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Xylanase Production by Mixed Culture Using Crude Hemicellulose from Rice Straw Black Liquor and Peat Moss as an Inert Support

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Abstract Black liquor (BL) is a by-product of rice straw pulping process. It is a low costs raw material for production value-adding proteins and enzymes, which has been paid more and more attention to reduce its environmental pollution. Mixed cultures of micelial fungi, *Trichoderma reesei* Northern Regional Research Laboratory (NRRL)11236, *Trichoderma reesei* NRRL 6165 and *Aspergillus niger strains* NRC 5A, NRC 7A, and NRC 9A were evaluated for their ability to produce xylanase using crude hemicellulose (CHC) prepared from BL and peat moss as an inert support under solid state fermentation (SSF). The most potent strains, *A. niger* NRC 9A (818.26 U/g CHC) and *T. reesei* NRRL 6165 (100.9±57.14 U/g CHC), were used in a mixed culture to enhance xylanase production by co-culturing under SSF. In the mixed culture, xylanase production (1070.52±12.57 U/g CHC) was nearly1.3 and 10.6-fold increases over the activities attained in their monocultures, *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively. Optimization of the culture parameters of the mixed culture SSF process, concentration of ammonium sulfate and corn steep liquor, CHC/peat moss ratio, inoculum size and ratios of the two strains, initial pH value, initial moisture content and incubation time, exhibited a significant increase (2414.98± 84.02 U/g CHC) in xylanase production than before optimization.

Keywords *Aspergillus niger* · crude hemicellulose · mixed culture · peat moss · solid state fermentation · *Trichoderma reesei* · xylanase production

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Introduction

Over the years, efforts have been made to solve pollution problem caused by pulp black liquor which has seriously increased. Lignin recovery from BL can significantly reduce the amount of environmental pollution. Furthermore, the remaining effluent (after lignin removal) which contains hemicellulose can be more easily degraded by biological treatment for production of xylanase (Lora and Glasser, 2002). Xylanase (endo-1, 4-β-D-xylanohydrolase; EC 3.2.1.8), the xylan-degrading enzyme, has been reported mainly from diverse group of microorganisms including bacteria, fungi, actinomycetes, yeast, protozoa, gastropods and arthropods (Shallom and Shoham, 2003; Collins et al., 2005). Fungal xylanases are more interesting from industrial point of view because their extracellular activities are much higher than those of yeast and bacteria. A number of fungal species are known for xylanases production such as *A. niger*, *Chaetomium thermophilum*, *Humicola lanuginosa,* and *Trichoderma harzianum* (Polizeli et al., 2005). In recent decades, the interest in cellulases and hemicellulases has increased due to ethanol production from lignocellulosic residues (De Almeida et al., 2011). Xylanases have been extensively studied for their usage in the production of hydrolysate from agro-industrial wastes, in nutritional improvements of lignocellulosic feeds, processing of food, in increasing animal feed digestibility, biobleaching of paper pulp, clarification of fruit juices and wine, extraction of plant oil, coffee and starch (Corral and Villaseñor-

Ortega, 2006). Xylanases in conjunction with other enzymes are used for bioconversion of agricultural wastes into easy fermentable sugars (Romdhane et al., 2010) and generation of biological fuels such as ethanol (Beg et al., 2001; Kuhad et al., 2011).

Solid state fermentation (SSF) is defined as a fermentation process occurring in absence of free flowing water, employing either a natural support or an inert support as a solid material (Ooijkaas et al., 2000).The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms. The use of an inert support impregnated with a liquid medium, with an almost constant physical structure throughout the process, facilitates reproducible and detailed physiological and kinetic studies in SSF, which will eventually be the basis for efficient process development, control strategies and reactor design (Gelmi et al., 2000; Ooijkaas et al., 2000). Several inert materials have been reported in the literatures, such as hemp, perlite, polyurethane foam, poly (styrenedivinylbenzene), sugarcane bagasse, amberlite, and vermiculite (Lareo et al., 2006).

