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ORIGINAL ARTICLE

Improvement of β-glucosidase production by co-culture of *Aspergillus niger* and *A. oryzae* under solid state fermentation through feeding process

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Abstract Eleven different *Aspergillus* strains were evaluated for their ability to produce β-glucosidase using sugar cane bagasse as a sole carbon source under solid state fermentation (SSF). The most potent strains, *A. niger* NRC 7 (674.6 U/g ds) and *A. oryzae* NRRL 447 (83 U/g ds), were used in a mixed culture to enhance β-glucosidase production by co-culturing under SSF. In mixed culture, β-glucosidase of the two strains (814 U/g ds) was nearly 1.2- and 9.8-fold than that of mono-cultures of *A. niger* NRC 7A and *A. oryzae* NRRL 447, respectively. Optimization of the culture parameters, initial pH value, moisture content, inoculum size and ratios of the two strains. and incubation time exhibited a significant increase in β-glucosidase production (1,893 U/g ds) than before optimization. Single feeding with citrate-phosphate buffer, succinate buffer, casein. and soybean flour individually after the third day of the fermentation time and controlling the moisture content at 90 % (w/w) induced β-glucosidase production. Maximum enzyme production increased up to 2.1-fold compared to 2,188 U/g ds during normal batch culture. Among nitrogen sources, soybean flour gave the highest β-glucosidase (4,578 U/g ds). while urea reduced β-glucosidase production (1,693 U/g ds). However, the combination of buffers with soybean flour through two fed cycles resulted in a decrease of the enzyme than single fed with buffers or soybean flour alone.

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Introduction

β-Glucosidase (EC 3.2.1.21) normally catalyzes the hydrolysis of β-1, 4-glycosidic bond and releases D-glucose from the non-reducing ends of cellobiose and oligosaccharides (Cai et al. 1999). It is also considered to be a part of the cellulase system, since it stimulates the rate and extent of cellulose hydrolysis by relieving cellobiose-induced inhibition of endo- and exo-β-glucanases (Parry et al. 2001; Wang et al. 2009). The enzymatic hydrolysis of cellulose involves three types of cellulase activities (cellobiohydrolases, endoglucanases, and β-glucosidases) working in synergy (Lynd et al. 2002). Endoglucanases (EC 3.2.1.4) randomly cleave the β-1,4 glycosidic linkages of cellulose, cellobiohydrolases (EC 3.2.1.91) attack cellulose chain ends to produce the cellobiose (a dimer of glucose linked by a β-1,4 glycosidic bond), and β-glucosidases. This enzyme is of considerable industrial interest due to its extensive applications in different fields (Bhatia et al. 2002).

Microbial sources have been widely exploited for β-glucosidase production by both SSF and submerged fermentation (Bhatia et al. 2002). *A. niger* and *A. oryzae* are two of the most important fungi worldwide for biotechnological applications and both have a long history with respect to strain improvement to optimize enzymes production. They are the most commonly used industrial *Aspergillus* species for the production of pharmaceuticals, food ingredients, and enzymes (Pandey et al. 1999). *A. niger* and *A. oryzae* produce a broad range of enzymes related to degradation of plant polysaccharides, such as cellulose, xylan, xyloglucan, galactomannan,

and pectin (de Vries and Visser 2001). They also have a significantly different set of polysaccharide-degrading enzymes in their genome (Coutinho et al. 2009). These enzymes are essential to convert the natural carbon sources of these fungi (mainly plant polymers) into small molecules that can be taken up into the cell. *A. niger* has been mainly used to produce β-glucosidases and endoglucanases, but limited information is available on the production of cellobiohydrolase and the induction and repression of cellulases (Ahamed and Vermette 2008). On the other hand, *A. niger* produces a strong activity of β-glucosidases, which causes deglycosylation of substrates and produces gentiobiose, which is a strong inducer of cellulases.

Substrate costs account for a major fraction of the costs of microbial products production, and the use of cheap biomass resources as substrates can help to reduce their prices. Also, the use of cheaper technologies like SSF, mixed cultures, and fed-batch technique can further improve the production economics of microbial products. SSF technology results in an enzyme preparation, which is more concentrated and hence best-suited for biomass conversion applications (Chahal 1985). Sugar cane bagasse is a low-cost and abundant biomass material (Pandey et al. 2000) containing about 27–54 % (w/w) cellulose (Toit et al. 1984), which can serve as a potent substrate for cellulase production. There have been several studies on the use of sugar cane bagasse as a substrate in cellulase production both under submerged and SSF (Gutierrez-Correa and Tengerdy 1997; Bigelow and Wyman 2002; Mekala et al. 2008).

In the present work, efforts are made to optimize the cultural conditions for production of β-glucosidase by using co-culture of *A. niger* and *A. oryzae* under SSF. Supplementation of the fermented media with buffers solutions or some nitrogen sources individually enhanced β-glucosidase production and the results were found to be quite encouraging.

Materials and methods

Microorganisms and culture maintenance

Aspergillus niger NRC 2A, *A. niger* NRC 4, *A. niger* NRC 5, *A. niger* NRC 7A, *A. niger* NRC 18, *A.. niger* NRC HOD, and *A. oryzae* NRC ASO were obtained from the stock culture of Natural and Microbial Products Department, National Research Center, Dokki, Cairo, Egypt. *A. awamori* NRRL 3112, *A. foetidus* NRRL 337, *A. oryzae* NRRL 6583, and *A. oryzae* NRRL 447 were kindly provided by the Northern Regional Research Laboratory (NRRL), Peoria, IL, USA. Cultures were maintained on potato-dextrose agar (PDA) 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 PDA 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 for each 250-mL Erlenmeyer flask containing the solid medium. For pure culture, either *A. niger* strains or other *Aspergillus* sp. each flask containing 5 g dry substrate was inoculated with a 3-mL spore suspension (10^6–10^7 spores/mL). Spore count was measured by the dilution plate count method (Parkinson et al. 1971). Unless otherwise stated, for mixed culture, 1.5 mL of each strain was inoculated into each flask simultaneously.

