Chapter

Prospective Application of *Aspergillus* Species: Focus on Enzyme Production Strategies, Advances and Challenges

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Abstract

Fungal enzymes that catalyze different types of biochemical reactions play a significant role in modern industry by improving existing processes. Also, the use of enzymes to replace some traditional toxic chemical or mechanical approaches helps decrease energy demand and environmental pollution. However, enzymes must be able to compete commercially with relatively low-priced traditional approaches. Meeting economical and commercial feasibility criteria depends on a number of enzymatic properties including the specificity to the substrate, stability in industrial enzymatic reaction conditions and catalytic efficiency. Fungi used as an enzyme manufacture host should be appropriate for industrial scale fermentation. *Aspergillus* species are being developed as one of the best enzyme manufacture factories due to their capability to secrete high quantities of enzymes suitable for industrial applications. The industrial importance of *Aspergillus* species also includes the progress and commercialization of new products derived from genetically engineered modified strains. Hence, the main aim of this chapter investigation is to analyze the secreted and cellular proteins from *Aspergillus* species and their application in industries.

Keywords: filamentous fungi, *Aspergillus* species, fermentation, enzymes, intra- and extracellular secretion

1. Introduction

With the exponential increase in science and knowledge about biochemical processes; it would be fair to say that it is inconceivable to consider any biological process without an enzyme. They are biocatalysts that enhance the rate of reaction in numerous folds. Enzymes usually are reusable. In other words, they are not used up by the reaction and can be reused. Once an enzyme binds to a substrate and catalyzes the reaction, the enzyme is released, unchanged, and can be used for another reaction. This means that for each reaction, it is not necessary to have a ratio of 1:1 between enzymatic molecules and substrate molecules. Enzymes mostly

are proteinaceous-based in nature (there are a few RNA-based enzymes) and necessary for all living organisms [1]. A significant number of them have been recognized as safe from a biotechnological perspective. Fungi, as one of the simplest organisms, are often used to produce enzymes. In addition, factors such as low energy, low cost, non-toxic and environmentally friendly nature make them popular in many industrial processes [2]. Also, the need for gentle temperature and pressure for enzymes to function enables them to become a viable alternative to hazardous chemical catalysts [3]. Enzymes are commonly used to make wine, beer, bread, cheese, vinegar, and leather and textiles. However, the pure and clean form of enzymes has found wide applications in industry only a few decades ago [4]. Enzymes produced by the fungal system are commonly used in various sectors including food, chemicals, medicine, agriculture and energy [5]. Today, due to multiple applications, the demand for different kind of enzymes in various food sectors has increased greatly [6–18], as shown in **Table 1**. Additionally, the manipulation of strains through recombinant DNA techniques and protein engineering technology has made it possible to meet the growing demand for enzymes [19]. Fungi are metabolically dynamic, simple to ferment and can work on an industrial scale, require simple nutrients and can be used throughout the year and are not subject to seasonal conditions [20]. The genus *Aspergillus* has more than 340 officially known species [21]. These fungi are characterized by an extraordinary capability to produce and secrete large amounts of proteins, metabolites, and organic acids into their growth medium [22]. Notwithstanding the existence of different pathogenic *Aspergillus* strains, a significant number of them have been recognized from a biotechnological perspective [23]. Characteristics such as the existence of a secretory pathway, the eventuality of genetic manipulation, and high productivity using diverse fermentative processes are beneficial and positive for the make use of *Aspergillus* species [23]. Enzymes produced by *Aspergillus* species have been broadly investigated for their potential in the formulation of commercial products [24]. Hence, the main goal of this chapter investigation is to analyze the secreted and cellular proteins from *Aspergillus* species and their utilization in food industries.

Enzymes	Food sectors	Applications
Amylases	Brewing industry	Fermentation of alcohol by converting starch to sugars
	Baking industry	Breakdown of starch into simple sugars; thereby allowing the bread to rise and impart flavor
		Dough conditioning
		Generates additional sugar in the bread, which improves the taste, crust color and toasting quality
		Anti-staling effect during bread making; improves the softness and shelf-life
Cellulases	Fruit industry	Fruit and vegetable juice clarification
		Reducing the viscosity of nectars
		Alteration of fruit sensory properties
	Beverages industry	Concentrating purees
	Health food industry	Carotenoids extraction
	Edible oil extraction industry	Olive oil extraction
	Baking industry	Improvement quality of bakery products

Enzymes	Food sectors	Applications
Chitosanases	Seafood industry	The degradation of crustacean chitinous waste
	Agriculture industry	Biological activities such as antifungal effect
Galactosidases	Dairy industry	Production of low lactose/milk free lactose
		Production of prebiotics
		Prevents crystallization of lactose Improves the scoop ability and creaminess of the product
		Production of ice creams, sweetened flavor and condensed milks
		Improves the scoop ability and creaminess of the product
Invertases	Food sweetener market	Invert sugar production
	Confectionery food industry	Production of high fructose syrup
		Manufacturing of soft-centered candies
		Manufacture of artificial honey
Laccase	Wine industry	Removal of polyphenol, thereby providing stability to wines
		Preparation of cork stoppers of wine bottles
		Reduces cork taint generally imparted to aged wine bottles
	Brewing industry	Removal of oxygen at the end of beer fermentation process
		Prevent the formation of off-flavors (trans 2-nonenal)
	Fruit industry	Juice clarification
	Baking industry	Increase strength, stability and reduce stickiness
		Increase volume, improved crumb structure and softness of the product
Lipases	Fats and oils food industry	Production of mayonnaise and other emulsifiers, Triglycerides synthesis and trans-esterification of triglycerides in non-aqueous media; specially fat production
	Dairy industry	Development of flavoring agent in milk, cheese, and butter
		Hydrolysis of milk, fat, cheese ripening, and modification of butter fats
	Meat industry	Degumming during the refining of vegetable oil
		Flavor development, meat and fish product fat removal
	Baking industry	Flavor development, shelf-life prolongation
Naringinases	Fruit industry	Debittering of citrus fruit juices
	Wine industry	Enhances the aroma in the wine
		Production of pruning, a flavonoid
Pectinases	Fruit industry	Clarification of the fruit juices
		Enhanced levels of fruit juice volume when fruit pulps treated with pectinase
		Soften the peel of citrus fruits
		Enhances the citrus oil extraction such as lemon oil
	Beverages industry	Accelerates tea fermentation
		Reduces foam forming property in instant tea powders
		Remove mucilaginous coat from coffee beans
	Wine industry	Imparts stability of red wine

