β-Glucosidase production by mixed culture using crude hemicellulose from rice straw black liquor and peat moss as an inert support

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Background and objectives
Rice straw black liquor (RSBL) has no commercial significance and causes environmental pollution because of its poor disposal, although it holds promise as a cheap substrate for the production of β-glucosidase. The objective of the present work was to improve and optimize the cultural conditions of mixed cultures of *Trichoderma reesei* NRRL 6165 and *Aspergillus niger* NRC 9A under solid-state fermentation (SSF) for the production of β-glucosidase using crude hemicellulose (CHC) prepared from RSBL and peat moss as an inert support.

Materials and methods
Mixed cultures of the mycelial fungi *T. reesei* NRRL 11236 and *T. reesei* NRRL 6165 and *A. niger* strains (NRC 5A, NRC 7A, and NRC 9A) were evaluated for their ability to produce β-glucosidase using CHC prepared from RSBL and peat moss as an inert support under SSF. Optimization of initial pH (4–8), supplementation with different concentrations of corn steep liquor and ammonium sulfate, the initial moisture content (63.6–87.4% v/w), inoculum size and ratio of microorganisms, CHC/peat moss ratio, and incubation time (0–14 day) were studied. The extracted enzyme was assayed using p-nitrophenyl-β-d-glucopyranoside as a substrate. Data analysis was performed by one-way analysis of variance using computer software Minitab 16.

Results and conclusion
Mixed cultures of the mycelial fungi *T. reesei* NRRL 11236 and *T. reesei* NRRL 6165 and *A. niger strains* (NRC 5A, NRC 7A, and NRC 9A) were evaluated for their ability to produce β-glucosidase using CHC prepared from RSBL and peat moss as an inert support under SSF. The most potent coculture composed of *A. niger* NRC 9A (192.39 ± 7.37 U/g CHC) and *T. reesei* NRRL 6165 (12.56 ± 0.42 U/g CHC) was used in a mixed culture to enhance β-glucosidase production by coculturing under SSF. In mixed culture, β-glucosidase of the coculture (265.32 ± 0.25 U/g CHC) was nearly 1.3-fold and 10.6-fold than that of monocultures of *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively. Optimization of the environmental and culture parameters, different solid supports, concentration of nitrogen sources (ammonium sulfate and corn steep liquor), initial pH value, CHC/peat moss ratio, inoculum size and ratios of the two strains, moisture content, and incubation time exhibited a significant increase (466 ± 5.42 U/g CHC) in β-glucosidase production compared with before optimization. The study demonstrated that a substrate that does not find any commercial significance and causes environmental pollution because of its poor disposal holds promise as a cheap substrate for the production of β-glucosidase.

Keywords:
Aspergillus niger, crude hemicellulose, β-glucosidase, mixed culture, peat moss, solid-state fermentation, *Trichoderma reesei*

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Introduction

Rice straw black liquor (RSBL) represents a large potential bioresource for producing bio-based chemicals, materials, and energy. It contains a variety of components, including undigested crude fiber, lignin, hemicelluloses, silica, and minerals [1]. Although there have been some reports about the recovery of black liquor components by chemical treatments (mainly hemicelluloses, silica, and lignin) [2], limited efforts have been focused on the microbial utilization of the hemicellulose of RSBL [3]. Hemicelluloses mainly comprise hexoses and pentoses; the major component is hexoses, whereas pentoses constitute the minor part. Hexoses are composed of mannose, rhamnose, galactose, and glucose, whereas pentoses consist of arabinose and xylose. The efficiency of cellulose hydrolysis requires the synergistic action of a cellulase system containing the following: endocellulase (EC 3.2.1.4), which cleaves internal glycosidic bonds; exoglucanase (EC 3.2.1.91), which cuts the cellulose chain from either the reducing or the nonreducing end; and β-glucosidase (β-d-glucoside glucohydrolase, EC 3.2.1.21), which hydrolyzes cellobiose to produce glucose [4]. A high

level of β-glucosidase is then necessary to avoid the accumulation of cellobiose, which is a strong inhibitor of endocellulase and exoglucanase. β-Glucosidases are a group of enzymes mainly involved in the hydrolysis of β-glycosidic bonds connecting carbohydrate residues in different classes of β-d-glycosides such as aryl, alkyl and amino-β-d-glycosides, short oligosaccharide chains, and disaccharides. They have been regarded as a component of the cellulase system. Even though they are not directly acting on cellulose, they convert cellobiose and cello-oligosaccharides produced by the endoglucanase and exoglucanase to glucose [5]. This deficiency in β-glucosidase is common to most strains of *Trichoderma* spp. and several approaches have been attempted to overcome this deficiency. For example, a temperature and pH cycling strategy was applied to the culture *Trichoderma reesei* RUT-C30 to increase β-glucosidase production [6]. In another study, the mutant *Trichoderma* E12 was grown on microcrystalline cellulose with peanut cake used as a nitrogen source to obtain a high C/N ratio. As a result, a well-balanced ratio of β-glucosidase activity to filter paper activity was observed [7]. *Trichoderma* spp. could also be cocultured with fungi such as *Aspergillus niger* [8], *Aspergillus phoenicis* [9], and *Aspergillus oryzae* [10], which are good β-glucosidase producers.

The objective of the present work was to improve and optimize the culture conditions of *T. reesei* NRRL 6165 and *A. niger* NRC 9A under solid-state fermentation (SSF) for the production of β-glucosidase using crude hemicellulose (CHC) prepared from RSBL and peat moss as an inert support.

Materials and methods

Microorganisms and culture maintenance

A. niger NRC 5A, *A. niger* NRC 7A, and *A. niger* NRC 9A were obtained from the stock culture of the Natural and Microbial Products Department, National Research Center (Cairo, Egypt). *T. reesei* NRRL 11236 and *T. reesei* NRRL 6165 were kindly obtained from Northern Regional Research Laboratory (NRRL, Peoria, Illinois, USA). Cultures were maintained on potato dextrose agar slants and stored at 4°C in a cold cabinet and transplanted into fresh slants every 2 weeks.

