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Review Paper

Fungal Glucoamylase Production and Characterization: A Review.

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ABSTRACT: Fungal enzymes are preferred over other microbial sources owing to their widely accepted Generally Regarded As Safe (GRAS) status. Glucoamylase (GA) is an exohydrolase, which releases β-glucose units from the nonreducing ends of starch and used in the manufacture of glucose, fructose syrups and other industrial purposes. They can be produced from various substrates by different methods, including submerged, semi-solid and solid-state fermentation processes. Optimum production of glucoamylase was observed in previous studies at a range of pH from 4.0 to 5.0 and temperature 30 to 40°C with the incubation period of 4 to 5 days. Optimum catalytic activity was also recorded in previous experimental studies at a wide range of pH 4 to 8 and temperature 40 to 70°C. In previous literature, it was observed that most of the fungi produced several isoenzymes that had different molecular weights ranges from 40 to 125 kDa. The majority of fungal glucoamylase was multidomain consisting of the N-terminus catalytic domain (CD) and the C-terminus starch-binding domain (SBD). The catalytic domain folds as a twisted (α/α) 6-barrel containing a hex-helical hairpin toroidal structure while starch binding domain folds as an antiparallel β-barrel having two independent substrate binding sites. The present review focuses on recent findings on glucoamylase production, characteristics, structure, mechanism of action and industrial applications.

Key words: Fungus, Glucoamylase, Characteristics, Structure, Industrial uses

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INTRODUCTION

Starch is the most widely dispersed natural polysaccharides, from plants[1]. It is composed of two high molecular weight polymers, amylose and amylopectin[1]. Amylose is a straight chain of glucose unit connected with α-1,4 glycosidic bonds[1]. On the other hand, amylopectin is branched polysaccharides of glucose unit, having both α-1,4 glycosidic linkage and α-1,6 glycosidic linkage, where the α-1,6 glycosidic bonds are found after every 17-26 glucose units from the linear chain[1,2]. A large number of microorganisms can produce starch hydrolyzing enzymes such as the α-amylases, glucoamylase (GA), isoamylases, β-amylases and pullulanase that are able to hydrolyze the amylose, amylopectin and other polysaccharides[1]. The 1,4-α-D-glucan glucohydrolase is the chemical name of glucoamylase (GA), which is an exo-acting enzyme that yields β-D-glucose from the nonreducing ends of starch and related polysaccharide chains by hydrolyzing α-1, 4 and α-1, 6 linkages[3,4]. This enzyme is also able to completely hydrolyse starch if incubated for extended periods of time and hence called the saccharifying enzyme. Amylase or glucoamylase

has a significant role in starch processing in the food industries, such as for the fructose and glucose syrup that are produced from liquefied starch with an action of amylase and glucoamylase[5,6]. Amylase is also used in bioethanol, confectionary, pharmaceuticals, beverage and different fermented food in industries[6,7]. Glucoamylase belongs to the family of the glycoside hydrolase 15 (GH15) due to its structural similarity of that enzyme group or family[8]. A huge number of microorganisms, including fungi, yeast and bacteria are capable of glucoamylase production[9]. Filamentous fungi are the main sources for glucoamylase[10]. In the enzyme industry exclusive use of glucoamylase was recorded from, *Rhizopus oryzae*, *Aspergillus niger* and *Aspergillus oryzae*[11-13], and the fungal sources of enzyme is regarded as safe[14].

A fungal fermentation system is recognized as a complicated multi-phase, multicomponent process. Cell growth and product formation are determined by a wide range of parameters, including culture medium, fermentation pH, temperature, dissolved oxygen tension, shear stress, and fungal morphology[15]. The potential of glucoamylase research is obvious from the large number of research publications and reviews that appeared in the recent past[2,7,16-20]. An attempt has been made to review the recent progress in understanding the fungal glucoamylase in this article.

Factor Affecting Glucoamylase Production from the Native Host

Glucoamylase production is greatly affected by several factors such as strains, method of fermentation, composition of cultivation media, moisture content, fermentation temperature and pH, incubation and etc.

