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SHORT COMMUNICATION

Phenotypic and molecular characterization of *Aspergillus* species for the production of starch-saccharifying amyloglucosidase

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Abstract The continuous exploration of new microbial cell factories in diverse environmental and geographical locations has led to pivotal developments in the production of industrially important extracellular metabolites. Filamentous fungi are used for large-scale production of various enzymes, and amylolytic enzymes in particular. Among them, amyloglucosidase is known to be secreted extracellularly in copious amounts. The current investigation was based on molecular phylogeny and taxonomy of filamentous fungi from the genus *Aspergillus* that are capable of producing amyloglucosidase under submerged fermentation conditions. Optimization of physical and chemical cultivation parameters were also investigated. Among the various natural isolates identified in the current study, *Aspergillus fumigatus* KIBGE-IB33 was selected based on maximum enzyme production. Parametric optimization resulted in the secretion of maximum amyloglucosidase in a modified starch medium. High enzyme yield was achieved after 4 days of fermentation at 30 °C in a starch-based medium (pH 7.0). The mesophilic nature of this isolate, with its broad pH range and reduced fermentation time, renders *A. fumigatus* KIBGE-IB33 an attractive candidate for large-scale production of amyloglucosidase for starch saccharification.

Keywords *Aspergillus fumigatus* · Bioprocessing · Starch saccharification · Submerged fermentation

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Findings

Starch is a glucose polymer linked by α,1→4 and α,1→6 glycosidic bonds, and requires appropriate combinations of hydrolases for the depolymerization of its complex structure into various oligosaccharides. The hydrolyzed products of starch have a wide range of applications (Pandey et al. 2000; Gupta et al. 2003; Norouzian et al. 2006; Michelin et al. 2008). Amyloglucosidase (EC: 3.2.1.3) is used extensively in starch bioprocessing exclusively for the saccharification of liquefied starch. A wide variety of microbes are capable of producing amyloglucosidase; among them, various filamentous fungi have shown great potential for extracellular secretion of this enzyme (Zambare 2010). On a commercial scale, *Aspergillus* and *Rhizopus* species are commonly used due to their low level of transglycosylation activity and therefore, after starch saccharification, the yield of glucose is approximately 100 % (Mertens and Skory 2007). Other features, including their ubiquitous nature, non-fastidious requirements, and ability to secrete copious amounts of proteins, give these fungal species greater prominence. Therefore, in light of the aforementioned factors, several starch-hydrolyzing *Aspergillus* species have been isolated and identified from biologically diverse locations. Selection of a potential natural isolate to achieve a maximum enzyme yield is a tedious task. Studies related to various physical and chemical culture cultivation conditions have also been undertaken in order to achieve maximum amyloglucosidase yield that can be further utilized by industries for the bioconversion of starch into glucose.

In the current study, soil samples were collected aseptically from different vegetative fields located in different areas of Karachi, Pakistan, and different species were isolated using the spread plate technique. Initially, seven *Aspergillus* isolates were identified after serial plating onto potato dextrose agar (PDA). The starch–iodine plate assay method was used for preliminary screening of these natural isolates based on

hollow zones around the colonies. Among them, only four isolates exhibited prominent starch hydrolyzing capability. These selected *Aspergillus* species were completely characterized based on taxonomical and molecular identification using 18S rDNA sequence analysis. PDA and Czapek-Dox agar were used for macroscopic characterization, and microscopic studies (Online Resource 1 and 2) were conducted utilizing lactophenol blue staining (St. Germain and Summerbell 1996). For 18S rDNA analysis, fungal DNA was extracted using a cetyltrimethyl ammonium bromide (CTAB)-based method (Karthikeyan et al. 2010), and it was amplified using 18SF: GTAGTCATATGCTTGTCTC and 18SR: TCCGCAGGTTCACCTACGGA as a primer set. Thermal cycling was performed according to the following program: initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1.5 min, and final extension at 72 °C for 10 min. The amplified PCR product was purified and sequenced. The sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank, and the following accession numbers were assigned: KF905648 (*A. fumigatus* KIBGE-IB33), KF905649 (*A. flavus* KIBGE-IB34), KF905650 (*A. terreus* KIBGE-IB35), and KF905651 (*A. niger* KIBGE-IB36). For comparison of the current 18S rDNA sequences with other available sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/), multiple sequence alignment was performed and a phylogenetic tree was constructed using the MEGA 5.0 software program. Neighbor-joining analysis was conducted with 100 replicates. Selected sequences of genus *Aspergillus* were retrieved from the NCBI database and compared with sequences used in the current study. The constructed dendrogram illustrates that the isolated strains appeared in separate clades according to their respective groups (Online Resource 1).