Fungal co-cultivations have been previously described for the production of ligninolytic enzymes under SSF. *A. niger* and *T. reesei* were co-cultivated for cellulases production (Brijwani et al., 2010), while *A. niger* and *A. oryzae* were co-cultivated for β-glucosidase production (Hu et al., 2011; Noor El-Deen et al., 2014). Co-cultivation of *Pleurotus ostreatus* and *Phanerochaete chrysosporium* resulted in higher production of ligninolytic enzymes (Verma and Madamwar, 2002), which was also found for a *Trametes* and a *Trichoderma* strain (Zhang et al., 2006).

In the present work, efforts are made to optimize the cultural conditions of the co-culture of *A. niger* and *T. reesei* under SSF for xylanase production using crude hemicellulose (CHC) prepared from RSBL and peat moss as an inert support. Optimization of the factors affecting xylanase production such as the initial pH, inoculum size and ratio of the two organisms, corn steep liquor (CSL) concentration and CHC/peat moss ratio individually enhanced xylanase production. To the best of our knowledge, it is the first paper that reports the production of xylanase by mixed culture using CHC prepared from RSBL and peat moss as an inert support and the results were found to be quite encouraging.

Materials and Methods

Microorganisms and culture maintenance. *A. niger* NRC 5A, *A. niger* NRC 7Aand *A. niger* NRC 9A were obtained from the stock culture of Natural and Microbial Products Department, National Research Center, Egypt. *T. reesei* Northern Regional Research Laboratory (NRRL) 11236 and *T. reesei* NRRL 6165 were kindly obtained from NRRL, USA. The 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 three milliliters 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 *T. reesei* strains each flask containing 4 gram (2 g CHC and 2 g peat moss) dry substrate was inoculated with 4mL 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, 2 mL of each strain was inoculated into each flask simultaneously.

Preparation of CHC from RSBL. Rice straw black liquor (pH 12) obtained from Nobaria rice straw pulping mill (Nawwar et al., 2008) was treated with CaO (18 g/L w/v) 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, air-dried over night followed by oven drying at 80°C to remove the moisture content and obtain constant weight. The obtained brown precipitate affords the crude polysaccharides with the following constituents: Natural detergent fiber, 47%; Acidic detergent fiber, 21.4%; Acidic detergent lignin, 17.72%.

Xylanase production under SSF. Unless otherwise stated, SSF was carried out in 250 mL Erlenmeyer flasks, each having 2 g of CHC and 2 g of peat moss moistened with 8 mL mineral salt solution (g/L: 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; $ZnSO_4$, 0.0014, and pH 5.5) to attain final solid-to-moisture ratio of 1:3 (w/v). The flasks were sterilized by autoclaving at 120°C (15 psi), cooled to room temperature and then inoculated with the desired volume of inocula. 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 sterilized glass rod to distribute the inoculum throughout the substrate and incubated at 30°C.

Screening for xylanase activity. Three strains of *A. niger* and two strains of *T. reesei* (*T. reesei* NRRL 11236, *T. reesei* NRRL 6165) were used for the primary screening for their ability to grow and produce xylanase activity in a solid medium containing CHC as the main carbon source and peat moss as an inert support (2 g/ flask) and impregnated with mineral medium. Flasks were autoclaved and inoculated by 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 duplicates and the average values were reported.

Optimization of process parameters for xylanase production. The medium described above was taken as a 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 to 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) using 50 mM citrate-phosphate buffer and initial moisture content (63.6–87.4% v/w) were optimized for xylanase production. The procedure adopted for optimization of various process parameters influencing xylanase production was to evaluate the effect of individual parameters (keeping all other parameters as constant) and to incorporate it at the optimized level in the experiment before optimizing the next parameter. All the experiments were carried out in duplicate and the mean values are reported.

Enzyme extraction. A 20 mL aliquot of citrate-phosphate buffer (50 mM, pH 6.0) was added to each flask and the mixture 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 in the enzyme assay. Each batch was prepared in duplicate and average values plus percentage standard deviations of the mean were obtained.

