Chapter 19
Amylolytic Enzymes

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Contents

Abstract Amylolytic enzymes act on starch and related oligo- and polysaccharides. The recent wealth of information on the DNA sequence, structural analysis and catalytic mechanism led to the extensive research on starch hydrolyzing enzymes which led the concept of the alpha amylase family. Amylolytic enzymes are extensively used in starch liquefaction, paper industries, food, pharmaceutical and sugar industries which demands a specific hydrolysis profile. To fulfill the industrial requirements, the primary concern is the formulation of a simple indigenous and cost effective system for producing high titers of amylases. One alternative low cost and feasible production method is the use of agro-industrial residues as fermentation substrates. These residues represent one of the best reservoirs of fixed carbon in nature. Considerable research has been carried out in the effective utilization of these residues in large scale production of enzymes. This chapter gives a brief overview on the wide range of naturally occurring agricultural by products explored so far for the production of amylolytic enzymes.

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19.1 Introduction

Starch represents one of the most abundant storage polysaccharides in nature and the most popular ingredient in food. It is composed exclusively of α-glucose units that are linked by α-1,4- or α-1,6-glycosidic bonds. The two high-molecular-weight components of starch are amylose (15–25%), a linear polymer consisting of α-1,4-linked glucopyranose residues with their molecular weights varying from hundreds to thousands, and amylopectin (75–85%), a branched polymer containing, in addition to α-1,4 glycosidic linkages, α-1,6-linked branch points occurring every 17–26 glucose units with molecular weight as high as 100 million (Bertoldo and Antranikian 2002). Because of the complex structure of starch, cells require an appropriate combination of enzymes for its depolymerization to oligosaccharides and smaller sugars, such as glucose and maltose. Amylolytic enzymes play an important role in the degradation of starch and are produced in bulk from microorganisms representing about 25–33% of the world enzyme market. Microbial enzymes are preferred for their stability over plant and animal enzymes which increases their spectrum of industrial applications. They also have the advantages of cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization. The amylolytic enzymes find a wide spectrum of applications in food industry for production of glucose syrups, crystalline glucose, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices, solubilization and saccharification of starch for alcohol fermentation in brewing industries, retardation of staling in baking industry, in detergent industry used as an additive to remove starch based dirts, in paper industry for the reduction of viscosity of starch for appropriate coating of paper, in textile industry for warp sizing of textile fibers and in pharmaceutical industry they are used as a digestive aid (Sivaramakrishnan et al. 2006).

The vast research on whole genome sequencing and the accumulated protein sequence databases since the last two decades led to the study of a full range of starch hydrolyzing enzymes. The homology among alpha amylases from different origins was first studied by (Friedberg 1983). The detailed study of the amylolytic enzymes proved the existence of four highly conserved regions in eleven different α-amylases which is related to the catalytic and substrate binding sites (Nakajima et al. 1986). Thus the structural similarity and common catalytic mechanism among most of the amylases led to the concept for one enzyme family, 'the alpha amylase family'. The family included enzymes acting on α-glucosidase linkage to produce α-anomeric mono and oligosaccharides or form α-glucosidic linkages by transglycosylations, they possess four conserved regions containing the catalytic (Asp-206, Glu-230 and Asp-297) and substrate binding sites and a $(\beta/\alpha)_8$ or TIM barrel catalytic domain (Kuriki and Imanaka 1999). Thus the amylolytic and related enzymes

have been classified into the families of glycoside hydrolases (GHs) and almost one hundred GH families have been reported. Amylolytic enzymes of microbial origin are divided into exo-acting, endo-acting, debranching and cyclodextrin producing enzymes.

19.2 Amylolytic Enzymes

19.2.1 Exo Acting Amylases – Glucoamylases and β-Amylases

Glucoamylases (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) catalyse hydrolysis of α-1,4 and α-1,6 glucosidic linkages to release β-D-glucose from the non-reducing ends of starch and related poly- and oligosaccharides. They have widely been reported to occur in a large number of microbes, including bacteria, yeast and fungi. Filamentous fungi, however, constitute the major source among all microorganisms and strains of genera *Aspergillus* and *Rhizopus* are mainly used for commercial production (Pandey 1995). β-amylases are known to be produced only by plants and certain bacteria mostly by several species of the genus *Bacillus*, including *B. polymyxa*, *B. cereus*, *B. megaterium*, and also by *Clostridium thermosulfurogenes* (Selvakumar et al. 1998). They hydrolyze α-1,4 bonds but cannot bypass α-1,6 linkages in amylopectin and glycogen and they produce maltose from amylose and maltose and a β-limit dextrin from amylopectin and glycogen.