Media Composition

Carbon and Substrate Sources

For the production of glucoamylase, different types of starch such as soluble starch, raw sago starch, cassava starch, potato starch, cellulose, corn starch and simple sugar such as glucose, fructose and sucrose are commonly used as the source of carbon. Wheat bran, paddy husk, rice processing waste, tea and copra waste, pastry waste, and bagasse of wheat bran and sugar cane are also used as substrate for enzyme production[21-23].

Different concentrations of starch are used by fungi to give maximum extracellular enzymes such as 4% starch from *Aspergillus fumigates*[24], 2% waste bread as substrate (W/V) by *Arachniotus* sp25 in the medium and 3% oat bran from *A. fumigates*[26]. Glucoamylase productions decreases with the presence of glucose[27], and at the same time addition of fructose and glucose inhibit the enzyme secretion by *Aspergillus* sp. JAN-25[28]. Ominyi Matthias[29], reported that *Rhizopus* spp, *Aspergillus* spp and *Mucor* spp can produce maximum glucoamylase with 8% gelatinized potato starch compared to gelatinized cassava, corn, soluble starch and raw starch. Higher glucoamylase production was observed in wheat bran medium[9,30,31]. Some of the carbon sources or substrates and their concentrations, influences the production of glucoamylase of the specific fungi strain, and some carbon sources inhibit the production of glucoamylase of certain strains.

Source of Nitrogen

The production of glucoamylase is intensely affected by the concentration and nature of nitrogen and carbon sources. Inorganic or organic compounds such as $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, yeast extract, beef extract, peptone, casein, urea and other compounds were commonly used as the source of nitrogen in the fermentation media by many researchers to find the best nitrogen source either in single or combination of both organic and inorganic compounds[9,32]. The production of glucoamylase from fungi on the nitrogen source depends on species of fungi, the amount of supplementation of nitrogen compounds, sources of compounds and the substrate composition in the culture media. Some of the nitrogen compounds influence the production of enzyme, while other may have negative effect on enzyme synthesis of the certain fungi[33]. For example, organic nitrogen has negative effects on enzyme production from *Rhizopus* sp. MKU 40, may be due to the formation of protease[33]. Presence of both inorganic and organic source of nitrogen enhanced the production of raw starch degrading enzyme (RSDE)[34]. Bhatti et al.,[9] found that the addition of urea as a nitrogen source exhibited better GA production followed by peptone and yeast extract, which was a similar result from Ellaiah et al.,[35]. In addition, it was observed that yeast extract was the better source of organic nitrogen for glucoamylase production according to many previous studies[21,24,32,36,37].

Incubation Temperature, pH and Incubation Periods

The growth of microorganism and enzyme production is greatly affected by temperature, and varies from microorganism to microorganism[38,39]. Microbial growth and enzyme production are inhibited at elevated temperature, due to the formation of metabolic heat in the media[40]. At the same time, enzyme becomes inactivated or hydrolysis of some proteins may occur at the higher temperature[30]. The optimum production of glucoamylase was observed at 30 to 40°C from many fungi [9,28,35,41], for example, from *Fusarium solani* at 35±1°C9, 30ºC by Alva et al.,[42], 32ºC from *Arachniotus* spp[25], 35ºC for *Aspergillus niger*[43], marine endophytic *Aspergillus* sp. JAN-25 at 30-37°C[28] and thermophilic fungi *Aspergillus fumigates* at 30°C[41]. Neverthless some fungi can produce glucoamylase at the higher temperature such as *A. oryaza* at 60°C by Parbat and Singhal[22], 45°C from *Aspergillus terreus*[6] and *Rhizopus* sp at 45°C[44].

The physical parameter, like pH has also significant effects to the growth and enzyme production[9]. Most of the fungi and yeasts are active at acidic environment (pH 4.0 - 6.5) for enzyme production[28], but the optimal production of glucoamylase was observed at pH 4.0 to 5.0 from many fungi[9,28,35,36]. The optimum glucoamylase production by *Aspergillus oryzae* was found at pH 5.830 and pH 5.4 by *C. gloeosporioides*[45].

