use of pancreatin at the soaking step enables the tanner to handle fat skins. Furthermore, protease promotes subtle modifications on the proteins, favoring water absorption by the skin. A mixture of alkaline protease and lime in dehairing the skin promotes hair detachment from the roots (a smooth leather is thus obtained). Dehairing by enzymes leads to a decrease in sulfured chemicals needed, contributing to diminish the environmental pollution near the tannery because less polluted effluents and unpleasant odors are emitted, respectively, into water streams (or urban sewage system) and in the air. Dewooling involves two steps: **1st)** dehaired skin is embedded in a water solution containing hydrated lime, sodium chloride and alkaline bacterial protease; and **2nd)** the flesh side of the skin is evenly sprinkled with a powder constituted by sodium sulphate, sodium sulfite, ammonium chloride, ammonium

sulphate and alkaline or neutral protease. Next, the skin is hung in a conditioning room at 25-30°C for 24 h before the wool is pulled out. Since 1908, pancreatic trypsin substitutes animal excrements (used by millenniums) in bating. The action of trypsin, in combination with the added chemicals, is to remove any hair residues, allow water penetration deswelling the collagen fibers, and have a minimal influence on collagen. The proteases used for bating are selected for their pattern of specificity to the various proteins of the skin. Thereby, trypsin, fungal acid protease, bacterial neutral and alkaline proteases are active on muscles; bacterial super-alkaline protease acts on muscles and keratin; papain acts on muscles, collagen and elastin; ficin acts on muscles, collagen, elastin and keratin; and bromelain acts on collagen and elastin. By using adequate activity combinations of pancreatin, plant proteases (papain, bromelain and ficin) and microbial proteases (acid, neutral, alkaline or super-alkaline), the tanner can perform a weak, medium or strong bating.[12]

In fact, the incorporation of enzymes into some steps of leathering has proved very successful both in improving leather quality and reducing environment pollution.

TEXTILES

During the production of fabrics, sizing the treads with starch combined or not with polyvinyl alcohol, gelatin, gums or carboxymethyl cellulose is fundamental to obtain a good weaving. In weaving, starch paste is applied for warping aiming to provide strength to the textile and prevent the loss of string by friction/cutting and generation of static electricity on the string. The desizing of the treads – to allow an efficient adsorption of dyes, bleaches and texture enhancer on the cloth – is accomplished by using a thermal (85-110°C) and a chemical resistant microbial α-amylase. The operating pH ranges from 5.0 to 7.5, and the addition of calcium ions (about 0.5 g/L) for stabilizing the enzyme is required when very soft water (hardness lower than 50 ppm) is used as solvent. The starch hydrolysis during desizing must occur in the shortest time possible.

The desizing process can be divided as follows: **a) prewashing** (removing of waxes or other additives by passing the fabric through boiling water and surfactant at 0.5 g/L; moreover, the starch glued on the treads swallows in, facilitating the α-amylase hydrolysis in the next stage); **b) impregnation** (starched-treads are treated with the enzyme solution (0.05% w/v) so that the hydrolysis begins slowly, but consistently); **c) starch hydrolysis** (the polymer breakdown can be programmed to occur for 12-16 h at 35-40°C or for 1-4 h at 70-75°C

depending on the fabric desired. Long reaction times at low enzyme levels can be economic if stability is ensured, otherwise it is better to use high enzyme levels at high temperatures, reducing the duration of the hydrolysis); and **d) after-wash** (the complete removal of the hydrolysis products (low MW oligosaccharides) is pivotal for the success of bleaching, the step following desizing. In this stage, hot water containing synthetic detergent (5 g/L) and sodium hydroxide (10 g/L) is added aiming the complete removal of oligosaccharides following the neutralization of alkalis with acid and the thorough rinsing with water).[11]

In denim processing, a mixture of α-amylase and cellulase is used aiming the substitution of pumice stones. The stones are used for removing the excess of indigo blue adsorbed by twill cotton in the production of the fabric directed to jeans confection. This procedure is carried out by suspending the tinted-blue cotton with stones in a water-loaded tumbling machine. After an intense agitation, the excess of indigo dissolves into the water, leading to a color-faded denim jean. This procedure creates problems such as the disposal of the sand – resulting from stone eroding –, which, if not adequately treated, affects the environment surrounding the facility; the decrease of the operational half-life of the tumbler; the production of a fabric with a low tensile strength, and difficulty for reproducing a particular combination of fade and abrasion to create very large consignments of identical products.[11][13]