Xylanase assay. The enzyme activity was determined according to Sanghi et al. (2008) with some modifications by measuring the release of reducing sugars during the enzyme-substrate reaction using 3, 5 dinitrosalicylic acid (DNS) reagent (Miller, 1959). The reaction mixture (1.0 mL) containing 0.5 mL of 1% w/v Birchwood xylan (prepared in 50 mM citrate-phosphate buffer pH 5.0) as substrate, 0.025 mL of suitably diluted enzyme extract and 0.475 mL citrate-phosphate buffer (50 mM, pH 5.0) was incubated at 50°C for 20 min and then the reaction was terminated by adding 2.0 mL DNS reagent. A control was run simultaneously which contained all the reagents but the reaction was terminated prior to the addition of enzyme extract. The contents were placed in a boiling water bath for 10 min and then cooled to room temperature. The absorbance of the resulting color was measured against the control at 540 nm in a spectrophotometer. One unit of xylanase activity was defined as the amount of enzyme catalyzing the release of 1 µmol of reducing sugar equivalent to xylose per minute under the specified assay conditions. Xylanase production was expressed as units (U) per gram CHC. All the experiments were carried out independently in duplicate and the results presented are the mean of the two values.

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

Screening of xylanase producing *Aspergillus* and *Trichoderma* strains. In the present study, screenings of some different *A. niger* strains, *T. reesei* NRRL 11236 and *T. reesei* NRRL 6165 and the co-cultures of them for their production of xylanase activities by SSF were carried out using CHC prepared from RSBL and peat moss as an inert support incorporated in the basal salt medium. After an incubation period of 7 days, *A. niger* strains (*A. niger* NRC 5A, *A. niger* NRC 7A, and *A. niger* NRC 9A) generally produced more xylanase (312.62, 384.97, and 818.26 U/g CHC, respectively) than the other *Trichoderma* strains [*T. reesei* NRRL 11236 (230.73 U/g CHC) and *T. reesei* NRRL 6165 (100.90 U/g CHC)] as indicated in Fig. 1. Similar findings were reported by other researchers under the optimized conditions by *A. niger* LPB 326 cultivated on lignocellulosic substrate composed of sugarcane bagasse and soybean meal in SSF (Maciel et al., 2008; Brijwani et al., 2010).

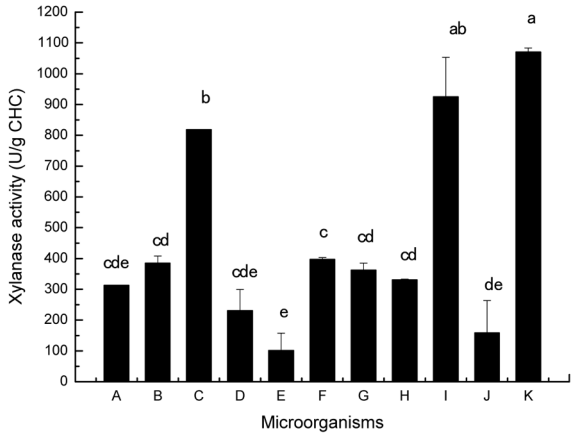

Fig. 1 Screening of mono-culture and co-culture of some *A. niger* strains, *T. reesei NRRL 11236* and *T. reesei* NRRL 6165 and the co-cultures of them for their production of xylanase by SSF. A *A. niger* NRC 5A; B *A. niger* NRC 7A; C *A. niger* NRC 9A; D *T. reesei NRRL 11236*; E *T. reesei* NRRL 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* NRRL6165. Y-error bars indicate the standard deviation (±SD) among the replicates. Means, in each bar, followed by the same letter are not significantly different ($p \leq 0.05$).

The synergistic interaction of *A. niger* NRC 5A, *A. niger* NRC 7A and *A. niger* NRC 9A with *T. reesei* NRRL 11236 and *T. reesei* NRRL 6165 led to a greater efficiency for xylanase production [*A. niger* NRC 5A and *T. reesei* NRRL 11236 (393.83±6.1U/g CHC), *A. niger* NRC 5A and *T. reesei* NRRL 6165 (362.34±22.86 U/g CHC), *A. niger* NRC 7A and *T. reesei* NRRL 6165 (924.93±127.99 U/g CHC) and *A. niger* NRC 9A and *T. reesei* NRRL 6165 (1070.52±12.57 U/g CHC)]. The highest yield of xylanase activity produced from the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 represent about 1.3 and 10.6 fold more than that of the monocultures of *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively. These results are in accordance with those reported by other researchers (Ahamed and Vermette, 2008; Brijwani et al., 2010). Hu et al. (2011) mentioned that most mixed cultivations resulted in increased enzyme activities compared to the single cultures, although not always for all enzymes tested. Compared with the corresponding pure cultures, the enzyme levels produced