The incubation period is another factor that infuences the enzyme production due to a difference in the lag and log phases of growth of different organisms[9,28,46]. The optimal glucoamylase production from most of different fungi was observed after 4 to 5 days of incubation periods, although most of the fungi can produce the glucoamylase either in solid state fermentation (SSF) or submerged fermentation (SmF) in the range of 2 to 6 days of incubation with few exceptions and depending on substrate types and concentration. Many studies reported that maximum glucoamylase was produced on the 5th day of cultivation[22,29,36,47,48]. But in a few studies, it was observed after 4 days of cultivation; like glucoamylase produced by *Fusarium solani*[9], *Aspergillus niger*[49], *Fusarium moniliforme*[46] and *Aspergillus* sp. JAN-25[28]. Long duration of cultivation periods resulted in a decrease in the secretion of the enzyme by the culture of *Aspergillus* sp. due to the formation of other by-products and deficiency of nutrients in the media[43,50]. The optimum temperature and pH for the production of glucoamylase from various fungi species are shown in Table 1.

Table 1. Optimum pH and temperature for glucoamylase production by fungi

Name of fungi	pH	Temperature (°C)	References
Arachniotus spp	4.0	32	Asghar *et al.*,[25]
Monascus purpurens ATCC 16437	4.2	-	Sayed *et al.*,[51]
Aspergillus niger	6	35	Feroza *et al.*,[43]
Aspergillus awamori		42	Moreira *et al.*,[52]
*Aspergillus niger(*wild and mutant variety)	4.0	40	Imran *et al.*,[53]
Aspergillus sp.	5.0	30	Puri *et al.*,[48]
Fusarium solani	5	35 ±1	Bhatti *et al.*,[9]
Candida famata	5	30	Mohamed *et al.*,[37]
Aspergillus oryaze	5	60	Parbat and Singhal, [22]
Aspergillus fumigatus	7.0	37	Cherry *et al.*, [24]

table continued...

Aspergillus sp.	4	30	Ominyi Matthias, [29]
Aspergillus oryaze	6	35	Kumar *et al*., [31]
Aspergillus sp.	5.0	30	El-Glendy, [28]
Aspergillus terreus	4.0	45	Abdalwahab *et al*., [6]
Rhizopus sp.	4.5	45	Naher *et al*., [44]
Rhizopus delemar	-	35	Soccol *et al*., [54]

Mode of Fermentation for the production of Glucoamylase

Submerged, semi-solid and solid state fermentations are used for the production of glucoamylase which is influenced by the bioreactor design and operation mode. Different fermentation vessels from flasks to bioreactors (airlift and stirred-tank) and strategies, including batch, fed-batch and continuous fermentations have been employed for the production of glucoamylase[55-57]. For example, glucoamylase production by Thermomyces lanuginosus was found to be 2.5-fold higher in shake flasks compared to static cultures[58], and batch cultivation was better compared to continuous and fed batch operation[55].

Traditionally, glucoamylase has been produced by submerged fermentation (SmF). In recent years, however the solid-state fermentation (SSF) processes have been increasingly popular for the production of this enzyme[35]. SSF compared to SmF is more simple, requires lower capital, has superior productivity, reduced energy requirement, simpler fermentation media and absence of rigorous control of fermentation parameters, uses less water and produces lower waste water, has easier control of bacterial contamination and requires low cost for downstream processing[40]. Agro-industrial residues are generally considered as the best substrates for the SSF processes and enzyme production[35]. In liquid fermentation, the secreted proteins are released into the culture medium resulting in substrate degradation in the whole culture. In SSF process, synthesis of glucoamylase depends on the culture conditions and type of nutrients available to the organism[13,30,33,45,59]. Based on research with rice kernel solid-state fermentation (SSF) with *Aspergillus oryzae*, it was shown that high levels of glucoamylase B, an enzyme that is clearly different from glucoamylase A[60], are due mainly to induction of the glucoamylase B gene[61]. Ishida et al.,[62] found that the promoter region of glaB mediates the induction of transcription by starch, high temperature, low A_w (water activity), and physical barriers to hyphal extension.