Inoculum preparation

The spores from a fully sporulated fungal strain slant grown on potato dextrose agar slants at 28°C for 7 days were dispersed in 3 ml of sterile distilled water by dislodging them with a sterile loop under aseptic conditions. The spore suspension was used as inoculum in each 250 ml Erlenmeyer flask containing the solid medium. For pure culture of either *A. niger* strains or *Trichoderma* strains, each flask containing 4 g (2 g CHC and 2 g peat moss) of dry substrate was inoculated with 4 ml spore suspension (10^6–10^7 spores/ml). Spore count was measured by the dilution plate count method [11]. Unless otherwise stated, for mixed culture, 2 ml of each strain was inoculated into each flask simultaneously.

Preparation of crude hemicellulose from rice straw black liquor

RSBL (pH 12) obtained from the Nobaria rice straw pulping mill [12] was treated with CaO (18 g/l) while stirring for 20 min, and left to stand overnight. The obtained precipitate was filtered and discarded while the effluent was acidified with H_2SO_4 (50% v/v) to pH 2–3. The precipitate was then washed several times with cold tap water to remove the excess sulfate and air-dried overnight, followed by oven drying at 80°C overnight, to remove the moisture content and obtain constant weight [13]. The obtained brown precipitate affords the crude polysaccharides with the following constituents: natural detergent fiber, 47%; acidic detergent fiber, 21.4%; and acidic detergent lignin, 17.72%.

Screening for β-glucosidase activity

Three strains of *A. niger* (NRC 5A, NRC 7A, and NRC 9A) and two strains of *Trichoderma* spp. (*T. reesei* NRRL 11236 and *T. reesei* NRRL 6165) were used for the primary screening for their ability to grow and produce β-glucosidase activity in a solid medium containing CHC as the main carbon source and peat moss (nitrogen, 0.5–1.5%; carbon, 53–55%; phosphorus, 0.02–0.3%; potassium, 0.015%; ash, 5%) [14] as an inert support (2 g/flask) and impregnated with mineral medium (g/l) containing the following: corn steep liquor (CSL), 5 ml (40% w/v); $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2; $CaCl_2$, 0.5; $MgSO_4$, 0.3; $FeSO_4$, 0.005; $MnSO_4$, 0.002; and $ZnSO_4$, 0.0014 (pH 5.5). Flasks were autoclaved and inoculated with 4 ml of spore suspension previously prepared as mentioned above. Unless otherwise stated, for mixed culture, 2 ml of each strain was inoculated into each flask simultaneously. The cultures were incubated for 10 days at 28–30°C. All experiments were run in parallel in duplicate and average values were reported.

Screening of solid supports for solid-state fermentation

Different solid supports (urethane, vermiculite, Avicel, cotton fiber, filter paper, peat moss, and rice straw as a control) were used as solid materials with or without CHC for the production of β-glucosidase under SSF.

SSF was carried out in 250 ml Erlenmeyer flasks, each having 2 g of each dry solid support material with or without 2 g of CHC as control and moistened with 8 ml of liquid medium (g/l) containing the following: CSL, 5 ml (40% w/v); $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2; $CaCl_2$, 0.5; $MgSO_4$, 0.3; $FeSO_4$, 0.005; $MnSO_4$, 0.002; and $ZnSO_4$, 0.0014 (pH 5.5); finally, distilled water was added to attain a final solid-to-moisture ratio of 80% (w/v). The flasks were sterilized by autoclaving at 120°C (15 psi) and thereafter cooled to room temperature and inoculated with the desired volume of inocula.

β-Glucosidases production under solid-state fermentation

SSF was carried out in 250-ml Erlenmeyer flasks, each having 2 g of CHC with 2 g of dry peat moss moistened with distilled water, supplemented with mineral salts (8 ml of the above-mentioned liquid medium) to the required moisture content and pH 5.5 and mixed thoroughly. The flasks were sterilized by autoclaving at 120°C (15 psi) and thereafter cooled to room temperature and inoculated with the desired volume of inocula. Unless otherwise stated, the moisture content of the CHC after pretreatment and supplementation with peat moss and addition of nutrients and inocula was 80% (w/w) in SSF. Sterilized water was added if required to obtain the desired moisture content of the substrate in the fermentation medium. The contents of the flasks were mixed well under aseptic conditions with a sterilized glass rod to distribute the inoculum throughout the substrate, and incubated at 28–30°C.

Optimization of process parameters for β-glucosidase production

The medium described above was taken as the basal medium and the process parameters under study were varied. Incubation time (0–14 day), supplementation with different concentrations of nitrogen sources (ammonium sulfate, CSL), different ratios of CHC and peat moss, inoculum concentration and ratios (spore concentration ranging from 0.5×10^6–10^7 and 3.5×10^6–10^7 spores for *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively), initial pH of the moistening medium (4–8), and initial moisture content (63.6–87.4% v/w) were optimized for β-glucosidase production. The various process parameters influencing β-glucosidase production were optimized by evaluating the effect of each parameter (keeping all other parameters constant) and incorporating it at the optimized level in the experiment before optimizing the next parameter. All experiments were carried out in duplicate and the mean values are reported.

Enzyme extraction

At the end of the incubation period, unless otherwise stated, 20 ml aliquot of sodium phosphate buffer (0.05 mol/l, pH 6.0) was added to each flask and the mixture was incubated at 30°C on an orbital shaker, at 200 rpm, for 30 min. The suspended slurry was filtered by squeezing through a wet muslin cloth and centrifuged at 4000 rpm for 20 min. The clear filtrate thus obtained was used for β-glucosidase. Each batch was prepared in duplicate and average values plus %SDs of the mean were obtained.

Assay of β-glucosidase activity

β-Glucosidase activity was performed using p-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate according to De Vries *et al.* [15] with some modifications. One unit of β-glucosidase is defined as 'the amount of enzyme that catalyzes the hydrolysis of pNPG to liberate 1.0 mmol/l of p-nitrophenol in 1 min under standard assay conditions'. It is expressed as U/g CHC, where g CHC is the gram of initial CHC used for growth.

