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Optimization of Fermentation Parameters for β-Glucosidase Production by *Aspergillus niger*

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Abstract: β-glucosidase produced by *Aspergillus niger* AS 3.4309 was carried out under Solid State Fermentation (SSF). The effects of medium composition and incubation conditions on the enzyme activity were investigated and suitable solid medium components and optimum incubation conditions for fermentation process were therefore established. The maximum β-glucosidase production (508 U g^{-1} dry matter) occurred after 72 h of incubation at 28°C in optimized solid medium containing wheat bran 100%, ammonium chloride 2%, $MgSO_4 \cdot 7H_2O$ 0.5% and KH_2PO_4 1.5% at the optimal conditions including moisture content of solid substrate 70% (w/w) initial pH 6.0 and inoculum level at 1×10^7 spores g^{-1} of solid substrate.

Key words: β-glucosidase, *Aspergillus niger*, solid state fermentation, medium, condition, China

INTRODUCTION

The β-glucosidase (β-D-glucoside-o-glucohydrolase, EC.3.2.1.21) can hydrolyze β-glucosidic links of oligosaccharides and other glucosides conjugates such as soybean isoflavones which are phenolic compounds comprised of aglucones (Daidzein, genistein and glycitein), β-glucosides (Genistin, daidzin and glycitin) and β-glucosides conjugated with malonyl and acetyl groups. When soybean isoflavones were present as aglucones (Genistin, daidzin and glycitin) rather than as β-glucosides (Genistin, daidzin and glycitin), they have biological properties including oestrogenic, antioxidant and antitumoral activities (Liggins *et al*., 2000; Hsieh and Graham, 2001; Brouns, 2002). Fermentation technique, with reference to β-glucosidase influences the type and level of aglucones remaining in the final product (Brouns, 2002; Daroit *et al*., 2007; Elyas *et al*., 2010). Fermented soybean foods showed higher levels of aglucones (Daidzein and genistein) than non fermented soybean products i.e., non fermented products, retain high concentrations of the unhydrolysed daidzin and genistin (Ng *et al*., 2010).

Thereby, soybean used as a food and feed has many advantages for human and animal health because of the importance of its nutritional properties and the functional characteristics of the aglucones released from isoflavones compounds by β-glucosidase. The β-glucosidase has been found in plants (Ribeiro *et al*., 2007; Yu *et al*., 2007; Han and Chen, 2008) as well as in microorganism sources (Miura *et al*., 2002; Harnpicharnchai *et al*., 2009; Joo *et al*., 2010; Qian and Sun, 2009). β-glucosidase activity in soybean cultivars of different maturity groups ranged from 61.10-88.97 U g^{-1} of whole soybean flour on dry weight and soybean isoflavones could not be sufficiently hydrolyzed into aglucones by soybean endogenesis β-glucosidase (Pushalkar *et al*., 1995). Although, the apparent bioavailability of isoflavones in soy protein was very low, the aglycones (Genistein, daidzein and glycitein) were liberated primarily by the actions of β-glucosidase present in microbes. Accordingly, the β-glucosidases from microorganisms are of considerable interest to scientists because of the potential of this enzyme to enhance the nutritional value of soybean in food and feed industries.

Many bacteria, yeast and filamentous fungi have been shown to produce β-glucosidase. The bacteria like *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Bifidobacterium breve*, *Bifidobacterium thermophilum* and *Termitomyces clypeatus* could produce β-glucosidase (Choi *et al*., 2002; Pyo *et al*., 2005; Pal *et al*., 2010). Among yeasts, *Candida molischiana*, *C. wickerhamii* possessed activities towards various β-glucosides (Gunata *et al*., 1990). Fungi like *Aspergillus oryzae*, *A. niger*, *A. carbonarius* and *Penicillium purpurogenum* are reported to be the enzyme producers (Brumbauer *et al*., 2000; Tsao *et al*., 2000; Dhake and Patil, 2005; Zhang *et al*., 2007).

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Among those microbes, the most particular of industrial fungi with GRAS (Generally Regarded As Safe) status as enzyme producers is the *A. niger* due to its easily cultivated with agro-products under the processes of Submerged Fermentation (SmF) and Solid-State Fermentation (SSF). Some researcher showed that different strain of *A. niger* and various culture conditions could affect the β-glucosidase production. The maximum yield of β-glucosidase by *A. niger* KK2 mutant was 514 U g^{-1} ground rice straw in SmF (Yang and Ren, 2005) and 100 U g^{-1} rice straw in SSF (Hernandez *et al*., 2003) and the levels of β-glucosidase produced by a mutant strain of *A. niger* was 169.2 U g^{-1} under SSF (Yang and Ren, 2005). Although, higher enzyme level in SmF than in SSF system was observed (Kang *et al*., 2004), the latter process is often cited that enzyme production are higher than in SmF, when comparing the same strain and fermentation medium (Viniegra-Gonzalez, 1998). Accordingly, there is a significant interest in using SSF technique to produce a wide variety of enzymes, mainly from fungus origin (Viniegra-Gonzalez *et al*., 2003), due to the economical advantages over SmF and the possibility of using the cheap and abundant agro-products as substrates and the crude fermented product directly as the enzyme sources. Large amounts of agro-products are generated every year increasing the biotechnological interest on the utilization of these materials as substrates in SSF processes. Although, the β-glucosidase produced by *A. niger* have been studied, different strain under various fermentation techniques should be carried out to enhanced the enzyme activity.