19.2.2 Endo Acting Amylases

α-amylases (E.C. 3.2.1.1.) hydrolyse α-1,4 bonds and bypass α-1,6 linkages in amylopectin and glycogen. In spite of the wide distribution of amylases in microbes, animals and plants, microbial sources, namely fungal and bacterial amylases are preferred in industries. Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production while fungi belonging to the genus *Aspergillus* are most common (Sivaramakrishnan et al. 2006). They are classified in two categories depending on the extent to which they hydrolyze starch. Liquefying α-amylases hydrolyze 30 to 40% of starch and saccharifying α-amylases hydrolyze 50 to 60%.

19.2.3 Debranching Amylases

Isoamylases and pullulanases are debranching enzymes that hydrolyze only α-1,6 linkages. On the basis of substrate specificity and product pattern, pullulanase (pullulan α-glucano-hydrolase; EC 3.2.1.41) have been classified into two groups: type I and type II. As they hydrolyze the α-glucosidase-resistant α-1,6 linkages in dextrins, they improve the starch saccharification rate and yield when used in combination with α-glucosidases. Many mesophilic (*Aerobacter aerogenes*, *B. macerans*, *B. acidopullulyticus* and *Bacillus* sp), thermophilic and hyperthermophilic bacteria

and archae (*B.stearothermophilus*, *Clostridium thermosulfurogenes, Pyrococcus* and *Thermococcus* genus) have been reported to produce pullulanase (Gomes et al. 2003, Kunamneni and Singh 2006).

19.2.4 Cyclodextrinases

Cyclodextrin glycosyltransferase (α-1,4-D-glucan,α-4-D-(α − 1,4-D-glucano)-transferase, EC 2.4.1.19) produces a series of non-reducing cyclic dextrins (α-, β- and γ-cyclodextrins) from starch, amylose, and other polysaccharides. α-, β- and γ-cyclodextrins contain six, seven and eight glucose units, respectively, that are linked by α-1,4-bonds. *Thermococcus* sp., *B. coagulans, C. thermohydrosulfuricum* 39E, *B. sphaericus and* alkalophilic *Bacillus* sp are the most reported microorganisms producing these enzymes.

19.3 Production of Amylolytic Enzymes – Effective Utilisation of Agro Residues

Commercial production of enzymes is generally carried out by submerged (SmF) and solid state fermentation (SSF). The physico-chemical and nutritional requirements are unique for a particular microorganism. The composition and concentration of media and fermentation conditions greatly affect the growth and production of extracellular enzymes from microorganisms. The production of amylolytic enzymes in submerged fermentation employing synthetic media have been largely exploited (Tigue et al. 1995 and Hamilton et al. 1999) but is limited by the high cost of production. In the case of Smf the abundance of water gives more control of environmental factors such as temperature, oxygen concentration and pH and also provides ease in handling. However few reports have suggested agro residues as an alternative for synthetic basal media for the production amylase (Crueger and Crueger 2000, Hernandez et al. 2006, Gangadharan et al. 2008). Large scale production of enzymes would require formulation of a cost effective media and the commercial success of amylases is linked to the utilization of starchy biomass as an industrial raw material. The most inexpensive and highly energy rich substrates for fermentation is represented by agro-industrial residues.

The utilization of agro residues for the production of enzymes has gained renewed interest from researchers for the use of SSF as it solves solid waste disposal problem and also produce lesser waste water (Pandey 2003). Initially SSF was considered to be suitable for fungi and yeast considering the low water activity but there has been continous exploitation of bacterial cultures (Nampoothiri and Pandey 1996, Gangadharan et al. 2006 and Selvakumar 1999). Several naturally occurring agricultural by products such as wheat bran, coconut oil cake, groundnut oil cake, rice bran, wheat and paddy straw, sugar beet pulp, fruit pulps and peels, corn cobs, saw dust, maize bran, rice husk, soy hull, sago hampas, grape marc, coconut coir pith, banana

waste, tea waste, cassava waste, aspen pulp, sweet sorghum pulp, apple pomace, peanut meal, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch etc. could be used in one or the other industrial bioprocess for the production of value added products through SSF (Pandey et al. 2001).