Data analysis

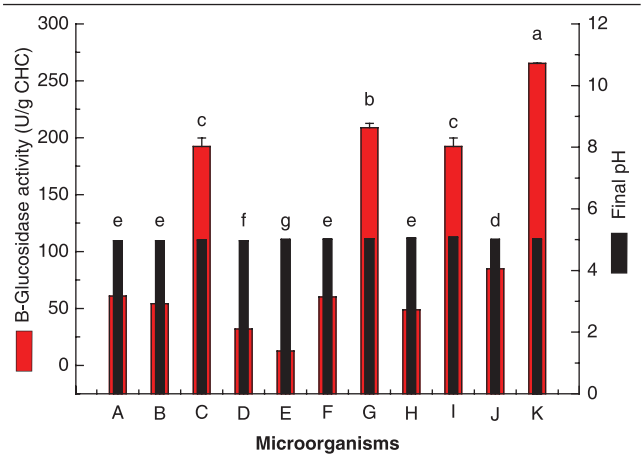
Treatment effects were analyzed, the mean comparison was performed by one-way analysis of variance using computer software Minitab 16 (Minitab Inc., State College, Pennsylvania, USA), and the average values were reported. Significant differences among the replicates have been presented at the 95% confidence level ($P \leq 0.05$).

Results and discussion

Screening of β-glucosidase producing *Aspergillus* and *Trichoderma* strains

In the present study, screening of different local *A. niger* strains, *T. reesei* NRRL 11236, and *T. reesei* NRRL 6165 and their mixed cultures for production of β-glucosidase activities by SSF was carried out using CHC previously prepared from RSBL and with peat moss as an inert support incorporated in the basal salt medium (Fig. 1). After an incubation period of 10 days, *A. niger* strains (*A. niger* NRC 5A, *A. niger* NRC 7A, and *A. niger* NRC 9A) generally produced more β-glucosidase (60.82 ± 2.87, 54.20 ± 1.20, and 192.38 ± 7.37 U/g CHC, respectively) compared with *Trichoderma* strains *T. reesei* NRRL 11236 (31.83 ± 0.51 U/g CHC) and *T. reesei* NRRL 6165 (12.56 ± 0.42 U/g CHC). Sørensen *et al.* [4] reported that β-glucosidases are widely produced by different genera and species of the fungal kingdom, including Ascomycetes and Basidiomycetes, where especially the Ascomycete genus *Aspergillus* has been widely studied for β-glucosidase production. *A. niger* has been setting

Figure 1



Screening of monoculture of *Aspergillus niger* strains, *Trichoderma reesei* NRRL 11236, and *T. reesei* NRRL 6165 and their cocultures for production of β-glucosidase by solid-state fermentation. A, *A. niger* NRC 5A; B, *A. niger* NRC 7A; C, *A. niger* NRC 9A; D, *T. reesei* NRRL 11236; E, *T. reesei* NRRL 6165; F, *A. niger* NRC 5A and *T. reesei* NRRL 11236; G, *A. niger* NRC 5A and *T. reesei* NRRL 6165; H, *A. niger* NRC 7A and *T. reesei* NRRL 11236; I, *A. niger* NRC 7A and *T. reesei* NRRL 6165; J, *A. niger* NRC 9A and *T. reesei* NRRL 11236; K, *A. niger* NRC 9A and *T. reesei* NRRL 6165. *Y*-error bars indicate the ± SD among the replicates. Means, in each bar, followed by the same letter are not significantly different (*P*£0.05). CHC, crude hemicellulose.

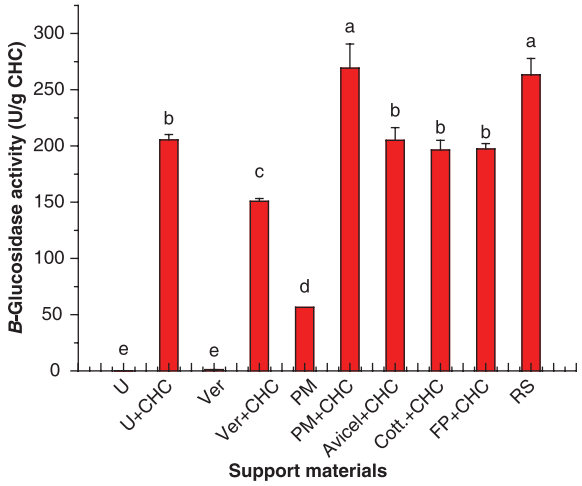
the standard in commercial β-glucosidase production, but within the last few years more research papers have been published on efficient β-glucosidases from other *Aspergillus* spp. and *Penicillium* spp. The synergistic interaction of *A. niger* NRC 5A, *A. niger* NRC 7A, and *A. niger* NRC 9A with *T. reesei* NRRL 6165 led to greater efficiency in β-glucosidases production [*A. niger* NRC 5A and *T. reesei* NRRL 6165 (208.86 ± 4.91 U/g CHC), *A. niger* NRC 7A and *T. reesei* NRRL 6165 (192.39 ± 7.37 U/g CHC), and *A. niger* NRC 9A and *T. reesei* NRRL 6165 (265.32 ± 0.25 U/g CHC)]. The highest yield of β-glucosidase activity produced from the mixed culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 represents about 1.38-fold and 21.12-fold more than that of single cultures of *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively. Compared with the corresponding pure cultures, the enzyme levels produced by the mixed culture depended on the fungus species, substrate used, and substrate physicochemical properties [16]. When *T. reesei* LM-UC4 and *A. phoenicis* QM 329 were grown on bagasse, the filter paper activity and β-glucosidase activity from the mixed culture were much higher than those of the corresponding pure cultures [17]. In contrast, in the coculture of *Thermoascus aurantiacus* and *A. niger* [18] the obtained β-glucosidase activity was 3.76 times higher than that of the *A. niger* monoculture in SSF grown on oats straw. Hu *et al.* [19] also mentioned that most mixed cultivations resulted in increased enzyme activities compared with single cultures, although not always for all enzymes tested. In their work, β-glucosidase activity was increased for all combinations except for *A. niger* with *Phanerochaete chrysosporium*.