Table 1 Xylanase production under SSF by different co-cultures and different substrates

Microorganisms	Substrate	Xylanase U/g	References
T. reesei LM-1 and *A. niger* ATCC 10864	Sweet sorghum silage	187	Castillo et al. (1994)
T. reesei LM-UC4 and *A. phoenicis* QM 329	Sugar cane bagasse	854.1	Dueñas et al. (1995)
T. reesei LM-UC4E1 and *A. niger* ATCC 10864	Sugar cane bagasse and soy meal	2600	Gutierrez-Correa and Tengerdy (1998)
T. reesei LM-UC4E1 and *A. phoenicis* QM 329	Sugar cane bagasse and soy meal	2800	Gutierrez-Correa and Tengerdy (1998)
T. reesei QM 9414 and *A. niger*	Water hyacinth	168	Deshpande et al. (2008)
T. reesei ATCC 26921 and *A. oryzae* ATCC 12892	Soybean hulls and wheat bran	505	Brijwani et al. (2010)
Penicillium oxalicum SAUE-3.510 and *Pleurotus ostreatus* MTCC 1804	Sugarcane bagasse and black gram husk	8205.31	Dwivedi et al. (2011)
A. niger F7 and *Fusarium oxysporum* F8	*Toona ciliat*a	901.15	Kaushal et al. (2012)
A. niger F7 and *Fusarium oxysporum* F8	Celtris australis	1012.95	Kaushal et al. (2012)
A. niger F7 and *Fusarium oxysporum* F8	Cedrus deodara	204.72	Kaushal et al. (2012)
A. niger F7 and *Fusarium oxysporum* F8	Pinus roxburghii	311.32	Kaushal et al. (2012)
*A. nige*r NRC 9 and *T. reesei* NRRL 6165	CHC and CSL	2414.98	This work (after optimization)

by the mixed culture depended on the fungi species and substrate used (Table 1). Maximum levels of xylanase was obtained by co-cultivation of mutant *Penicillium oxalicum* SAU(E)-3.510 and *Pleurotus ostreatus* MTCC 1804 using 4 g of solid supported with 80% (v/w) of moisture content compared to their monocultures. A combination of sugarcane bagasse and black gram husk in a ratio of 3:1 (w/w) was found to be the most ideal solid substrate and support for fungal colonization and enzyme production during co-cultivation (Dwivedi et al., 2011). The co-culture of *A. niger* and *Fusarium oxysporum*, produced more xylanase activity in pretreated *Celtris australis* wood compared with mono culture individually (Kaushal et al., 2012). Also, β-xylosidase activity was increased in the combinations of *A. niger* and *Phanerochaete chrysosporium* and *A. oryzae* and *Magnaporthe grisea*, while the activity in the *A. niger–A. oryzae* co-cultivation was approximately the average of the two single cultures (Hu et al., 2011).The advantage of co-culture is more pronounced in SSF condition because the colonization of the substrate may be accomplished better in symbiotic association i.e. each species having its own niche for growth and substrate degradation (Selby, 1968). It is postulated that cellulases from different species are closely related to each other and that endoglucanase enzyme component from one fungal species can operate with exoglucanase of another at least when both fungi have endoglucanase-exoglucanase system. The explanation may be that the two enzymes in order to achieve a synergistic effect have to work together in the form of a base complex. The advantage of co-culture can also be due to the production of both cellulase and xylanase enzymes in the co-culture (Kaushal et al., 2012).