Moisture Content and Inoculum density In Glucoamylase Production

The amount of moisture is an important determinant factor for the production of enzymes in the solid-state fermentation. The natural moisture (7-13%) in bran or any substrate is too low to support the metabolic activities of fungi; therefore the solid substrate needs to be moistened during preparation. High moisture content is known to reduce porosity of substrate and causes particles to stick together and adversely impacts oxygen transfer[63]. In contrast, a low moisture level reduces water activity to levels that are not conducive to supporting good fungal growth and metabolism[2]. In many reports maximum glucoamylase productions were found in 45% to 80 % at initial moisture levels. Maximum amylases yielded by *Penicillium* sp. X-1 was found at moisture level 65%, which was 4.1-fold higher than that obtained at a moisture level of 50%[59]. Several other studies also found similar results at the moisture level 70% such as *Aspergillus* sp. MK07 with wheat bran[47], *F. solani*[9] and *Aspergillus niger*[53]. The highest glucoamylase at 80% moisture was observed by Puri et al.,[48] from *Aspergillus orayza*.

Inoculum density is another important consideration for SSF since higher inoculum levels are inhibitory factors for good growth and metabolite production while lower inoculum levels require more time for fermenting the substrates in SSF [9,64]. On the other hand, not much difference in the production was found in different inoculum sizes compared to the production at optimum inoculum size[21]. Bhatti et al.,[9] tested the different inoculum levels on GA production by *F. solani*. Under optimum conditions, GA production

increased with an initial increase in inoculum size and maximum GA activity was noted in the medium receiving 15 % (by mass per volume) inoculum level. A further higher spore density caused a decrease in GA synthesis in SSF of wheat bran. Similar results were also found by Kumer et al., [31] at the 15% inoculum level, but 10 % inoculum level for GA production in SSF of wheat bran by *Aspergillus* sp. A3 has previously been reported as optimum[35]. Puri et al.,[48] found that an increase in inoculum size from 1×10^5 to 1×10^7 spores/ml, there was an increase in glucoamylase production from 2.37 to 4.14 IU for 5 g of rice bran.

Characteristics of Glucoamylase

Types of Glucoamylase

More than one form of glucoamylase enzyme have been observed from fungi, and their properties vary due to the difference in molecular mass, amino acid sequence or composition, the stability of protein, the percentage of glycosylation, and with and without starch binding domain[7]. Five to six different forms of fungal glucoamylase was found[7,65], and two to four isoforms of glucoamylase occurs from different *Aspergillus* sp.[66-68]. Most of fungi, comprising of the commercially used *A. niger* and *A. awamori* has two forms of glucoamylase (GAI and GAII)[7]. According to Norouzian et al.,[2], the majority of fungal glucoamylase are multidomain consisting the N-terminus catalytic domain and the C-terminus starch-binding domain. But with an exception, glucoamylase without starch binding domain structure are also available in *Rhizopus oryzae*[69], *A. niger*[70], *A. oryzae*[60], *A. flavus* NSH9[71].

Glucoamylase differs in activity on soluble and raw starches depending on its structure and domain, but both (with and without SBD) are equally active on soluble starch[7,68,72].

Two different forms of glucoamylase, with and without starch binding domain, were produced from one gene or two different genes depending on mRNA splicing[73], posttranslational processing[74] and limited proteolysis[75]. For example, *A. awamori* var. kawachi and *A. niger* that produced two GA from the same gene [76], but for *A. oryzae* from two different genes[72].

Molecular Weight of Glucoamylase

The subunits present in the enzyme molecule are separated according to their molecular weights, which show different bands on SDS-PAGE gel. The monomeric glucoamylase usually shows single band on SDS-PAGE. For example, Bhatti et al.,[77] reported monomeric glucoamylase from *F. solani*, and found the molecular weight was 41 kDa based on gel filtration chromatography and 40 kDa by the SDS-PAGE. The two isoenzymes (GA1 and GA2) were identified (GA) from *Aspergillus flavus* HBF34 strain and one had two subunits that were 64, 70 and another was 125 kDa[1]. At the same time some glucoamylase was secreted as dimeric form, for example the molecular weight of glucoamylase from *Colletotrichum* sp. KCPI was found as 162.18 kDa by native-PAGE but in SDS-PAGE, the two sub-units with a molecular mass of 94.62 and 67.60 kDa, respectively were observed[78].