For the quantification of maximum amyloglucosidase production, selected filamentous fungi were cultivated in a designed production medium consisting of (g l^{-1}): soluble starch, 10.0; yeast extract, 10.0; peptone, 5.0; magnesium sulphate, 1.0 and dipotassium hydrogen phosphate, 1.0. *A. fumigatus* KIBGE-IB33 secreted the highest amount of enzyme titers among the four natural isolates (Fig. 1a). This strain was selected for enzyme production studies. Fungal biomass was harvested after 4 days of incubation by centrifugation of the fermented broth at $40248\times g$ for 15 min at 4 °C. The supernatant was filtered through a 0.45-μm filter under a vacuum in order to remove any remaining spores. Specific amyloglucosidase activity was determined as described earlier (Ghani et al. 2013). Estimation of liberated glucose was calculated using the GOD-PAP method (Trinder 1969a, b). A unit of amyloglucosidase is defined as the "amount of enzyme that liberates 1.0 μM of glucose per minute under standard assay conditions". Units expressed are presented in terms of kilo units per mg of protein (kU mg^{-1}). Total protein

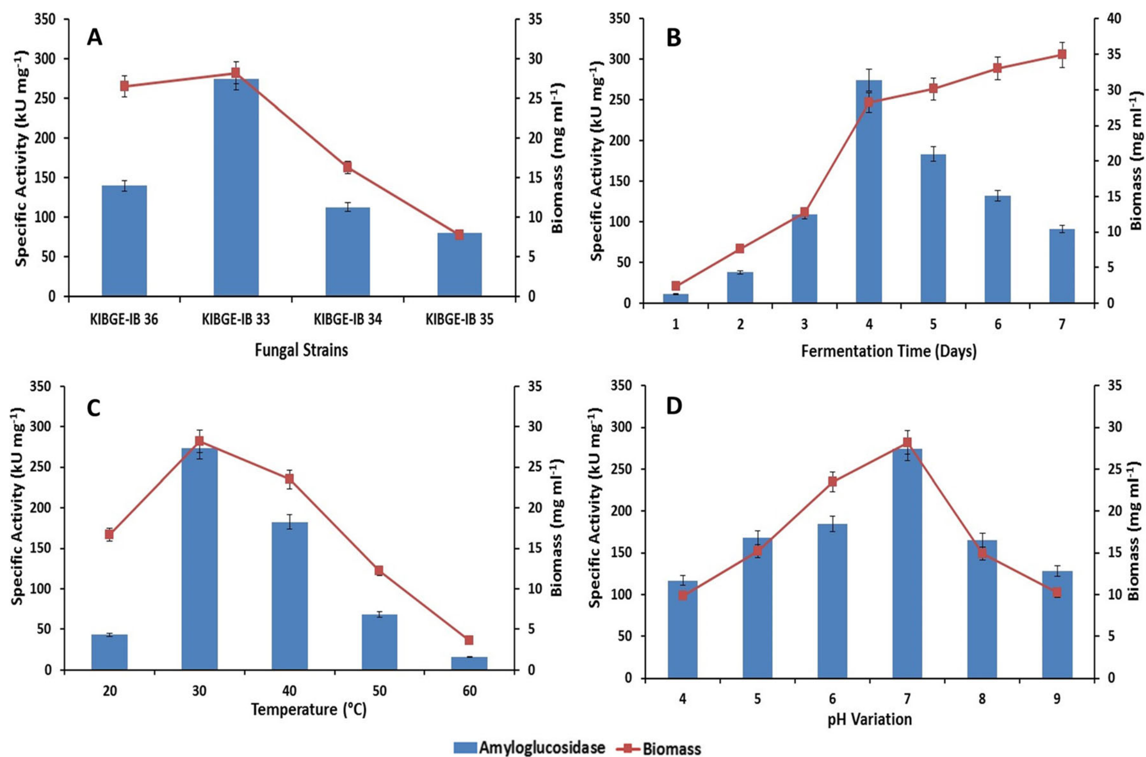

Fig. 1 Quantification of amyloglucosidase production and fungal growth profile. **a** Amyloglucosidase production using various filamentous fungi. **b** Fermentation time. **c** Temperature selection. **d** pH variation for amyloglucosidase production using *Aspergillus fumigatus* KIBGE-IB33

was calculated using the Lowry et al. (1951) method, with bovine serum albumin as standard.