Enzymes	Food sectors	Applications
Phytases	Baking industry	Reduction of phytate content in dough & fresh breads
		Shortening of formulation time without any change in pH
		Increase in bread volume and an improvement in crumb texture
		Softer bread crumbs were obtained
		Other texture parameters like gumminess and chewiness were also decreased
Proteases	Dairy industry	Prevent coagulation of casein during cheese production
		Flavor development
	Meat industry	Meat tenderization
	Baking industry	Assures dough uniformity
		Improve dough consistency
		Gluten development
		Improve texture and flavor
		Reduce mixing time
Tannase	Brewing industry	Removal of polyphenolic compounds
	Beverages industry	Manufacture of instant tea

Table 1.
The main applications of enzymes in different food sectors [6–18].

2. History and background

The use of various *Aspergillus* species dates back to almost a century ago, when it was discovered that the fungus *Aspergillus niger* was able to produce citric acid, which is a food and beverage additive and is normally extracted from citrus fruits [25]. Nowadays, *A. niger* is preferable to other microbes for the commercial production of citric acid because of its improved manufacturing yield. *Aspergillus* species are easily manipulated and can ferment different low-priced raw materials and offer high yields [26]. Thus, *Aspergillus* strains can be improved to generate industrial strains for application in the commercial manufacture, and mutagenesis and strain choice have been performed for such improvement. Through the application of different mutagenic agents, including various radiation for example ultraviolet, gamma and X-rays radiation, various chemicals for example, ethyl methane sulphonate and diethyl sulphonate have been commonly used to induce the mutation of *Aspergillus* species [26]. However, the industrial applications of *Aspergillus* species are not limited to the manufacture of citric acid. They have the capability to produce other organic acids such as gluconic, malic and itaconic acids, secondary metabolites and industrially important enzymes [26]. For example, *A. niger* has been used to manufacture different extracellular enzymes such as glucose oxidase, pectinase, α-amylase and glucoamylase, organic acids, and recombinant proteins [26]. *A. oryzae is a* fungus that widely plays an indispensable role in Asian food industrialized, such as soy sauce (sake and shoyu) and soybean paste (miso). Moreover, it has been applicable in the manufacture of industrial enzymes used in food processing [27]. *A. terreus* has attracted interest due to its ability to produce a group of secondary metabolites called statins that are used in the manufacture of cholesterol-lowering drugs [28]. *A. nidulans* is a capable fungal cell factory that can

produce different industrial enzymes such as amylase, cellulases, cutinases, glucosidases, etc. [29]. There are many companies (such as Genencor, Novozymes, Pfizer, Amano Enzyme, Verenium, etc.) that used enzymes, proteins and secondary metabolites produced by different species of *Aspergillus* on a large scale to produce commercial products [30].

3. Enzymes production process

A general overview of the enzyme production process has been shown in **Figure 1**. Fermentation has two parts, upstream processes (UsP) and downstream processes (DsP). The UsP for the enzyme manufacture include the selection of *Aspergillus* strain, inoculum and sterilization of media [31]. For primary culture, the frozen culture of the *Aspergillus* strain is inoculated into the medium. The culture media are sterilized before inoculations for primary and secondary culture. Once the strain is inoculated, primary cultivation is acceptable for primary fermentation [31, 32]. The primary culture is used as inoculum during the secondary fermentation process [32]. The DsP of the fermented product depends on the nature of the secretion of an enzyme (intracellular/extracellular) [31]. If the enzymes are intracellular, the cells are first collected with the help of centrifugation and other filtration methods [32]. Disruption of cells by diverse techniques such as sonication, french press, enzymatic lysis and freezing/thawing process is necessary to get the intracellular content [31, 32]. After the cell breaking, the cell residue is excluded by filtration. In the case of extracellular proteins, the enzymes are secreted in the culture medium, so they are easier to purify. The enzymes produce using both intracellular and extracellular fermentation are subsequently concentrated using concentrators. The fermentation is generally followed by cell lysis or cell extraction then elimination of cells-residue from the culture, either using centrifugation, filtration or sedimentation. The cell-free culture is then concentrated using ultra-filtration, ammonium sulphate precipitation or liquid extraction with organic solvents [33]. About 80% of the purification processes employed thus far have used a precipitation step, with 65% of these using ammonium sulphate and 35% using ethanol, acetone or an acid (usually hydrochloric) followed by a combination of ultra-filtration system and various chromatographic techniques (20%) for example gel filtration and affinity chromatography [33]. The purification of an enzyme leads to the procedure of formulation [34]. The formulation may include methods such as drying, vacuum drying, spray drying, and freezedrying [34]. These processes make