On the other hand, results in Fig. 1 show also the synergistic interaction of *A. niger* NRC 7A and *A. niger* NRC 9A with *T. reesei* NRRL 11236 produced xylanase activity lower than their mono culture alone. Similar results were also observed in the mixed culture of *T. reesei* and *A. terrus* on bagasse (Massadeh et al., 2001). The decrease in enzymes activities maybe 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 in sufficient 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 ammonium sulfate and CSL on xylanase production. The effects of supplementation of fermentation medium with different concentrations of ammonium sulfate and CSL individually as nitrogen sources on xylanase production are evaluated. The selection of ammonium sulfate was based on ammonium salts in the form of sulfate facilitate cellulase, β-glucosidase and xylanase production in *Penicillium funiculosum*, *Chaetomium cellulolyticum*, *T. reesei*, *A. niger* and *A. terreus* (Gutierrez-Correa et al., 1998; Raghavarao et al., 2003).The results in Figs. 2A and B) indicate that CSL alone is more suitable for xylanase production by the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165. The highest xylanase levels were detected in the presence of CSL (2003.42±87.38 U/g CHC) at 6 mL/2 g CHC and $(NH_4)_2SO_4$ (1203.13±96 U/g CHC) at 5.6 g/L, respectively. The better production of xylanase activity obtained in the media containing CSL was probably due to the presence of certain nutrients that were absent in the other formulations. It is generally a rich source of nitrogen, water soluble vitamins, amino acids, minerals and other growth stimulants. The richness of CSL with these nutrients (Kulp and Ponte, 2000) may be enough to stimulate growth and enhancing xylanase production.

Effect of initial pH. The pH of the medium is the other most important factor for a fermentation process, which influences the microbial growth and enzymes activities. Optimum pH required for maximal xylanase production during SSF using CHC was evaluated using various initial pH levels (4-8) adjusted in the moistening solution using 50 mM citrate-phosphate buffer. Results presented in Fig. 3 showed that maximal enzyme production (2110.36±178.12 U/g CHC) was recorded with initial pH value of the mineral salt solution as 5.0 for the mixed culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165. It was observed that if the initial pH value of the fermentation medium was between 5 and 7, no significant difference was observed on xylanase production. However, any further increase (more than pH 7) or decrease (less

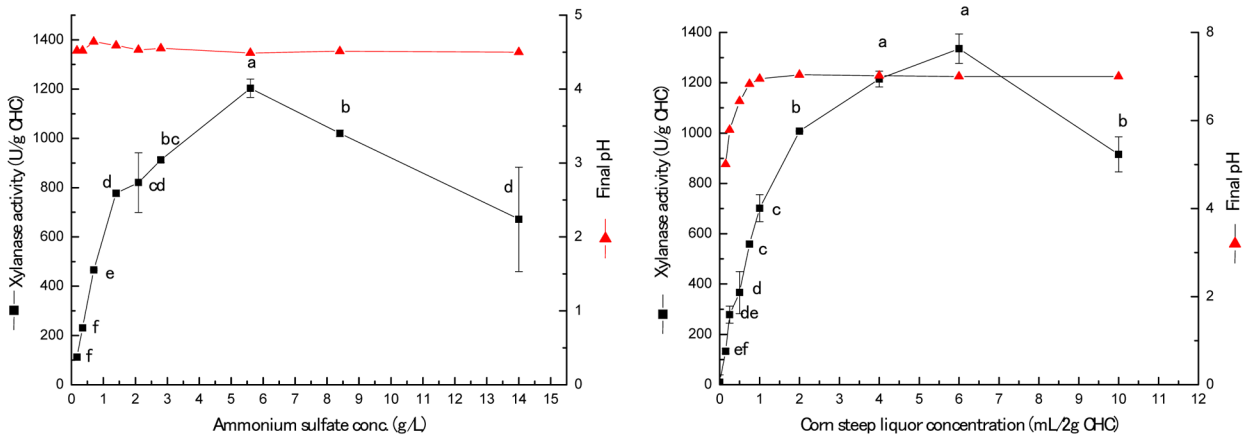

Fig. 2 Effect of different concentrations of ammonium sulfate (A) and corn steep liquor (B) on the production of xylanase activity under SSF by the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 using CHC as a carbon source and peat moss as an inert support. Y-error bars indicate the standard deviation (±SD) among the replicates. Means, in each bar, followed by the same letter are not significantly different ($p \leq 0.05$).

