Chapter 12

Enzymes and Phytohormones from Micromonospora

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Additional information is available at the end of the chapter

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Abstract

Actinobacteria produce diverse and huge amounts of enzymes that are widely used in different industrial purposes. Specific properties of enzymes allow to run the reactions under milder conditions with improved yield and reduced wastes. Further redesign for natural enzymes is very essential because they are often not suitable for biocatalytic processes. Recently, new microbial natural and creating enzymes are discovered synchronous with the late advanced technologies of genomics, metagenomics, proteomics, efficient expression systems and emerging recombinant DNA. The ongoing development of enzyme biotechnology aids in the improvement of the industrial biocatalysis field. Thermophilic actinobacteria produce thermostable enzymes that are widely used in industrial processes. In contrast, psychrophilic actinobacteria grow well at low temperatures and subsequently their enzymes are more effective at low temperatures. The advanced DNA sequencing technique allows determining and identifying the sequences and functions of all the genes that synthesize proteins that are widely use in the industry. Recombinant strains can be obtained by using certain biotechnological tools to potentially increase enzymes production on a large scale. The ongoing development in this field will lead to the improvement of different industrial purposes such as food, chemicals, textiles, leather, pharmaceuticals, and so on.

Keywords: Actinomycetes, biocatalysts, enzyme biotechnology, industrial microbiology

1. Introduction

Actinobacteria are recognized by their morphology thin, elongated cells with branching filaments, as its name denotes. Nevertheless, morphology alone is not enough to add an organism to the actinobacterial group, so the advanced identification depends on other techniques such as cultural, physiological, biochemical and molecular characteristics. More-

over, phylogenetic analysis is necessary to distinguish between true actinobacteria and those that have closely phylogenetic affinities, and at the same time to precisely determine the similarity among different species of the same genus. A lot of enzymes produced from actinobacteria are used in industries thus they are called "industrial enzymes". These enzymes employ in the industry of paper, leather, detergents, textiles, pharmaceuticals, chemicals, foods, beverages, biofuels, animal feed, personal care, and others [1]. The sustainable industry based on enzymes technology depends on the biotechnological development, which is represented in the modern molecular techniques such as metagenomics and genomics. The latter allows the discovery of new potential recombinant-biocatalytic enzymes used in different industries needed by society. These industrial enzymes are employed to yield over 500 products [2, 3]. Although chemical and organic methods are used in industries, they have several disadvantages non-economic, unfriendly to the environment, lacking of enantiomeric specificity for chiral synthesis, need for high temperature, low pH and high pressure and leads to organic waste and pollutants. On the other hand, enzymes are more useful in industrial applications because they act at mild reaction conditions, have a long half-life, and are friendly to the environment [4]. Moreover, gene-encoded enzymes can be modified to improve their properties stability, substrate specificity and specific activity. There are many competitive companies that participate in the enzyme industry Novozymes being the largest one followed by DSM and DuPont among others. The companies' competition relies on certain criteria such as product quality, performance, use of intellectual property rights, and the ability to innovate.

2. Enzymes detection

Recently, new enzymes can be detected by using certain biotechnological tools such as metagenome screening [5, 6], microbial genome mining [7, 8], and exploring the diversity of extremophiles [9, 10].

2.1. Metagenome screening

Metagenome is a new manner for microbial screening based on the function and/or sequence of the gene [6, 11, 12]. Function-based screening is a method by which gene isolation is carried out depending on the desired gene function through phenotype detection, heterologous complementation, and induced gene expression [13]. Whilst sequence-based screening is a method performed by either polymerase chain reaction (PCR) or hybridization procedures. Strains producing industrial enzymes are isolated from different environments such as volcanic vents, arctic tundra, cow rumen [14], marine environments [15], and termite guts [16] such as lipase [17, 18], oxidoreductase [19, 20], amidase [21], amylase and nitrilase [22], beta-glucosidase [23, 24], decarboxylase, and epoxide hydrolase [25]. Function-based screening method has main disadvantages due to its inefficiency and biased expression of foreign genes in *Escherichia coli* as an alternative host [12]. To overcome these problems, alternative bacterial host and expression systems are currently being examined including *Streptomyces lividans*, *Pseudomonas putida* and *Rhizobium leguminosarum*, among others [26, 27]. Target rate depends on other factors such as gene size and the assay method. Enzyme activity can be assayed by

agar plate method, which has a sensitivity developed by using cell lysates [28], toxics resistant genes [29, 30], correlation of enzyme activity with a phenotypic characteristic such as green fluorescent protein (GFP) [31], or β-galactosidase [32]. Also, development of flow cytometry-based screens as SIGEX are leading the way as they enable more rapid screening of large metagenomic libraries [33].