Cellulase was introduced into the denim processing in order to fade homogeneously the blue color of the fabric with a wider range of color tones. The enzyme is directly added to the fabric as soon as it is completely desized and fully water rinsed.[13]

During the last years, the textile industry has been pressured to move away from chemical bleaches to meet ecological demands of the society. The response resulted on the substitution of chlorine-based chemicals for hydrogen peroxide in bleaching (a less environment damaging chemical; the excess is removed by catalase).[14]

The textile industry is intensely pressured to minimize the consumption of treated water, the pollution of water streams surrounding the facility, and the overall volume of dischargeable effluents resulting from processing operations. The use of enzymes may be expected to play a relevant role in helping the textile industry with its effort to meet all such demands.

PULP AND PAPER

The invention of writing (about 3,300 BC) was fundamental for the humankind, insofar as the registration of administrative acts and the knowledge accumulated along the centuries were made possible.

However, with the advent of writing, it was peremptory to find a suitable material on which the words could be written. The first writings were recorded on solid surfaces (wood, animal bones and stones), followed by papyrus leaves (prepared with the stalk of leaves of the plant *Cyperus papyrus*), parchment (prepared with animal skin), and, finally, paper (made of cellulose fibers prepared from trees of hardwood and softwood). The development of printing led to the consolidation of the paper industry worldwide.

The paper production involves the feedstock, handling, pulping, refining, dewatering, bleaching, and papermaking. Regardless of the fiber source, physical handling is carried out to obtain cleanliness and a particle size suitable for pulping. This processing takes the form of debarking, washing and screening to remove the foreign matter, and mechanical chipping for most pulping processes.[11]

The use of enzymes in the paper industry – more precisely, in pulp refining/dewatering, bleaching and papermaking – has grown rapidly since the mid-1980s.

Pulping is carried out by either mechanical (for newsprint and other bulk papers) or chemical (writing and wrapping papers) processes.

In the mechanical process, the fiber feedstock is cleaned and chipped to a uniform size. The raw fiber is torn apart by mechanical means during pulping (the process by which the macroscopic structure of raw wood fiber is broken apart, rendering a pliable fiber). Closely associated with the pulping is refining, which improves the strength of the pulp. Then, the pulp dewatering (removing the water retained within the wooden lattice) is made in presence of enzymes (cellulases and hemicellulases).[15] A careful control of the enzyme dose is important; excessive enzyme treatment can weaken the pulp because of cellulose hydrolysis. In addition, the excessive creation or destruction of fines (particles constituted by cellulose and hemicellulose that pass through a 200-mesh screen) can affect the opacity of the sheet. Following dewatering/refining, the pulp is treated with peroxide for bleaching, from which a pulp ready for papermaking is obtained.

In chemical pulping (designed for dissolving lignin), the wood chips are cooked between 160°C and 190°C for 3 h in either a concentrated solution of sodium hydroxide and sodium sulfide (Kraft pulp) or sulfurous acid (sulfite pulp).[11]

Today, recycled paper is becoming an important feedstock for paper industry, being known as secondary fiber processing. In this case, the feedstock is shredded and pulped again. This raw material comes from discharged printed paper. Therefore, the removal of ink (deinking process) precedes bleaching. Deinking is carried out by pulping again at a 3-4% solids consistency. The slurry is diluted into a 1% solid consistency followed by the addition of flocculating surfactants, ink solvents and cellulase (used for releasing ink particles from fiber fines). The ink particles float in the surface and are collected and removed.[11]

Bleaching – the process by which the pulp is brightened or made completely white by oxidizing chemicals. It involves the complete removal of lignin without any alteration on the amount and structure of cellulose. Xylanase is added into Kraft pulp slurry to change the pulp structure and facilitate the action of oxidizing bleaching chemicals (chlorine gas, alkali extraction and chlorine dioxide), and diminish the amount needed by 20%.[11] Xylanase preparation – which must be devoid of cellulase activity, otherwise cellulose would be hydrolyzed and papermaking would be hindered – is characterized by a range of pH and temperatures from 3.0 to 8.0 and from 30°C to 60°C, respectively.[16]

