Exogenous Enzymes

Saqib Farooq, Manzoor Ahmad Shah, Tariq Ahmad Ganaie, and Shabir Ahmad Mir

Introduction

Enzymes, also known as biocatalysts are the large biomolecules required for the numerous chemical inter-conversions that sustain life. They accelerate all the metabolic processes in the body and carry out a specific task (Gurung et al. 2013). Certain enzymes are of special interest and are utilized as organic catalysts in numerous processes on an industrial scale. Enzyme-mediated processes are rapidly gaining interest because of reduced process time, intake of low energy input, cost effective, nontoxic and eco-friendly characteristics (Li et al. 2012; Choi et al. 2015).

Enzymes can be obtained from different sources such as plants, animals and microorganisms. Microbial enzymes are known to be superior enzymes, particularly for applications in industries on commercial scales. Many enzymes from microbial sources are already being used in various commercial processes. Selected microorganisms including bacteria, fungi and yeasts have been globally studied for the bio-synthesis of economically viable preparations of various enzymes for commercial applications (Pandey et al. 1999).

Global market for industrial enzymes was estimated about $4.2 billion in 2014 and expected to develop at a compound annual growth rate of approximately 7% over the period from 2015 to 2020 to reach nearly $6.2 billion (Markets and Markets Watch 2015). Part of this market is ascribed to enzymes used in large-scale

S. Farooq · T. A. Ganaie (✉)
Department of Food Technology, Islamic University of Science and Technology, Awantipora, Jammu and Kashmir, India

M. A. Shah
Department of Food Science and Technology, Government PG College for Women, Gandhi Nagar, Jammu, Jammu and Kashmir, India

S. A. Mir
Department of Food Science and Technology, Government College for Women, M. A. Road, Srinagar, Jammu and Kashmir, India

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applications, among them are those used in food and feed applications (Binod et al. 2008). These include enzymes used in baking, beverages and brewing, dairy and meat industry, dietary supplements, as well as fats and oils (Berka and Cherry 2006; Kirk et al. 2002).

Moreover, with the advent of recombinant DNA technology and protein engineering a microbe can be manipulated and cultured in large quantities to meet increased demand (Liu et al. 2013). Associated driving factors that motivate the use of microbial enzymes in industrial applications are increasing demand of consumer goods, need of cost reduction, natural resources depletion, and environmental safety (Choi et al. 2015).

Production of Exogenous Enzymes

Microorganisms are being the most important source of commercial enzymes today. Enzyme manufacturers have optimized microorganisms for the production of enzymes through natural selection and classical breeding techniques (Agarwal and Sahu 2014). A few years later, for the first time, an enzyme (a protease) was produced by fermentation of *Bacillus licheniformis.* In this, way, large-scale production of enzymes became possible, thus facilitating the industrial application of enzymes (Chaudhary et al. 2015). The primary source of industrial enzymes is microorganisms, out of which, 50% originate from fungi and yeast, 35% from bacteria, while the remaining 15% are either of plant or animal origin (Anisa and Girish 2014).

Amylolytic Enzymes

Amylase can be obtained from different species of microorganisms, but for commercial use, *α*-amylase derived from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* has number of applications in different industries such as in food, fermentation, textiles and paper industries (Konsoula and Liakopoulou-Kyriakides 2007; Pandey et al. 2000). Fungal enzymes are limited to terrestrial isolates, mostly to *Aspergillus and Penicillium* (Kathiresan and Manivannan 2006). *Aspergillus oryzae* is considered to be the favorable host for the production of commercial enzymes including *α*-amylase (Jin et al. 1998).

Several bacterial isolates were isolated from Egyptian soil and were capable to grow and produce amylases. Among these isolates, *Baccilus amyloliquefaciens* was found to produce the highest amylases activity. For the production of amylases, nine agro-industrial residues were added as carbon sources to the basal medium. The medium supplemented with potato starchy waste as the sole carbon source enhanced the enzyme activity more than soluble starch as control for α, β and γ amylases activity, as it increased by *B. amyloliquefaciens* about 1.26 and 4 and eightfold, respectively after 48 h at 50 °C using rotary shaker at 150 rpm. *B. amyloliquefaciens*

gave the maximum values of α, β and γ amylases activity on medium supplemented with 2% potato starchy waste after 30, 30, and 36 h of fermentation periods at 50 °C using shake flasks technique as a batch culture. These values were 155.2 U mL^{-1}, 1.0 U mL^{-1} and 2.4 U mL^{-1}, respectively. It could be stated that productive medium supplemented with 2% potato starchy waste as a low price substrate could be more favorable than basal medium containing 1% starch for amylases production in submerged fermentation, as it increased α, β and γ amylase activity by 1.98, 7.69 and 12-fold than that produced in basal medium (control), respectively (Abd-Elhalem et al. 2015).