2.2. Microbial genome mining

New enzymes particularly used in industries are explored from huge genome sequence databases [34]. The advanced genome sequencing programs, such as 454 from Roche, Solexa from Illumina, or SoLiD from ABI, reduce time and cost. Using these programs support the complete and accurate reading of multiple whole genomes in less than two weeks [35]. Two approaches are being followed to discover new enzymes [36].

2.3. Exploring the diversity of extremophiles

More than 30 papers were published on extremophiles in a special issue [37]. Extremophiles are promising microorganisms for enzyme production because they can grow under extreme physical conditions such as temperature (2-12°C, 60-110°C), pressure or radiation, and/or geochemical conditions such as salinity (2-5% NaCl) and pH (< 2, > 9). High biodiversity for extremophiles is present under all extreme conditions mentioned [10, 38]. For example, there are some thermostable enzymes such as proteases, lipases, cellulases and amylases that are successfully used in different industrial applications [10, 39]. Thermostable enzymes are isolated from different thermophilic bacteria such as *Clostridium*, *Thermus*, *Thermotoga*, and *Bacillus*, as well as Archaeabacteria such as *Pyrococcus*, *Thermococcus* or *Methanopyrus*. The most common thermostable enzyme is Taq DNA polymerase which isolated from *Thermus aquaticus* [40]. Moreover, thermostable enzymes are greatly used for detergent manufacturing. Polymer-degrading enzymes such as cellulases and xylanases are used for pulp and paper industries, and are also used with other applications such as extraction and clarification of fruit juices, improvement of bakery products, polishing and stone-washing of textiles, and bioremediation of waters contaminated with oils or hydrocarbons [10, 41 - 43]. Halophites are produced for specific enzymes that tolerate salt stress by containing a considerable number of negatively-charged amino acids that prevent precipitation [44]. The most common halophites that used in industrial applications are *Halobacterium*, *Halobacillus* and *Halothermothrix* [45, 46]. Thermoalkaliphilic enzymes, such as proteases and lipases, are produced by microorganisms that grow under extreme pH values and used as additives in laundry and dishwashing detergents [10, 47].

3. Improvement of microbial enzymes

Improvement of enzyme properties can be carried out by creating a growing demand [40]. Industrial enzymes usually need further fine tuning to achieve industrial scale production [48]. Using recombinant DNA technology in large industrial scales is very urgent due to the increase

in production by 100-fold [49]. There are two main manners for enzymes modification to adapt their functions rational redesign and combinatorial methods.

3.1. Rational redesign

This strategy is based on using direct mutation to hit amino acid substitutions, which require complete information about the 3D structure and chemical mechanism of the enzymatic reaction. Databases include protein structures and sequences, so the sequence comparison of a new enzyme with the references can identify related enzymes whose functions or/and structures are already known [50 - 54].