β-glucosidases production under SSF

Solid-state fermentation was carried out in 250-mL Erlenmeyer flasks, each having 5 g of dry sugar cane bagasse (1–5 mm) moistened with 15 mL mineral salt solution (g/L: $(NH_4)_2SO_4$, 0.2; KH_2PO_4, 0.5; $CoCl_2$, 0.001; $CaCl_2$, 0.01; $MgSO_4$, 0.001; lactose, 0.02, and pH 5.5) to attain the final substrate-to-moisture ratio of 1:3 (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 inoculums. Unless otherwise stated, the moisture content of the substrates after pretreatment addition of nutrients and inoculum 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.

Screening for β-glucosidase activity

The *Aspergillus* sp. were used for the primary screening for their ability to grow and produce β-glucosidase activity in a solid medium containing sugar cane bagasse (1–5 mm) as the main carbon source (5 g/flask). Flasks were autoclaved and inoculated by 3 mL of spore suspension previously prepared as mentioned above. The cultures were incubated for 10–14 days at 28–30 °C. All experiments were run in parallel in duplicates and the average values were reported.

Optimization of SSF conditions

Various process parameters influencing the enzymes production during SSF were studied by the conventional method (by varying one parameter at a time) including moisture content, initial pH value, inoculum sizes with different ratios of the selected strains, and incubation periods. In order to study the influence of the initial moisture content on enzymes production,

the solid medium was set at different moisture levels (61, 72, 82, 84, 86, 88, and 90 % w/w) using distilled water after autoclaving and inoculum addition. To determine optimal initial pH, *A. niger* and *A. oryzae* were cultivated in a 250-mL conical flask containing solid medium with different initial pH ranges from 3.0 to 7.0 using 50 mM citrate-phosphate buffer. The effect of the inoculum size on β-glucosidase production was studied by adding different inoculum sizes (1, 2, 3, and 4 mL/5 g ds) of 7-day old cultures (10^6–10^7spore/mL) with different ratios (1:1, 1:2, 2:1,1:3, 3:1, 1:5, 5:1, 3:7, and 7:3 v/v) of *A. oryzae* and *A. niger*. Fermentation period was an important parameter for enzymes production by the co-culture of *A. niger* and *A. oryzae*. In this study, the fermentation experiment was carried out up to 14 days and enzyme yields were measured at 24-h intervals. For each experimental variable, all other parameters were kept at their optimal levels. Data were expressed as the average of replicates.

Effect of additional nutrients

The effect of additional nutrients on β-glucosidase production was carried out by supplementing the fermented bagasse under aseptic conditions after the third day, separately with 1 mL/flask of sterilized nitrogen sources solutions (10 % w/v), such as casein, defatted soybean flour, and urea (sterilized by filtration), and buffers such as 50 mM citrate-phosphate, pH 4.8, and 50 mM succinate buffer, pH 4.8. In another set of experiments, combination of buffer solutions and the best nitrogen source was also tested. In this experiment, 0.5 mL of each buffer (100 mM) was mixed with 0.5 mL of the nitrogen source (20 % w/v) and the mixture was fed under aseptic conditions for two successive additions into the fermented media at the third and fifth day. Fermentation was carried out for 14 days and all other parameters were kept at their optimal levels.

Enzyme extraction

After the incubation period, 7 mL/g ds of 0.05 M citrate-phosphate buffer (pH 4.8) were added to the fermented substrate in each flask. The flasks were rotated on a rotary shaker at 100 rpm for 1/2 h at room temperature. The fermented broth was centrifuged at 6,000 rpm for 10 min to get a clear supernatant which was analyzed for β-glucosidase and cellulase activities.

Beta-glucosidase assay

Beta-glucosidase analysis was performed using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate according to de Vries et al. (2004) 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 μM of *p*-nitrophenol in 1 min under standard assay conditions". It is expressed as U/g ds (where, g ds=gram of initial substrate used for growth).

Determination of cellulase activities

The cellulase activity was measured using the filter paper activity (FPA) assay, expressed in filter paper units (FPU) according to the method of Ghose (1987). This method measured the release of reducing sugars produced in 60 min from a mixture of dilute enzyme solution (0.5 mL) and of citrate-phosphate buffer (0.05 M, pH 4.8, 1 mL) in the presence of 50 mg Whatman No. 1 filter paper (1 cm×6 cm strip) and incubated at 50 °C for 60 min. The cellulase activity of endo-β-1-4-glucanase was measured using a carboxymethyl cellulase assay (Ghose 1987). This method measured at 50 °C the release of reducing sugars produced in 30 min from a mixture of diluted enzyme solution (0.5 mL) and 0.5 mL of a 2 % (w/v) carboxymethyl cellulose (sodium salt; Acros Organics, NJ, USA) solution made in 0.05 M citrate-phosphate buffer (pH 4.8). The filter paper and carboxymethyl cellulase assays were based on the same principle of estimating the amount of total reducing sugars formed from the relevant substrate. The released sugars were analyzed by the dinitrosalicylic acid method (Miller 1959). One unit of enzyme activity was defined as the amount of enzymes releasing 1 μmol of reducing sugars in 1 min. The samples were analyzed in replicates and mean values were calculated. They are expressed as U/g ds (where g ds=gram of initial substrate used for growth).

Data analysis

Treatment effects were analyzed and the mean comparison was performed by ANOVA one-way analysis of variance using computer software Minitab 16 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

The cost of cellulolytic enzymes is a major factor in the process economics of biomass to ethanol technology and other industrial process (Gregg et al. 1998). The production cost of cellulases can be brought down if cheaper substrates are used, as the cost of substrate account for a major fraction of the production cost (Wen et al. 2005). Considering these, the present study was undertaken to explore the potential of using sugar cane bagasse, a cheap substrate, for β-glucosidase production by the filamentous fungus *A. niger* and *A. oryzae*. SSF technology needs less space per unit biomass used as substrate and is less demanding on infrastructure and running costs (Pandey et al. 1999). Environmental factors such as temperature,

pH, moisture content, and concentration of nutrients and products in the medium can significantly affect microbial growth and product formation under SSF.