The finished pulp directed to papermaking is combined with chemicals (clay or starch) to improve paper properties such as strength, stiffness and erasability (important in good typing paper), as well as the performance of paper machines. In this phase, α-amylase (removes excess of starch), lipase (removes pitch, constituted of highly water insoluble lipids, which adheres to the paper machine) and levan-hydrolase (eliminates slime – such as levan, a bacterial β-2,6-linked fructose polymer – that accumulates in the paper machine) are used.

In short, enzymes are used in pulp and paper industry to modify substrates such as fiber constituents (cellulose, lipids and hemicellulose) and paper contaminants and additives (starch, pitch and slime).

EDIBLE OILS

Pulp and seeds of oleaginous plants (palm, olive, soybean, sunflower, cottonseed, canola, rapeseed etc.) are raw materials for the extraction of edible oils. The oils from the pulp of

oleaginous fruits (olive and palm oils) and from seeds (rapeseed and cottonseed oils) are extracted by water and organic solvent, respectively.

As edible oils are sold at low prices, the use of enzymes tends to increase their market price. Thereby, the use of enzymes is justified if the oil is sold at a high price (olive oil) or if a production increase is achieved (palm oil).

The production of olive oil consists on olive milling in presence of water to make a paste with an adequate texture for pressing. The pressed slurry has a solid phase (composed by 3-5% of residual oil, 50% of water and 43-45% of solids), an aqueous phase (composed by less than 1% of residual oil, 90-95% of water and 5-10% of solids), and an oil phase (composed by 98-99% of oil and water and solids less than 1%).[11] By carrying out pressing in presence of cellulase and pectinase, less water will appear in the oil phase. Consequently, less water separates from the oil during the storage period (obligatory step for oil maturation). Such procedure leads to an increase of oil yield by 1%.[17] Considering that thousands of cubic meters of olive oil are processed, any volume close to 1% of water over the whole oil volume will certainly represent some cubic meters more of oil bottled and sold.

In oleaginous seeds and fishes, the oil is often bound to proteins. Therefore, the use of proteases increases the extraction yield.

When processing oleaginous seeds, it is common to obtain oil contaminated by phospholipids (e.g., lecithin) and/or phosphatides, which confer gumminess to the final product. Although degumming could be made with enzymes (phospholipases A1, A2, C and D), the industry prefers removing them with organic compounds or hot water for economic reasons.

Microbial lipases can be used in inter-esterification processes – such as the conversion of palm oil (rich in lauric acid but poor in stearic and palmitic acids) into cocoa butter (rich in stearic and palmitic acids) – and in the hydrolysis of oily triglycerides.[4][11]

The trends for using enzymes in edible oil technology are promising despite their high cost. Environmental concerns, pushed over by humans each day more conscious on better ecological environments, will arise due to the huge wasting of water during oil processing as well as the high volume of pollutant effluents generated.

ENZYMES IN ANIMAL FEEDING

Enzymes can be added in animal food aiming to improve the digestibility of raw materials (starches, proteins, fats, fibers etc.) and to reduce the excretion of nitrogenous and phosphorous substances to the environment.

The main feed raw materials in terms of fiber composition can be divided as follows: **group I** (materials rich in β-glucan, e.g., barley and oats), **group II** (materials rich in pentosans – arabino-xylans –, e.g., wheat, rye and triticale), **group III** (materials not susceptible to the enzyme decomposition; e.g., white sorghum and maize), and **group IV** (vegetable protein sources with pectic and galactosaccharide substances in their fiber structure).[11] The raw materials belonging to the groups I, II and IV are susceptible to enzymatic attacks.