The objective of the present investigation was to optimize the cultural parameters including media composition composed of some agro-byproducts and growth conditions for maximum production of β-glucosidase by *A. niger* AS 3.4309 in solid state fermentation. The effects of medium composition and incubation conditions on the enzyme activity were investigated and suitable solid medium composition and optimum incubation conditions for fermentation process were therefore established.

MATERIALS AND METHODS

Microorganism: A strain of *Aspergillus niger* AS 3.4309 maintained on potato dextrose agar slants at 4°C was used in the present study.

Materials: Wheat bran, rice bran, ground corncob, rice bran, glucose, sucrose, lactose, maltose, potato starch, dextrin, corn starch, cassava starch, cellulose, ammonium sulfate, ammonium nitrate, ammonium chloride, urea, peptone, yeast extract, soybean meal, cottonseed meal, rapeseed meal, $MgSO_4 \cdot 7H_2O$, KH_2PO_4 and other chemical reagents were all obtained locally and p-Nitrophenyl-β-D-Glucoside (pNPG), p-nitrophenol were from Sigma (St Louis, MO, USA).

Inoculum preparation: Inocula were prepared by transferring 1 mL spore suspension of *A. niger* AS 3.4309 from 72 h old wheat bran culture into 250 mL Erlenmeyer flasks containing 10 g of sterile solid substrate medium and cultivated at defined condition used to be optimized.

Optimization of solid state medium for β-glucosidase: Solid state medium composition affecting the β-glucosidase production by *A. niger* AS 3.4309 by SSF were studied such as Wheat bran, rice bran, ground corncob, rice bran, glucose, sucrose, lactose, maltose, potato starch, dextrin, corn starch, cassava starch, cellulose, ammonium sulfate, ammonium nitrate, ammonium chloride, urea, peptone, yeast extract, soybean meal, cottonseed meal, rapeseed meal. The experiments were conducted in 250 mL Erlenmeyer flasks containing 10 g solid medium supplemented with $MgSO_4 \cdot 7H_2O$ 0.5% and KH_2PO_4 1.5% dissolved in distilled water. Distilled water was added in such a way that the Initial Moisture Content (IMC) in medium was 70% (w/w). After sterilization by autoclaving at 121°C for 30 min, the medium were cooled to room temperature and inoculated with a 10^7 spores g^{-1} inoculum at 28°C for 72 h under various experimental conditions.

Optimization of incubation condition for β-glucosidase: In order to optimize the incubation conditions for β-glucosidase under SSF by *A. niger* AS 3.4309, the optimum solid medium with $MgSO_4 \cdot 7H_2O$ 0.5 and KH_2PO_4 1.5% dissolved in distilled water was adopted. Various incubation conditions (Initial moisture content initial pH incubation temperature and fermentation period) affecting enzyme activities were analyzed. The experiments were conducted in 250 mL Erlenmeyer flasks containing 10 g solid medium with a certain pH value and moisture content. After sterilization by autoclaving at 121°C for 30 min, the medium was cooled to room temperature and inoculated with the inoculum and incubated at a certain temperature for defined time under different experimental conditions.

Initial Moisture Content (IMC): The fermentation was carried out under various IMC (55, 60, 65, 70 and 75%). IMC were adjusted with distilled water. Other conditions were natural pH inoculum level at 10^7 spores g^{-1} solid medium and incubation for 72 h at 28°C.

Inoculum level: Various inoculum levels at 10^3, 10^5, 10^7 and 10^9 spores g^{-1} were used to evaluate their effects on enzyme activities. The fermentation was performed at 28°C for 72 h in optimum medium at its natural pH.

Initial pH: Different initial pH levels of the solid medium at 2, 3, 4, 5, 5.5, 6 and 7 adjusted with 1 mmol L^{-1} HCl or 1 mmol L^{-1} NaOH were employed to investigate their effects on β-glucosidase production in the optimum medium under optimum IMC and initial pH. The fermentation was carried out at 28°C for 72 h.

Incubation temperature: The fermentation was carried out at various temperature (24, 28, 30, 32 and 36°C) for 72 h to study their influence on enzyme production. All other conditions were kept at their optimum levels.

Fermentation period: Various incubation periods (12, 24, 36, 48, 60 and 72 h) were adopted and the fermentation was performed with all other parameters kept at their optimum levels.

Analytical methods

β-glucosidase assay: At the end of cultivation or at defined intervals, 0.2 g of dried fermented power were sampled and the enzyme was extracted with 10 mL citrate phosphate buffer (0.1 mol L^{-1}, pH 5.0) at 4°C for 3 h then the liquid was centrifuged (12,000×g for 5 min) and the supernatant was used as enzyme source. β-glucosidase activity was assayed by a modified procedure based on the method of Daroit *et al.* (2007). The p-Nitrophenyl-β-D-Glucoside (pNPG) dissolving in 0.1 mol L^{-1} citrate phosphate buffer (pH 5.0) was used as a substrate for assaying activity of β-glucosidase. β-glucosidase activity was analyzed at 45°C for 30 min with a mixture of 1.2 mL of citrate phosphate buffer (0.1 mol L^{-1}, pH 5.0), 0.4 mL of p-NPG (8 mmol L^{-1}) and 0.4 mL of enzyme solution. The reaction was stopped by adding 2 mL of cold sodium carbonate buffer (0.5 mol L^{-1}, pH 10). The activity of β-glucosidase was measured by reading the absorbance of the liberated p-Nitrophenol (pNG) at 400 nm. All the assays were performed in triplicate.