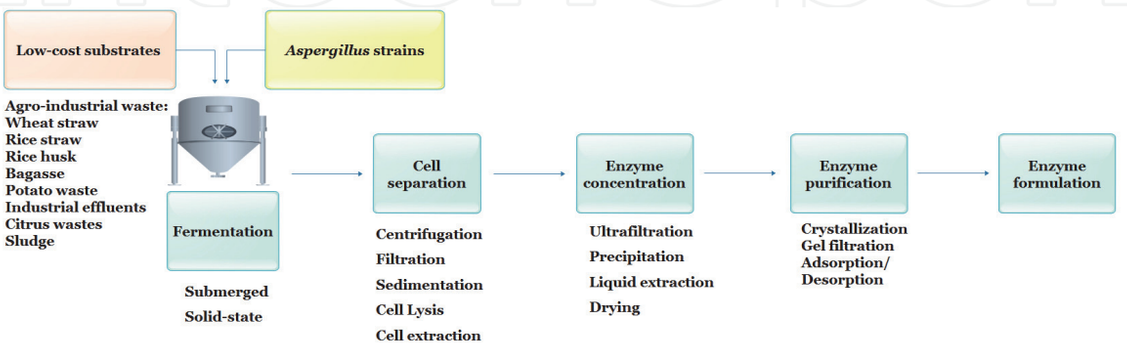

Figure 1.
Process steps for conversion of low-cost substrate to various enzymes by Aspergillus strains. The fermentation (submerged or solid-state) is generally followed by cell-lysis/extraction, then elimination of cells-residue from the culture, either using centrifugation, filtration, sedimentation. The cell-free culture is then concentrated using ultra-filtration, ammonium sulphate precipitation or liquid extraction with organic solvents. Then followed by a combination of various chromatographic techniques for example gel filtration and affinity chromatography.

a decision how an enzymatic product will be launched in the market. It can be a mixture of two enzymes or more of them. The formulation is exclusively based upon the research and development of an industry or a lab. Formulation development is a fundamental factor not only in the early stages of enzymes/protein development, but also in the future marketing success of a promising investigational product. This is the operation of discovering novel sciences which can be used to generate novel and valuable products. The development part comes after the study and analysis and is the act of turning the discovered science into a precious product that the company can commercialize and sell. All the UsP and DsP lead to the formation of the final product.

3.1 Fermentation

Both submerged (SmF) and solid-state (SSF) fermentation are used for making different enzymes by fungi [35–99]. Due to easy measurement and of control fermentation parameters, reduction fermentation time and basic ways for harvesting and refining enzymatic products, more attention has been paid to SmF [62–65]. In recent time, intensive investigate on SSF has been conducted and has gained reliability due to low water consumption, low energy necessities, less contamination and high manufacture yields [35, 52]. However, *Aspergillus* species have shown potential for producing enzymes under both SSF and SmF (**Table 2**).

3.2 Use of low-cost/economical substrates for enzyme cost-effective/ commercial production

A large amount of agro-industrial waste like wheat straw, rice straw, rice husk, bagasse, potato waste, industrial effluents, citrus wastes, sludge, etc., are produced annually. They are rich sources of sugars, mineral elements, vitamins, fiber and different phenolic compounds, etc. Consequently, they can be used for the manufacture of commercially important products like enzyme, due to their nutritional potential (**Table 2**). Enzymes like amylases, celluloses, chitosanases, galactosidases, invertases, laccase, lipases, naringinases, pectinases, phytases, proteases, tannase, etc., have industrial significance and are broadly used in different industries such as pulp and paper, textile, wine and brewery, food processing, laundry and detergent, agricultural industries and bio-ethanol production [6–18]. The cost of these enzymes is a big subject faced by these industries, and efforts are going on to decrease the cost through strain improvement, better fermentation and recovery system and utilization of easily available low-cost substrates [60]. These agro-industrial wastes have exposed potential for the production of various kinds of enzymes using *Aspergillus* species [46, 60, 100].

3.3 Recovery purification and formulation

Enzymes are recovered from fermentation through chemical engineering operations that are broadly used to produce enzymes [101]. When the enzyme is intracellular, the cells must be broken down to release the enzyme. This can be done using mechanical methods (such as high-pressure press, grinding, or ultrasound) or non-mechanical methods (such as drying or lysis). In the case of extracellular enzymes, an early stage of isolation (centrifugation, filtration or both) is often used to eliminate residue of the cells [101]. Then the dissolved enzyme is concentrated by eliminating the water (cross-flow filtration or evaporation), resulting in an enzyme concentrate. Alternatively, a whole enzyme preparation containing inactivated cells or cell debris may be suitable where the resulting food undergoes further

Enzymes	*Aspergillus* species	Substrate	Type of fermentation	Fermentation conditions				Yield of enzymes	Reference
				pH	T (°C)	D	M (%)		
Amylase	*A. niger* JGI 24	Wheat bran	SSF	4.5–9.0	22–40	4	43–81	74 U/mgds	[34]
			SmF				—	58.06 U/ml	
	A. terreus NCFT4269.10	Pearl millet	SSF	7.0	30	4	70	19.19 U/gds	[35]
	A. fumigatus	Maltose	SmF	6.0	30	8–10	—	60–130 U/mgds	[36]
	A. tamarii	Starch or maltose	SmF	4.0–10.0	25–42	6	—	ND	[37]
	A. oryzae LS1	Wheat bran	SSF	6.0	28–30	7	50	14,249 U/gds	[38]
	A. oryzae	Groundnut oil cake, coconut oil cake, sesame oil cake	SSF	4.5	32.5	4–5	64	9868.12U/gds	[39]
	A. flavus AUMC 11685	Mandarin (*Citrus reticulata*) peel	SmF	4.0–5.5	28–40	4–5	—	26.90 U/ml	[40]
	A. awamori ATCC 22342	Rice flour	SmF	6.5	30	2	—	0.18 U/ml	[41]
	A. niger							0.08 U/ml	
	A. awamori nakazawa MTCC 6652	Wheat bran	SSF	5.5	35	4	85	4528.4 ± 121 U/gds	[42]
	A. carneus SA 1326	Ground millet, starch or carboxymethylcellulose	SmF	5.6	28	4	—	ND	[43]
	A. sydowii IMI 502692	Cassava root fiber	SSF	3.62	ND	2	ND	1.327 U/ml	[44]
Cellulases	*A. niger* NS-2	Agricultural and kitchen waste residues	SSF	3.0–8.0	20–50	4	57–67	17–310 U/gds	[45]
	A. heteromorphus	Wheat straw	SmF	5.0	30	5	—	3.2 U/ml	[46]
	A. fumigatus	Rice straw and wheat Bran	SSF	5.0–6.0	40	4	75	0.68–42.7 U/gds	[47]
	A. terreus	Lantana leaves	SmF	5.0	25	7	—	213.3 U/ ml	[48]
	A. niger	Wheat bran, rice bran, rice husk, coir waste and saw dust	SSF	6.0	30	4	50	29.11 U/gds	[49]
			SmF					2.04 U/ml	
	A. niger USM AI 1	Corn steep liquor	SSF	7.0	30	5	70	3. 4 U/gds	[50]