The rationale for using enzymes in animal food assumes that catalysts, when added, must degrade soluble fibers – normally with an anti-nutritional effect – and/or to supplement the animals' own digestive enzymes. In the latter case, the pig pancreas at weaning is incapable of producing a pancreatic juice with amylase and protease activities enough to carry out an efficient digestion of the meal. This physiological condition lasts four weeks. After the full pancreas functioning is reached. Thereby, during the enzyme insufficiency period, the supplementation with exogenous amylases and protease is beneficial to the development of the animal. Besides the supplementation of diets for young animals, enzymes are also important in the degradation of non-starch polysaccharides found in cereals and vegetable proteins.

Diets for poultry can be based on barley, maize, wheat and/or vegetable protein sources. Soybean meal is the most largely used. Broilers are negatively affected by diets containing more than 10% (w/w) of barley due to their susceptibility to the high β-glucan content of barley. Therefore, the feed for them must be added with β-glucanases. In the case of wheat-based diets – rich in water-soluble pentosans –, the addition of pentosanases or endo-xylanases will improve meal use by broilers. Laying hens and turkeys are also positively affected by the cited enzymes. For laying hens, egg cleanliness is lost when diets with high non-starch polysaccharides levels (barley and wheat) are used because these cereals promote egg dirtiness with fecal material. This problem can be minimized by using β-glucanases and xylanases in the feed formulation. In addition, when the overall diet composition is considered in terms of nutrition evaluation, the metabolized sugars resulting from the enzyme

action generate an extra metabolic energy, which must be considered in formulating the diet.[18]

Diets for pigs formulated with barley, wheat and agricultural and milling byproducts require essentially the same enzymes as poultry diets. Poultry and pigs present physiological differences (such as in secretion of endogenous enzymes) regarding the response to their needs for exogenous enzymes. The activity peaks of amylase, lipase, pepsin and trypsin in the small intestine of pigs are, respectively, reached after 33, from birth, 49 and 25 days after birth, whereas for broilers, the peaks are reached after 5, 7, 10 and 15 days after birth, respectively.[19]

Enzymes for animal feeding are additives whose assessment must match that of substrates – whose contents in raw materials vary regarding the crop site, time of harvesting, climatic conditions and species of cereal – in order to present a high efficiency. Consequently, a large variety of enzyme preparations are available in the market. Thereby, the user can only compare different enzyme preparations based on feeding trial, i.e., under practical conditions. The enzyme producer, in turn, must guarantee that the enzyme is resistant to thermal treatment (during the feed processing: 90^{o}C/30 min) and transiting the digestive system intact to the point of action (during animal feeding).

Cattle rearing generate huge amounts of manure rich in nitrogen and phosphorous compounds, which ends up reaching subsoil and water supplies, rivers and the ocean coast after being dissolved by rainwater. To minimize the environmental damage, the best way is to optimize the metabolism of nitrogen and phosphorous by the animal, assuring that only a small portion of these compounds is present in the manure. This can be achieved by improving feed digestibility, adding amylases, proteases, xylanases, β-glucanase, β-galactosidase and phytase to the animal feed. Phytase is an enzyme that decomposes phytate salts in which the phosphorous is retained. By decomposing such salts, the phosphorous element is freed and fully metabolized by the animal. A huge amount of phytase is needed to meet the increase in animal rearing worldwide. Undoubtedly, the main trend on developing enzymes for animal feeding is the optimization of the phytase production in large scale.[20]

ENZYMES AS ANALYTICAL TOOLS

Enzymes have been important reagents in analytical techniques used in clinical chemistry, food and chemical analysis since 1960.

Enzyme assays are often the method of choice in analysis because of their high specificity and sensitivity. Because of their high specificity, samples often require little or no purification prior to analyses. Enzymatic reactions can be run rapidly at or below room temperature, often close to pH neutrality and in a few minutes. Under these conditions, instability of compounds and/or enzyme is not a problem. Side reactions do not occur when purified enzymes are used.

Enzymes can be used in soluble or insoluble forms.[21]

The soluble enzymes can be used either as catalysts for the determination of a compound concentration (any inorganic or organic substance that serves as substrate, activator or inhibitor) even at the order of ng/mL or pg/mL, or as a target for the substrate in order to measure its catalytic activity. The latter approach leads to measuring the enzyme activity present in a biological tissue, becoming a diagnostic tool to determine the state of health or illness of humans. Moreover, enzyme activities are fast indicators of the quality of foods (for example, zero activity of peroxidase and alkaline phosphatase, respectively, in blanched vegetables and pasteurized milk is an indication that the heat treatment was properly executed). Another use of enzymes is for the determination of absolute stereochemistry and/or the primary, secondary or higher order structures of complex chemicals.