One Unit (U) of β-glucosidase was defined as the amount of enzyme required for the release of 1 μmol of pNG per minute, under the assay conditions.

Assay of moisture content: About 5 g of solid medium or fermented product were dried to constant weight at 40°C and the dry weight was recorded. The moisture content was calculated as follows:

$$\text{Mouisture content of solid medium or fermented product (\%)} = \frac{(\text{Wt. of solid medium or fermented product} - \text{Dry wt.}}{\text{Wt. of solid medium or fermented product}} \times 100$$

Calculation of dry matter weight loss and Fermentation Productivity (FP): The growth of microbe was evaluated by the method previously described by Terebiznik and Pilosof (1999). The dry matter weight loss was calculated as follows:

$$\text{Dry matter weight loss (\%)} = \frac{(\text{Dry wt. of initial solid substrate} - \text{Dry wt. of fermented product})}{\text{Dry wt. of initial solid substrate}} \times 100\%$$

The FP were calculated using the following equation:

$$\text{FP(\%)} = \frac{(\text{Dry wt. of fermented product})}{\text{Dry wt. of initial solid substrate}} \times 100\%$$

Determination of pH value: About 10 mL of distilled water were added into 1.0 g of medium and then it was agitated violently. After 15 min, the pH of the supernatant was measured with a pH meter.

Statistical analysis: One-way ANOVA followed by LSD post-hoc test was used to determine significant differences among treatment groups. For all analysis, differences were considered to be significant at $p<0.05$ (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Effects of wheat bran and ground corncob levels on β-glucosidase production: Six ratios of wheat bran to ground corncob (100/0, 90/10, 80/20, 70/30, 60/40 and 50/50/) were used to study their effects on the growth of *A. niger* and β-glucosidase activity (Fig. 1). When the ratios were 100/0, 90/10 and 80/20, microbes grew well and produced higher β-glucosidase. FP and enzyme activity were 61.54% and 215 U g^{-1} at 100/0 level, 62.53% and 203 at 90/10 level and 63.97% and 204 U g^{-1} at 80/20 level, respectively.

When wheat bran content decreased to 70-60% and ground corncob content increased to 30-40%, the microbial growth (FP 63.83-63.50%) was at the same level at 80/20 but enzyme production (186-173 U g^{-1}) dropped. When both wheat bran and ground corncob content were 50%, the FP was up to 70.79% and β-glucosidase activity declined to 149 U g^{-1}, significantly lower than those of other wheat bran levels. Wheat bran, as a solid support, loosened the solid medium and overcame the agglomeration of the substrate so, it was helpful for air diffusion. The results showed that the optimum ratios

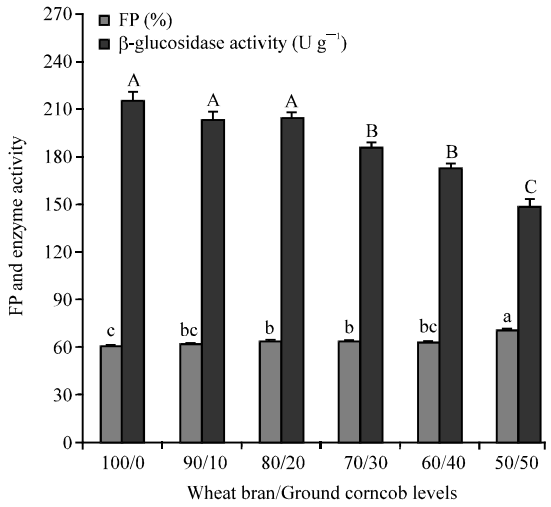

Fig. 1: Effects of wheat bran and ground corncob levels on β-glucosidase production; same pattern with various letters were significant differences ($p<0.05$)

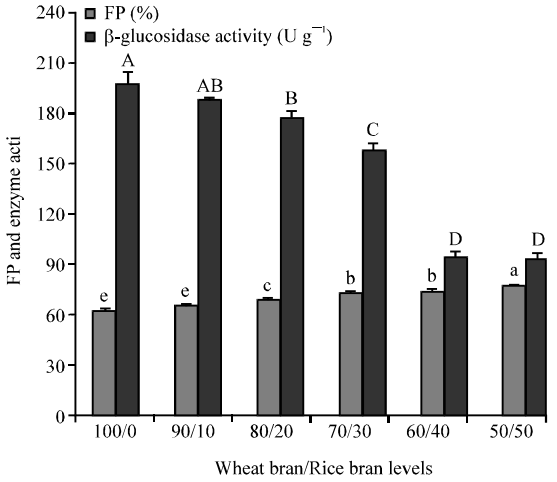

Fig. 2: Effects of wheat bran and rice bran levels on β-glucosidase production; same pattern with various letters were significant differences ($p<0.05$)

of wheat bran to ground corncob for β-glucosidase production in solid medium was 100/0, 90/10 or 80/20 and the high β-glucosidase activity could be achieved when using wheat bran alone. The result also was identical with the result given by Tsao *et al.* (2000) which *A. niger* NRRL3 was cultivated in a moist wheat bran and ground corncob solid medium, a high concentration of enzyme (215 U g^{-1} of solid substrate) was obtained after 96 h of incubation.