In contrast, results in Fig. 1 show also that the synergistic interaction of *A. niger* NRC 5A and *A. niger* NRC 7A with *T. reesei* NRRL 11236 and *T. reesei* NRRL 6165 was not significantly higher than that of monocultures. Similar results were also observed in the mixed culture of *T. reesei* and some *Aspergillus* spp. on bagasse and starch substrate [20]. Wan Mohtar and Thayan [21] reported that mixed culture of *T. reesei* and *A. niger* in submerged culture fermentation gave good cellulase production with increased amounts of β-glucosidase but the synergism of β-glucosidase and cellulase from different species was lower than that observed in the pure culture of *T. reesei*. They suggested that the hydrolytic potential of cellulolytic enzymes in SSF could not be enhanced synergistically by supplementing components of enzyme (i.e. β-glucosidase) from different microorganisms but rather the complete system of hydrolytic enzymes should originate from the same microorganism. Wen *et al.* [9] also mentioned that the reason might be that the nutrients contained in the solid medium were insufficient for the growth of both fungi together and, as a result, competition between nutrients existed between the two fungal species.

Screening of different solid materials on β-glucosidase production

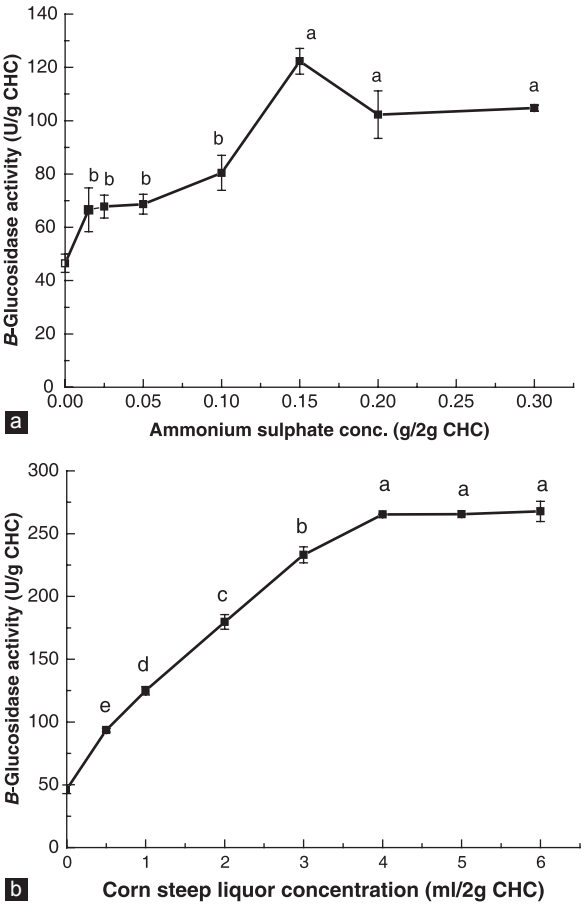
The effect of different solid materials (urethane, vermiculite, Avicel, cotton fiber, filter paper, peat moss, and rice straw as a control) supplemented with 2 g CHC as a carbon source on β-glucosidase production by the coculture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 is shown in Fig. 2. From the inert support materials (urethane, vermiculite, and peat moss) the highest yield of β-glucosidase (269.31 ± 21.49 U/g CHC) production was observed with peat moss. Peat moss is a natural, organic conditioner (>95% w/w organic matter), with a unique structure that provides a good balance of air and water for healthy fungal growth. It has a pore volume of more than 96%, which makes it an excellent support material for SSF. It decomposes slowly compared with other types of organic matter [14] that can be used for repeated fermentation. Therefore, it provides better aeration, less compaction problems, and greater growth surface for spore and metabolite production [13]. The presence of Avicel, cotton fiber, and filter paper as solid materials with CHC produced an appreciable amount of β-glucosidases (205.07 ± 11.35, 196.47 ± 8.59, 197.39 ± 4.7 U/g CHC) by the mixed culture

Figure 2



Effect of different solid support materials [urethane (U), vermiculite (Ver), Avicel, cotton fiber (Cott), filter paper (FP), peat moss (PM), and rice straw (RS) as a control] supplemented with 2 g crude hemicellulose (CHC) as a carbon source on β-glucosidase production by the coculture of *Aspergillus niger* NRC 9A and *Trichoderma reesei* NRRL 6165. *Y*-error bars indicate the ± SD among the replicates. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$).

Figure 3



Effect of different concentrations of ammonium sulfate (a) and corn steep liquor (b) on the production of β-glucosidase under solid-state fermentation by the coculture of *Aspergillus niger* NRC 9A and *Trichoderma reesei* NRRL 6165 using crude hemicellulose (CHC) as a carbon source and peat moss as inert support. *Y*-error bars indicate the ± SD among the replicates. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$).

of *A. niger* and *T. reesei* but less than peat moss with CHC. These solid materials (Avicel, cotton fiber, and filter paper) contain a higher percentage of crystalline cellulose and thus possibly supports Avicel-adsorbable endoglucanase production [22]. It is apparent from the literature that *T. reesei* cellulases are particularly active toward crystalline cellulose; however, enzymes from *Aspergillus* spp. lack the ability to degrade crystalline cellulose. In mixed culture fermentation wherein *A. oryzae* was dominant, filter paper and endocellulase activities were reduced because of the inability of *A. oryzae* to digest crystalline substrate [16]. However individually, Avicel, cotton fiber, and filter paper failed to support high β-glucosidase production. Masui *et al.* [23] also reported that Avicel reduced β-glucosidase production by the thermophilic fungus *Humicola brevis* var. thermoidea under SSF by about 40%. Another reason is the compaction of these materials, which may lead to low aeration and less penetration of the coculture compared with peat moss.