Substrate concentrations are determined enzymatically in two ways, i.e., end-point method (in which the substrate is converted completely) and measurement of reaction rate (useful when the reaction reach rapidly the equilibrium and/or the product is insoluble or volatile).

Some examples of reactions are:

1. Determination of glucose with glucose oxidase (***GO***), peroxidase (***PER***) and a chromogen (pyrogalol, o-dianisidine; CH):

$GLUCOSE + O_2 + H_2O \text{ — } (\boldsymbol{GO}) \rightarrow GLUCONATE + H_2O_2$

$H_2O_2 + CH_{REDUCED} \text{ — } (\boldsymbol{PER}) \rightarrow H_2O + CH_{OXIDIZED}$

2. Determination of cholesterol with cholesterol esterase (***CE***), cholesterol oxidase (***CO***), peroxidase (***PER***) and a chromogen (**CH)**:

$CHOLESTEROL_{\textbf{esterified}} + H_2O \text{ — } (\boldsymbol{CE}) \rightarrow CHOLESTEROL + FATTY\ ACID$

$CHOLESTEROL + O_2 \text{ — } (\boldsymbol{CO}) \rightarrow CHOLESTENONE + H_2O_2$

$H_2O_2 + CH_{REDUCED} \text{ — } (\boldsymbol{PER}) \rightarrow H_2O + CH_{OXIDIZED}$

3. Determination of glucose with hexokinase (***HK***), glucose 6-phosphate dehydrogenase (***G6PDH***) and NADP:

GLUCOSE + ATP — (***HK***) → GLUCOSE 6-PHOSPHATE + ADP

GLUCOSE-6-PHOSPHATE + NADP — (***G6PDH***) → GLUCONO-δ-LACTONE + NADPH

4. Determination of triglycerides with lipase (***LIP***), glycerol kinase (***GK***), pyruvate kinase (***PK***), lactate-dehydrogenase (***LAD***), ATP and phosphoenolpyruvate (PPP):

TRIGLYCERIDE + 3 H_2O — (***LIP***)→ GLYCEROL + 3 FATTY ACID

GLYCEROL + ATP — (***GK***) → GLYCEROL 3-PHOSPHATE + ADP

ADP + PPP — (***PK***) → ATP + PYRUVATE

PYRUVATE + NADH+ H^+ — (***LAD***) → L-LACTATE + NAD

5. Determination of the activity of alkaline phosphatase (***AP***) using the 4-nitrophenylphosphate (4-NPP):

4-NPP + H_2O — (***AP***) → PHOSPHATE + 4-NITROPHENOLATE

Enzymes in the immobilized form[21] can be used as analytical tools, presenting advantages over the soluble form such as the repeated use for many assays and increased sensitivity and stability. Moreover, immobilized enzymes are pivotal in diagnostic procedures because they are components of auto-analyzers, test strips and biosensors.

Auto-analyzers incorporate in their configuration devices such as electronic circuit and column filled with an immobilized enzyme. There are auto-analyzers for measuring ethanol (**alcohol dehydrogenase:** ethanol + NAD → acetaldehyde + NADH), ammonia (**L-glutamate dehydrogenase**: 2-oxoglutarate + ammonia + NADPH → glutamate + NADP), glucose (**glucose oxidase/peroxidase:** glucose + O_2 + $2H_2O$ → gluconic acid + H_2O + O_2), and uric acid (**uricase/peroxidase:** uric acid + $2H_2O$ + O_2 → allantoin + CO_2 + H_2O + O_2).