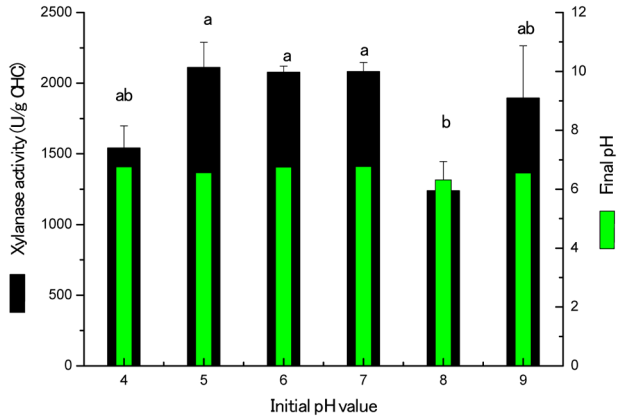

Fig. 3 Effect of different initial pH values on the production of xylanase under SSF by the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 using CHC as a carbon source and peat moss as an inert support. Y-error bars indicate the standard deviation (±SD) among the replicates. Means, in each bar, followed by the same letter are not significantly different ($p \leq 0.05$).

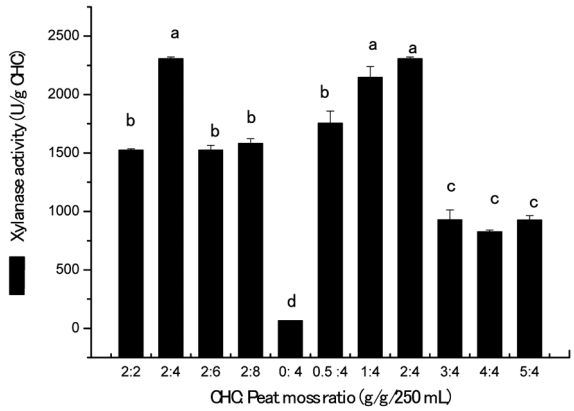

Fig. 4 Effect of different ratios of CHC and peat moss on xylanase production by the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165under SSF. Y-error bars indicate the standard deviation (±SD) among the replicates. Means, in each bar, followed by the same letter are not significantly different ($p \leq 0.05$).

than pH 4) in the initial pH value of the mineral salt solution reduced the enzyme production. These findings are in line with the work of Ellaiah et al. (2002) who found pH 5 was the optimal culture condition for enzyme production by fungal strains including *Aspergillus* sp. under SSF. This acidic condition in the SSF was acceptable since CHC prepared from RSBL used as a substrate in this study is acidic by nature and it was favorable for the fungus to grow and produce enzyme. On the other hand, the results also show that water produced appreciable amount of xylanase (1894.11±369.67 U/g CHC) without adjustment or buffering the fermentation medium. Generally, fungi prefer low pH levels for good growth. The natural pH of the substrates, which was around 6.0 (without adjustment or buffering), was the best for development of the fungi and xylanase production by *A. niger* in SSF (Maciel et al., 2008).

Effect of CHC and peat moss ratio. In the present work, the study was also undertaken to optimize the critical CHC and peat moss ratio in SSF for xylanase production by co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165. Different ratios of CHC and peat moss were used for xylanase production in SSF. The initial pH of all experiments was adjusted at pH 5 as mentioned before using 50 mM citrate-phosphate buffer. The results (Fig. 4) show that xylanase activity were maximal (2146.00±94.1 and 2305.22±16.8 U/ g CHC) in the culture comprised of CHC: peat moss ratio of 1:4 and 2:4 (g CHC/g peat moss/250 mL conical flask), respectively. However, the xylanase activity was diminished with CHC and peat moss ratio producing the lowest xylanase activity at the higher peat moss ratios (2:6 and 2:8 w/w/250 mL conical flask). These results are in a good agreement with that obtained by Lee et al. (2011) and Ibrahim et al. (2012). They reported that a significant reduction in filter paper activity (FPase) production was detected with an increasing use of the amount of solid materials in the fermentation process. The level of a solid

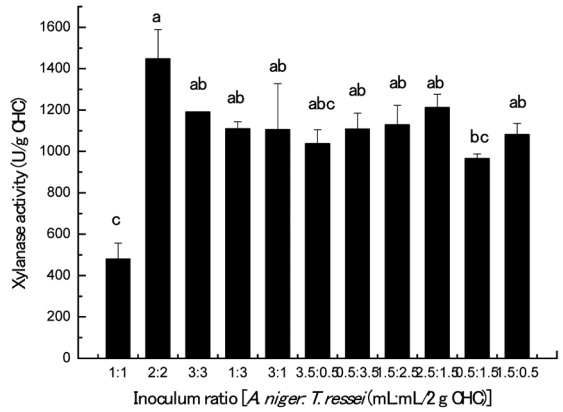

Fig. 5 Effect of different inoculum sizes and ratios of *A. niger* NRC 9A and *T. reesei* NRRL 6165 on the production of xylanase under SSF using CHC as a carbon source and peat moss as an inert support. Y-error bars indicate the standard deviation (±SD) among the replicates. Means, in each bar, followed by the same letter are not significantly different ($p \leq 0.05$).