Optimization of medium components and operating conditions is the most important element in producing an enzyme in feasible quantities. In starch hydrolyzation industries, the aforementioned factors not only govern the cost of the process, but they also facilitate the development of new starch saccharification methodologies. In this study, various physical and chemical parameters were studied in a stepwise manner. The approach used for the selection of various parameters was based on one variable at a time in a batch fermentation process. Fungal biomass was estimated using mycelial mats, which were dried to a constant weight at 80 °C. The effect of various supplementary macronutrients (carbon and nitrogen sources) and micronutrients (mineral salts) were studied (Table 1). The concentration of each nutrient was varied in order to determine the maximum yield of amyloglucosidase. A myloglucosidase secretion was induced by all of the carbon sources however, potato starch was found to be the most effective in terms of enzyme yield (274 kU mg^{-1}) and cell mass (28.2 mg ml^{-1}). In addition to its inductive effect, potato starch is also an inexhaustible source of energy compared to other simple carbon sources (McTigue et al. 1994). This carbon source is also reported to provide stability to the enzyme after its production in the fermentation medium (Santamaria et al. 1999; Aguilar et al. 2000). In the present study, the concentration of potato starch was varied (0.0-25.0 g l^{-1}), and maximum enzyme yield was obtained at a concentration of 10 g l^{-1} (Table 1). Higher starch

Table 1 Production of amyloglucosidase and fungal biomass yield in the presence of various macronutrients and micronutrients

Nutrient components	Specific activity (kU mg^{-1})	Dry cell mass (mg ml^{-1})	Nutrient components	Specific activity (kU mg^{-1})	Dry cell mass (mg ml^{-1})
Carbon source[b]			Yeast Extract[a]		
Starch (rice)	229.5±6.45	13.8±0.07	0.0	164.6±2.45	4.90±0.03
Starch (commercial)	208.6±4.89	10.6±0.05	5.0	241.3±7.01	17.5±0.08
Starch (potato)	274.0±7.32	28.2±1.19	10.0	274.0±7.32	28.2±1.19
Starch (sago)	206.8±4.21	9.50±0.05	15.0	210.0±4.54	24.7±0.09
Maltose	93.22±3.98	7.90±0.03	20.0	186.8±3.11	23.6±0.09
Starch (wheat)	200.0±4.01	11.0±0.05	Peptone[a]		
Sucrose	35.06±2.52	6.50±0.03	0.0	171.5±3.21	9.70±0.05
Wheat bran	15.45±1.23	14.0±0.07	5.0	274.0±7.32	28.2±1.19
Bagasse	5.810±0.05	9.50±0.05	10.0	259.0±6.75	16.6±0.08
Rice bran	25.98±1.91	10.5±0.05	15.0	210.3±4.23	19.7±0.09
Barley	75.61±3.23	8.70±0.04	20.0	191.6±3.75	20.0±0.09
Potato starch[a]			Trace elements[c]		
0.0	7.430±0.64	3.00±0.01	$MgSO_4$	274.0±7.32	28.2±1.19
5.0	205.2±4.14	13.0±0.07	K_2HPO_4	274.0±7.32	28.2±1.19
10.0	274.0±7.32	28.2±1.19	NaCl	122.8±3.23	15.4±0.08
15.0	233.5±6.98	10.8±0.05	$MnSO_4$	23.82±0.98	8.30±0.05
20.0	202.3±4.22	8.6±0.04	$CaCl_2$	30.53±1.02	15.0±0.09
25.0	98.57±3.98	7.4±0.04	Magnesium sulphate[a]		
Nitrogen source[b]			0.5	148.7±4.23	13.2±0.07
Peptone	259.0±6.75	16.6±0.08	1.0	274.0±7.32	28.2±1.19
Tryptone	109.0±3.23	11.0±0.07	1.5	132.5±3.78	10.2±0.05
Ammonium nitrate	18.60±1.11	5.00±0.03	2.0	102.6±1.11	7.50±0.04
Potassium nitrate	Nil	3.80±0.02	Dipotassium hydrogen phosphate[a]		
Yeast extract	274.0±7.32	28.2±1.19	0.5	225.7±4.54	19.7±0.09
Sodium nitrate	Nil	4.20±0.03	1.0	274.0±7.32	28.2±1.19
Urea	64.27±2.45	5.50±0.04	1.5	232.1±5.87	21.0±0.09
			2.0	185.1±3.23	18.7±0.09