Enzymes	*Aspergillus* species	Substrate	Type of fermentation	Fermentation conditions				Yield of enzymes	Reference
				pH	T (°C)	D	M (%)		
Chitosanases	*A. niger*	Wheat bran	SSF	6.6	28	5	65	41.33 U/gds	[51]
	Aspergillus sp. QD-2	Yeast glucose	SmF	4.0	30	1	—	85.816 U/ml	[52]
	A. fumigates ATCC13073	Vogel's medium	SmF	6.0	37	1	—	8.80 U/mg	[53]
	A. oryzae SU-B2	Yeast-peptone glucose	SmF	5.0	30	4	—	352 mg/l	[54]
Galactosidases	*A. niger* ATCC 9142	Rice straw and wheat straw	SSF	7.0	30	6	70	4681 U/mg	[55]
	A. oryzae	Red gram and waste-wheat bran	SSF	5.5	35	6	50	ND	[56]
	A. oryzae ATCC 20423	Lactose and wheat bran	SmF	4.8	30	7	—	ND	[57]
	A. oryzae	Wheat bran and rice husk	SSF	5.0	30	7	90	146.6–386.6 U/ml	[58]
Invertases	*A. caespitosus*	Wheat bran	SSF	4–6	30–40	3	70	117.4 U/gds	[59]
			SmF				—	19.1 U/ml	
	A. niger MTCC 282	Orange fruit peel	SSF	5.0	30	4	80	43 U/ml	[60]
	A. nidulans	Rye flour	SmF	6.0	30	3	—	30–33.6 U/ml	[61]
Laccase	*A. flavus*	Starch and yeast extract	SmF	7.0	35	14	—	17.39 U/ml	[62]
	A. sydowii NYKA 510	Banana peel and peptone excelled	SmF	5.2	31	7	—	15.1 and 2.60 g/l	[63]
	A. nidulans	Glucose and straw	SmF	5–7	28	2	—	0.052 and 0.0677 U/ml	[64]
Lipases	*A. niger* NCIM 1207	Wheat bran + synthetic oil based	SSF	5.5	30	6	40	630 U/gds	[65]
	A. niger AS-02	Sheanut cake	SSF	7.0	30	7	60	49.37 U/gds	[66]
	A. oryzae RBM4	Sorghum, wheat bran	SmF	5.5	30	3	—	5.66 U/ml	[67]
	A. flavus PW2961	Bran-wood flour-olive oil, bran-soy bean	SSF	5.0	28	3–4	50	37.4 U/gds	[68]
	A. niger MTCC 872	Rice husk, cottonseed cake and red gram husk	SSF	6.0	40	1	75	28.19 U/gds	[69]

Enzymes	*Aspergillus* species	Substrate	Type of fermentation	Fermentation conditions				Yield of enzymes	Reference
				pH	T (°C)	D	M (%)		
Naringinases	*A. niger* van Tieghem MTCC 2425	Citrus wastes	SmF	3–5	26–30	6–8	—	426.4–545.2 U/gds	[70]
	A. foetidus	Orange and grapefruit rind	SSF	5.4	35	8	ND	2.58 U/ml	[71]
	A. aculeatus JMUdb058	Yeast extract, naringin	SmF	6.0	28	7	—	1.16 U/ml	[72]
	A. oryzae 11,250	Orange peel	SmF	5.0	45	4	—	2194.62 U/mgds	[73]
	A. niger MTCC 1344	Rice bran, wheat bran, sugar cane bagasse, citrus peel, and press mud	SSF	4.0	27	4	50	58.1 U/gds	[74]
	A. brasiliensis 1344	Cassava waste	SSF	5.0	27	5	ND	889.91 U/mg	[75]
	A. tubingensis MN589840	Mildew pomelo peel	SSF	4.0	30	5	ND	808.85 U/mg	[76]
	A. sojae	Soybeans	SmF	4.5	28	6	—	1.5 U/mgds	[77]
Pectinases	*A. niger*	Wheat bran	SSF	4.0	30	3	63	68 U/gds	[78]
	A. carneus NRC1	Orange peels and pulps	SmF	5.0–5.5	30–55	5	—	40 U/ml	[79]
	A. flavipes FP-500	Lemon peel	SmF	4.2	37	6	—	ND	[80]
	A. tamarii	Wheat bran, banana peel, sugarcane bagasse, lemon peel, coffee pulp and orange peel	SSF	6.0	30	4	70	101.05 U/ml	[81]
	A. flavus CECT-2687	Agroindustrial residues and polysaccharides	SmF	3.5–9.0	37	5	—	1.35–7.89 U/ml	[82]
	A. japonicus	Polygalacturonic acid, citrus pectins	SmF	4.0–5.5	30	ND	—	805 and 839 U/mg	[83]
Phytases	*A. niger* CFR 335	Wheat bran, rice bran, and groundnut cake	SSF	2.0–7.5	30	8	10–80	60.6 U/gds	[84]
			SmF			10	—	9.6 U/mL	
	A. ficuum SGA 01		SSF			8	10–80	38 U/gds	
			SmF			10	—	8.2 U/mL	