Among the above substrates amylolytic enzyme production have been carried out mainly with wheat bran, rice bran, rice husk, oil cakes, tea waste, cassava, cassava bagasse, sugarcane bagasse (Mulimani et al. 2000). Banana waste, corn flour, saw-dust, soybean meal, sweet potato, potato, rice hull, sugar beet pulp have also been tried by some of the researchers. The utilization of agro residues such as wheat bran, molasses bran, maize meal, millet cereal, wheat flakes, barley bran, crushed maize, corncobs and crushed wheat have been exploited for the production alpha amylase by thermophilic fungus *Thermomyces lanuginosus* under solid state fermentation. Among the amylolytic enzymes commercial production of alpha amylase and glucoamylase utilizing agro residues are well studied and production of β amylases and pullulanase have been studied to a some extent. There are limited reports on the production of cyclodextrinases and research on the utilization of agro residues are yet to be explored in detail Thus the agro residues popularly employed for amylolytic enzyme production can be broadly classified as cereal brans, oil cakes and other starchy and non starchy substrates.

19.3.1 Cereals and Cereal Bran

Cereals are the fruits of cultivated grasses belonging to the genus *Gramineae.* The main cereals grown are wheat, rice, corn, barley, oats, sorghum and millet. Cereal grains contain 60–70% starch (Dendy and Dobraszczyk 2001). They have similar structure which include a hull (husks) and a kernel (caryopsis) and the kernel contains three components – bran, germ and endosperm. The bran is separated from cleaned and scoured cereals during milling. Among different agricultural by-products evaluated, wheat bran was found to be the best basal and standardized medium for optimal production of alpha amylase (Haq et al. 2003, Baysal et al. 2003, Balkan and Ertan 2007, Sivaramakrishnan et al. 2007). The strains of *Bacillus sp*. AS-1 and *Aspergillus sp.* AS-2 colonized well on the wheat bran based solid media and exhibited high production of α amylase and glucoamylase (Soni et al. 2003).

Table 19.1 Chemical composition of wheat bran and rice bran

Content	Wheat-bran (%)	Rice bran (%)
Moisture	6.4	12
Protein	16.4	16
Fat	6.8	22
Ash	6.5	10
Total dietary fibre	44.5	25
Starch	11.1	10–20

The physico- chemical properties of wheat bran (soft white) and rice bran are tabulated (Table 19.1). Corn gluten meal (CGM), a by-product of corn wet milling which are traditionally used for animal feed was found to be a promising substrate for the production of α amylase by *B. amyloliquefaciens* due to its high content of proteins (≥ 60%), vitamin and other minerals (Tanyildizi et al. 2007). Agricultural raw starches such as pearl millet, rice, gram, hordium, corn and wheat starches at 1% levels were tested for the production of alpha amylase by *B. licheniformis* (Haq et al. 2005). Barley and oat brans are chiefly composed of beta glucans.Comparatively higher production was found in the case of pearl millet, which is represented by 56–65% starch, 20–22% amylose, free sugars ranging from 2.6–2.7% and a total protein content of 8–19% (Dendy and Dobraszczyk 2001). Spent brewing grain was found to be a good substrate for the production of α-amylase by *A.oryzae* under solid-state fermentation (Francis et al. 2003). Spent grain is the by product of breweries left after the grain (barley, corn, wheat, rice, and other grains) is fermented and the alcoholic solution drawn off. It is normally wet, with 80 to 85% moisture content and relatively high protein content (27–30%).