Effects of wheat bran and rice bran levels on β-glucosidase production: Rice bran as one of the most popular cereal by products was an easily available substrate like wheat bran in Asian. Six ratios of wheat bran to rice bran (100/0, 90/10, 80/20, 70/30, 60/40 and 50/50) were used to evaluate their effects on the growth of *A. niger* and enzyme production. As shown in Fig. 2, when the solid medium contained 100-90% wheat bran with 0-10% rice bran i.e., wheat bran to rice bran was 100/0 and 90/10, there was significant difference in FP at the levels of 62.80 and 65.42% ($p<0.05$), respectively but their enzyme activities reached the same levels, each of them was 197 and 189 U g^{-1}, respectively. With the wheat bran decreased or rice bran increased, enzyme activity declined and FP increased, the result was not identical with the reported by Kang *et al.* (2004) which β-glucosidase (94-107 U g^{-1}) activities were similar irrespective of the ratio of wheat bran and rice straw. Although, the ratios of wheat bran to rice bran at 90/10 and 80/20 had different effects on the growth of *A. niger*, no significant differences in β-glucosidase activity were observed. With the rice bran level rising from 30-50% and the corresponding decrease in wheat bran levels from 70-50%, the β-glucosidase production were dramatically descent from 159-93 U g^{-1} with their FP increasing from 73.56-78.16% although, there were no significant differences in FP between ratios 70/30 and 60/40 and in enzyme activity between ratios 60/40 and 50/50.

Mixed with wheat bran, rice bran enlarged valid surface and interspace among solid substrates causing better oxygen supply for the growth of *A. niger* and easier removal of CO_2 and heat generated during SSF but Higher rice bran level caused the increase in water loss due to quick volatilization during fermentation and hence inhibited the growth of microorganism, subsequently declined the enzyme production. The results concluded that <10% of rice bran level was enough for *A. niger* producing β-glucosidase by SSF.

Effects of various carbon sources on β-glucosidase production: The solid medium having wheat bran 100% was adopted to estimate the effects of various carbon sources on enzyme activity (Fig. 3). Some carbon sources including glucose, sucrose, lactose, maltose and potato starch each at the 0-5% level, did not have any significant effects on enzyme production compared with the control group (without any carbon source supplement). The results were not consistent with the *Acremonium persicinum* β-glucosidase, subject to carbon source control by readily metabolizable sugars (Pitson *et al.*, 1999). When dextrin, corn starch, cassava starch and cellulose were used as carbon source supplement, each at 0-5% level, they had inhibition influences on β-

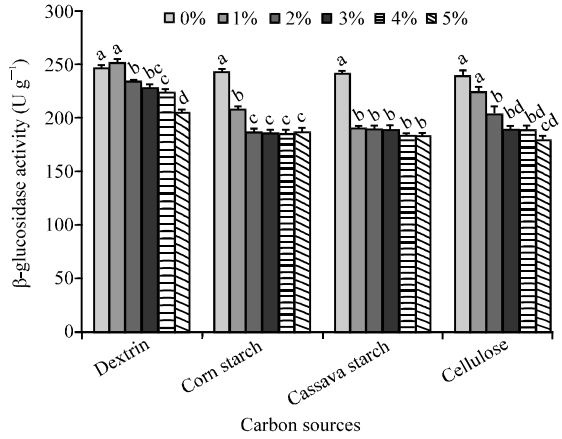

Fig. 3: Effects of various sources on enzyme production; same pattern with various letters were significant differences ($p<0.05$)

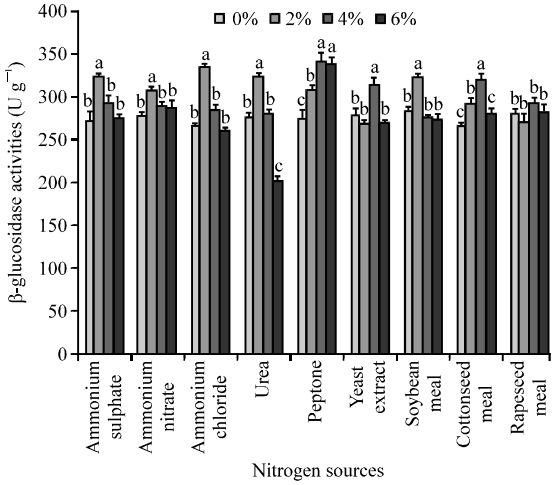

Fig. 4: Effects of various nitrogen sources on enzyme production; same pattern with various letters were significant differences ($p<0.05$)

glucosidase activities ($p<0.05$) on the whole although, no obvious negative effect was found at the present of 0.1% dextrin and 0.1% cellulose ($p>0.05$), respectively. In view of the economical efficiency of fermentation, wheat bran can meet the need for β-glucosidase production by SSF.

Effects of various nitrogen sources on β-glucosidase production: Solid medium was supplemented with different levels of nitrogen sources having inorganic and organic nitrogen at 0, 2, 4 and 6% as shown in Fig. 4. Results indicated that each of inorganic nitrogen sources including ammonium sulfate, ammonium nitrate, ammonium chloride and urea, each at 2%, improved the enzyme activities reaching at levels of 308-336 U g^{-1}. The