Effect of ammonium sulfate and corn steep liquor on β-glucosidase production

The effects of supplementation of different concentrations of ammonium sulfate and CSL individually as nitrogen sources on β-glucosidase production are evaluated. The results in Fig. 3a and b indicate that CSL alone is a more suitable supplement for β-glucosidase production by the mixed culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165. The highest level of β-glucosidase was detected in the presence of CSL (292.34 ± 16.31 U/g CHC) and $(NH_4)_2SO_4$ (122.29 ± 4.82 U/g CHC). Earlier it was reported that ammonium salts in the form of sulfate facilitate cellulase and β-glucosidase production in *Penicillium funiculosum*, *Chaetomium cellulolyticum*, *T. reesei*, *A. niger*, and *A. terreus* [24,25]. The better production of β-glucosidase obtained in the media containing CSL was probably due to the presence of certain nutrients that were absent in the inorganic nitrogen sources. CSL is generally a rich source of nitrogen, water-soluble vitamins, amino acids, minerals, and other growth stimulants [26]. Constituents of CSL ash include magnesium, phosphorus, potassium, calcium, chlorine, sodium, sulfur, and essential trace elements such as iron, manganese, boron, copper, and zinc [27]. All of these constituents provide natural nutrition for normal cell metabolism and are not a cause for concern. The richness of CSL with these nutrients may be enough to stimulate growth and enhance β-glucosidase production. This cheap residue (CSL) has been

successfully used for a variety of fermentations such as solvents, antibiotics, and enzyme production [28,29]. The low cost of by-products such as CSL facilitated its replacement of more expensive source of nutrients. Thus, CHC allied to CSL seems a very attractive combination for β-glucosidase, xylanase [13], and other microbial enzyme productions as well.

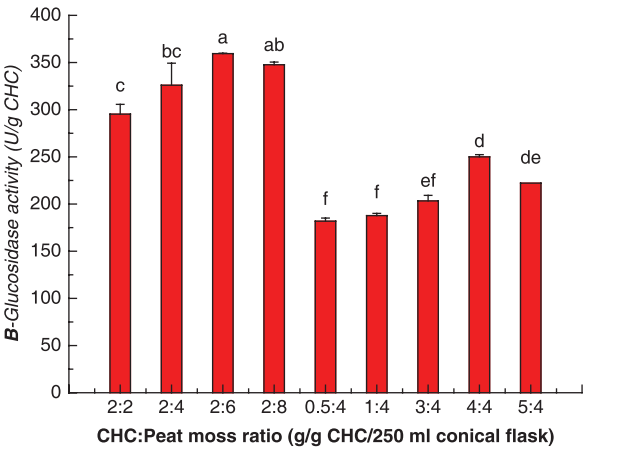
Effect of initial pH

The pH of the medium is one of the most important factors for a fermentation process, influencing the microbial growth and enzyme activity. In the present experiment, β-glucosidase production during SSF using CHC and peat moss was evaluated using citrate–phosphate buffer at various initial pH levels (4–8). Another experiment was carried out using distilled water in which the initial pH was adjusted at pH 5.5–6 using 0.1 mol/l HCl and 0.1 mol/l NaOH. Maximum enzyme production (294.9 U/g CHC) was recorded with medium supplemented with distilled water (data not shown). In contrast, when citrate–phosphate buffer was used as the moistening agent at different pH values (4–8), the results showed that maximum β-glucosidase activity (235.41 U/g CHC) was recorded at initial pH 6.0, which might indicate a higher stability of the enzyme protein under this pH range as the final pH values ranged between 6 and 6.6. However, any further increase or decrease from the initial pH 6 value of the mineral salt solution had little effect on β-glucosidase activity within the experimental range. These findings are in line with the work of Lynd *et al.* [30] and support their finding that the pH optimum of β-glucosidase is between 5 and 6. Brijwani *et al.* [10] found pH 4.5 to be the optimal culture condition for cellulase and β-glucosidase production from mixed-culture SSF of *T. reesei* and *A. oryzae*.

Effect of crude hemicellulose and peat moss ratio on β-glucosidase production

The present study was undertaken to optimize CHC and peat moss ratio in SSF for the production of β-glucosidase by coculture of *A. niger* NRC 9A and *T. reesei* NRRL 6165. Different ratios of CHC and peat moss (2 : 2, 2 : 4, 2 : 6, 2 : 8, 0.5 : 4, 1 : 4, 3 : 4, 4 : 4, and 5 : 4 w/w) were used for β-glucosidase production in SSF. The initial pH values of all experiments were adjusted at pH 5.5–6 as mentioned before using 0.1 mol/l HCl and 0.1 mol/l NaOH. The results in Fig. 4 show that β-glucosidase activity was maximal (359.37 ± 1.02 U/g CHC) in the culture comprising CHC–peat moss ratio of 2 : 6 (g CHC/g peat moss/250 ml conical flask). It was reported that, at higher bed thickness, the oxygen availability decreased in the middle and bottom area of the substrate. This condition is due to the fast colonization of the fungus

Figure 4



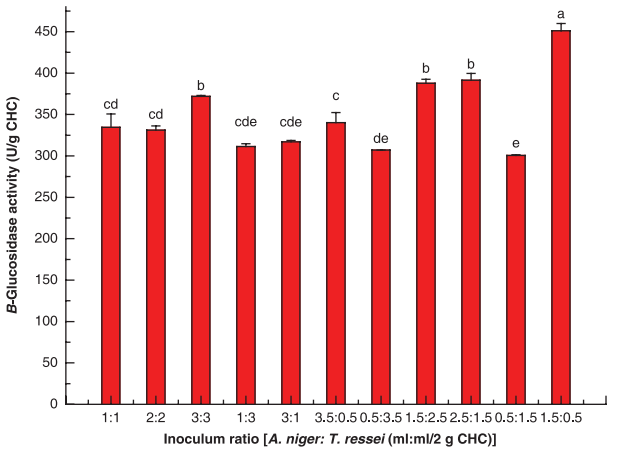
Effect of different ratios of crude hemicellulose (CHC) and peat moss on β-glucosidase production by the coculture of *Aspergillus niger* NRC 9A and *Trichoderma reesei* NRRL 6165 under solid-state fermentation. *Y*-error bars indicate the ± SD among the replicates. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$).

on the surface area, which promotes high density of the substrate. This condition caused the exhaustion of oxygen, which affected the fungal growth as well as the enzyme activity. Moreover, it was also reported that the oxygen availability in the tray system correlated with the substrate thickness [25]. Therefore, thicker than optimum bed height leads to undesirable situations like cell lysis and anaerobic conditions. In contrast, a thinner bed height is usually chosen in a tray system as it is easily fermented [31] and permits better oxygen supply and heat removal [32]. However, the results showed that β-glucosidase activity was diminished with changes in the CHC and peat moss ratio, producing the lowest β-glucosidase activity at CHC–peat moss ratios of 0.5 : 4 and 1 : 4 (w/w). This might be because nutrients (CHC) contained in the solid medium were insufficient for the growth of both fungi together and, as a result, a nutrient competition existed between the two fungal species.