Screening of cellulases producing *Aspergillus* strains

In the present study, screenings of some different *Aspergillus* strains and the co-cultures of the most potent strains for their production of β-glucosidase and cellulases (FP-ase and CMC-ase) activities by SSF were carried out using sugar cane bagasse as the main carbon source incorporated in the basal salt medium. After an incubation period of 10 days, *A. niger* strains (*A. niger* NRC 5, *A. niger* NRC 7A, *A. niger* NRC 4, and *A. niger* NRC HOD) generally produced more β-glucosidase (749.18±17.579, 674.60±17.579, 474.24±3.102 an,d 374.80±7.238 U/g ds, respectively) than the other *Aspergillus* species [(*A. awamori* NRRL 3112 (45.03±2.068 U/g ds), *A. foetidus* NRRL 337 (46.49±14.477 U/g ds), *A. oryzae* NRRL 6583 (46.71±13.443 U/g ds), *A. oryzae* NRRL 447 (83.33±2.068 U/g ds), and *A. oryzae* NRRL ASO (31.87± 4.136 U/g ds)] as indicated in Table 1. The synergistic interaction of *A. niger* NRC 7A with *A. oryzae* NRRL 447 and *A. niger* NRC HOD with *A. oryzae* NRRL 447 led to a greater efficiency for production of β-glucosidases (814.25 ±12.41and 583.93 ±13.44 U/g ds, respectively). In the present study, β-glucosidase of *A. niger* NRC 7A and *A. oryzae* NRRL 447 was nearly 1.2- and 9.8-fold more than that of monocultures of *A. niger* NRC 7A and *A. oryzae* NRRL 447, respectively. The interactions of *A. niger* NRC 7A with *A. oryzae* NRRL 447, *A. niger* NRC HOD with *A. oryzae* NRRL 447, and *A. niger* NRC 5 with *A. oryzae* NRRL 447 generally resulted in increased FP-ase activity (0.858±0.040, 0.605±0.155 a,nd 0.445±0.021 U/g ds, respectively) compared with using each mono-culture alone. On the other hand, a synergistic interaction of the above-mentioned *Aspergillus* sp. decreased CMCase activity more than each mono-culture alone as indicated in Table 1. In the co-culture of *Thermoascus aurantiacus* and *A. niger*-A (Stoilova et al. 2005) the obtained β- glucosidase activity was 2.5 times higher when compared to the total activity of *A. niger*-A mono-culture. Hu et al. (2011) also mentioned that most mixed cultivations resulted in increased enzyme activities compared to the single cultures, although not always for all enzymes tested. In their work, β-glucosidase activity was increased for all combinations except *A. niger* with *Phanerochaete chrysosporium*, while cellobiohydrolase and α-arabinofuranosidase activities were only increased in the combination *A. niger* with *Magnaporthe grisea*. Also, β-xylosidase activity was increased in the combinations of *A. niger–P. chrysosporium* and *A. oryzae–M. grisea*, while the activity in the *A. niger–A. oryzae* co-cultivation was approximately the average of the two single cultures. Furthermore, co-cultivations of *A. oryzae* with either *P. chrysosporium* or *M. grisea* resulted in increased β-galactosidase activities. The presence of *P. chrysosporium* resulted in an increase in laccase activity for both *A. niger* and *A. oryzae*. On the other hand, *M. grisea* produced very high laccase activity, which was reduced in combinations with *A. niger* and *A. oryzae*. Co-cultures of *A. niger* with *A. awamori* or *A. oryzae* resulted in increased β-glucosidase activities, whereas the combination of the three strains led to a reduction of the enzyme production (Raza et al. 2011). Ahamed and Vermette (2008) also reported that the synergistic interaction of *Trichoderma reesei* and *Aspergillus* strains in submerged fermentation leads to a greater efficiency of cellulose degradation than using *T. reesei* alone. They mentioned that this is certainly due to the complementary interactions of *T. reesei* cellulases and the β-glucosidases of the *Aspergillus* strain, which together achieve a more complete cellulose hydrolysis. This was also observed in a different fermentation process in which the simultaneous inoculation of two microorganisms into the production medium resulted in higher cellulase activity and β-glucosidase activity when compared to mono-cultures (Duff et al. 1986). On the other hand, results in Table 1 show also the synergistic interaction of *A. niger* NRC 5 and *A. oryzae* NRRL

Table 1 Screening of mono-culture and co-culture of some *Aspergillus* sp. for β-glucosidase production under SSF

Microorganism	β-Glucosidase activity U/g ds	FP-ase activity U/g ds	CMC-ase activity U/g ds
A. niger NRC 18	ND	0.142±0.010^{e}	2.619±0.083^{b}
A. niger NRC 4	474.24±3.102^{d}	0.115±0.035^{e}	1.597±0.004^{c}
A. niger NRC A2	121.80±8.582^{e}	0.255±0.005^{de}	0.839±0.0^{de}
A. niger NRC 5	749.18±17.579^{ab}	0.169±0.047^{e}	0.650±0.002^{ef}
A. niger NRC 7A	674.60±17.579^{bc}	0.291±0.010^{cde}	1.047±0.00^{d}
A. niger NRC HOD	374.80±7.238^{d}	0.126±0.005^{e}	0.435±0.00^{f}
A. awamori NRRL 3112	45.03±2.068^{ef}	0.177±0.022^{e}	2.913±0.00^{b}
A. foetidus NRRL 337	46.49±14.477^{ef}	0.131±0.058^{e}	ND
A. oryzae NRRL 6583	46.71±13.443^{ef}	0.146±0.004^{e}	1.021±0.00^{d}
A. oryzae NRRL 447	83.33±2.068^{ef}	0.493±0.066^{bc}	4.642±0.280^{a}
A. oryzae NRRL ASO	31.87±4.136^{ef}	0.230±0.015^{e}	2.898±0.00^{b}
A. niger NRC 7A+*A. oryzae* NRRL 447	814.26±12.408^{a}	0.858±0.040^{a}	1.061±047
A. niger NRC HOD+*A. oryzae* NRRL 447	583.93±13.443^{c}	0.605±0.155^{b}	0.953±0.037
A. niger NRC 5+*A. oryzae* NRRL 447	402.59±3.412^{d}	0.445±0.021^{bcd}	1.046±0.110