In previous studies, many researchers reported the molecular mass of glucoamylase in fungi to be about 25 to 112 kDa[2,66,71,79]. While glucoamylase from *Tricholoma matsutake* which had MW 11.5 kDa[80]; glucoamylae from *Aspergillus niger* by Suresh et al.[81] and Slivinski et al.,[82] reported that MW was of 125 and 118.17 kDa, respectively. The molecular weights of glucoamylase from different fungi are given in Table 2. The molecular weight of protein depends on the number of amino acids and the percentage of glycosylation. The fungal glucoamylase usually has 10-20% carbohydrate of the molecular weight[68,83], and the major sugar in glucoamylase was mannose (62%)[5].

Table2: Molecular weights of glucoamylase from different source of fungi

Fungi	Molecular weight (kDa)	References
Aspergillus flavus	51.3	El-Abyad *et al.*,[84]
Aspergillus flavus HBF3	64, 70, 125	Koc and Metin,[1]
Aspergillus niger	125	Suresh *et al.*,[81]
Aspergillus niger	118.17	Slivinski *et al.*,[82]
Aspergillus niger	52, 66	Jebor *et al.*,[79]
Aspergillus niger	60	Imran *et al.*,[53]
A. niger Bo-1	91, 73, 59	Aalbaek *et al.*,[85]
A. fumigates	42	da Silva and Peralta,[86]
A. awamori var. *kawachi*	90, 83, 57	Pandey,[16]
A. awamori nakazawa MTCC 6652	109.64, 87.1, 59.43	Negi and Banerjee,[66]
Arthrobotrys amerospora ATCC 34468	44.7, 71.0, 74.5	Norouzian *et al.*,[87]
Curvularia lunata	66	Feng *et al.*,[88]
Chaetomium thermophilum	64	Chen *et al.*,[89]
F. solani	40/41	Bhatti *et al.*,[77]
Humicola sp.	72.75	Riaz *et al.*,[90]
Rhizopus sp.	74, 58.6, 61.4	Takahashi *et al.*,[91]
Tricholoma matsutake	11.5	Hur *et al.*,[80]
Thermomyces lanuginosus	75	Nguyen *et al.*,[5]