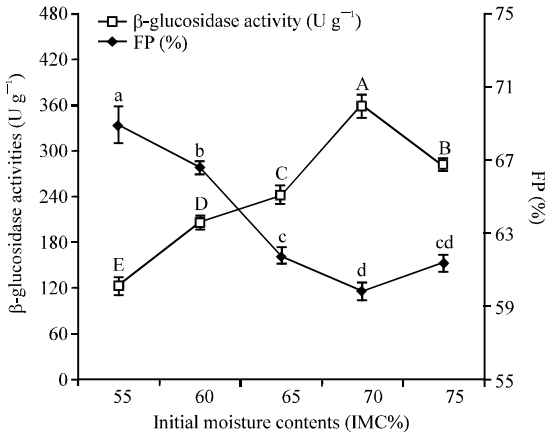

Fig. 5: Effects of Initial Moisture Content (IMC) on enzyme production and Fermentation Productivity (FP), dots of the same pattern with various letters were significant differences ($p<0.05$)

result was agreed with the report that ammonium sulfate was one of the best nitrogen sources for the production of enzymes by *A. niger* NCIM 1207 in submerged fermentation (Gokhale *et al*., 1991). However, the utilization of ammonium chloride did not support high β-glucosidase production by *Monascus purpureus* NRRL1992 on submerged fermentations (Daroit *et al*., 2007). With the rising of the inorganic nitrogen sources, β-glucosidase production deceased significantly ($p<0.05$) compared with the control group (without any nitrogen source supplement). When organic nitrogen sources containing peptone, yeast extract, soybean meal, cottonseed meal and rapeseed meal were used as nitrogen supplement, they had various effects on enzyme activities. Higher production of β-glucosidase (308-342 U g^{-1}) was optimally achieved when *A. niger* was grown in solid substance medium, containing 2-6% peptone, 4% yeast extract, 2% soybean meal or 4% cottonseed meal, respectively. Rapeseed meal had no influence on the enzyme activity ($p>0.05$) compared with the control group (without any nitrogen supplement).

These results indicated that the nitrogen sources at proper levels used here except rapeseed meal was the suitable for β-glucosidase biosynthesis by *A. niger* and also exhibited that the solid substrate medium composed of wheat bran alone can not meet the demand of nitrogen for β-glucosidase biosynthesis during SSF.

Effects of Initial Moisture (IMC) Contents on β-glucosidase production: Moisture content of solid medium is a very important factor affecting SSF. The effect of IMC on β-glucosidase activity was shown in Fig. 5. Below 70% IMC, the medium fermentation by

A. niger was incomplete, resulting in significant increase in FP ($p<0.05$) and decrease in β-glucosidase activity ($p<0.05$). At 70% level, the FP and enzyme activity were 59.87% and 358 U g^{-1}. The best moisture for β-glucosidase production from solid substrate by *A. niger* NRRL3 was also found to be 70% (Tsao *et al*., 2000). Above 70% IMC with further increased moisture, β-glucosidase activity ($p<0.05$) decreased in spite of the little effect on the growth of *A. niger* ($p>0.05$). The optimal IMC for *A. niger* growth and β-glucosidase activity appeared to be 70%. The low moisture content may reduce the solubility of nutrients of the substrate and degree of swelling increase water loss due to quick volatilization during fermentation and hence inhibite the growth of microorganism. Higher moisture content caused the agglomeration of the substrate, subsequently restricted the supply of oxygen for microorganism growth to some extent and decreased the valid inner pinholes for the vent of CO_2 and transfers of heat and oxygen which was beneficial for growth of microorganism among particles in solid substrate (Lonsane *et al*., 1985).

Inoculum level: Inoculum level was also an important factor for β-glucosidase production. The effects of inoculum level on β-glucosidase production by SSF were shown in Fig. 6. When the medium were mixed with the inoculum of *A. niger* AS 3.4309 at the level of 1×10^3 spores g^{-1} of solid state medium, the β-glucosidase activity was low (73 U g^{-1}) and FP was 80.63%. With the spore concentration increasing, the yield of β-glucosidase was enhanced while FP was decreased. As the spore concentration was up to 10^7 cell g^{-1}, the β-glucosidase production reached the maximum of 355 U g^{-1} and FP decreased to 62.67%. A further increase in spore concentration did not affect the β-glucosidase production ($p>0.05$) and FP ($p>0.05$). This result suggested that the optimum inoculum level was 10^7 cell g^{-1} of solid state medium.

Effects of initial pH on β-glucosidase production: The effect of initial pH on β-glucosidase production by SSF was shown in Fig. 7. Because the metabolic activities of microorganism were very sensitive to pH changes, the optimum initial pH for β-glucosidase production and FP was at 6.0. This result was different from some reports given by researchers who found that 3.0-5.5, 4.0-5.0 and 4.0 were the optimum pH for the production of enzymes by *A. niger* NCIM 1207 in submerged fermentation (Gokhale *et al*., 1991). The β-glucosidase production by *A. niger* AS 3.4309 was found to be affected when pH level was deviated from the optimum value (Fig. 7). There was poor growth of *A. niger* AS 3.4309 and low

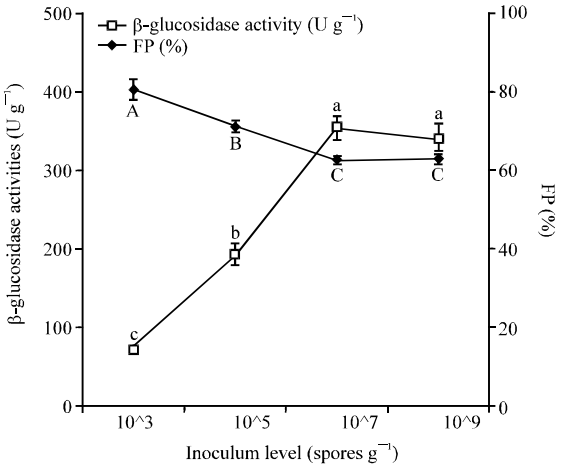

Fig. 6: Effects of inoculum level on enzyme production and Fermentation Productivity (FP), dots of the same pattern with various letters were significant differences ($p<0.05$)