Means followed by different letters within each column differ significantly at $p \leq 0.05$

ND not detected; ± indicates standard derivation among replicates

447 produced β-glucosidases activity lower than *A. niger* NRC 5 alone. Similar results were also observed in the mixed culture of *Trichoderma reesei* and *A. terrus* on bagasse (Massadeh et al. 2001). When *T. reesei* RUT-C30 and *A. phoenicis* were grown on starch substrate, both filter paper activity and β-glucosidase activity were lower than those of each pure culture (Duff et al. 1987). All the above reports suggest that β-glucosidase and cellulases production from mixed fungi culture are species-specific and dependent on the different substrates being used (Wen et al. 2005). They also mentioned that the decrease in filter paper activity and β-glucosidase activity may be due to the lack of synergism of the enzymes produced from the two fungi species. Another reason might be that the nutrients contained in the solid medium were insufficient for the growth of the two fungi and, as a result, a nutrients competition existed between the two fungi species (Wen et al. 2005).

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 substrate, the requirements of microorganism, and the type of end product (Kalogeris et al. 2003). The results of this study have shown that an increase in the solid:liquid (w: v) ratio from 1:1.6 to 1:8.6 (w:v), representing moisture content of 61 to 90 % w/w, greatly enhanced the enzyme production of β-glucosidase, FP-ase, and CMC-ase (Fig. 1). The substrate to moisture ratio of 1:8.6 (w: v) resulted in maximum production of β-glucosidase (1,035.01 U/g ds), CMC-ase (1.43 U/g ds) and FP-ase (0.77 U/g ds). However, any further increase in moisture level in SSF caused free water in the fermentation medium which may lead to limited gas exchange and higher vulnerability to bacterial contamination, while low moisture leads to reduced solubility of nutrients and substrate swelling (Hamidi-Esfahani et al. 2004). The critical importance of moisture level in SSF media and its influence on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles. Lonsane et al. (1985) reported that higher moisture level decreases porosity, changes substrate particle structure, promotes development of stickiness, reduces gas volume and exchange, and decreases diffusion, which results in lowered oxygen transfer and enhanced formation of aerial mycelium. On the other hand, lower moisture content reduces the solubility of nutrients present in solid substrate, decreases the degree of swelling, and increases water tension. Pandey et al. (1994) reported that, with low water availability, fungi suffer modification in their cell membranes leading to transport limitations and affecting microbial metabolism. Additionally, a reduction in enzyme production was recorded at high initial moisture content and this may be due to a reduction in substrate

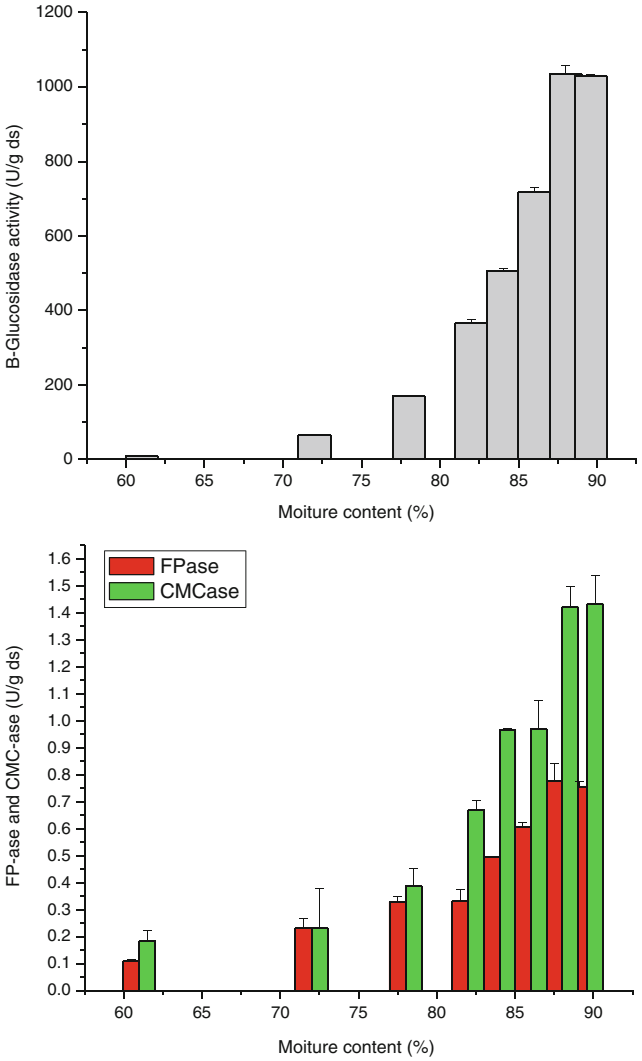

Fig. 1 Effect of different moisture contents on the production of β-glucosidase, FP-ase, and CMC-ase activities under SSF by the co-culture of *A. niger* NRC 7A and *A. oryzae* NRRL 447. *Error bars* standard deviation (±SD) among the replicates, which differ significantly at $p \leq 0.05$

porosity, changes in the structure of substrate particles, and reduction of gas volume.

Effect of initial pH

The pH of the medium is one of the most critical environmental parameters affecting the mycelia growth, enzyme production, and the transport of various components across the cell membrane (Kapoor et al. 2008). β-glucosidase production by the co-culture was also tested at different pH ranging from 3 to 7 using 0.05 M citrate-phosphate buffer. The co-culture of *A. niger* NRC 7A and *A. oryzae* NRRL 447 produced maximum β-glucosidase (1,289 U/g ds), FP-ase (1.00 U/g ds), and CMC-ase (1.38 U/g ds) at initial medium pH 6 (Fig. 2). Increasing the initial pH of the medium from 6 to 7 showed