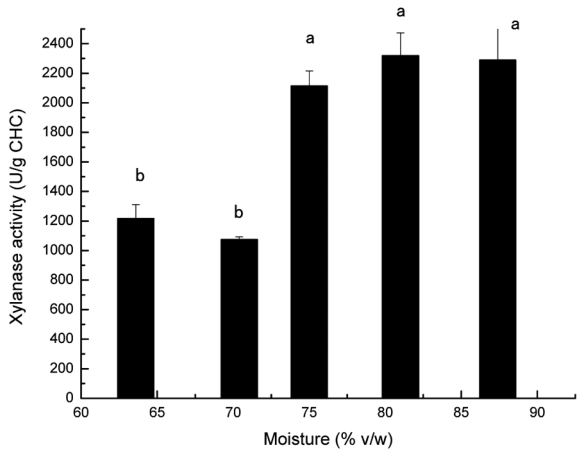

Fig. 6 Effect of different moisture contents on the production of xylanase under SSF by the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 using CHC as a carbon source and peat moss as an inert support. Y-error bars indicate the standard deviation (±SD) among the replicates. Means, in each bar, followed by the same letter are not significantly different ($p \leq 0.05$).

support per unit area of working volume of the flask influences the porosity and aeration of the substrate. In SSF, solid height is important as fungal growth on the surface of substrate was almost similar, but the growth within the solid height varied depending on the solid thickness or also known as the bed height. Annuar et al. (2010) attributed this observation to the heat generated by the fungal fermentation process which should be removed or controlled, since fungal growth is sensitive to temperature rise. It is further suggested that heat is better dissipated from the surface than from within the bed, and a thinner bed height allows for better heat removal than a thicker bed height. Raghavarao et al. (2003) reported that at larger bed thicknesses, the oxygen concentration at the bottom of the bed falls to zero within first 24 h of the fermentation depending on the height of the bed which leads not only to inefficient use of the substrate but also to undesirable situation like anaerobiosis and cells lysis. On the other hand, a thinner bed height is usually chosen in the tray system since it easily to be fermented and permits better oxygen supply and heat removal (Ibrahim et al., 2012).

Effect of inoculum size. The effect of mixed inoculum sizes (2–6 mL/2 g CHC) with different ratios of *A. niger* NRC 9A and *T. reesei* NRRL 6165 on the production of xylanase using CHC is shown in Fig. 5. All experiments were carried out at initial pH 5. The maximum production of xylanase (2305.22±16.8 U/g CHC) was obtained in the fermentation medium that was inoculated with 4 mL of mixed spore suspension (2 mL of *A. niger* NRC 9A and 2 mL of *T. reesei* NRRL 6165). Further increase or decrease in the ratio of both organisms at the above-mentioned inoculum size; however, resulted in a decrease of xylanase production. However, inoculum size less than 4 mL resulted also in a decrease of xylanase production. In SSF inoculum should be distributed homogeneously and high enough to assure predominance of the strain. Most applications of SSF involve the use of a fungal spore inoculum. Spore inocula are more evenly distributed and give better interparticle translocation (Gawande and Kamat, 1999).The microbial (fungal/bacterial) particles which initially will be on the outer surface of the substrate particles, will slowly grow, multiply and penetrate into the macro and micro pores of the solid (Raghavarao et al., 2003). They consume the available energy sources and secrete enzymes that can also break down the starch and cellulose in the substrate to provide energy. Most of the previous studies (Mahanama et al., 2011) mentioned a strong influence of high inoculum size on the production of microbial metabolites. Laukevics et al. (1984) 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. Dueñas et al. (1995) reported also 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. However, 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 initial moisture content. Moisture content has a profound influence on the production of xylanase by the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 in the SSF system consisting mainly from CHC as the main carbon source and peat moss as impregnated support. All experiments were carried out at initial pH 5 and excess distilled water was added to get the desired moisture percent. Fig. 6 shows the yields of xylanase under five different moisture contents. The moisture content of 87% (w/w) provides the best environment for xylanase production (2318.51±154.26 U/g CHC). However, any further increase in moisture level in SSF causes free water in the fermentation medium which may lead to limit gas exchange and higher vulnerability to bacterial contamination, while low moisture leads to reduce solubility of nutrients and substrate swelling. The