Residues of wheat processing were used as substrate for amylase production. The medium was supplemented (in g L^{-1}) of: peptone 24; urea 1.9; glycerol 1.5; KH_2PO_4 0.6 MgSO4 0.5; $(NH_4)_2SO_4$ 0.25 and distilled water. The initial pH was 5.0, and the medium was inoculated with 1.10^6 spores mL^{-1} of a spore suspension from *Aspergillus oryzae* NRRL 695. Submerged fermentation was carried out in a rotary shaker (150 rpm) at 30 °C for 96 h (Kammoun et al. 2008). In another study Ramachandran et al. (2004) investigated the production of α-amylases under solid-state fermentation (SSF) by *Aspergillus oryzae* using coconut oil cake (a by-product obtained after oil extraction from dried copra) as substrate. It contains starch, soluble sugars, soluble proteins, lipids and trace amounts of nitrogen. They achieved 3388 U gds^{-1} when coconut oil cake was supplemented by 1% of peptone in 72 h of fermentation at 30 °C, carried out with 2 mL spore suspension (6.108 spores mL^{-1}) with the initial moisture content of 66%.

Anto et al. (2006) analyzed starch content of the raw materials and correlated it with the glucoamylase production under solid-state fermentation. Wheat bran (75.6% of starch content) and coarse waste (71.1% of starch content) presented highest glucoamylase production (264 U gds^{-1} and 211.5 U gds^{-1} respectively) compared to the enzyme production with rice powder (55.8%), medium waste (48.6%) and fine waste (34.2%). Higher enzyme production using wheat bran and coarse waste can be correlated with their starch content. The production media contained solid substrate and mineral solution (1:2 m/v), pH 7.0, inoculate with 106 spores mL^{-1} collected from 72 h grown culture of *Aspergillus* sp. HA-2.

Proteolytic Enzymes

Proteases are important industrial enzymes synthesized by different types of microbes like fungi, bacteria and yeasts. Because of their rapid growth, less space requirement for their cultivation, microbes serve as a preferred source of protease enzymes (Anwar and Saleemuddin 2000). Microbial proteases represent one of the largest classes of industrial enzymes, accounting for 40% of the total worldwide sales of enzymes by value Santhi (2014). *Bacillus* sp. is one among the best protease producers. Several *Bacillus* species involved in protease production are *B. stero-thermophilus*, *B. cereus*, *B. megaterium, B. mojavensis*, and *B. subtilis* (Anwar and Saleemuddin 2000).

The microbial production of protease by *Bacillus Cereus* using cassava waste water was studied by (Santhi 2014) and it was reported that maximum protease production was observed at 48 h with pH 7; increase in production was observed when glucose and peptone were used as carbon and nitrogen sources in the production medium respectively. Further, cassava waste water was also used as an alternative carbon source for enzyme production, showing a maximum of protease production when supplemented with 6% in the production medium.

An alkaline protease produced by a thermophilic bacteria *Bacillus subtilis*DM-04 was studied by Mukherjee et al. (2008) in SSF using potato peel (51.7% of carbon; 2.6% of nitrogen and 19.9% of C/N ratio) achieving 400 U gds^{-1}. The higher alkaline protease (2382 U gds^{-1}) was obtained with a mixture of potato peel and *Imperata cylindrica* grass in the proportion 1:1 in tray-type bioreactor recovered with aluminum foil, incubated at 50 °C during 24 h. Low levels of acid and alkaline proteases were produced during fermentation using residues (cassava peel, corn cob, corn husk, oat husk and sugar cane bagasse) by *Penicillium janthinellum* CRC87M-115 (Oliveira et al. 2006). In another study alkaline protease production under SSF was investigated using isolated alkalophilic *Bacillus sp*. and green gram husk as substrate. Maltose and yeast extract supplementation increased protease production. The fermentation was conducted at 33 °C, pH 9.0, and moisture content (140%) during 60 h. Protease activity reached more than 35,000 U g^{-1}(Prakasham et al. 2006). Futhermore, SSF was conducted with rice bran and *Rhizopus oligosporus* ACM 145F, incubated at 37 °C for 72 h, achieved a maximal production of an acid protease (1.6 U mL^{-1}) at pH 2.0, decreasing its activity in pH values above 5.0 (Ikasari and Mitchell 1996). In addition to this *Pseudomonas aeruginosa* was a strain isolated from the tannery wastewater for its ability to produce alkaline protease (1160–1175 U mL^{-1}) (Kumar et al. 2008).