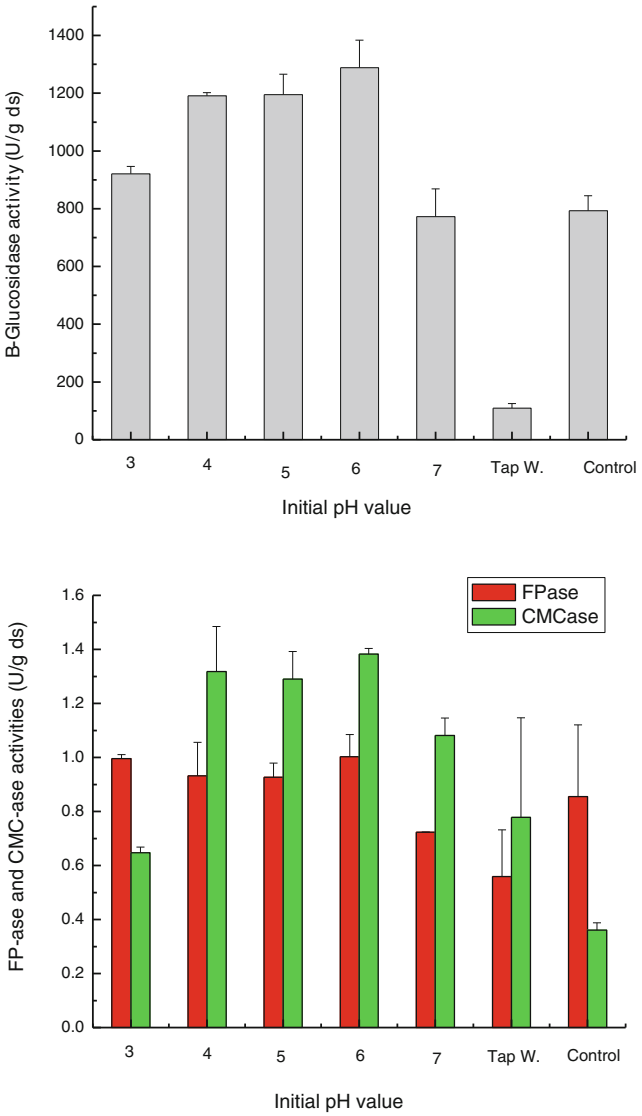

Fig. 2 Effect of different initial pH values on the production of β-glucosidase, FP-ase, and CMC-ase activities under SSF by the co-culture of *A. niger* NRC 7A and *A. oryzae* NRRL 447. *Error bars* standard deviations (±SD) among replicates, which differ significantly at $p \leq 0.05$

a significant decrease in the production of β-glucosidase. A loss of more than 37.7 % in β-glucosidase production was observed at initial medium pH 7. The decrease in initial pH of the medium from 6 to 3 also caused a slight decrease in β-glucosidase, FP-ase, and CMC-ase. The optimum initial pH for maximum fungal cellulase production has been reported to be variable in the majority of the cases (Niranjane et al. 2007). Kalogeris et al. (2003) also reported a decrease in the β-glucosidase by *Thermoascus aurantiacus*, when the pH of the production medium was shifted from an acidic to an alkaline range. The effect of pH on β-glucosidase production by the co-culture of *A. oryzae* NRC 7A and *A. niger* NRRL 447 supported the findings of Lynd et al. (2002) who reported that the pH optimum of β-glucosidase between 5 and 6.

Effect of inoculum size

The effect of mixed inoculum sizes (1–4 mL/5 g ds) with different ratios of *A. niger* NRC 7A and *A. oryzae* NRRL 447 on the production of β-glucosidase is shown in Table 2. The maximum production of β-glucosidase (1,701.98±5.170 U/g ds) was obtained in the fermentation medium that was inoculated with 3 mL of mixed spore suspension (2 mL of *A. niger* NRC 7A and 1 mL of *A. oryzae* NRRL 447) and 1,692.23±70.662 U/g with 4 mL (2 mL of each organism). Further increase or decrease in the ratio of both organisms at the above-mentioned inoculum sizes (3 and 4 mL), however, resulted in the decrease of β-glucosidase production. However, inoculum size less than 3 mL resulted in decrease of β-glucosidase production. On the other hand, further increase or decrease in the size and ratio of mixed inoculum has no significant effect on CMC-ase production. Maximum activity of CMC-ase (1.73±0.010 U/g ds) was recorded when the inoculum size was 4 mL/5 g ds with a ratio of 1:1 (v/v) of *A. oryzae* NRRL 447 and *A. niger* NRC 7A. The trends of

Table 2 Effect of different inoculum sizes and ratios of *A. oryzae* and *A. niger* on production of β-glucosidase, CMC-ase and FP-ase under SSF

Inoculum size (ml/5 g ds)	*A. oryzae*: *A. niger* (v/v)	β-Glucosidase activity (U/g ds)	CMC-ase activity (U/g ds)	FP-ase activity (U/g ds)
1	0.5:0.5 (1:1)	895.21±8.617^{e}	1.47±0.104^{a}	0.57±0.03^{ef}
	0.7:0.3 (7:3)	840.37±24.128^{e}	1.51±0.043^{a}	0.64±0.03^{def}
	0.3:0.7 (3:7)	857.51±10.340^{e}	1.57±0.031^{a}	0.49±0.04^{f}
2	1:1 (1:1)	784.31±34.469 ^{e}	1.44±0.033^{a}	0.59±0.00^{def}
	1.5:0.5 (3:1)	774.57±6.894^{e}	1.57±0.112^{a}	0.59±0.02^{def}
	0.5 :1.5 (1:3)	917.15±5.170^{e}	1.59±0.149^{a}	0.53±0.00^{f}
3	1.5 :1.5 (1:1)	1244.97±37.916^{cd}	1.38±0.154^{a}	0.74±0.01^{bcd}
	0.5:2.5 (1:5)	1519.17±15.511^{b}	1.37±0.029^{a}	0.69±0.04^{cde}
	2.5:5.0 (5:1)	1110.92±58.597^{d}	1.48±0.166^{a}	0.87±0.00^{ab}
	1.0:2.0 (1:2)	1701.98±5.170^{a}	1.32±0.077^{a}	0.87±0.03^{ab}
	2.0:1.0 (2:1)	1270.57±60.32^{cd}	1.38±0.166^{a}	0.89±0.02 ^{a}
4	3.0:1.0 (3:1)	1331.52±77.556^{c}	1.34±0.024^{a}	0.84±0.08^{abc}
	1.0:3.0 (1:3)	1342.47±37.916^{c}	1.56±0.068^{a}	0.84±0.04^{abc}
	2.0:2.0 (1:1)	1692.23±70.662^{a}	1.73±0.010^{a}	0.80±0.07^{abc}