19.3.2 Oil Cakes

It is the solid residue that are usually extracted from various types of oily seeds like soya bean, pea nuts, linseed, cotton seed, cotton seed and sunflower by being pressed and removing the oil. They are valued for being rich in minerals and protein. They are rich in fibre and have high concentration of non-starch polysaccharides (NSP). Their chemical composition varies due to the differences in the extraction methods of oil. They are obtained by extraction of oil by means of a solvent from the expeller pressed oil cake. The meal may also be obtained directly from seeds after a preliminary treatment. The expeller pressed oil cake used for extraction are obtained by pressing clean and sound seeds. The meal are subjected to heat and steam treatment under controlled and regulated conditions so as to prevent denaturation of the protein and removal of traces of solvent. The material are in the form of either flakes or powder and are free from harmful constituents and castor cake or husk, rancidity, adulterants, insects or fungus infestation and from musty odour. The moisture content, crude protein and crude fibre (weight percent) of ground nut, cotton seed, linseed, mustard, sesame, coconut and safflower oil cakes have been tabulated (Table 19.2). Soya bean are also included under oil seeds and they are composed of oil and protein accounting to 60%, 35% carbohydrate and 5% ash.

The production of a thermostable pullulanase of *Thermoactinomyces thalpophilus* was studied in shake-flask cultures. Maximum production of pullulanase was obtained with 5% (w/v) soybean meal, 2% (w/v) yam starch (Odibo and Ob 1990). Oil cakes such as coconut oil cake (COC), sesame oil cake (SOC), groundnut oil cake (GOC), palm kernel cake (PKC) and olive oil cake (OOC) were screened to be used as substrate for the alpha amylase production by *A. oryzae* and they were also compared with wheat bran (WB). It was found that GOC and its combination with WB (1:1) resulted in higher enzyme titres (Ramachandran et al. 2004). Arasarat-

nam et al. have reported glucoamylase production by *A. niger* using rice bran and paddy husk as alternative substrates against wheat bran. Paddy husk was reported to enhance the nutrient utilization when mixed with the substrates like rice bran, corn flakes, soya flour and soy meal powder by *A. niger* CFTRI 1105 during SSF thereby increasing glucoamylase production (Arasaratnam et al. 2001).

19.3.3 Other Starchy and Non Starchy Substrates

Cassava (*Manihot esculenta* Crantz) is a root crop of tropical American origin, and the fourth most important staple crop in the tropics. Its starchy roots produce more raw starch per unit of land than any other staple crop. It is grown almost exclusively in arid and semiarid tropics, where it accounts for approximately 10 percent of the total caloric value of staple crops. Cassava starch is composed of unbranched amylose (20±5%) and branched amylopectin (20±5%. Cassava fibrous residue (CFR) contains about 10–15% crude fibre, 55–65% starch and very low ash content (1–1.2%) (dry weight basis) (Jyothi et al. 2005). Because of its low ash content, CFR could offer numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash contents, respectively, for uses in bioconversion processes using microbial cultures (Pandey et al. 2000a, Pandey et al. 2000b). A study was carried out to investigate the α-amylase production by *B. subtilis* strain CM3 in SSF using CFR as the substrate (Swain and Ray 2007). Gonzalez et al. determined the optimal nutritional and operative conditions for amylolytic enzymes (α amylase and glucoamylase) production by *S. fibuligera* DSM-70554, cultured with cassava starch as the sole carbon source under different fermentation strategies, in order to improve cassava starch utilization. They described 97% degradation of cassava starch with a remaining 3% likely related to limit dextrins when grown under batch culture mode (Gonzalez et al. 2008).

Molasses, a by-product of sugar industry, is one of the cheapest sources of carbohydrates. Besides a large amount of sugar – 50% (sucrose 33.5%, invert sugar 21.2%), molasses contain nitrogenous substances (0.4–1.5%), vitamins such as thiamine (830μg per 100 g dry weight), pyridoxine (650μg per 100 g), folic acid (3.8μg per 100 g), biotin (120μg per 100 g), pantothenic acid (2140μg per 100 g), and trace elements (CaO 0.1–1.1%; MgO 0.03–0.1%; K_2O 2.6–5.0%) (Pandey 2003).