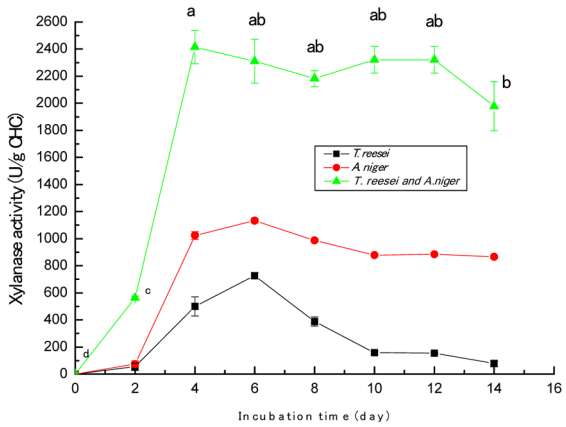

Fig. 7 Time course of xylanase production under SSF by the co-culture of *A. niger* NRC 9A and *T. reesei* NRRL 6165 using CHC as a carbon source and peat moss as an inert support. Y-error bars indicate the standard deviation (±SD) among the replicates. Means, in each bar, followed by the same letter are not significantly different ($p \leq 0.05$).

free water of the substrate determines the void space which is occupied by air. Since the transfer of oxygen affects the growth and metabolism, the substrate should contain suitable amount of water to enhance mass transfer. Furthermore, higher moisture level leads to higher in humidity that would make the solid substrate clump together due to the interference of moisture with the physical properties of the solid particles which results in a decrease of inter-particle space leading to decrease diffusion of nutrients(Pandey et al., 2001; Hamidi-Esfahani et al., 2004).

Effect of incubation period. The co-culture of *A. niger* NRC 9Aand *T. reesei* NRRL 6165 was grown in time course studies to determine the optimum time for production of xylanase under SSF using CHC as substrate and peat moss as an inert support. Xylanase production started earlier (i.e. during lag phase) for the mixed culture. Maximum xylanase (2414.98±84.02 U/g CHC) production by the co-culture was observed after 4 days (Fig. 7). Further incubation after this time did not show any increment in the level of enzyme production. Unlike mixed culture, the highest levels of xylanase by single culture for both *A. niger* NRC 9A (1133.58±15.68 U/g CHC) or *T. reesei* NRRL 6165 (727.31± 23.86 U/g CHC) were obtained after 6 days (Fig. 7). The results shown indicate that mixed culture results in a better xylanase production than either single culture. The highest xylanase activity produced by mixed culture of *A. niger* and *T. reesei* after medium optimization represents about 2.13 and 3.32-fold increase over the activities attained in single-culture SSF for *A. niger* NRC 9A and *T. reesei* NRRL 6165, respectively. Similar trends were also reported in xylanase production by fungal mixed culture and single culture SSF on lignocellulosic materials (Gutierrez-Correa and Tengerdy, 1998; Shahi et al., 2011).

Conclusion. With regard to the optimization of xylanase production by both fungal strains, agro-industrial waste (CHC) obtained from treatment of RSBL, which is inexpensive and abundant, was found to be good. Economically, CHC, peat moss as an inert support and CSL are cheaper than pure xylan as a substrate for xylanase production. Our investigation has indicated the bioconversion of CHC from rice straw pulping manufacturing may be an added benefit to the process. This approach is also used to decide that peat moss could be used for fungal growth, spore, organic compounds and enzymes production in SSF because of its excellent physical and chemical properties. Peat moss is a natural, organic conditioner (more than 95% organic matter); with a unique structure that provides a good balance of air and water for fungal growth. It has a pore volume of more than 96%, which makes it an excellent support material for solid state fermentation. It decomposes slowly compared to other types of organic matter which can be used for repeated fermentation. Therefore, it provides better aeration, less compaction problems and greater growth surface for spores and metabolites production.

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