Means followed by different letters within each column differ significantly at $p \leq 0.05$

± indicates standard derivation among replicates

filter paper activity were in a different pattern with CMC-ase activity. The FP-ase activity increased with increase of inoculum size up to 3 mL. However, the ratio of mixed inocula generally has no significant effect on FP-ase production. These results are more or less similar with those reported by other researchers. Dueñas et al. (1995) reported that the enzyme production appears to be growth-associated, where doubling the amount of inoculums doubled the activities of endoglucanases and β-glucosidase and significantly increased filter paper cellulase and xylanase activities. Niladevi and Prema (2008) reported that decreased enzyme production at high inoculum levels might be due to the production of inhibitory metabolites that interfere with the enzyme production.

Effect of incubation period

The co-culture of *A. oryzae* and *A. niger* was grown in time course studies to determine the optimum time for production of β-glucosidase and other cellulases (FP-ase and CMC-ase) under SSF. The co-culture started producing all the three enzymes on day 2 of incubation. Maximum β-glucosidase (1,893 U/g ds) production was observed on the 10th day (Fig. 3). Further increase in incubation time did not favor any increase in the enzyme production. The trends of filter paper and CMC-ase activities were in a similar pattern and increased in parallel with incubation time. However, maximum CMC-ase (1.49 U/g ds) and FP-ase (1.18 U/g ds) activities were shown after 8 and 14 days, respectively. The exact comparison of β-glucosidase and cellulases production by different microorganisms reported in the literature may not be possible because many laboratories estimate the enzymes under different conditions (Deswal et al. 2011).

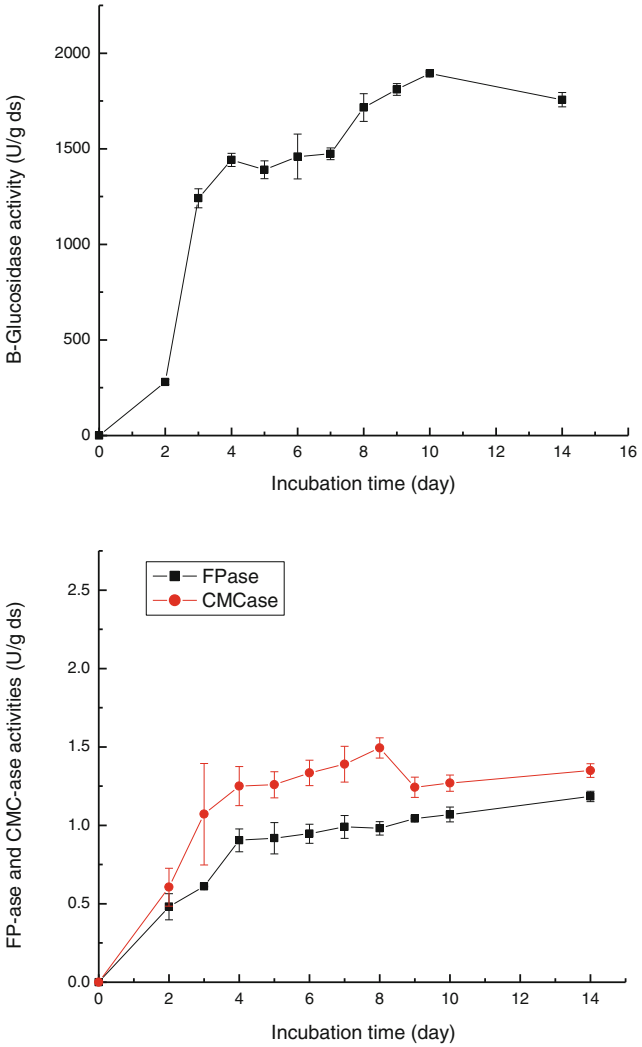

Fig. 3 Time course of β-glucosidase and cellulases (FP-ase and CMC-ase) production by the co-culture of *A. niger* NRC 7A and *A. oryzae* NRRL 447 under SSF. *Error bars* standard deviations (±SD) among the replicates, which differ significantly at $p \leq 0.05$

Effect of supplementation of some different nutrients on β-glucosidase production by the co-culture of *A. oryzae* and *A. niger* under SSF

Extracellular enzyme production depends greatly on the composition of the medium especially carbon and nitrogen sources, minerals, and physical factors such as pH, temperature, and moisture (Lynd et al. 2002). In order to obtain maximum enzyme production, development of a suitable medium and culture conditions is obligatory. Being one of the factors affecting the growth and β-glucosidase and other cellulases by mixed culture, the pH of cultivation is of major interest. During the fermentation process, pH may drop to the acidic range due to acid formation. From the previous results (Fig. 3), it can be noticed that production of β-glucosidase increased gradually up to 1,440 U/g ds at the 4th day and continued to increase at a slower rate in the normal batch SSF for 14 days. Therefore, five different supplementation solutions (50 mM citrate-phosphate buffer, pH 4.8; 50 mM succinate buffer, pH 4.8; 10 % w/v urea, 10 % w/v casein, and 10 % w/v defatted soybean flour) were selected according to the previous reports on enhancement of β-glucosidase production (Ferreira et al. 2009; Gutierrez-Correa and Tengerdy 1997; Deswal et al. 2011). The feeding process was carried out at the 3rd day as mentioned before in "Materials and methods". In order to study the influence of different buffer systems on β-glucosidase and other cellulase production by the co-culture of *A. oryzae* and *A. niger*, the fermented solid medium was supplemented individually with 1 mL of two different buffers (citrate-phosphate and succinate buffer). The control medium was supplemented with the same volume of water to control the moisture content. The results summarized in Fig. 4 show that the highest β-glucosidase activities (4,287 and 4,254 U/g ds) were obtained when fermented medium was supplemented with 1 mL of 50 mM citrate-phosphate or 50 mM succinate buffer, respectively. β-glucosidase activity increased by about 1.66- and 1.64-fold as