Effect of inoculum ratio and size of coculture on β-glucosidase production

Most applications of SSF involve the use of a fungal spore inoculum. Spore inocula are more evenly distributed and give better interparticle translocation [32]. The effect of mixed inoculum sizes (2, 4, and 6 ml/2 g CHC) (10^6–10^7 spores/ml) with different ratios (1 : 1, 2 : 2, 3 : 3, 1 : 3, 3 : 1, 3.5 : 0.5, 0.5 : 3.5, 1.5 : 2.5, 2.5 : 1.5, 0.5 : 1.5, and 1.5 : 0.5 v/v) of *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively, on the production of β-glucosidase using CHC is shown in Fig. 5. The initial pH of all experiments was adjusted at pH 5.5–6 as mentioned

Figure 5



Effect of different inoculum sizes and ratios of *Aspergillus niger* NRC 9A and *Trichoderma reesei* NRRL 6165 on β-glucosidase production under solid-state fermentation using crude hemicellulose (CHC) as a carbon source and peat moss as inert support. *Y*-error bars indicate the ± SD among the replicates. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$).

before using 0.1 mol/l HCl and 0.1 mol/l NaOH. Maximum production of β-glucosidase (451.25 U/g CHC) was obtained in the fermentation medium that was inoculated with 2 ml of mixed spore suspension (1.5 ml of *A. niger* NRC 9A and 0.5 ml of *T. reesei* NRRL 6165). Further increase or decrease in the ratio of both organisms at the above-mentioned inoculum size and ratio, however, resulted in the decrease of β-glucosidase production. However, inoculum size greater than 2 ml (10^6–10^7 spores/ml) also resulted in a decrease in β-glucosidase production. Most of the previous studies [33,34] mentioned a strong influence of high inoculum size on the production of microbial metabolites. They reported that decreased enzyme production at high inoculum levels might be due to the production of inhibitory metabolites that interfere with enzyme production. An increase in the number of spores in inoculum size lead to rapid proliferation and biomass synthesis. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity [35]. A balance between the proliferating biomass and available nutrient would yield an optimum condition at which the enzyme synthesis would be maximal [36]. Laukevics *et al.* [37] found that good colonization of the substrate mass by *T. reesei* depended on the inoculum size used, which must be large enough for all the substrate particles to be colonized. However, it was reported that lower inoculum size required longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzymes. Lower inoculum sizes also shortened the microbial lag phase stage, whereas inoculum size beyond the optimum value increased the moisture factor that caused lower levels of enzyme formation due to the overcrowding of fungal spores [38].

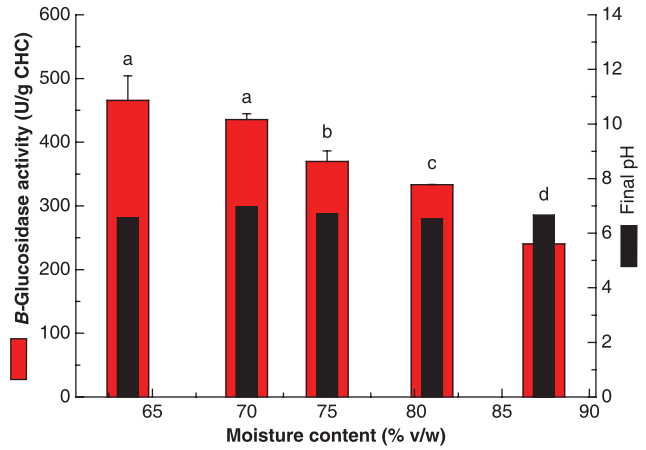
Effect of initial moisture content

The moisture content of the growth medium is a critical variable affecting the SSF. The optimal moisture content in SSF depends mainly on the nature of the substrate, the requirements of the microorganism, and the type of end product [39]. In the present work moisture content had a profound influence on the production of β-glucosidase by the mixed culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 in the SSF system consisting mainly of CHC as the main carbon source and peat moss as the impregnated support. All experiments were carried out at initial pH 5.5–6 and excess distilled water was added to get the desired moisture percentage. Figure 6 shows the yields of β-glucosidase under five different moisture contents (63.6, 70, 75, 80, and 87% w/w). A moisture content of 63.6% (w/w) provides the best environment for β-glucosidase production (465.65 ± 6.06 U/g CHC). Liu and Yang [40] mentioned that moisture enables better utilization of the substrate by microorganisms, and the efficiency of mass transfer in the solid-phase particles is enhanced depending on the substrate characteristics and the appropriate moisture. However, further increase in moisture content influences the enzyme production negatively. It reduces the surface area of the particles and makes the water film thicker, which affects the accessibility of air to the particles. Lonsane *et al.* [41] reported that higher moisture level decreases porosity, changes substrate particle structure, promotes the development of stickiness, reduces gas volume and exchange, and decreases diffusion, which result in lowered oxygen transfer and enhanced formation of aerial mycelium. In contrast, lower moisture content reduces the solubility of nutrients present in the solid substrate, decreases the degree of swelling, and increases water tension. Pandey *et al.* [42] reported also that, with low water availability, fungi suffer modification in their cell membranes, leading to transport limitations and affecting microbial metabolism. The optimal moisture content in SSF depends mainly on the nature of the substrate, the requirements of the microorganism, and the type of end product [39].