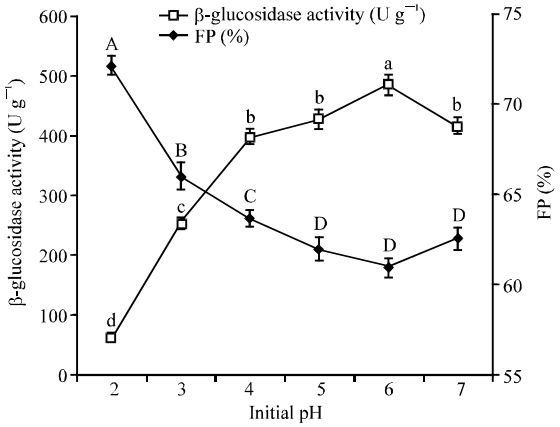

Fig. 7: Effects of initial pH on enzyme production and Fermentation Productivity (FP); dots of the same pattern with various letters were significant differences ($p<0.05$)

β-glucosidase production at pH 2.0. The β-glucosidase production increased to 251, 397, 426 and 482 U g^{-1} at pH 3.0, 4.0, 5.0 and 6.0, respectively. Meanwhile the FP decreased to 66.02, 63.66, 61.98 and 60.97%, respectively. Further increasing pH, β-glucosidase production and the growth of *A. niger* AS 3.4309 declined to 414 U g^{-1} and 62.57%, respectively. These results indicated there was no need for the solid medium to adjust the pH of the solid medium since its inherent initial pH 6.0 at which was optimum for *A. niger* AS 3.4309 growth and producing β-glucosidase.

Effects of incubation temperatures on β-glucosidase production: Incubation temperature had significantly

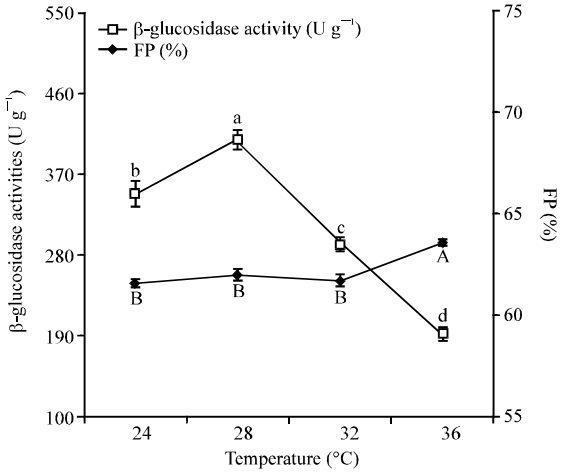

Fig. 8: Effects of incubation temperatures on enzyme production and Fermentation Productivity (FP), dots of the same pattern with various letters were significant differences ($p<0.05$)

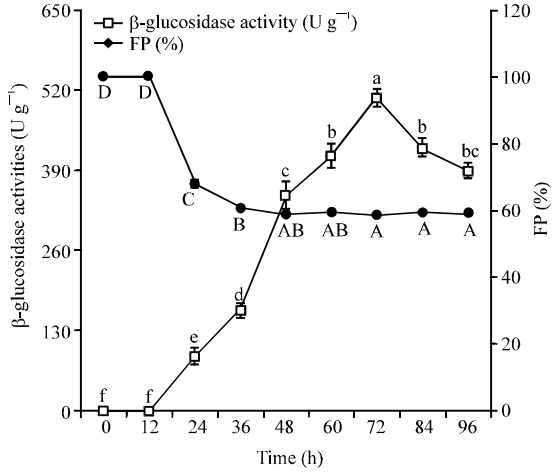

Fig. 9: Time course of solid-state fermentation by *A. niger*; The solid medium was kept at 28°C for different periods of fermentation. Samples of the fermented substrate were dried at 40°C and then were analyzed for contents of dry matter weight loss of solid substrate and enzyme activities. Dots of the same pattern with various letters were significant differences ($p<0.05$)

influences on β-glucosidase production (Fig. 8). When *A. niger* were cultivated at the temperature from 24-36°C the Fermentation Productivities (FP) were in the range of 60.51-63.51% displaying a little better growth at 24-32°C and no significant differences between 24, 28 and 32%($p<0.05$). Although, these temperatures were favorable for stable cultivation in solid medium, the optimal temperature for β-glucosidase biosynthesis (408 U g^{-1}) was found to be 28°C which was in accord with the condition for β-glucosidase production from solid substrate by *A. niger* NCIM 1207 (Gokhale *et al*., 1991) but different from the temperature (35°C) for *A. niger* NRRL3 and *A. niger* SA58 (Elyas *et al*., 2010; Tsao *et al*., 2000). Temperatures deviate from the optimum resulted in the decrease of enzyme production, especial in higher temperatures at 32 and 36°C and β-glucosidase activity was markedly lower at 32-36% than at 24°C.