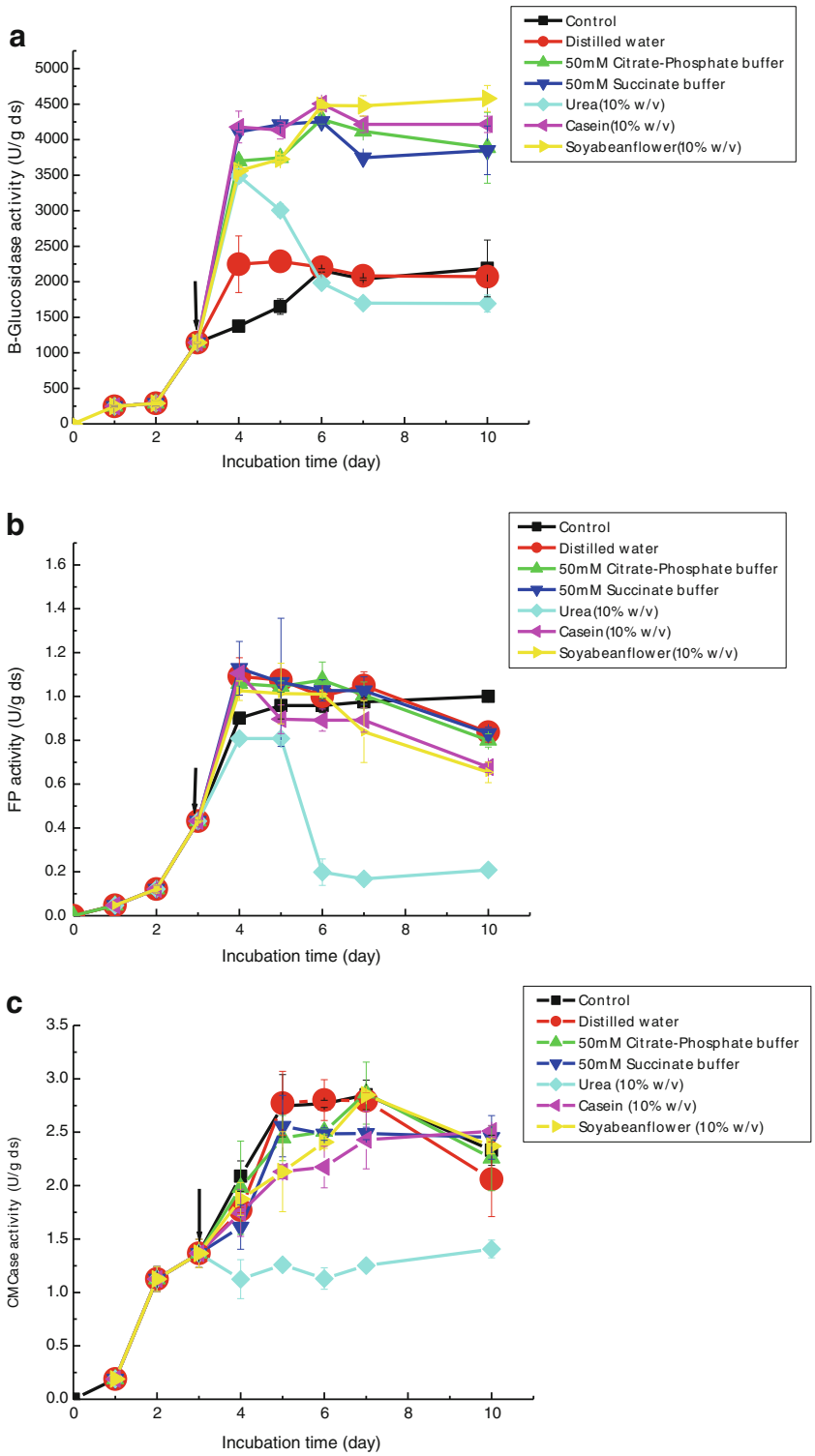

Fig. 4 Time course of β-glucosidase (**a**) ,FP-ase (**b**), and CMC-ase (**c**) production by the co-culture of *A. niger* NRC 7A and *A. oryzae* NRRL 447 under SSF and nutrient [50 mM citrate-phosphate buffer, 50 mM succinate buffer, urea (10 % w/v), casein (10 % w/v) and soybean flour (10 % w/v)] supplementation individually after the 3rd day

compared to control without supplementation. These results are in accordance with Juhasz et al. (2004) who reported a high cellulase production in submerged culture with succinate buffer at 0.1 M, pH 5. Ferreira et al. (2009) mentioned that medium supplementation with citrate buffer or 100 mM succinate buffer at pH 4.8 led to 3.3- to 3.8-fold and 4- to 5.7-fold higher filter paper activity compared with non-buffered cultures and at the same time led to highest β-glucosidase activity. This phenomenon may be related to the regulation capacity of the buffers studied, which are capable of preventing medium pH from lowering below the optimum pH for cellulase production which in turn could avoid the loss of enzyme activities (Ferreira et al.

2009). On the other hand, in the present study, citrate-phosphate and succinate buffers presented no significant effect on either FP-ase or CMC-ase activities.

An important requirement in SSF is the ratio between carbon and nitrogen (C/N). The ratio of C/N is most crucial for a particular process to obtain a specific product. Sugar cane bagasse is a good source of carbon, due to the cellulose and hemicellulose content, and supplementation with soybean flour, casein, and urea may improve the C/ N ratio, presenting good conditions for fungal growth and cellulase production. Also, hemicellulose as a whole is also a good inducer of cellulolytic enzyme system (Babu and Satyanarayana 1996). In the current study, it can be noticed that supplementation of fermented sugar cane bagasse with 1 mL of soybean flour (10 % w/v) or casein (10 % w/v) was conducive for higher β-glucosidase in the mixed culture of *A. niger* and *A. oryzae* under SSF (Fig. 4). Supplementations with 1 mL soybean flour (10 % w/v) or casein (10 % w/v) resulted in 1.77- and 1.74-fold increase of β-glucosidase activity, respectively. It was also observed that supplementation of fermented medium with 1 mL (10 % w/v) urea decreased β-glucosidase production compared with the control. A similar study by Camassola and Dillon (2007) on mixed culture fermentation of sugar cane bagasse supplemented with wheat bran as a nitrogen source strengthened our results of nitrogen sources addition. Also, a number of authors (Ahamed and Vermette 2008; Gutierrez-Correa and Tengerdy 1997) have reported that the type and concentration of the nitrogen source exert some influence on cellulase and β-glucosidase yield. Gautam et al. (2011) reported that municipal solid waste residue (4–5 % w/v), peptone, and yeast extract (1.0 % w/v) were found to be the best combination of carbon and nitrogen sources for the production of cellulase by *A. niger* and *Trichoderma* sp. Crolla and Kennedy (2004) mentioned that the increase in enzyme production after the feeding is an indication that the culture may be able to divert more of its metabolism to enzyme production or that the culture is remetabolizing the cellular stationary phase by products or a combination of both phenomena.