3.2. Combinatorial methods

Combinatorial methods rely on certain factors relevant to an enzyme molecule such as chirality, biocatalytic effect, catalytic rate, solubility, specificity and stability. Combinatorial methods are faster and cheaper than methods for finding new enzymes it acts better than natural methods under specific conditions [3, 55 - 57]. Combinatorial methods, also called "directed evolution" have random mutagenesis in the protein-encoding gene using different techniques such as PCR [58], repeated oligonucleotide directed mutagenesis [59], and chemical agents [60]. The PCR technique introduces both random and point mutations in a large number of enzymes. These techniques perform random recombinant *in vitro*, typically between parent genes with homology higher than 70% [61]. The point mutation is carried out by the target active site residues (about 10–15 amino acids) and those closest to it (another 20–30 amino acids) [62]. There is another way, called CAS Ting, which is based on a combinatorial active site testing in which libraries are generated from groups of two or three residues made from the active site residues [63]. While running PCR, the amino acid alphabet is reduced and new proteins composed of only 12 amino acids are synthesized [64 - 66]. Further techniques can create shuffle exons or domains [67], loop regions [68], random truncations [69], or insertions and deletions of codons [70]. Moreover, random redesign technology is used to create new and improved enzymes that have the highest activity, are more stable at different pH values and temperatures [71], have increased chirality [72], altered substrate specificity [73], stability in organic solvents [74], novel substrate specificity and activity [75] and increased biological activity of protein pharmaceuticals and biological molecules [76, 77]. Directed evolution method could improve the activity of glyphosate-N-acetyltransferase by 10,000-fold and thermostability by 5-fold [78]. Directed evolution work has resulted in the presence of new and improved enzymatic proteins in the market since 2000 [79, 80].

4. Production of recombinant microbial proteins

Molecular biology techniques, particularly recombinant DNA, have a great effect in enzyme production from different microorganisms such as *Bacillus* spp, *Ralstonia eutropha*, *Pseudomonas fluorescens*, *Saccharomyce cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Aspergillus* spp, *Trichoderma* and *E. coli* [81]. The latter is most common bacteria used in this purpose due to its

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accurate genome modification, rapid growth, and good growth on different media [82, 83]. Moreover, the *Pseudomonas fluorescens* bacteria of the Pfenex Company can produce 20 g/L of protein [84]. On the other hand, *Saccharomyces cerevisiae* is more useful than bacteria because it usually used as a cloning host, it has high cell density, produces heterologous proteins, and its genetics are more advanced than any other eukaryote. Despite all the benefits mentioned above, this yeast is not convenient for use in the mammalian proteins industry at the large-scale because drawbacks may occur such as hyperglycosylation, antigenic response in patients due to accumulation of α-1,3- mannose residues, and absence of strong and tightly-regulated promoters [85]. *P. pastoris* is regarded as one of the most common industrial microorganism and could produce over 700 proteins [81, 86, 87]. Recombinant protein production had 22 g/L for intracellular proteins [88] and 14.8 g/L for secreted proteins [89]. *P. pastoris* can produce up to 30 g/L of recombinant proteins [90 - 94]. Although DNA recombination technology is more easy and available, gene insertion and deletion remain difficult [95, 96]. The problem at industrial level is that non-fungal proteins production is low compared to homologous proteins. There are many strategies used to overcome this problem such as the establishment of weak protease strains [97], insertion of a considerable number of gene copies [98], use of strong fungal promoters, efficient secretion signals [98, 99], and gene fusions with a gene that encodes part of or an entire well-secreted protein [99]. *Chrysosporium lucknowense* was found to have a great ability for protein production (50–80 g/L), and from which low-viscosity and low-protease mutants have been obtained [100, 101].

5. Biocatalysts

Biocatalysts are widely used in different economic industries such as food industry [102]. Biocatalysis can be carried out by using intact cells, immobilized cells, cell free extracts, purified enzymes, or immobilized enzymes [103,104]. Microbial industry has been developed rapidly due to due to using the recent techniques such as genome sequencing, directed evolution, protein expression, metabolic engineering, and structural biology [105,106].

5.1. Enzymes applications

Enzymes have great importance in industrial processes [107, 108]. These enzymes are used in the detergent, textiles, pulp, paper, leather, and biofuels industries. Biofuels have the highest sales [109, 110]. In the textiles industry, enzymes are used as cleaners, reducing the use of raw materials and waste production [111]. Pectate lyase is used in the cotton industry [112]. Lipases, xylanases, and laccases are used in removing pitch in the pulp industry [113 - 115]. Cellulases are widely used in the textiles industry and are also used in the degradation of lignocellulosic feed stocks. There are several cellulases such as endoglucanases that degrade cellulose randomly, cellobiohydrolases that release glucose dimers from both ends of cellulose chains, and beta-glucosidases that hydrolyze oligomer chains to liberate glucose molecules [116 - 118]. The main microbial source of cellulases is *Trichoderma reesei* which depolymerizes plant blocks to free sugar molecules [119 - 124].