Nil no enzyme activity; ± standard deviation; n=3

[a] g l^{-1}

[b] 10 g l^{-1}

[c] 1.0 g l^{-1}

concentrations may also be responsible for the suppression of amyloglucosidase yield due to excessive glucose formation that results in catabolite repression of enzyme substrate reaction.

Enzyme productivity and fungal growth was also greatly influenced by the type and concentration of nitrogen source used. Different organic and inorganic nitrogen sources were tested, and a combination of yeast extract (10 g l^{-1}) and peptone (0.5 g l^{-1}) exhibited maximum amyloglucosidase production as compared to inorganic nitrogen sources. Yeast extract is reported to have a stimulating effect on the growth of microbes, as it contains most of the essential amino acids and ammonium salts (Djekrif-Dakhmouche et al. 2006). However, high concentrations of yeast extract or peptone may have a negative impact on amyloglucosidase secretion, as both of these nutritive sources are also responsible for facilitating the secretion of various proteolytic enzymes that ultimately may result in proteolytic digestion of amyloglucosidase (Fogarty and Kelly 1990; Babu and Satyanarayana 1993). Dipotassium hydrogen phosphate and magnesium sulphate displayed a significant effect on enzyme secretion relative to other salts used. However, higher concentrations of both salts resulted in suppression of enzyme production. This effect was also reported by Shaku et al. (1980) and Yoon et al. (1989) for the secretion of protein by magnesium and inorganic phosphate.

In industrial enzymology, optimum growth conditions regulate the overall cost of upstream bioprocessing. Hence, in order to produce maximum amyloglucosidase yield, various physical parameters were optimized in a stepwise manner. Microbial incubation time is the most important physical factor affecting enzyme secretion. *A. fumigatus* KIBGE-IB33 was incubated for different time intervals, and maximum enzyme secretion was achieved after 4 days of fermentation (Fig. 1b). Thereafter, a gradual decline in enzyme production was observed. This may be due to the simultaneous secretion of secondary metabolites, which eventually alters the pH of the production medium. This finding suggests that, in parallel to the microbial growth, extra secretion of amyloglucosidase occurred during the late exponential phase of fungal growth. The metabolic rate of any microbial cell factory is directly influenced by the cultivation temperature, which is also responsible for regulating the secretion of extracellular metabolites, especially enzymes (Patil and Dayanand 2005). Optimum cultivation temperature for *A. fumigatus* KIBGE-IB33 was achieved at 30 °C. The selected temperature suggests the mesophilic nature of this organism as when the temperature was increased from 30 to 50 °C, a deleterious effect on fungal biomass and enzyme productivity was noted (Fig. 1c). Another important factor affecting the growth and metabolic activities of microorganisms is the pH of the cultivation medium. Microbes are sensitive to the concentration of hydrogen ions present in the fermentation broth, which also plays a critical role in determining the nature of the end-product. The secretion of an enzyme either under acidic or alkaline conditions will govern its application in different industries. Therefore, the pH of production medium was also studied with respect to amyloglucosidase secretion in both acidic and alkaline environments. Maximum enzyme secretion was achieved at a physiological pH of 7.0. However, *A. fumigatus* KIBGE-IB33 was capable of producing amyloglucosidase under both acidic and alkaline conditions (Fig. 1d). The secretion of amyloglucosidase at a wide range of pH levels renders *A. fumigatus* KIBGE-IB33 a plausible aspirant for commercial production. In addition, its starch depolymerization ability was determined with regard to utilization of this amyloglucosidase in starch bioprocessing. Its starch saccharification property was confirmed by the enzymatic treatment of potato starch, and thin-layer chromatography data revealed that amyloglucosidase efficiently saccharified potato starch into glucose (data not shown).

Low nutritional requirements, broad cultivation conditions, and high amyloglucosidase yield suggest strong applicability of *A. fumigatus* KIBGE-IB33 in several industrial sectors, whereas amyloglucosidase secreted by this organism has strong potential in starch saccharification processes.

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