Cellulases

Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi andbacteria during their growth on cellulosic materials (Kubicek 1993; Lee and Koo 2001). These microorganisms can be aerobic, anaerobic, mesophilic or thermophilic. Among them, the genera of *Clostridium, Cellulomonas, Thermomonospora, Trichoderma,* and *Aspergillus* are the most extensively studied cellulose producers (Sun and Cheng 2002; Kuhad et al. 1999). Cellulolytic microbes are primarily carbohydrate degraders and are generally unable to use proteins or lipids as energy sources for growth (Sukumaran et al. 2005).

Rodrigues (2011) reported that different concentrations of cellulose, ranging from 20 $g{\cdot}L^{-1}$ to 60 $g{\cdot}L^{-1}$, were assayed as the sole carbon source of the growth medium of *Aspergillus terreus* A-1 and N-Y strains. The activity of cellulases produced by *Aspergillus terreus* A-1 strain had a maximum (13.2 U/mL) at the concentration of 30 $g \times L^{-1}$ of cellulose. On the other hand, the maximum production of cellulases by N-Y strain (10.2 U/mL) was obtained on 20 $g \times L^{-1}$ of cellulose. For

both strains the maximum cellulasic activity occurred between 72 h and 96 h of incubation at 30 °C.

Waseem et al. (2014) reported that CB-2 and CB-3 strains of *Bacillus subtilis* were used for the flask-scale production of cellulase through submerged fermentation. Results revealed the highest cellulolytic activity (CMCase) with 120.321 U/ml and filter paper activity (FPase) with 1.076 U/ml by CB-2 strain followed by CB-3. Optimum temperature and pH of the medium for cellulase production was 37.5 °C, pH and 9 respectively, with 2% untreated cotton stalk as carbon source, yeast as organic nitrogen source and ammonium sulphate as inorganic nitrogen source with 3% inoculum size.

Ariffin et al. (2006) conducted a study to produce cellulase by local isolate *Bacillus pumilus* EB3, using carboxymethyl cellulose (CMC)as substrate. Following that, cellulase produced from *Bacillus pumilus* EB3 was purified using ion exchange chromatography with anion exchanger (HiTrap QXL) for characterization of the cellulase. Cellulase was successfully produced in 2 L stirred tank reactor (STR) with the productivity of 0.53, 3.08 and 1.78 U/L h and the maximum enzyme activity of 0.011, 0.079 and 0.038 U/mL for FPase, CMCase and β-glucosidase, respectively. Purification of cellulase from *Bacillus pumilus* EB3 using ion exchange chromatography showed that 98.7% of total CMCase was recovered.

Xylanases

Xylanase production has been documented in a wide spectrum of microorganisms, including bacteria, actinomycetes, yeasts and filamentous fungi (Nascimento et al. 2003; Bakri et al. 2008). Studying the effect of orange pomace, orange peel, lemon pomace, lemon peel, apple pomace, pear peel, banana peel, melon peel and hazelnut shell on the production of xylanase, using *Trichoderma harzianum* 1073-D3, Seyis and Aksoz (2005) discovered that molasses are able to reduce the time of production in 50% when used as an additional carbon source. The maximum activity has been observed on 2.5% melon peel medium (26.5 U mg^{-1} of protein)incubated at 30 °C for 7 days on a rotary shaker (150 rpm).

Rose and Van Zyl (2008) optimized xylanase production using a recombinant strain of *Aspergillus niger* D15[xyn2]pyrG⁻. The highest xylanase activities of 226 and 209 U mL^{-1} were produced with 20 and 30% molasses, respectively, at 30 °C, pH 6.5, agitation of 100 rpm and a spore inoculum of 1.106 spores mL^{-1}. In another study Maciel et al. (2009) also produced xylanase with sugarcane bagasse under SSF by *Aspergillus niger* LPB 326. The highest xylanase activity was 2327 U gdm^{-1} using 65% of sugarcane bagasse and 35% of soybean meal supplemented with a mineral salt solution, 85% initial moisture, 106 spores gdm^{-1},at 30 °C for 4 days. Dobrev et al. (2007) also used corn cobs in medium composition for increasing xylanase production by *Aspergillus niger* B03. The optimization process was performed. The fermentation was carrying out in flasks inoculated with 10% inoculums, cultivated at 28 °C for 64 h at 180 rpm shaking. The xylanase activity obtained

with the basic nutrient medium was 750.37 U mL^{-1}. After optimization, xylanase activity attained 996.3 U mL^{-1}, which was 33% higher than the activity obtained with the basic medium. The nutrient medium optimized was composed (in g L^{-1}) by (NH4)2HPO4 2.6, urea 0.9, corn cobs 24, wheat bran 14.6 and malt sprout 6.