Effect of incubation period

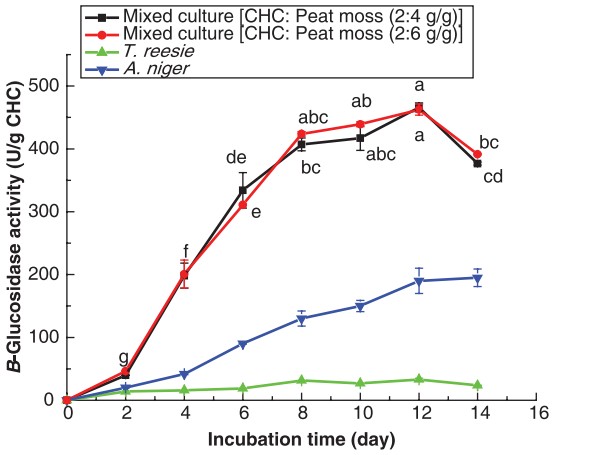
The coculture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 and the two pure cultures were grown in time–course studies to determine the optimum time for production of β-glucosidase under SSF using CHC as the substrate and peat moss as the inert support

Figure 6



Effect of different moisture contents on β-glucosidase production under solid-state fermentation by the coculture of *Aspergillus niger* NRC 9A and *Trichoderma reesei* NRRL 6165 using crude hemicellulose (CHC) as a carbon source and peat moss as inert support. *Y*-error bars indicate the ± SD among the replicates. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$).

Figure 7



Time–course of β-glucosidase production under solid-state fermentation by the coculture of *Aspergillus niger* NRC 9A and *Trichoderma reesei* NRRL 6165 using crude hemicellulose (CHC) as a carbon source and peat moss as inert support. *Y*-error bars indicate the ±SD among the replicates. Means, in each bar, followed by the same letter are not significantly different ($P \leq 0.05$).

with two different ratios (2 : 4 and 2 : 6 g CHC/g peat moss). β-Glucosidase production started earlier (i.e. during lag phase) for the mixed and monocultures. Maximum β-glucosidase yields (466.02 ± 5.42 and 462.44 ± 6.63 U/g CHC) obtained by the mixed culture were observed after 12 days for 2 : 6 and 2 : 4 g CHC/g peat moss, respectively (Fig. 7). Further incubation after this time did not show any increment in the level of enzyme production. The highest β-glucosidase activity produced by mixed culture of *A. niger* and *T. reesei* after optimization represents about 2.38-fold and 19.66-fold increase over the maximum activities attained in single-culture SSF for *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively. Similar trends were also reported in β-glucosidase production by fungal mixed culture and single-culture solid substrate fermentation or submerged fermentation on lignocellulosic materials [8–10]. However, Chandra *et al.* [43] also observed maximum production of β-glucosidase after 3 days of fermentation on wheat bran under SSF. Kang *et al.* [44] recorded maximum β-glucosidase production from *A. niger* after 5 days of fermentation when grown on rice straw during SSF. We can conclude that the duration needed for incubation of mixed culture might depend mainly on the growth rate of the fungal species, on the substrate used, on substrate physicochemical properties, and on the enzyme production pattern.

Conclusion

This study established that CHC prepared from RSBL, which does not have any significant commercial use and causes pollution, especially in developing countries, could serve as a good substrate for the production of β-glucosidase. Supplementing CHC with CSL and peat moss as inert support obviates the need of addition of inorganic nitrogen sources and results in optimum β-glucosidase activity. Hence, the technology using these cheap and readily available substrates for production of β-glucosidase in optimum quantities in about 12 days holds promise for the future. The results are significant for the study on β-glucosidase production and show potential for large-scale production.

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Conflicts of interest

There are no conflicts of interest.

References

1 Nada AMA, Hamzawy EMA, Omara HI, El Gohary S. Thermal treatment of the precipitated solid material (PSM) from the waste black liquor (WBL). Bull Mater Sci 1999; 22:1067–1107.

2 Minu K, Jiby KK, Kishore VVN. Isolation and purification of lignin and silica from the black liquor generated during the production of bioethanol from rice straw. Biomass Bioenergy 2012; 39:210–217.

3 Xiong Z, Zhang X, Wang H, Ma F, Li L, Li W. Application of brown-rot basidiomycete *Fomitopsis* sp. IMER2 for biological treatment of black liquor. J Biosci Bioeng 2007; 104:446–450.

4 Sørensen A, Lübeck M, Lübeck PS, Ahring BK. Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials. Biomolecules 2013; 3:612–631.

5 Kumar R, Singh S, Singh OV. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 2008; 35:377–391.

6 Tangnu SK, Blanch HW, Wilke CR Enhanced production of cellulase, hemicellulase and β-xylosidase by *Trichoderma reesei* (RUT-C30). Biotechnol Bioeng 1981; 23:1837–1849.

7 Cochet N. Cellulase of *Trichoderma reesei*: influence of culture conditions upon enzymatic profile. Enzyme Microb Technol 1991; 3:104–109.

8 Ahamed A, Vermette P. Enhanced enzyme production from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA grown as fed batch in a stirred tank bioreactor. Biochem Eng J 2008; 42:41–46.

9 Wen, Z, Liao W, Chen S. Production of cellulase/β-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. Process Biochem 2005; 40:3087–3094.

10 Brijwani K, Oberoi HS, Vadlani PV. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Process Biochem 2010; 45:120–128.

11 Parkinson D, Gray TRG, Williams ST. *Methods for studying the ecology of soil microorganisms*. Oxford, UK: Blackwell; 1971. 116.

12 Nawwar AM, EL-Katib M, Zawawy WK. Paper pulp from rice straw using solar energy. Egyptian patent no. 2008030442; 2008

13 Shata HMA, Noor El-Deen AM, Nawwar GAM, Farid MA. Xylanase production by mixed culture using crude hemicellulose from rice straw black liquor and peat moss as an inert support. J Appl Biol Chem 2014; 57. [in press].