5.2. Enzymatic food industry

A certain group of enzymes plays an important role in the food industry and subsequently achieves great revenue [125]. The mechanism of feed enzyme action supports nutrient digestion and therefore leads to a much easier feed utilization. Moreover, they hydrolyze complex components that can be deleterious or have no value [126]. The most common commercial feed enzymes are phytases, proteases, α-galactosidases, glucanases, xylanases, α-amylases, and polygalacturonases, which are used in swine and poultry products industries [127, 128]. Lipase is a distinguishable group of enzymes that is used in the food industry. The maximum yield obtained from lipases requires optimization of enzyme concentration, pH, temperature, and emulsion content. The most common recombinant fungal lipases are produced from *Rhizomucor miehi*, *Thermomyces lanuginosus* and *Fusarium oxysporum* [129, 130]. Protease is also an important group of enzymes that is mainly used in the dairy industry, such as cheese manufacturing where it hydrolyzes a specific peptide bond that generates para-k-casein and macropeptides [131]. Recombinant protease of *A. niger var awamori* can produce 1 g/L of chymosin after nitrosoguanidine mutagenesis and the selection for 2-deoxyglucose resistance [132, 133]. There are four recombinant proteases that have been registered by the FDA for cheese production [128, 134]. Although all enzymes mentioned above are the most common in the food industry, there are others used in this application such as invertase used for candy and jam manufacture, β-galactosidase (lactase) used for hydrolysis of lactose from milk or whey, and galactosidase used for crystallization of beet sugar [135 - 137].

5.3. Enzymatic processing of chemicals and pharmaceuticals

The chemicals industry depends on enzyme technology that is low cost, has easy methods, and high quality [138]. Enzymatic processing requires lower energy, which has many benefits such as high yield, high catalytic activity, low releasing of wastes and byproducts, and lower volumes of wastewater streams [139]. Genomic and proteomic technology development improved enzyme properties which in turn improved the chemicals industry [140, 141]. L-tyrosine is a main compound for protease conversion [142, 143]. Beta-lactam antibiotics manufacturing is one of the common pharmaceuticals industries that depend on enzymes technology [144]. Esterases, lipases, proteases and ketoreductases are used in the industry of chiral alcohols, carboxylic acids, amines or epoxides [110, 145, 146]. Sometimes, recombinant microbial enzymes raise the yield percentage up to 100% [147]. A combination of random gene mutagenesis and ProSAR analysis increase the chirality of ketoreductase enzyme toward tetrahydrothiophene-3-one from 63 to 99% [148 - 152]. The improved enzymatic biotechnology supports the production of 2-methyl pentanol as an important intermediate for pharmaceuticals manufacture [153 - 155]. Also, an improved acyltransferase aids the conversion of cholesterol-lowering agent, lovastatin, to simvastatin [156, 157]. Moreover, lipase is used in stereoselectivity for acetylation of asymmetrical diol during an antifungal agent production [158]. Enzymatic biotechnology has many advantages such as higher substrate solubility, reversal of hydrolytic reactions, and modified enzyme specificity, which result in new enzyme activities [159].

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6. Industrial enzymes

6.1. Pectinases

Actinobacterial pectinases are widely used in various industrial applications such as food and beverages industries, as well as fruit treatment including fruit maturation, viscosity rising, decreasing of must, preliminary treatment of must for wine industries, extraction of tomato pulp, and tea and chocolate fermentation [160, 161]. Pectinases are also used in the textile and paper industries in plant fiber degumming [162 - 165]. Moreover, the combination of pectinase and β-glucosidase supports the scent and volatile substances of fruits and vegetables, and raise the content of antioxidants [166, 167]. Pectinases are produced from several genera of microorganisms such as *Bacillus, Aspergillus, Rhizopus, Trichoderma, Pseudomonas, Penicillium* and *Fusarium* [168, 169].