Changes of microbial growth and β-glucosidase production during SSF: In this experiment, the Fermentation Productivity (FP) was used to estimate biomass growth according to a previous report that dry matter weight loss was highly correlated with the biomass (Weng and Sun, 2006). Results showed that the β-glucosidase production and the growth of *A.niger* were different with the changes of incubation time (Fig. 9). At the beginning of fermentation (0-12 h), the spores germinated resulting in near 100% of FP due to no changes in the dry matter weight loss and no enzyme activities were found. During 12-36 h fermentation, *A. niger* grew fast, resulting in rapid increase of dry matter weight loss in other words in marked decreased of FP. With the fermentation course prolonged, the FP decreased but there were no significant difference in the FP ($p>0.05$) between 48, 60, 72, 84 and 96 h incubation. About 41% of initial dry matter weight i.e., its corresponding 59% FP was found at the end of fermentation. β-glucosidase production by *A. niger* depended on the growth of microorganism, i.e. Fermentation Productivity (FP). With *A. niger* growing quickly or with the FP falling rapidly, β-glucosidase production was biosynthesized promptly. At 24 h of fermentation only 89 U g^{-1} was produced. When the strain of *A. niger* was cultivated from 24-72 h, large amount of β-glucosidase was produced by *A. niger* and enzyme reached to the maximal peak (508 U g^{-1}) at 72 h. This best cultivation time for the *A. niger* AS 3.4309 β-glucosidase was distinctly different from the same enzyme reported from other sources where *A. niger* NRRL3 was able to produce a high concentration of enzyme (215 U g^{-1} of solid substrate) after 96 h of incubation from solid substrate (Tsao *et al*., 2000), the maximum yield of β-glucosidase by *A. niger* KK2 mutant, grown on the basal medium for 7 days, was 514 I U g^{-1} ground rice straw (Hernandez *et al*., 2003) and the production of β-glucosidase by *A. terreus* reached the peak in liquid shake cultures on the 7th day of growth (2.18 U mL^{-1}) (Pushalkar *et al*., 1995). Further increase of incubation time from 72-96 h, the levels of β-glucosidase declined to 390 U g^{-1}. In view of the fermentation period shown in Fig. 9, the course could be divided into early-stationary phase, logarithmic growth phase, steady phase and decline phase according to FP and enzyme production.

CONCLUSION

Filamentous fungi are suitable microorganisms for Solid State Fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate. The present research demonstrated that the production of β-glucosidase could be affected by the solid medium and incubation condition used in fermentation. The results showed that *A. niger* AS 3.4309 could be cultivated under SSF for the β-glucosidase biosynthesis using wheat bran as major solid substrate. Some medium components and fermentation parameters were found to play very significant roles in enhancing the growth of microbes and the production of β-glucosidase.

The optimal solid medium was determined to be 90-100% wheat bran, 0-10% rice bran, a small quantity of nitrogen sources including inorganic source (one of ammonium sulfate, ammonium nitrate, ammonium chloride or urea at 2% level, respectively) or organic source (2-6% peptone, 4% yeast extract, 2% soybean meal or 4% cottonseed meal, respectively), 0.5% $MgSO_4 \cdot 7H_2O$ and 1.5% KH_2PO_4. The optimal conditions turned out to be 70% Initial Moisture Content (IMC) of solid substrate initial pH 6.0 inoculum level at 1×10^7 spores/g of solid substrate incubation temperature at 28°C and incubation time 72 h. Under these optimum conditions, the β-glucosidase production in solid substrate by *A. niger* AS 3.4309 reached the maximum of 508 U g^{-1} dry matter. It could be concluded that *A. niger* AS 3.4309 was able to produce a higher activity of β-glucosidase in SSF by using agro-products and had great potential for producing β-glucosidase in enzyme fermentation. This was a significant advantage from the viewpoint of practical application to food and feed industry.

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REFERENCES

Brouns, F., 2002. Soya isoflavones: A new and promising ingredient for the health foods sector. Food Res. Int., 35: 187-193.

Brumbauer, A., G. Johansson and K. Reczey, 2000. Study on heterogeneity of -glucosidase from *Aspergillus* species by using counter-current distribution. J. Chromatogr. B: Biomed. Sci. Appl., 743: 247-254.

Choi, Y.B., K.S. Kim and J.S. Rhee, 2002. Hydrolysis of soybean isoflavone glucosides by lactic acid bacteria. Biotechnol. Lett., 24: 2113-2116.

Daroit, D.J., S.T. Silveira, P.F. Hertz and A. Brandelli, 2007. Production of extracellular β-glucosidase by *Monascus purpureus* on different growth substrates. Process Biochem., 42: 904-908.

Dhake, A.B. and M.B. Patil, 2005. Production of β-glucosidase by *Penicillium purpurogenum*. Braz. J. Microbiol., 36: 170-176.

Elyas, K.K., A. Mathew, R.K. Sukumaran, P.P. Ali, K. Sapna, S.R. Kumar and K.R. Mol, 2010. Production optimization and properties of beta glucosidases from a marine fungus *Aspergillus*-SA 58. N. Biotechnol., 27: 347-351.

Gokhale, D.V., S.G. Patil and K.B. Bastawde, 1991. Optimization of cellulase production by *Aspergillus niger* NCIM 1207. Applied Biochem. Biotechnol., 30: 99-109.

Gunata, Y.Z., C.L. Bayonove, R.E. Cordonnier, A. Arnaud and P. Galzy, 1990. Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wickerhamii* β-glucosidases. J. Sci. Food Agric., 50: 499-506.

Han, Y. and H. Chen, 2008. Characterization of β-glucosidase from corn stover and its application in simultaneous saccharification and fermentation. Bioresour. Technol., 99: 6081-6087.

Harnpicharnchai, P., V. Champreda, W. Sornlake and L. Eurwilaichitr, 2009. A thermotolerant â-glucosidase isolated from an endophytic fungi, *Periconia* sp., with a possible use for biomass conversion to sugars. Protein Expression Purif., 67: 61-69.