Paddy husk was used as support to optimize xylanase production by SSF using *Bacillus pumilus*. The medium contained 200 g of paddy husk with 800 mL of liquid fermentation medium (20 g L^{-1} of xylan; 2 g L^{-1} of peptone; 2.5 g L^{-1} of yeast extract and mineral solution at pH 9.0). The highest xylanase activity of 142 U gdm^{-1} was obtained after 6 days of fermentation at 30 °C. The xylanase activity was highest (147.3 U gdm^{-1}) with a 2:9 ratio of paddy husk and liquid fermentation medium on the sixth day at 40 °C achieving 177.5 U gdm^{-1} (Kapilan and Arasaratnam 2011). In another study Bocchini et al. (2005)used *Bacillus circulans* D1 under Submerged Fermentation containing mineral medium and hydrolysated of bagasse with initial sugar concentration of 2.5 g L^{-1}, agitation of 200 rpm, incubated at 45 °C. The authors achieved 8.4 U $mL^{-1}$of xylanase in 24 h of cultivation.

A mutant *Pseudomonas* sp. WLUN024 grown on xylosidic materials, such as hemicellulose, xylan, xylose, and wheat bran was used for xylanase production by Xu et al. (2005). Batch fermentations were carried out on a rotary shaker at 220 rpm, 37 °C for 24 h. After optimization the maximum activity of xylanase reached 1245 U mL^{-1}. The optimized medium consisted of 70 g wheat bran, 8 g $(NH_4)_2\ SO_4$ and 4 g L^{-1} K_2HPO_4 and initial pH adjusted to 8.5 before autoclaving.

Meshram et al. (2008) produced xylanase by Submerged Fermentation using *Penicillium janthinellum* NCIM in 50 mL of Mandels–Weber medium, sugarcane bagasse and beef extract, incubated at 28–30 °C at 180 rpm. The best parameters observed were: carbon source 1.63%, nitrogen source 0.16%, pH 4.1, and inoculum 5.5%, achieving a maximum xylanase activity of 28.98 U mL^{-1}. In addition to this Antoine et al. (2010) tested five agro-industrial wastes (soya oil cake, soya meal, wheat bran, whole wheat bran, and pulp beet) for xylanase production. The fungus, *Penicillium canescens* was investigated in SSF. A xylanase production level of 18,895 U g^{-1} in Erlenmeyer flasks and 9300 U g^{-1} in plastic bags were reached after 7 days of incubation with 5 g of soya oil cake crushed (5 mm particle size) supplemented with 20 mL of distilled water, 3% of casein peptone, 4% $Na_2HPO_4\ 2H_2O$ at 30 °C and 80% of initial moisture.

Pectinases

Many bacteria, fungi and higher plants are known to produce pectinolytic enzyme called pectinase, that breakdown pectin, a polysaccharide substrate that is found in the cell wall of plants. Microbial pectinases account for 25% of the global food enzymes sales (Singh et al. 1999). Almost all the commercial preparations of pectinases are produced from fungal sources. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Jayani et al. 2005).

Orange peel could be an attractive and promising substrate used in pectinase production by submerged fermentation using *Aspergillus niger*. Results indicate that at pH 2.2, a pectin yield of 15.5% was obtained from orange peels at 70 °C, and of the three pectinolytic fungi isolated from natural environment and induced with pectin from orange peels as a sole carbon source for pectinase production; *Aspergillus niger* produced more pectinase than others. Upon partial purification, a twofold increase in pectinase activity whose pH and temperature optima were 5.0 and 40 °C was obtained (Ezike et al. 2014). Furthermore, Patil and Dayanand (2006) reported pectinase production using deseeded sunflower head under Submerged Fermentation by *Aspergillus niger* DMF 27 and *Aspergillus niger* DMF 45 in SSF. In both fermentations processes the pH of 5.0 and temperature of 34 °C were ideal. Under optimum conditions, maximum production of exo-pectinase was 34.2 U g^{-1} SSF (65% initial moisture) and endo-pectinase was 12.6 U mL^{-1} in Submerged Fermentation.

Pectinases production by *Thermoascus aurantiacus* in SSF was reported by Martins et al. (2002) using wheat bran. The authors reached 43 U g^{-1} of polygalacturonase at the 4th day of fermentation while pectin lyase (11,600 U g^{-1}) was produced in the 14th day. The same authors reported the production using orange bagasse (composed in dry material 11.8% fibre, 6.4% protein, 63% nitrogen, 6.7% ash, 19% total sugar (9% reducing sugar) and 0.1% pectin), reaching 43 U g^{-1} of polygalacturonase after 6 days and 19,320 U g^{-1} of pectin lyase. Solid State Fermentation was conducted with initial moisture of 67%.