Enzymes	*Aspergillus* species	Substrate	Type of fermentation	Fermentation conditions				Yield of enzymes	Reference
				pH	T (°C)	D	M (%)		
	A. niger NCIM 563	Chickpea flour	SmF	7.0	35	4	—	164 U/mL	[85]
	A. ficuum	Potato waste	SSF	6.1	27	6	79	12.93 U/gds	[86]
	A. terreus	Rice bran	SmF	4.5	30	4	—	ND	[87]
Proteases	*A. niger*	Wheat bran	SSF	8.0	40	8	3.3	30.21 U/mg	[88]
	A. oryzae LBA 01	Wheat bran	SSF	5–5.5	23	3	50	3961.30 U/gds	[89]
	A. clavatus	Vogel medium with glucose	SmF	9.5	37	10	—	38 U/ml	[90]
	A. flavus IMI 327634	Wheat bran	SSF	7.5–9.5	32	2	63	6.8 U/ml	[91]
Tannase	*A. tamarii*	Tannic acid, gallic acid and methyl gallate	SmF	5.0	30	2	—	20.6 U/ml	[92]
	A. ruber	Jamun (*Syzygium cumini*) leaves	SSF	5.5	30	4	50	69 U/gds	[93]
	A. niger MTCC 5898	Cashew testa	SSF	3.0–8.0	32–35	3–5	60	97.32–301.7 U/gds	[94]
	A. heteromorphus MTCC 8818	Rosewood (*Dalbergia sissoo*) sawdust—a timber industry waste	SSF	5.5	30	4	70	1.84 U/gds	[95]
	A. ochraceus	Khanna medium	SmF	5.0	40	3	—	0.92 U/mgds	[96]
	A. melleus	Achachairu seed powder	SSF	5.5	40	2	60	452.55 U/ml	[97]
	A. aculeatus DBF9	Wheat bran, rice bran, saw dust, rice straw dust, sugarcane pith	SSF	5.0	30	3	80	1.32–3.95 U/gds	[98]

ND = not determined.
SSF: solid-state fermentation; Smf: submerged fermentation; T: incubation temperature; D: day incubation time of fermentation; M: moisture content in case of SSF.
U/ml = μmoles of fatty acids released per minute per ml of crude enzyme; U/gds = μmoles of fatty acids released per minute per gram of dry solid substrate from which enzyme has been extracted; U/mgds = μmoles of fatty acids released per minute per milligram of dry solid substrate from which enzyme has been extracted; mg/l = milligram per liter is a unit of measurement of mass concentration that shows how many milligram of a certain substance are present in one liter of a mixture; g/l = gram per liter is a unit of measurement of mass concentration that shows how many gram of a certain substance are present in one liter of a mixture.

Table 2.
Production of industrial enzymes from various Aspergillus *species.*

refinement, for example in potable alcohol production. In all cases, the prepared enzyme is free of viable fungi [101]. The concentrate is then formulated using the correct ingredients to stabilize and standardize the enzyme [101]. The raw materials used for recovery and formulation require to be of suitable purity for the future use and require to be used according to good manufacturing Practices, i.e., in the minimal quantities required to achieve the desired effect [101]. The utilize of potential allergens in the process of producing food enzymes must be addressed and, if necessary, included in the enzyme preparation. At the end of the manufacturing procedure, the last formulated enzyme generate is introduced in the market after testing to verify agreement with qualifications for contaminants (microbial and chemical) established for enzyme preparations by the Food Chemicals Codex and FAO/WHO's JECFA. In other words, enzymes come in three diverse forms. Firstly, there is the enzymatic protein itself, which is a pure substance that is used in labs [102]. Secondly, enzymatic concentrates are products produced following fermentation or extraction [102]. They contain the enzyme produced in much smaller amounts from other substances obtained during the fermentation process, like other (secondary) enzymes or the remainder of the fermentation [102]. These enzymatic concentrates are evaluated for safety prior to being approved for marketing. Finally, there are enzymatic preparations, which are formulations containing one or more enzymatic concentrates with added stabilizers, preservatives, and diluents to stabilize enzymes and maintain activity. These formulas are sold commercially. In general, a proven quality feature of enzymes produced by microorganisms is the lack of viable cells. In addition, other specific features of microbial enzymes include their ability and significant activity under abnormal conditions, mostly temperature and pH. For example, some microbial enzymes are produced in thermophilous, acidophilic or alkalophilic forms.

4. Recombinant DNA (rDNA) technology

In industries, the rDNA technique will contribute to the manufacture of chemicals of commercial importance, to the advancement of existing fermentation processes and protein/enzyme production from waste materials. For this purpose, more effective strains of microorganisms can be developed. Thus, the technology of rDNA has many useful applications in crop betterment, medication and industry.

The rDNA in microorganisms occurs through three different parasexual processes namely conjugation, transduction, and transformation [103]. Internal genetic rearrangements can also occur via translocatable DNA segments (insertion sequences or transposons) [103]. Conjugation implies DNA transfer through cell-to-cell contact. Transduction occurs from the host cell to the recipient cell through bacteriophaging mediation. Transformation involves the absorption and expression of bare DNA by the appropriate cells. Competence occurs naturally but can also be induced by changes in the physical and chemical environment. In the laboratory, it can be induced by cold calcium chloride treatment, protoplasting, electroporation and heat shock [103]. After 1980, there was a heightened interest in the application of genetic recombination to the production of important microbial products such as antibiotics. The use of rDNA technology has made it possible to produce new enzymes appropriate for specific food-processing conditions [104]. Various substantial enzymes (lipases, pectinases, cellulases, amylases, etc.) are useable for the specific manufactures because of their exclusive roles and utilization in food and feed industries. Manufacture of microbial strains is another vast accomplishment that became feasible with the assist of rDNA technology. Various microbial strains have been expanded to manufacture superior enzymes by particular engineering.

Specific strains of fungi have been modified in order that their capability of manufacturing toxic and hazardous materials could be decreased. Wide ranges of recombinant proteins/enzymes have been expressed in different species of fungi to be used as enzymes in industries [105]. *Aspergillus* species are regarded as promising candidates for developing large-scale heterologous protein production platforms. However, production yields of heterologous proteins are usually significantly lower than those detected for native proteins. Failure to achieve the favorable protein amounts in *Aspergillus* cultures can be ascribed to limitations associated to transcription, translation, and the post-translation processing and modifications during protein manufacture. Furthermore, bottlenecks in the fungal secretion system and the problem of extracellular breakdown further impede the effective production of foreign proteins in *Aspergillus* species [106].