Table 19.2 Chemical composition of oil cakes

Oil cake	Moisture (%)	Crude protein (%)	Crude fat(%)	Crude fibre (%)
Groundnut	10	51	1.0	10
Cotton seed	8.0	40	8.0	10
Linseed	10	29	8.0	10
Mustard	10	35	8.0	9.0
Sesame	10	37	8.0	7.0
Coconut	10	21	8.0	12
Safflower	8.0	41	8.0	13

The enzyme titre and cost of α-amylase production by *Geobacillus thermoleovorans* using cane molasses and synthetic media has been compared. The enzyme titres were found to increase by 2.5 fold and cost reduced by nearly 22 fold when molasses was employed (Uma Maheswar Rao and Satyanarayana 2007). Cane molasses served as an excellent carbon and energy source for the economical production of glucoamylase by alginate-immobilized *Thermomucor indicae-seudaticae*, which was almost comparable with that in sucrose yeast-extract broth (Kumar and Satyanarayana 2007).

Potato is grown and consumed all over the world, and a large number of processed food industries market potato-based products. Although potato peel does not pose serious disposal and environmental problems, meaningful utilization of this nutrient-rich waste has not drawn much attention. Interestingly potato peel was found to be a superior substrate for solid state fermentation, compared to wheat bran, for the production of α-amylase by two thermophilic isolates of *B. licheniformis* and *B. subtilis* (Shukla and Kar 2006). Potato starch was found to be superior to other starch grains and tubers (amaranthus, wheat, sago, cassava, rice, maize etc) for the production of alpha amylase by *B. licheniformis* SPT 27 (DharaniAiyer 2004). High titres of β amylase production with 16.5% potato starch was reported in the case of *C. thermosulfurogenes* (Reddy et al. 2003). The composition of starch from *Amaranthus paniculatas* was reported to be 66.4%, which was utilized in the production of alpha amylase by *A. flavus* under SSF (Viswanathan and Surlikar 2001).

Brewery (BW) and meat processing (MPW) wastewaters, were used as a base of the culture media in the production of amylase by *A.niger* UO – 1 under submerged fermentation. BW contained (g/L): total sugars- 1.98, reducing sugars – 1.46, total nitrogen – 0.095, total phosphorous – 0.034 and MPW (g/L) was- total sugars – 1.82, reducing sugars – 0.99, total nitrogen – 0.172, total phosphorous – 0.028 (Hernandez et al. 2006). Tea waste is composed of approximately 19% crude protein, 5.4% calcium and 0.84% of phosphorous. They have been popularly used as cattle feed. SSF experiments with *A. niger* for the synthesis of glucoamylase production concluded tea waste, enriched with minerals, as a potential solid substrate (Selvakumar et al. 1998).

Banana is one the most consumed fruits in the world and India is one of the largest producing countries of this fruit. Each hectare of banana crop generates nearly 220 ton of plant residual waste that consists mainly of lignocellulose material and the waste disposal often causes serious environmental problem. The main residual wastes of the banana crop are leaves and pseudostem, both containing high levels of lignocelluloses (Shah et al. 2005). *A. oryzae*, produced amylase when banana fruit stalk was used as substrate in a solid state fermentation system. Banana waste has been exploited as an SSF substrate for α amylase production by *B. subtilis* (Krishna and Chandrasekaran 1996). An attempt was also made to utilize the food waste, kind of organic waste discharged from households, cafeterias and restaurants, which accounts for a considerable proportion of municipal solid garbage in China for the production of glucoamylase by *A. niger*. Wang et al. characterized the food waste and carbon content 53.68%, nitrogen 2.54%, reducing sugar 13.65%, total

sugar 50.23%, starch 46.12%, crude protein – 15.56%, crude lipid – 18.06%, crude fiber – 2.26% (Wang et al. 2008).

19.4 Conclusion

The cost and availability of the substrates play an important role in the development of efficient processes. The feasibility of agricultural by products for the commercial production of amylolytic enzymes has been well explored. The effective use of these residues has served dual purpose of value addition and waste management. Even-though wheat bran was given the prime position among agro residues as substrate, extensive research on the compositional analysis of the individual substrates has proved their high nutritive and productive value.

Abbreviations

SSF:	Solid state fermentation
SmF:	Submerged fermentation
CGM:	Corn gluten meal
NSP:	Non-starch polysaccharides (NSP)
COC:	Coconut oil cake
SOC:	Sesame oil cake
GOC:	Groundnut oil cake
PKC:	Palm kernel cake
OOC:	Olive oil cake
WB:	Wheat bran
CFR:	Cassava fibrous residue
BW:	Brewery wastewater
MPW:	Meat processing wastewater

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