Mannanases

Mannanses are enzymes produced mainly from microorganisms but mannanases produced from plants and animals have also been reported. Bacterial mannanases are mostly extracellular and can act in a wide range of pH and temperature, though acidic and neutral mannanases are more common (Dhawan and Kaur 2007). Galactomannan-rich substrate locust bean gum (LBG) has been used widely as an inducer of β-mannanase (Kote et al. 2009; Kim et al. 2011). Other substrates like konjac powder, copra meal and wheat bran have also been practiced for the same purpose, since they offer significant benefit due to their cheaper cost and abundant availability (Zhang et al. 2009; Meenakshi et al. 2010; Chauhan et al. 2012).

Various microbes require different incubation times for maximum β-mannanase production. In case of bacteria, it ranges from 24 h in *Acinetobacter* sp. ST 1-1 (Titapoka et al. 2008) to 96 h in *Bacillus* sp. MG-33 (Meenakshi et al. 2010). In contrast to bacteria, fungi require 3 days in case of *Streptomyces* sp. PG-08-03 (Bhoria et al. 2009) to 11 days in *Aspergillus* ATCC 20114 (Mohamad et al. 2011). The optimum temperature for mannanase production has been reported in the mesophilic range in most of the cases, and it corresponds with the growth temperature of the respective microorganism. In general, bacteria prefer neutral to alkaline pH and

fungi acidic pH for best growth and mannanase production (Mabrouk and El Ahwany 2008; Abdeshahian et al. 2010).

The most potent mannanase producer was reported to be *Aspergillus niger,* which produced the highest extracellular mannanase activity (2.90 U/mL), followed by *Aspergillus flavus* (2.54 U/mL) and *Aspergillus ochraceous* (2.16 U/mL). The optimal operating conditions for β-mannanase activity by *Aspergillus niger* arising from this study are as follows: temperature of 30 °C, 6 days incubation period, initial pH 5.0 and inoculum size of 3×10^6 spore/mL (Alsarrani 2011). In another study mannanase production was carried out with *Aspergillus siydowii* grown on 1.0% (w/v) banana stem as the carbon source, producing 1.229 U mL^{-1}, and *Emericella nidulans* grown on 1.0% (w/v) dirty cotton residue producing 0.455 U mL^{-1} of mannanase activity. Fermentation was conducted using as inoculum 108 spores mL^{-1} during 7 days and agitation of 120 rpm (de Siqueira et al. 2010).

Heck et al. (2005) optimized the mannanase production by *Bacillus circulans* in SSF using industrial fibrous soy residue and nutritive solution in a 500 mL cylindrical bioreactor achieving 0.54 U mg^{-1}. Bacteria of *Bacillus amyloliquefaciens* produce the mannanase enzyme and the activity of the mannanase enzyme with a substrate that is a combination of coconut and tofu waste is not much different compared to with a substrate using locust bean gum, with only a 13.34% difference and the optimum pH and temperature are the same. The results showed that the best conditions were a substrate ratio of 80% coconut waste to 20% tofu waste, a 48 h incubation time, a pH of 6.5 and a temperature of 40 °C yielding a mannanase enzymatic activity of 5.13 U mL^{-1}. When locust bean gum was added to the substrate composed of coconut and tofu waste, the best conditions were a dose of 0.6%, a 48 h incubation time, a pH = 6.5 and a temperature of 40 °C yielding a 5.92 U mL^{-1} mannanase enzymatic activity (Zurmiati et al. 2017).

Safety of Exogenous Enzymes

Safety concerns associated with food enzymes in general are possible allergenic, irritative and otherwise toxic properties. Oral toxicity is especially relevant to consumers of food enzymes. The regulation of enzymes internationally is quite varied between countries, with specific country either requiring a full approval process, a notification of enzyme or no requirement thereof. Pre-market approval may depend on whether an enzyme is classified as processing aid or a food additive, though the point of consideration regardless of classification is that the safety of the enzyme must be assured. Challenges to regulators and industry arise from unresolved issues and from lack of harmonization of both legislation and safety evaluation (Agarwal and Sahu 2014).