Several *Aspergillus* species, in particular *A. niger* and *A. oryzae*, are widely used as protein-production hosts in a variety of biotechnology applications. To improve the expression and secretion of recombinant proteins from these filamentous fungi, several novel genetic engineering strategies have been developed over the past few years. Yu et al. reported construction and application of a novel genetically engineered *A. oryzae* for expressing proteases [107]. Their results showed different degrees of improvement in the protease activity when compared with wild-type *A. oryzae*. A major improvement in the polypeptide yield was achieved when these strains were used in soybean meal fermentation. The polypeptide conversion rate of transformants *A. oryzae* reached 35.9%, which was approximately twofold higher than that exhibited by wild-type *A. oryzae* [107]. Amino acid content analysis showed that the essential amino acid content and amino acid composition of the fermentation product significantly improved when engineered *A. oryzae* strains were used for soybean meal fermentation [107]. Prathumpai et al. reported lipase production by recombinant strains of *A. niger* expressing a lipase-encoding gene from *Thermomyces lanuginosus* [108]. Their heterologous lipase was expressed using the TaKa amylase (the *A. oryzae* amylase is known as Taka-amylase) promoter from *A. oryzae*. Their results showed the transformants strain was found to be the best producer. Record et al. reported expression of the *Pycnoporus cinnabarinus* laccase gene in *A. niger*. *A. niger* preprosequence allowed an 80-fold increase in laccase production [109]. Bohlin et al. reported heterologous expression of *Trametes versicolor* laccase in *A. niger*. Their results showed recombinant laccase from *A. niger* harboring the lcc2 cDNA was purified to homogeneity and it was found to be a 70 kDa homogeneous enzyme with biochemical and catalytic properties similar to those of native *T. versicolor* laccase [110]. Dragosits et al. reported recombination for the production and purification of two previously uncharacterized β-galactosidases from *A. nidulans* as well as one β-galactosidase from *A. niger*. Their results showed that *A. niger* and *A. nidulans* are suitable for various glycobiological and biotechnological applications [111].

5. Enzymes production by *Aspergillus* species and their application

5.1 Amylases

The *Aspergillus* species produce a wide range of intra- and extracellular enzymes, and amylases are one of the main enzymes used in industrial markets (**Table 2**). Amylases are broadly employed in food manufacturing such as brewing, baking, preparation of digestive aids, manufacture of cakes, fruit juices and starch syrups [112]. They have been extensively used in the baking industry. Amylases can be added to the dough of bread which degrade the starch in the flour into smaller

dextrins, and these smaller dextrins are subsequently fermented by the yeast. The addition of these enzymes to the dough results in improving the rate of fermentation and the reduction of the viscosity of dough, resulting in progresses in the volume and texture of the product. Moreover, they produce additional sugar in the dough that improves the flavor, crust color and toasting qualities of the bread [112]. In addition to producing fermentable compounds, they also have an antistaling effect in baking bread which advances the retention of the softness of bakery products, increasing the shelf life of these products. These enzymes are also used for the clarification of fruit and veggie juices or beer or for the pretreatment of animal feed to improve the digestibility of fiber [6, 34–44].

5.2 Cellulases

Cellulases are groups of enzymes that are secreted using a wide range of *Aspergillus* species (**Table 2**). According to current enzyme market information, the main areas of the industry where cellulases enzymes are progressively being applied are textile, pulp and paper, laundry detergents, healthcare, food and beverages. Their extensive application in coffee and tea processing, fruit juice production and wine making is associated to food and beverage segment. In other industrial applications, it is widely used to generate laundry detergents and cleaning and washing agents. These enzymes are also being highly known as an effective alternative to available various antibiotics and antifungal. Lemnaru Popa et al. reported bacterial cellulose in skin wound treatment is very attractive due to its unique characteristics. Their results confirmed the drugs' presence in the bacterial-cellulose dressing's structure as well as the antimicrobial efficiency against *Staphylococcus aureus* and *Escherichia coli* [113]. In another study, Limón et al. reported antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain was increased [114]. Consequently, the potential of these enzymes is a wonderful trend to fight against antibiotic-resistant bacteria which may overcome problems in the healthcare sector [7]. Furthermore, in the food market these enzymes have many applications. Fruit and veggie juice clarification, carotenoid extraction, reducing the viscosity of nectars, alteration of fruit sensory properties, concentrating purees, olive oil extraction, and the quality betterment of bakery products are among the different processes in food industry and biotechnology where cellulases are exploited worldwide. Consequently, these enzymes can cause a huge economic impact. However, there are some significant bottlenecks of employing this enzyme in the industry such as loss of enzyme activity, immobilization of enzyme in undesired conformation and subsequent loss of activity, cost of carrier and additional preparation materials and methods, as well as laborious training strategies, mass transfer limitations, laborious and time-consuming immobilization processes [7, 46–50].

5.3 Chitosanases

Chitosanases can degrade chitin and it can generate using various *Aspergillus* species (**Table 2**). These enzymes play a significant role in nutrition and defense. Due to its antimicrobial activity against a wide range of filamentous fungi, yeasts and bacteria, they have attracted much attention as a potential food preservative [8]. These enzymes potentially used in preparation of N-acetyl d-glucosamine and chitooligosaccharides. Chitooligosaccharides and N-acetyl glucosamine are currently of enormous relevance to pharmaceutical, nutraceutical, cosmetics, food, and agriculture industries due to their wide range of biological activities, which include antimicrobial, antitumor, antioxidant, anticoagulant, wound healing,

immunoregulatory, and hypocholesterolemic effects. Furthermore, the control of pathogenic fungi in agriculture could be done using chitonases. The degradation of crustacean chitinous waste in seafood industry could be enhanced using them. It is also valuable for the preparation of single-cell protein and also for the isolation of protoplasts from fungi and yeast, etc. [8, 51–55].