Effect of combination of citrate-phosphate buffer and succinate buffer with soybean flour

Based on the results of the previous experiments, further sets of experiments were carried out to evaluate the effect of combinations of citrate-phosphate buffer with soybean flour and succinate buffer with soybean flour on the production of β-glucosidases under SSF by the co-culture of *A. oryzae* NRC 7A and *A. niger* NRRL 447. The supplementation process was carried out at the 3rd and the 5th day. Figure 5 shows that the production of β-glucosidases increased by about 10.7 and 38.8 % and 25.5 and 69.6 % after the first and the second supplementation with citrate-phosphate and succinate buffers individually with soybean flour, respectively, compared to the control without supplementation. But the

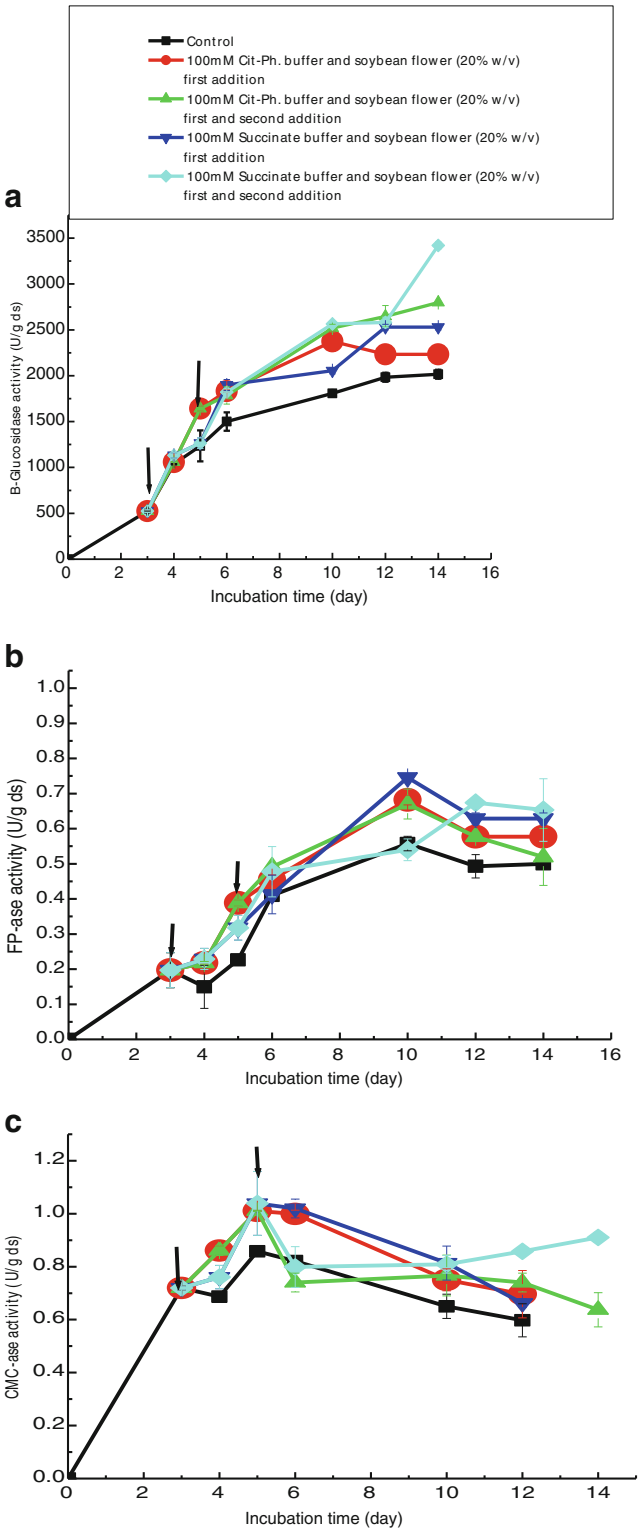

Fig. 5 Time course of β-glucosidase (**a**) FP-ase (**b**), and CMC-ase (**c**) production by the co-culture of *A. niger* NRC 7A and *A. oryzae* NRRL 447 under SSF and mixed nutrients [100 mM citrate-phosphate buffer with soybean flour (20 % w/v) and 100 mM succinate buffer with soybean flour (20 % w/v)] supplementation after the 3rd and 5th days

produced enzymes were, however, less than obtained from the single supplementation with buffers or soybean flour alone.

These results indicate that the combination of buffers with soybean flour on the 3rd and 5th days did not lead to improvements of β-glucosidases production. The reason is that the combination of buffers with soybean flour may enhance the growth of fungal cultures more than enzyme production. Xiaoyan et al. (2007) mentioned that nutrient feeding can prolong the duration of the exponential growth period (log phase) and the static period, and subsequently increase biomass and metabolites production.

Conclusion

Improvement of β-glucosidases production by the co-cultures of *A. oryzae* NRC 7A and *A. niger* NRRL 447 under SSF and intermittent fed-batch technique is a process with great potential for application, since the production of β-glucosidases was improved through this process and high yield was obtained. However, further studies are required to optimize the process. This is the first report on the production of β-glucosidases by the co-culture of *A. niger* and *A. oryzae* under SSF and fed-batch conditions, and the results are very promising.

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