In occupational contexts enzyme exposure includes mainly to dust or liquid aerosols that are set free while handling enzyme preparation in either manufacturing of the enzyme itself or using enzyme preparations in other industrial contexts. This is generally true for all enzymes regardless of the particular end-use. The particles are

deposited on the skin or on the mucous membranes of the respiratory tract. When an enzyme comes into contact with the respiratory tract or the skin, the body's immune system may be stimulated to produce antibodies resulting in respiratory allergy or contact urticaria, respectively. And because skin has a protein structure, enzymes which catalyse breakdown of proteins such as proteases, are potential skin irritants. The individuals who are exposed to a possible antigen (here: the enzyme) for the first time may develop antigen-specific IgG and/or IgE antibodies. The formation of IgG indicates exposure, and IgE antibodies indicate allergic sensitization but not allergic disease. Once an individual has developed an immune response as a result of inhalation or skin contact with the enzyme, re-exposure produces increasingly severe responses becoming dangerous or even fatal (Spök 2006; Chabane et al. 1994). The enzyme manufacturing industry introduced quite extensive measures to diminish and monitor the exposure of workers, including encapsulation of enzymes, using immobilized preparations, avoiding direct contact, introduction of safe working practices and training of workers, replacement of older products by antigenically distinct proteases, and exclusion of potentially pre-disposed and especially sensitive workers from directly working with enzyme preparations (Spök et al. 1998).

The micro-organisms used in the production of enzymes may themselves be sources of hazardous materials and have been the chief focus of attention by the regulatory authorities. Microbial toxins that are active via the oral route may be produced by certain bacteria or certain filamentous fungi (molds). Yeasts, by contrast, are not known to produce such toxins. The safety of the production strain should be the primary consideration in evaluating enzyme safety. The primary issue in evaluating the safety of a production strain is its *toxigenic potential*, specifically the possible synthesis by the production strain of toxins that are active via the oral route. *Pathogenic potential* is not usually an area of concern for consumer safety because enzyme preparations rarely contain viable organisms. Pathogenicity is, however, important to worker safety (Pariza and Johnson 2001).

Applications of Exogenous Enzymes in Different Food Systems

With the advancement of technology, exogenous enzymes with wide range of applications have great utility in food systems which are as under.

Food Industry

Enzymes have always been important to food technology because of their ability to act as catalysts, transforming raw materials into improved food products. Food processing enzymes are used as food additives to modify food properties. In the twentieth century, enzymes began to be isolated from living cells, leading to a large-scale commercial production and with wider application in the food industry. Food

processing enzymes are used in starch processing, meat processing, dairy industry, wine industry and in manufacture of pre-digested foods (Chaudhary et al. 2015).

The use of rennet in cheese manufacturing was among the earliest applications of exogenous enzymes in food processing. In recent years, proteinases have found additional applications in dairy technology, for example in acceleration of cheese ripening, modification of functional properties and preparation of dietic products (IDF 1990). Animal rennet (bovine chymosin) is conventionally used as a milk-clotting agent in dairy industry for the manufacture of quality cheeses with good flavour and texture. Rennin acts on the milk protein in two stages, by enzymatic and by non-enzymatic action, resulting in coagulation of milk (Bhoopathy 1994). Microbial coagulants are low-cost substitutes of rennet, since they are easily produced by fermentation. They have higher resistance to heat and stronger proteolytic activity during cheese making and ripening (Jacob et al. 2011). Lipases in dairy industry are used for flavour enhancement in cheese products (e.g. enzyme-modified cheese flavour) and acceleration of cheese ripening and lipolysis of milk/vegetable oil/fat to obtain specific flavours (Jooyandeh et al. 2009).

The supplementation of flour and dough with enzyme improvers (technical enzymes) is a usual practice for flour standardization and also as baking aids. Enzymes are usually added to modify dough rheology, gas retention and crumb softness in bread manufacture, to modify dough rheology in the manufacture of pastry and biscuits, to change product softness in cake making and to reduce acrylamide formation in bakery products (Cauvain and Young 2006). The enzymes most frequently used in breadmaking are the α-amylases from different origins (Penella et al. 2008). Amylases can degrade starch and produce small dextrins for the yeast to act upon. Enzymes such as hemicellulases, xylanases, lipases and oxidases can directly or indirectly improve the strength of the gluten network and so improve the quality of the finished bread. The addition of certain types of pentosanases or xylanases at the correct dosage can improve dough machinability yielding more flexible, easier-to-handle dough. The addition of functional lipases modifies the natural flour lipids so they become better at stabilizing the dough. The addition of lipases has been claimed to retard the rate of staling in baked products (Cauvain and Young 2006; Siswoyo et al. 1999). Lipoxygenases are also employed to improve mixing tolerance and dough handling properties (Cumbee et al. 1997).

Enzymes are processing aids used worldwide for fruit processing, particularly for the production of clear fruit juice and concentrate. Enzymes can increase the yield of solid recovery during pulp washing, facilitate the production of highly concentrated citrus bases, improve essential oil recovery from peel, de bitter juice, clarify lemon juice or increase the worth of waste products (Grassin and Fauquembergue 1996). Pectinases are one of the important upcoming enzymes of the commercial sector especially for fruit juice industry as prerequisites for obtaining well clarified and stable juices with higher yields (Lee et al. 2006; Sandri et al. 2011). Amylases are added together with pectinases at the start of the processing season when apples contain starch. Vegetable juice processing therefore requires

more cellulases in addition to pectinases to reduce viscosity sufficiently for juice extraction using a decanter (Chaudhary et al. 2015).