14 Canadian Sphagnum Peat Moss Association/CSPMA. Information about peat moss . Available at: *http://www.peatmoss.com/blog/education/horticultural-teaching-program/2014*. [Last accessed on 2014 Jan 2].

15 De Vries RP, Burgers K, van de Vondervoort PJ, Frisvad JC, Samson RA, Visser J. A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production.. Appl Environ Microbiol 2004; 70:3954–3959.

16 Brijwani K, Vadlani PV. Cellulolytic enzymes production via solid-state fermentation: effect of pretreatment methods on physicochemical characteristics of substrate. Enzyme Res 2011; 2011:860134. doi:10.4061/2011/860134.

17 Dueñas R, Tengerdy RP, Gutierrez-Correa M. Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. World J Microbiol Biotechnol 1995; 11:333–337.

18 Stoilova IS, Gargova SA, Krastanov AI. Production of enzymes by mixed culture from mycelial fungi in solid-state fermentation. Biotechnol Biotechnol Equipment 2005; 19:103–108.

19 Hu HL, Van den Brink J, Gruben BS, Wösten HAB, Gu JD, De Vries RP. Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi. Int Biodeterior Biodegrad 2011; 65:248–252.

20 Massadeh MI, Yusoff WMW, Omar O, Kader J. Synergism of cellulase enzymes in mixed culture solid substrate fermentation. Biotechnol Lett 2001; 23:1771–1774.

21 Wan Mohtar WY, Thayan R Synergism of β-glucosidase and cellulase in mixed culture fermentations. Malays Appl Biol 1991; 20:215–222.

22 Jatinder K, Chadha BS, Saini HS. Optimization of culture conditions for production of cellulases and xylanases by *Scytalidium thermophilum* using response surface methodology. World J Microbiol Biotechnol 2006; 22:169–176.

23 Masui DC, Zimbardi AL, Souza FH, Guimarães LH, Furriel RP, Jorge JA. Production of a xylose-stimulated β-glucosidase and a cellulase-free thermostable xylanase by the thermophilic fungus *Humicola brevis* var. thermoidea under solid state fermentation. World J Microbiol Biotechnol 2012; 28:2689–2701.

24 Chahal PS, Chahal DS, Lee GBB. Production of cellulase in solid state fermentation with *Trichoderma reesei* MCG80 on wheat straw. Appl Biochem Biotechnol 1996; 57:433–441.

25 Raghavarao KS, Ranganatha TV, Karanth NG. Some engineering aspects of solid state fermentation. Biochem Eng 2003; 13:127–135.

26 Filipovic SS, Ristic MD, Sakac MB. Technology of corn steep application in animal and their quality. Roum Biotechnol Lett 2002; 7:705–710.

27 White P, Johnson LA. *Corn: chemistry and technology* . 2nd ed. St. Paul, Minnesota, USA: American Association of Cereal Chemists; 2003.

28 De Azeredo LA, De Lima MB, Coelho RR, Freire DM. A low-cost fermentation medium for thermophilic protease production by *Streptomyces* sp. 594 using feather meal and corn steep liquor. Curr Microbiol 2006; 53:335–339.

29 Lima ALG, Nascimento RP, Bon EPS, Coelho RR. Cellulase activity produced by *Streptomyces drozdowiczii* using low cost agro-industrial by-products and tests for biotechnological application. Enz Microb Technol 2005; 37:272–277.

30 Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002; 66:506–577.

31 Suryanaran S. Current industrial practice in solid state fermentations for secondary metabolite production: the Biocon India experience. Biochem Eng J 2003; 13:189–195.

32 Gervais P, Molin P. The role of water in solid state fermentation. Biochem Eng J 2003; 13:85–101.

33 Mahanama R, Berenjian A, Talbot A, Biffin R, Regtop H, Dehghani F, Kavanagh J. Proceedings of the World Congress on Engineering and Computer Science 2011 Vol. II WCECS 2011, 19–21 October 2011; San Francisco, USA.

34 Niladevi KN, Prema P. Effect of inducers and process parameters on lactase production by *Streptomyces psammoticus* and its application in dye decolourization. Bioresour Technol 2008; 99:4583–4589.

35 Kashyap P, Sabu A, Pandey A, Szakacs G. Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid state fermentation. Process Biochem 2002; 38:307–312.

36 Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G, Pandey A. Coconut oil cake – a potential raw material for the production of alpha-amylase. Bioresour Technol 2004; 93:169–174.

37 Laukevics JJ, Apsite AF, Viesturs UE, Tengerdy RP. Solid substrate fermentation of wheat straw to fungal protein. Biotechnol Bioeng 1984; 26:1465–1474.

38 Iqbal HMN, Asgher M, Bhatti HN. Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of *Trametes versicolor*. Bioresources 2011; 6:1273–1287.

39 Kalogeris E, Christakopoulos P, Katapodis P, Alexiou A, Vlachou S, Kekos D, Macris BJ. Production and characterization of cellulolytic enzymes from the thermophilic fungus *Thermoascus aurantiacus* under solid state cultivation of agricultural wastes. Proc Biochem 2003; 38:1099–1104.

40 Liu J, Yang J. Cellulase production by *Trichoderma koningii* AS3.4262 in solid-state fermentation using lignocellulosic waste from the vinegar industry. Food Technol Biotechnol 2007; 45:420–425.

41 Lonsane BK, Ghildyal NP, Budiatman S, Ramakrishna SV. Engineering aspects of solid state fermentation. Enzyme Microb Technol 1985; 7:258–265.

42 Pandey A, Ashakumary L, Selvakumar P, Vijayalakshmi KS. Influence of water activity on growth and activity of *Aspergillus niger* for glycoamylase production in solid-state fermentation.. World J Microbiol Biotechnol 1994; 10:485–486.

43 Chandra MS, Viswanath B, Reddy BR. Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. Indian J Microbiol 2007; 47:323–328.

44 Kang SW, Park YS, Lee JS, Hong SI, Kim SW. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour Technol 2004; 91:153–156.