5.4 Galactosidases

Galactosidases can be generated by various *Aspergillus* species and being used in food industry for hydrolysis of lactose in milk and milk by-products (**Table 2**). They have attracted much attention in view of lactose intolerance in human population and due to importance of milk in human diet. These enzymes can hydrolyze galactopyranosides, that is, lactose, and form a wide range of trans-galactosylation products or galactooligosaccharides capable of providing some health benefits as prebiotics. Furthermore, these enzymes also find applications in production of lactose based sweeteners from high lactose containing effluents of cheese manufacturing industries. At present, these enzymes are mainly obtained from various *A. niger* strains [9, 56–59].

5.5 Invertase

Invertases are generated using plants, bees, and microorganisms [10], but the filamentous fungi belonging to the *Aspergillus* species are the most prominent organisms used for invertases manufacture (**Table 2**). They are used to hydrolyze sucrose and polysaccharides, which have the same type of β-d-fructofuranosyl bond, to obtain fructose and glucose as final products [10]. They are significant in the food biotechnology, especially in confectionery and candy manufacturers, as a catalytic factor in obtaining an artificial sweetener. Hence, they are used for the construction of formulations that prevent crystallization of certain sweet preparations, using in the chocolate industry markets. In some syrup, it is also used to growth its sweetening properties such as producing of soft caramel fillings. Honey is the most common form of this inverted sugar that is a supersaturated mixture of fructose and glucose. Furthermore, they are able to synthesize fructooligosaccharides through fructotransferase where sucrose is presented in high concentrations. The fructooligosaccharides are related to improve human health [9, 60–62].

5.6 Laccases

Fungi such as *Aspergillus* species have been used to generate Laccases (**Table 2**). Laccases are a broadly studied enzyme because of its potential use in some industries areas such as textile, paper and pulp [16]. They can be used in bioremediation, beverage processing (such as wine, fruit juice and beer), sugar beet pectin gelation, ascorbic acid determination, baking, and as biosensor and to progress food sensory parameters [17]. They can increase the productivity, efficiency and quality of food goods without costly investment, and this is an advantage. Wide areas of the food industry that benefit from processing with these enzymes include juice processing, wine stabilization, baking industry, and bioremediation of waste water [16, 17, 63–65].

5.7 Lipases

Lipases are one of the most important biocatalysts that perform different reactions in aqueous and non-aqueous media [11]. These enzymes usually catalyze the hydrolysis of long-chain triglycerides. They can operate on a diversity of substrates

counting natural oils, artificial triglycerides, and esters of fatty acids. They are manufactured using animals, plants, and microorganisms. Presently, fungal lipases are achieving much consciousness with the rapid development of enzyme technology. Fungi-produced lipases have played an interesting role in industrial biotechnology because many of them are stable in a wide range of pH, high temperatures, and organic solvents. They are signifying one of the most important groups of biocatalysts for industrial applications. *Aspergillus* species is an important chief manufacturer of lipases (**Table 2**). They are capable to modify the characteristics of lipids by altering the location of fatty acid chains in the glyceride and exchange one or more fatty acid with new ones. Cocoa butter is a crucial ingredient in chocolate that has a high butterfat value because it contains palmitic and stearic acids and has a melting point of almost 37°C. Melting of cocoa butter in the mouth creates a positive favorable cooling sensation in a product such as chocolate [11]. Lipases are used ex-situ to create taste and to change the formation using inter- or transesterification that obtain products of improved nutritional value, or appropriate for feeding [11]. They are also been used in food to modify taste using manufacture of esters of short-chain fatty acids and alcohol, which are known flavor and fragrance compounds. Lipases facilitate the elimination of fat from meat and fish products. They are used for the manufacture of maltose and lactose like sugar fatty acid esters. They have many applications in food and flavor industry and in the production of ice cream [11, 66–70].

5.8 Naringinases

Various microbial sources of naringinases have been reported worldwide by various investigators [12]. Production of naringinases has been very well studied in fungal sources. Among fungi, *Aspergillus* species (especially *A. niger*) have been reported as major producers of naringinases (**Table 2**). Naringin is a flavonoid naturally which present in citrus fruits (such as oranges, lemon, and grapefruit). Flavonoids may cause interference during the citrus fruit juice processing and are responsible for the bitter taste. The processing of citrus fruit juice has faced formidable problems in terms of bitterness and delayed onset of bitterness. The bitterness affects its consumer acceptability. In citrus juices two compounds namely flavonoids and limonoids are established responsible for bitterness. The bitterness in grapefruit juice can be reduced by using enzymes such as naringinases which hydrolyzes naringin into relatively non-bitter compounds. They are an enzyme which catalyzes the hydrolysis of naringin into pruning, rhamnose, glucose and then into naringenin, which is non-bitter and tasteless. This enzyme has two different enzyme activities (due to two different subunits). One is α-rhamonosidase which acts on naringin to release prunin and α-rhamnose. Second is β-D-glucosidase which acts on prunin to release naringenin and β-D-glucose. There are only few reports on the commercial manufacture of this enzyme. These enzymes have been used for elimination of bitter flavor in citrus fruit juice (due to naringin) [12, 71–78].

5.9 Pectinases

Pectinases have the most important role in fruit and veggie juice marketing by breaking the pectin (polysaccharide) structure present in the cell wall of plants. They are mainly manufactured using microorganisms and plants. Among microorganisms, fungi (especially *Aspergillus* species) have a high ability to secrete them (**Table 2**). Pectinases are a class of enzymes that catalyzes the degradation of pectic substances [13]. They have broad applications in food and agricultural industries. Pectinases can be used and commercially applied for the processing of fruit juices

and wines. In food industry, these enzymes are used especially for clarification, maceration, extraction and stabilization of fruit juices. They also enhance fruit juice yield and involved in fermentation of coffee, cocoa and tea and preparation of jams and jellies. They are used in oil extraction from plant but olive oil extraction is the most common. These enzymes are added for easy oil extraction during grinding of olives. They have capability to reduce feed viscosities which directly increase the nutrients absorption capability of animals. These nutrients are released from fibers using hydrolysis process and it also reduces animal defecation [13, 79–84].