The test strip consists on the immobilization of one or more enzymes in a flexible and porous material, such as filter paper, which is also impregnated with a chromogen. The glucose test strip for diabetics is by far the most sold device, in which glucose oxidase, peroxidase and o-dianisidine are adsorbed on the dried surface of filter paper or plastic strip, which becomes green when immersed in body fluids (blood or urine). High green intensity indicates high glucose concentration, alerting the person to take a shot of insulin.[22]

Biosensor or enzymatic electrode is a device whose sensing element is usually an enzyme which is immobilized in close proximity to an electrode capable of collecting electrons from or donating electrons to the enzyme reaction. The sensing element is reusable and does not require any reagent to make the measurement, except the buffer for flushing the sample chamber. The electrode allows carrying out dozens of determinations in a short period, reducing significantly the cost of the clinical/chemical analysis. Moreover, this device does not need sample preparation to remove colored compounds (e.g., whole blood) or solids (cell debris) from the sample.[23]

Enzymes such as horseradish peroxidase, alkaline phosphatase, glucose 6-phosphate dehydrogenase and lysozyme can be used as antibody markers in the enzyme-linked immunochemical assay (ELISA). Enzymes can be bound to antibodies using such bifunctional coupling reagents such as glutaraldehyde and 3-maleinimidobenzoyl-N-hydroxysuccinimide depending on the nature of the reactive groups of the enzyme. One of them consists of rabbit antibodies adsorption to the walls of a plastic micro-titer tray. Antigen, at a concentration lower than the concentration needed to bind to all antibody sites, is added and binds specifically to antibodies. Following washing, horse antibodies against the antigen are added to form the rabbit antibody-antigen-horse antibody complex. Then, the complex is treated with an enzyme coupled to rabbit anti-horse antibodies. The complex is washed and evaluated as to bound enzyme by adding substrate and buffer at the desired pH. Thereby, the components immobilized are rabbit antibodies, whereas the enzyme is the marker of the rabbit anti-horse antibody, but this complex is soluble. ELISA can be considered as a type of immobilization technique, in which the enzyme does not remain immobilized all the time.[24]

ENZYMES AS DRUGS

The use of enzymes in therapeutics is not a typical industrial use. However, it deserves a few words due to the significant commercial role enzymes play in healthcare, whose selling revenues surpass US$ 1 billion per year. Only the mucopolysaccharosidases – traded as Cerezyme® and Fabrazyme® and used to treat, respectively, Gaucher's and Fabry's syndromes, both of genetic origin – has sales (in a conservative estimative) of about US$ 400 million per year.

The production of therapeutic enzymes involve a large diversity of industrial unit operations to obtain them from cell culture, microbial fermentation or extraction from plants and

animals, and to purify them using a variety of downstream protocols (salt/solvent precipitation, filtration through ultra- or nanofiltration membranes, reverse osmosis, chromatographic techniques etc.). Moreover, therapeutic enzymes are needed at low amounts but at a high purity, contrarily to industrial enzymes, which are less purified preparations.

Enzymes in therapy resulted from attempts to take advantage of the specificity and efficiency characteristics of enzymatic reactions. As any drug, they must meet requirements regarding issues such as dosage, route of administration (intravenous or subcutaneous injection, pulmonary or gastrointestinal tract delivery. In any case, the enzyme must be protected against natural occurring proteases), bioavailability, mode of action (in the right tissue compartment and under physiological conditions with respect to ionic milieu, substrate and cofactor supply, and presence of endogenous inhibitors), and therapeutic value (outweighing the adverse reactions as immunogenicity and cross-reactivity).[25]

Perhaps the most significant approach regarding the therapeutic use of enzymes (powerful but delicate macromolecules) is the establishment of the adequate route for an effective administration.[26]

The main pathways for administering enzymes to the body are **a) nasal epithelium and lungs**: the challenge is to direct the enzyme to the alveoli and through them to enter the blood stream without being destroyed by macrophages. The use of spray containing enzymes molecules confined inside nanometer droplets seems a viable solution; **b) skin:** the enzyme molecules must cross the epidermis and the endothelium of blood vessels. Such barriers can be circumvented by using Iontophoresis (harmless electrical pulses) or ultra-sound vibrations (sonication);[27] **c) intestine:** the enzyme must cross the epithelium and avoid being hydrolyzed by proteases (abundant in the juice of intestinal lumen). To use this path, delivery technology methods have been used: **c1)** the particles containing enzyme molecules are covered with bio-adhesives, which adhere to the epithelium facilitating the crossing of the intestine wall; **c2)** increasing the enzyme affinity with surface cell receptors by inserting enzyme molecules within liposomes (encapsulated particles containing enzyme molecules). They are absorbed by lipoprotein-coated cells of the reticulum-endothelial system, making them reach the blood stream.[28] **c3)** linking the enzyme to a carrier molecule (salycilate, for example), which normally cross the intestinal wall.