Animal Feed

The use of exogenous enzymes as a feed additive strategy have attracted growing attention and proved to be useful in improving production efficiency of ruminants (Beauchemin et al. 2003). Morgavi et al. (2000) reported that enzymes improved fiber degradation in the rumen by acting synergistically with the rumen microflora, thereby increasing their hydrolytic capacity in the rumen (Beauchemin et al. 2004). Moreover, the use of fibrolytic enzymes in ruminant diets is generally characterized by an increases dry matter (DM)intake, cellulose degradation and/or nutrient digestibility, and consequently increase animal performance (Yang et al. 2000). The supplementation of exogenous fibrolytic enzymes milk yield was improved significantly (41.0 vs. 39.5 kg/cow/day) compared to untreated dairy cows. In addition, the energy corrected milk (40.6 vs. 39.4 kg) and feed efficiency in early lactating dairy cows were improved significantly compared to the control group (Mohamed et al. 2013). In another study Lopuszanska-Rusek and Bilik (2011) observed enhanced milk production with xylanase-esterase supplementation and a tendency of improving dry matter intake and milk production with xylanase and cellulase enzyme supplementation.

Apart from fibrolytic enzymes there is evidence that exogenous proteolytic enzyme could increase the total tract digestibilities of dry matter, organic matter, acid detergent fiber and neutral detergent fiber with larger increases in digestibility of cows though the feeding of proteolytic enzyme unexpectedly decreased feed intake of cows. As a result, milk production was suppressed, nevertheless, dairy efficiency, expressed as milk/dry matter intake, was increased. Supplemented proteolytic enzyme enhanced some milk composition factors such as milk fat and milk lactose percentages but decreased milk protein percentage (Eun and Beauchemin 2005).

In a study conducted by Gado et al. (2009), they found that addition of enzymes increased rumen microbial N synthesis. Intake of dry matter (DM) and organic matter (OM) was positively influenced by supplementation, and digestibility of all nutrients was higher in the total tract of supplemented cows, although the magnitude of the improvement varied among nutrients, with the highest improvement in neutral detergent fiber (aNDFom) and acid detergent fiber(ADFom) (418–584 and 401–532 g/kg respectively) than the other nutrients. Supplementation of enzymes also increased rumen ammonia N and total short chain fatty acid (SCFA) concentrations, and individual SCFA proportions were also altered with an increase in acetate (61.0–64.8 mol/100 mol) before feeding, and acetate and propionate increased 3 h post-feeding (60.0–64.0 and 18.3–20.8 mol/100 mol respectively).

Recent Advances in the Development of Food Enzymes

With the increase in market requirements for food packaging and preservation, the quest for optimum performance of enzymes has given enzyme engineering, particularly enzyme/cell immobilization or cell encapsulation, prime importance in production of biocatalyst with improved properties (Cao et al. 2003; Hamilton 2009).

Immobilization

Immobilization implies associating the enzymes or cells with an insoluble matrix so that it is retained for further economic use, i.e., giving the optimal immobilization yield and having the activity stability in long term (Miladi et al. 2012). Over the last few decades, intensive research in the area of enzyme technology has shown promise, i.e., the immobilization of enzymes (for extracellular enzymes) and cells (for intracellular enzymes). Immobilization enzymes and cells are widely used in the fermentation industry. Also biosensors are designed on the principle of immobilization of enzymes as it is convenient, economical and a time-efficient process of isolation and purification of intracellular enzymes (Mishra et al. 2016). Immobilization can be performed by several methods, namely, entrapment/microencapsulation, binding to a solid carrier, and cross-linking of enzyme aggregates, resulting in carrier-free macromolecules. The latter presents an alternative to carrier-bound enzymes, since these introduce a large portion of non-catalytic material. This can account to about 90% to more than 99% of the total mass of the biocatalysts, resulting in low space-time yields and productivities (Sheldon 2007).