5.10 Phytases

Phytases have a role in food and feed industry. They are synthesized using fungi, mainly from *Aspergillus* species (**Table 2**). They can reduce the antinutritional effect of phytate and improve the digestibility of phosphorous, calcium, amino acids and energy, as well as minimize the negative impact of inorganic phosphorous excretion on the environment [14]. The benefits of using phytase in animal feed are well recognized [14]. Especially, phytate-degrading enzymes from *Aspergillus* species offer industrial and economical feasibility for their manufacture and application. Phytates have been considered as a threat in human diet due to its antinutrient behavior, which is known as strong chelator of divalent minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}. There is a high potential for the employ of phytases in processing and developed of food for human consumption. Investigation in this aspect focuses on the progress of the nutritional value of plant-based food and feed as well as on the technical improvement of food processing. A diet rich in phytate leads to a significantly decreased absorption of dietary minerals and the dephosphorylation of phytate during food processing results in the formation of only partially phosphrylated myo-inositol phosphate esters with a lower capability to impair with the intestinal uptake of dietary minerals. Individual myo-inositol phosphate esters have been shown to have some important physiological functions in man. Consequently, phytases may find application in food processing to generate functional foods. Also, phytases were showing to be an excellent bread making improver [14, 85–88].

5.11 Proteases

Proteases are produce in all organisms, such as plants, animals, and microbes [15]. The peptide bond present in the polypeptide chain is hydrolyzed by proteases. They are degradative enzymes and demonstrate specificity and selectivity in protein modification. They are one of the most important industrial enzymes and their international market is significantly growing annually. Of the 60% of enzymes marketed worldwide, proteases account for 20%. Proteases have been successfully produced by researchers from various microbial sources [15]. Reports suggest that two-thirds of the world's commercial proteases are produced by microorganisms because of their greater yield, reduction in time consumption, reduction in space requirement, lofty genetic manipulation, and cost-effectiveness, which have made them suitable for biotechnological application in the market. Among microbes, *Aspergillus* species have been extensively studied for protease manufacture in a large scale (**Table 2**). Proteases are used on a large commercial scale in the production of baked goods, bread, crackers and waffles. Proteases produced by *Asperjillus oryzae* have been used to modify wheat gluten using limited proteolysis. They are also used in the dairy industry. Their main application in the dairy industry is in cheese manufacture. In cheese manufacture, the primary function of proteases is to

hydrolyze the certain peptide bond to generate para-k-casein and macro peptides [15, 89–92].

5.12 Tannase

Tannases are a group of enzymes that are employed in multitudinous industries such as food, brewing, and pharmaceutical [18]. They have an expansive range of scattering and are generated form animals, plants, and microbial sources. However, manufactured tannins of microbial origin are favored over other sources for industrial utilization. Fungi such as *Aspergillus* species have been used to generate tannases (**Table 2**). They operate upon hydrolyzable tannins by cracking the ester and depside bonds so as to release glucose and gallic acid. One of the most significant commercial applications of tannases is gallic acid manufacture. Besides that, they are widely employed in the food industry, especially in the manufacture of instant tea, where it increases the extractability and cold water solubility of key compounds [18]. Another significant applying of tannase is the elimination of haze formation and unflavored phenolic compounds from beer and wine [18]. Moreover, the quality of fruit juices can be enhanced using tannases enzymes. The turbidity and bitterness of fruit and veggie juices are minimized by using these enzymes. While using agro-industrial residues as animal feed, tannins-rich biomass are considered as anti-nutritional factors. De-tannification of feed using tannases enzymes treatment can extensively progress the quality of animal feed [18, 93–99].

6. Future perspectives and conclusions

At present, enzymes have become an important part of various industries [1]. The total enzymes market size in the worldwide is anticipated to reach over $13–14 billion by 2027. However, the manufacture of different enzymes has always been a challenge. They are produced from plants, animals and microorganisms [4]. Microbial enzyme production is generally accepted and occupies approximately 85–90% of the global enzyme market. In microbial enzyme manufacture, the localization of enzyme is a major aspect to be considered. If an enzyme is extracellular, the cost of downstream processing is reduced [100]. However, when it comes to intracellular enzymes, it becomes an expensive process to purify such enzymes. The degree of purification also varies according to the use of enzymes. Among microorganisms, fungi are especially used for the manufacture of various enzymes in a wide range [5]. Out of about 260 commercial enzymes, 60% are sourced from about 25 fungal genera [115]. Fungi can produce a number of industrial enzymes that are used in variety different industrial processes. Owing to their ability to use low-value substrates, their ability to handle and their ability to produce high enzymatic titres, fungi are the subject of extensive studies for industrial enzymes. Enzymes of fungal origin (especially from *Aspergillus* species) are employed in a wide range of industrial applications. The advantages of using *Aspergillus* species in the industry are multiple and they are a rich source of enzymes with valuable properties in industrial processes. A good example is their outstanding high stability, as they naturally evolved to work in a relatively harsh extracellular environment. They can use common “types of waste” as a source of carbon and energy and release the valuable enzymatic product from their cells into the medium. The cost of enzyme manufacture by *Aspergillus* species is another attractive parameter and an essential prerequisite for the exploitation of these catalysts in large-scale industrial settings. In addition, *Aspergillus* species will be able to produce strong, multifunctional (chimeric) enzymes using recombinant DNA technology, high-efficiency screening of the

latest, metagenomic screening, silicon enzyme engineering and site-to-site mutagenesis to meet future needs.

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Conflict of interest

Authors declare no conflict of interest.

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