A different form of delivering enzymes is the controlled liberation approach. It aims to maintain the enzyme at a desirable level in the blood without repeated administration. This can be achieved through implantable microchips containing reservoirs filled with enzyme, which are covered by a thin gold sheet. This sheet can be dissolved by applying an electric field, freeing the catalyst from time to time according to a delivering schedule.[27]

The enzyme therapy comprises controlling the activity of a metabolic enzyme – a specific inhibitor is administered to the patient (Table 1) – or the enzyme is a drug *per se*, i.e., it must be administered to the patient by pharmaceutical delivery form such as tablets, capsules, injections, inhalation sprays, creams, ointments etc. (Table 2).

Table 1: Drugs used as inhibitors of human metabolic enzymes.

INHIBITOR	TARGET ENZYME	EFFECT
Zileutine	5-lipoxygenase	Anti-asthma, anti-inflammatory
Omeprazol	H^+/K^+-ATPase	Inhibition of gastric juice secretion
Aspirine	Ciclooxigenase	Anti-inflammatory
Alopurinol	Xantine oxidase	Inhibition of uric acid synthesis
Acetazolamide	Carbonic anhydrase	Interfere on the $[(H_2O + CO_2)/H_2CO_3]$ equilibrium
Zidovudine (AZT)	Reverse transcriptase	Anti-HIV
Sildenafil citrate	phosphodiesterase	GMPc accumulation
Non steroidal anti-inflammatory	IK_B-cinase	Decreases the action of the promoter NFK_B on RNA polymerase II of immune cells

Table 2: Examples of enzymes used as drugs.

ENZYME	ILNESS
Cerezyme®	Gaucher's syndrome (type 1): congenital deficiency of β-glucocerebrosidase. Chronic, progressive and multi-systemic infirmity
Fabrazyme®	Fabry syndrome: congenital deficiency of α-galactosidase, leading to the accumulation of glycosphingolipids into the endothelium of blood vessels, damaging several tissues and organs.
Aldurazyme®	Mucopolysaccharidose I resulting from the congenital deficiency of α-L-iduronidase
Streptokinase	Acute myocardial infarction
Hyaluronidase	Diffusion of local anesthetics; dissolution of hyaluronic acid deposited inside the derma
Lysozyme	Anti-microbial
Pancreatin (trypsin, chymotrypsin, α-amylase and lipase),	Digestive disorder
Papain, trypsin, collagenase	Debridement of wounds
Snake venom protease	Blood rheology disorders
Urokinase	Thrombolysis
Thrombin	Blood clotting

Asparaginase	Malignancies
Chymopapain	Intervertebral disk herniation
Superoxide dismutase	Inflammation and reperfusion injury

ENZYMATIC BIOTRANSFORMATIONS

The potential use of enzymes in organic synthesis was recognized since the beginning of the last century. Probably the first reaction catalyzed by an enzyme was the hydrolysis of sucrose by invertase to produce inverted syrup (a mixture of glucose and fructose at a 1:1 ratio).[29]

The broadening of enzyme use in organic synthesis depends on circumventing technical handicaps such as cofactor regeneration, enzyme immobilization and enzyme stabilization. Nevertheless, enzyme-based synthetic chemistry has grown because enzymes operate under mild conditions of temperature and pH – so that sensitive substances can be handled. They can start reactions which are difficult to emulate using more conventional chemical methods, and they are able to distinguish an enantiomer in racemic mixture, and/or identify a functional chemical group in a prochiral molecule to generate an optically active compound (generally a pivotal intermediary in the synthesis of new pharmaceuticals, agrochemicals, fragrances, flavors etc.).