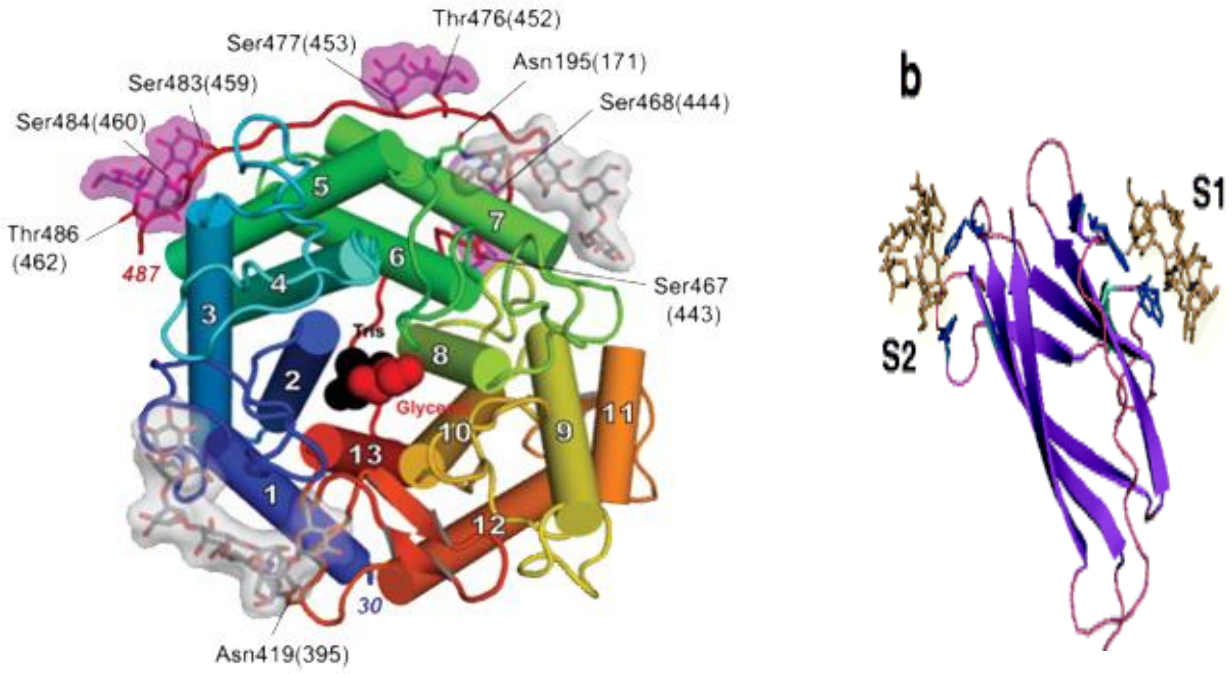

Figure 1: Structure of glucoamylase. A; The catalytic domain (CD) of *A. niger* GA from 30 (blue) to 487 (red) residue. 13 α-helices, indicated as tube-shaped, are counted from *N*-terminus. The active-site-bound Tris (black) and glycerol (red) are highlighted (Taken from Lee & Paetzel, 2011)[93]. B; Starch bnding domain (SBD) of *A. niger* glucoamylase, S1 and S2 are the two starch binding site (Taken from Marin-Navarro and Polaina, 2011)[118]

Structure of Glucoamylase

Most of the glucoamylase (glucoamylase with SBD) comprises three separate regions, the two functional globular domains, catalytic domain at N-terminal and starch binding domain at C-terminal joined by O-glycosylated linker region[7]. Each glucoamylase has a fundamental $(\alpha/\alpha)_6$ barrel catalytic domain, which has a six-helical hairpin toroidal structure[11,92.] The catalytic domain (CD) of *Aspergillus niger* glucoamylase has a mostly α-helical secondary structure made of thirteen α-helices and six 3_{10}-helices[93]. There are separate starch binding domain (SBD) in most of fungal glucoamylase, and classify in the family of carbohydrate-binding module (CBM20 and CMB 21, depending on the position of starch binding domain)[92]. The starch binding domain (SBD) of fungal glucoamylase, enclosed within catalytic domain by a linker region, has no affect on the stability of glucoamylase but has a significant contribution for hydrolysis of insoluble starch or polysaccharides[7,92].

The SBD of Glucoamylase has an important function in hydrolyzing insoluble starch[94]. Incomplete or entire proteolytic degradation of SBD of glucoamylase leads to formation of second glucoamylase (without SBD), which is more effective on soluble starch compare to raw starch[7,11]. Basically, the starch binding domain of glucoamylase is located at the C-terminal end[7]. But few exceptions are from *Rhizopus oryzae*[95] and *Mucor circinelloides*[96], which have starch binding domain at N-terminal[7]. Depending on the position of SBD in glucoamylase; can be classified as carbohydrate-binding modules family 20 (CBM20) having starch binding domain at the C-terminal like glucoamylase from *A. niger* and CBM21 family which has starch binding domain at N-terminal such as glucoamylase from *Rhizopus oryzae*[7,97]. There was negative correlation between the percentages of glycosylation of glucoamylase with the degree of proteolysis degradation by protease [2,11].

Physiochemical Properties

It has been reported that fungi glucoamylase is active at acidic pH [2], but the various forms have different pH optima. pH 4.0 was found to be optimum for the GA activity of *A. flavus*[84,98] and *Aspergillus tamari*[52], whereas the optimal pH values for other *Aspergillus* GAs were found to be between 4.5 and 7.0[1,36,99]. Most of the fungal starch-degrading enzymes have optimum pH values of 4.0–6.0[77,100,101]. Jebor et al.,[79] purified two types of glucoamylase, glucoamylase

(A & B) from *Aspergillus niger*, and observed the maximum activity at pH 8.0 and 6.5. The optimum catalytic activity of the recombinant glucoamylase at pH 4.0 to 5.0 expressed in *P. pastoris* was observed by many researchers; as reported by Chen et al.,[102] on recombinant glucoamylase in *Chaetomium thermophilum* at pH 4.5 -5.0, *Aspergillus flavus* NSH9 at pH 5.0[71] and in *Rhizomucor pusillus* at pH 4.0 [103]. The change in pH affects the ionization of essential active site amino acid residues, which are involved in substrate binding and catalysis, i.e. breakdown of the substrate into products. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity.