Entrapment/(micro)encapsulation, where the enzyme is contained within a given structure. This can be: a polymer network of an organic polymer or a sol-gel; a membrane device such as a hollow fiber or a microcapsule; or a (reverse) micelle. Apart from the hollow fiber, the whole process of immobilization is performed in-situ. The polymeric network is formed in the presence of the enzyme, leading to supports that are often referred to as beads or capsules. Still, the latter term could preferably be used when the core and the boundary layer(s) are made of different materials, namely, alginate and poly-l-lysine. Although direct contact with an adverse environment is prevented, mass transfer limitations may be relevant, enzyme loading is relatively low, and leakage, particularly of smaller enzymes from hydrogels (namely, alginate, gelatin), may occur. This may be minimized by previously cross-linking the enzyme with multifunctional agent (namely, glutaraldehyde) (Brady and Jordaan 2009; de Segura et al. 2003) or by promoting cross-linkage of the matrix after the entrapment (de Assis et al. 2004). The use of LentiKats, a polyvinyl-alcohol-based support in lens-shaped form, has been used for several applications in carbohydrate processing. Among these are the synthesis of oligosaccharides with dextransucrase (de Segura et al. 2003), maltodextrin hydrolysis with glucoamylase (Rebroš et al. 2006), lactose hydrolysis with lactase (Grosová et al.

2008), and production of invert sugar syrup with invertase (Rebroš et al. 2007). Flavourzyme, (a fungal protease/peptidase complex) entrapped in calcium alginate (Anjani et al. 2007), k-carragenan, gellan, and higher melting-fat fraction of milk fat (Kailasapathy and Lam 2005), was effectively used in cheese ripening, in order to speed up the process, while avoiding the problems associated with the use of free enzyme. These include deficient enzyme distribution, reduced yield and poor-quality cheese, partly ascribed to excessive proteolysis and whey contamination (Anjani et al. 2007). Calcium alginate beads were also used to immobilize glucose isomerase (Tumturk et al. 2008) and α-amylase for starch hydrolysis to whey (Rajagopalan and Krishnan 2008).

In a particularly favored technique immobilization of enzymes in liposomes, known as dehydration-rehydration vesicles (DRVs), small (diameters usually below 50 nm) unilamellar vesicles (SUVs) is prepared in distilled water and mixed with an aqueous solution of the enzyme to be encapsulated. The resulting vesicle suspension is then dehydrated under freeze drying or equivalent method. Upon rehydration, the resulting DRVs are multilamellar and larger (from 200 nm to a little above 1000 nm) than the original SUVs, and can capture solute molecules (Walde and Ichikawa 2001;Grosová et al. 2008). Recent work in this particular application has used lactase as enzyme model and has focused on the optimization and characterization of the liposome-based immobilized system (Rodríguez-Nogales and López 2006). Cocktails of enzymes, namely, Flavourzyme, bacterial proteases and Palatase M (a commercial lipase preparation), were immobilized in liposomes and successfully used to speed up cheddar cheese ripening (Kheadr et al. 2003). Encapsulation in lipid vesicles has been proved a mild method, providing high protection against proteolysis.

Binding to a solid carrier, where enzyme-support interaction can be of covalent, ionic, or physical nature. Curiously, the first reported application of enzyme immobilization was of invertase onto activated charcoal (Nelson and Griffin 1916). Another example is the immobilization of pectinase in egg shell for the preparation of low-methoxyl pectin. The immobilized biocatalyst could be reused for 32 times at 30 °C, and it was used in a fluidized-bed reactor, operated at an optimum flow rate of 5 mL h^{-1} and 35 °C (Nighojkar et al. 1995). Other examples are the surface immobilizations of α-amylase on alumina (Reshmi et al. 2006) and in zirconia (Reshmi et al. 2007).

Carrier-free macroparticles, where a bifunctional reagent (namely, glutaraldehyde), is used to cross-link enzyme aggregates (CLEAs) or crystals (CLECs), leading to a biocatalyst displaying highly concentrated enzyme activity, high stability and low production costs(Sheldon 2007; Roy and Abraham 2004). The use of CLEAs is favored given the lower complexity of the process. This approach is recent, as compared with entrapment and binding to a solid carrier, and there are still relatively few examples of its application to enzymes used in the area of food processing (Fernandes 2010) such as the immobilization of lactase for the hydrolysis of lactose, where, under similar operational conditions as for the free enzyme, the CLEA yielded 78% monosaccharides in 12 h as compared to 3.9% of the free form (Gaur et al. 2006).

Conclusion

Commercial-scale enzyme catalysis has been implemented in several industries such as food, chemical and pharmaceutical. Microbial enzymes are the preferred source to plants or animals due to their economic feasibility, high yields, consistency, ease of product modification and optimization, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity. In comparison with plant and animal enzymes, microbial enzymes can be produced very effectively by different fermentation techniques like solid-state and submerged fermentations. It is also easy to produce microbial enzymes on a large scale. The recombinant DNA technology has further improved production processes and helped to produce enzymes commercially that could not be produced previously. Global market for exogenous/commercial enzymes is expanding rapidly and this has given enzyme engineering particularly enzyme immobilization a prime importance in production of biocatalyst with improved properties.

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