Enzymes used in synthetic chemistry do not need to be extensively purified. Sometimes, intact cells such as *Saccharomyces cerevisiae*, *Pseudomonas putrida* and *Aspergillus niger* are used in the sucrose hydrolysis, conversion of methylbenzene into 1-methyl-2,3 dihydroxi-5,6 cycle hexane and progesterone into 11-hydroxi-progesterone, respectively. Moreover, enzymes may be used in immobilized forms (recovery and enhancing stability), and the catalysis can occur in aqueous (for most enzymes) or organic solvent-water (for few enzymes) medium.

The enzymes can be divided as follows: **a) enzymes not requiring coenzymes**: esterases, lipases, amidases (e.g., penicillinamidase), aldolases (e.g., fructose 1,6-diphosphate aldolase), lyases, hydrolases (e.g., α-amylase and thermolisin) and isomerases (e.g., glucose isomerase); **b) enzymes requiring coenzymes, but not cofactor regeneration systems:** the cofactor (flavins, pyridoxal phosphate, thiamine pyrophosphate, lipoamide, and metal ions) is bound tightly to enzyme molecule (e.g., glucose oxidase and peroxidase), often in a domain different of the active site, and regenerates during the course of the catalysis; **c) enzymes requiring added coenzymes:** approximately seventy percent of all enzymes require one coenzyme (nucleoside triphosphate, nicotinamide derivatives, or coenzyme A). As

coenzymes are expensive compounds, they must be regenerated *in situ*. For example, the xylose conversion into xylitol by xylose reductase (NADPH-dependent enzyme) coupled with the glucose 6-phosphate conversion into 6-phosphate gluconic acid by glucose 6-phosphate dehydrogenase (NADP-dependent enzyme) was recently described.[30] There are several types of organic synthesis reactions (Table 3).

Table 3: Examples of enzymes used in organic synthesis.

REACTION	ENZYME	EXAMPLE
Hydrolysis	Lipase	Ester → Alcohol + Acid
	Penicillinamidase	Penicillin G → 6-aminopenicillanic acid
	Nitrile hydratase	Acrylonitrile →Acrylamide
Esterification	Lipase	2-chloropropanoic acid + n-butanol → Butyl 2-chloropropanoate + 2-chloropropanoic acid
Transesterification	Lipase	2-methyl-6-hydroxy-2-heptene + trichloroethyl butanoate →2-methyl-2-heptenoyl butanoate + 2-methyl-6-hydroxy-2-heptene
Synthesis	Thermolisin	L-methyl-phenylalanine + N-carbobenzoxy-(L)-aspartic acid →N-carboxy aspartame
Reduction	Xylose reductase	Xylose → Xylitol
Oxidation	Glucose oxidase	Glucose →Gluconic acid + H_2O_2
Condensation	Fructose-1,6-diphospho aldolase	D-glyceraldehyde-3-phosphate + ketone dihydroxy-phosphate → Fructose-1,6-diphosphate
Isomerization	Glucose isomerase	Glucose ⇆ Fructose
Addition	Fumarase	Fumaric acid + H_2O →Malic acid Fumaric acid + NH_4^+ →L-aspartic acid

Complex and labile molecules of large use in immunology, endocrinology, intermediary metabolism, molecular genetics, plant and insect biology (pheromones) can be handled only by enzymes, which are enantiomer and regioselective catalysts. Undoubtedly, the classical synthetic organic chemistry techniques are well complemented by enzymology. Today 500 out of 3,000 known enzymes are used in industry, analysis, waste treatment, and therapy. However, the portfolio of enzymes marketed has been growing as recombinant DNA and cell fusion techniques develop. Moreover, progress in protein engineering has been remarkable in the production of synthetic catalysts.

CONCLUSION

Enzymes constitute a versatile group of catalysts as can be seen through the diversity of uses analyzed. Undoubtedly, this is a consequence of the incomparable qualities presented by these catalysts, i.e., specificity, enantiomer selectivity and plentiful activity under mild reaction conditions. Although only 500 out of 3,000 enzymes known are used, they share

about 20% (a conservative estimation) of the worldwide biotechnological market. Furthermore, the number of enzymes marketed has been growing because of the developments in genetic, protein engineering and immobilization techniques.

FUNDING: This work was supported by the National Council for Scientific and Technological Development CNPq (grant no. 303082/2015-1).

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