The stability of fungal glucoamylase is also pH dependent. For example, the GAs produced by *Aspergillus terreus* and *Aspergillus terreus* NA-170 were stable over the pH range of 3.0 to 7.0 [104], *A. flavus* was stable over a wide pH range with 100% stability at values of 5.0-9.0[98], GA from *A. flavus* HBF34 showed high stability in a wide range of pH (pH 3.0 - 8.0)[1] and also reported by a number of researchers[2,99]. Enzyme stability is an important criterion for long lasting industrial processes with extreme pH levels, making it highly desirable in the enzyme industry.

Most raw starch-digesting glucoamylase is known to exhibit optimum temperature between 50 and 70°C and are remarkably stable at high temperatures[2]. Many researchers investigated the optimum temperature for glucoamylase activity. For example, optimum temperature of purified GA was found 60°C from *A. flavus* HBF34[1], *A. flavus*[84], *Aspergillus niger* NRRL 3135[105]. In some cases, higher optimum temperatures were also reported. For example, the optimum temperature for glucoamylase activity of *A. flavus* A1.1[98], *Aspergillus fumigatus*[86] and *Aspergillus* sp GP-21 were reported to be 65°C. Additionally, an optimum temperature of 70°C was reported for *Thermomyces lanuginosus* A13.37[98], crude enzyme from *A. flavus* NSH9[36] and recombinant glucoamylase from *Aspergillus flavus* NSH9[71]. However, few exceptional cases, the optimum temperature of 40-45°C have been reported for glucoamylase from *Aureobasidium pulluan*[106], *Fusarium solani*[77] and *Aspergillus niger* ATCC 1015[107].

Industrial Application of Amylase/ Glucoamylase

Production of Sugar

Glucoamylase is an important enzyme in starch processing, which engaged second place after proteases of the industrial enzymes[1]. In starch industry, liquefaction and saccharification are two steps processing involved in conversion of starch into sugar. Thermostable Alpha amylase is commonly used in liquefaction process for hydrolysis of starch, it changes the starch molecules into short dextrins and cyclodextrins[108,109]. Thermostable α-amylase is used as thinning agent, which reduce the viscosity and partially hydrolyze the starch[110]. According to Prakash and Jaiswal, [111], α-amylases production by *Bacillus* species is most interesting due to the importance of thermostability. After the liquefaction (at 95-105°C), saccharification takes place. These steps involving the production of glucose, fructose, maltose, and glucose syrups through additional hydrolysis of liquefied molecules[111]. An exoamylase group of enzyme was used at this step in which they hydrolyzed α, 1-4 glycosidic bonds from the non-reducing end of the starch chain[112]. Glucoamylase from *A. niger* is commonly used in this process[112]. Fungal glucoamylase is active at acidic condition and also less thermostable, and the industrial saccharification process takes long time to desire yield[7,19,113]. Furthermore, isoamylase or pullulanase is used to speed up the starch processing[7].

Food Industry

In the food processing industry, amylase is comprehensively used in baking, brewing, starch syrups, juice and production of cakes[114]. Couto et al., [115] reported that α-amylase has been used in the baking industry, these enzyme were able to reduce the starch in the flour into smaller dextrin, when α-amylase was added to the dough of bread. The addition of the α-amylase to dough consequently increased the fermentation rate and reduced the thickness of the dough, therefore improved the texture and volume of the product[109]. Besides, amylase is also used in fruit juices or beer as the clarification and used for the treatment of animal feed to enhance the digestibility of the fiber[114,116].