Hernandez, L.F., J.C. Espinosa, M. Fernandez-Gonzalez and A. Briones, 2003. β-Glucosidase activity in a *Saccharomyces cerevisiae* wine strain. Int. J. Food Microbiol., 80: 171-176.

Hsieh, M.C. and T.L. Graham, 2001. Partial purification and characterization of a soybean â-glucosidase with high specific activity towards isoflavone conjugates. Phytochemistry, 58: 995-1005.

Joo, A.R., M. Jeya, K.M. Lee, K.M. Lee, H.J. Moon, Y.S. Kim and J.K. Lee, 2010. Production and characterization of β-1,4-glucosidase from a strain of *Penicillium pinophilum*. Process Biochem., 45: 851-858.

Kang, S.W., Y.S. Park, J.S. Lee, S.I. Hong and S.W. Kim, 2004. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour. Technol., 91: 153-156.

Liggins, J., L.J.C. Bluck, S. Runswick, C. Atkinson, W.A. Coward and S.A. Bingham, 2000. Daidzein and genistein content of vegetables. Br. J. Nutr., 84: 717-725.

Lonsane, B.K., N.P. Ghildyal, S. Budiatman and S.V. Ramakrishna, 1985. Engineering aspects of solid state fermentation. Enzyme Microb. Technol., 7: 258-265.

Miura, T., L. Yuan, B. Sun, H. Fujii, M. Yoshida, K. Wakame and K. Kosuna, 2002. Isoflavone aglycon produced by culture of soybean extracts with basidiomycetes and its anti-angiogenic activity. Biosci. Biotechnol. Biochem., 66: 2626-2631.

Ng, I.S., C.W. Li, S.P. Chan, J.L. Chir and P.T. Chen *et al*., 2010. High-level production of a thermoacidophilic β-glucosidase from *Penicillium citrinum* YS40-5 by solid-state fermentation with rice bran. Bioresour. Technol., 101: 1310-1317.

Pal, S., S.P. Banik, S. Ghorai, S. Chowdhury and S. Khowala, 2010. Purification and characterization of a thermostable intra-cellular β-glucosidase with transglycosylation properties from filamentous fungus *Termitomyces clypeatus*. Bioresour. Technol., 101: 2412-2420.

Pitson, S.M., R.J. Seviour and B.M. McDougall, 1999. Induction and carbon source control of extracellular β-glucosidase production in *Acremonium persicinum*. Mycol. Res., 103: 161-167.

Pushalkar, S., K.K. Rao and K. Menon, 1995. Production of β-glucosidase by *Aspergillus terreus*. Curr. Microbiol., 30: 255-258.

Pyo, Y.H., T.C. Lee, Y.K. Rhee and Y.C. Lee, 2005. Hydrolysis of isoflavone glycosides in soymilk fermented with some bacterial strains. Food Res. Int., 38: 551-559.

Qian, L.C. and J.Y. Sun, 2009. Effects of β-glucosidase as a feed supplementary on the growth performance, digestive enzymes and physiology of broilers. Asian-Aust. J. Anim. Sci., 22: 260-266.

Ribeiro, M.L.L., J.M.G. Mandarino, M.C. Carrao-Panizzi, M.C.N. de Oliveira, C.B.H. Campo, A.L. Nepomuceno and E.I. Ida, 2007. Isoflavone content and β-glucosidase activity in soybean cultivars of different maturity groups. J. Food Comp. Anal., 20: 19-24.

Steel, R.G.D. and J.H. Torrie, 1980. Principle and Procedure of Statistics. A Biochemical Approach. 2nd Edn., McGraw-Hill Book Company, New York.

Terebiznik, M.R. and A.M.R. Pilosof, 1999. Biomass estimation in solid state fermentation by modeling dry matter weight loss. Biotechnol. Tech., 13: 215-219.

Tsao, G.T., L. Xia, N. Cao and C.S. Gong, 2000. Solid-state fermentation with *Aspergillus niger* for cellobiase production. Applied Biochem. Biotechnol., 84-86: 743-749.

Viniegra-Gonzalez, G., 1998. Strategies for the Selection of Mold Strains Geared to Produce Enzymes on Solid Substrates. In: Advances Bioprocess Engineer II, Galiendo, E. and O.T. Ramirez (Eds.). Kluwer Academic Publishers, Dordrecht, Boston, pp: 123-126.

Viniegra-Gonzalez, G., E. Favela-Torres, C.N. Aguilar, S.D. Romero-Gomez, G. Diaz-Godinez and C. Augur, 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem. Eng. J., 13: 157-167.

Weng, X.Y. and J.Y. Sun, 2006. Biodegradation of free gossypol by a new strain of *Candida tropicalis* under solid state fermentation: Effects of fermentation parameters. Process Biochem., 41: 1663-1668.

Yang, J.H. and D.M. Ren, 2005. β-glucosidase-producing strain of breeding and solid fermentation conditions. J. Anhui Agric. Sci., 33: 1566-1568.

Yu, H.L., J.H. Xu, W.Y. Lu and G.Q. Lin, 2007. Identification, purification and characterization of β-glucosidase from apple seed as a novel catalyst for synthesis of O-glucosides. Enzyme Microbial Technol., 40: 354-361.

Zhang, C., D. Li, H. Yu, B. Zhang and F. Jin, 2007. Purification and characterization of piceid-β-d-glucosidase from *Aspergillus oryzae*. Process Biochem., 42: 83-88.