6.2. Lipases

Lipases are used in various commercial applications, such as in the detergents industry. The most common lipase-producing microorganisms are *Penicillium restrictum*, *Candida rugosa, Candida antarctica, Pseudomonas alcaligenes, Pseudomonas mendocina*, *Burkholderia cepacia* [72], *Geotrichum candidum* DBM 4013, *Pseudomonas cepacia, Bacillus stearothermophilus, Burkholderia cepacia*, *Candida lipolytica*, *Bacillus coagulans*, *Bacillus coagulans* BTS-3, *Pseudomonas aeruginosa* PseA, *Clostridium thermocellum* 27405, *Yarrowia lipolytica* and *Yarrowia lipolytica* CL180 [170 - 179].

6.3. Lactases

Lactases or β-galactosidases are hydrolyzing enzymes that catalyze the hydrolysis of lactose at terminal residues and produce glucose and galactose. The microbial source of lactases are yeasts such as *Kluyveromyces lactis*, *K. fragilis* and *Candida pseudotropicalis*; bacteria such as *Escherichia coli*, *Lactobacillus bulgaricus*, *Streptococcus lactis* and *Bacillus* sp; and fungi, such as *Aspergillus foetidus*, *A. niger*, *A. oryzae* and *A. Phoenecia* [180,181]. Beta-galactosidase is widely used in the dairy industry, and also used in dairy products crystallization such as milk candy, condensed milk, frozen concentrated milk, yoghurt and ice cream mixtures [182 - 185].

6.4. Cellulases

Cellulases are hydrolyzing enzymes for cellulose substances to produce cellobiose and then glucose [186]. The combination of cellulases and pectinases provides well in applications in juice and wine industries [187]. There are a wide array of microorganisms that produce cellulases, such as *Trichoderma*, *Penicillium*, *Aspergillus*, *Fusarium, Phoma*, *Acidothermus*, *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Streptomyces*, *Xanthomonas*, *Acetovibrio*, *Clostridium*, *Pseudonocardia,* and *Thermoanaerobacter* [188, 189].

6.5. Amylases

Amylases are used in the textiles, beer, liquor, bakery, infant feeding cereals, and animal feed industries. *Aspergillus* and *Rhizopus* are the most common amylases producers [190,191].

Amylases are used in the food industry in the conversion of starch into dextrin which in turn is converted to maltose, which used in the manufacture of soft drinks, beer, jellies, and ice cream. Maltose can be converted into glucose, which used in the soft-drinks and bakery industries [192,193]. Amylases are the most used enzymes in bread baking [194]. Amylases also play an important role in the pharmaceuticals industry [195 - 199].

6.6. Proteases

Proteases are used in the baking, feed and brewing industries. Proteases catalyze the splitting of peptide linkages in proteins. Proteases are classified into exopeptidases and endopeptidases according to the site of the concerned peptide bond to be cleaved. Recently proteases represent 60% of industrial enzymes on the market because they are easy to obtain and to recover. Proteases have high coagulation activity, with fewer risks [200]. Proteases are mainly used in the industry of soluble proteins, chemicals and pharmaceuticals [201]. Proteases are produced by *Bacillus*, *Thermus caldophilus*, *Desulfurococcus mucosus*, *Streptomyces* and *Escherichia coli* [202].

6.7. Glucose oxidase

Glucose oxidase can oxidize β-D-glucose with the formation of D-gluconolactone. The enzyme is used to remove harmful oxygen in the food industry to avoid toxicity [203].

6.8. Glucose isomerase

Glucose isomerase catalyzes the reversible isomerase from D-glucose and D-xylose into D-fructose and D-xylulose respectively. Glucose isomerase plays an important role in the food industry such as in the production of fructose-rich corn syrup [197]. The cloned gene xylA of *Thermus themophilus* is introduced to *Saccharomyces cerevisiae* to be expressed under the control of the yeast PGK1 promoter [205,206].

6.9. Invertase

Invertase is produced by *Saccharomyces cerevisiae* and other microorganisms. The enzyme catalyzes the hydrolysis from sucrose to fructose and glucose. The supplementation of an invertase to banana juice is to increase its sweetness and viscosity [207 - 210].

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