Other Applications

Amylase is also used in several fermented foodstuffs and pharmaceuticals industries for profitable production[6], and leather, detergent and textile industries[6,90,117]. Glucoamylase acted synergistically with α-amylases and some isoamylase, which are commonly used in the saccharification of starch to produce soluble sugars[118]. Favaro et al.,[119] reported that direct bioethanol could be produced from a natural starch substrate without any pretreatment using industrial yeast strains, by co-secreting both of glucoamylase and alpha amylase.

Cloning and Expression of Microbial Glucoamylase

Different expression host such as bacteria, fungi and yeast system are used for the expression and overexpression of foreign proteins. The glucoamylase-encoding gene was first sequenced from *Aspergillus niger*[73,120], followed by *A. awamori*[75]; both were found to be identical. After that, lots of glucoamylase encoding genes have been cloned from *A. awamori*[121,122,] *A. oryzae*[60,123], *A. terreus*[124], *A. tubingensis*[125], *Corticium rolfsii*[65], *Chaetomium thermophilum*[89], *R. oryzae*[126], *Neurospora crassa*[127], *Rhizomucor pusillus*[103], *Lentinula edodes*[27], *Sulfolobus solfataricus*[128], *T. lanuginosus*[129], *A. flavus* NSH9[71] and many of these have been successfully over-expressed. The cloning and expression of glucoamylase from different sources and also hosts are summarized in Table 3.

Table 3: Different sources of glucoamylase for cloning and the host used for expression

***Aureobasidium pullulans* NRRL 12974**	*Pichia pastoris*	Li *et al*.,[132]
Thielavia terrestris	*Aspergillus oryzae*	Rey *et al*.,[133]
Saccharomyces diastaticus	*Saccharomyces cerevisiae*	Cho *et al*.,[134]
***Thermoanerobacter tengcongensis* MB4**	*Eschericha coli*	Zheng *et al*.,[135]
***Bispora* sp. MEY-1**	*Pichia pastoris*	Hua *et al*.,[136]
A. awamori	*Saccharomyces cerevisiae*	Pavezzi *et al*.,122
A. tubingensis	*Saccharomyces cerevisiae*	Viktor *et al*.,[125]
Rhizomucor pusillus	*Pichia pastoris*	He *et al*.,[103]
***A. flavus* NSH9**	*Pichia pastoris*	Karim *et al*.,[71]

A number of features such as heterologous expression host strain, promoters, vectors, gene copy number, site of integration of gene, the recombinant strain stability and regulatory protein etc. are important in the effective overexpression/expression[7,130]. For example, in previous studies it was observed that the addition of extra copies of a gene to the host strain significantly increased the recombinant enzyme production[7,131].

CONCLUSIONS

Glucoamylase is an exo-acting enzyme that yields β-D-glucose from the non-reducing ends of polysaccharide by hydrolyzing both α-1,4 and α-1,6 linkages, and has great importance in starch processing industry. Solid-state fermentation is commonly used for glucoamylase production from different agricultural wastes and semi-solid state batch fermentation system yields the highest enzyme titer. GA can be produced using different substrates and nitrogen sources, however, wheat bran (as a substrate) and yeast extract (as a nitrogen source) are recommended for fungal glucoamylase production. Moreover, the optimal conditions for glucoamylase production are the pH 4.0 to 5.0, temperature 30 to 40°C and 4 to 5 days of incubation. The major form of glucoamylase contains three distinct regions, the two functional globular domains, N-terminal catalytic domain and C-terminal granular starch binding domain; interconnected by a linear, semi-rigid, bulky, and heavily glycosylated linker. The catalytic domain folds as a twisted (α/α)6-barrel with a central funnel-

shaped active site, while the starch-binding domain folds as an antiparallel □-barrel and has two binding sites for starch. Although glucoamylase from various fungus sources are reported to have optimum specific activities over a wide range of temperature and pH range, but most sources of GA are unstable at higher temperatures in industrial saccharication. The development of thermostable recombinant glucoamylase by site-directed mutagenesis and protein engineering from fungi will be worthy of performing industrial saccharafication at elevated temperatures (50-80°C) and neutral pH, which obviously has great importantance in the industry.

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