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Synergistic Saccharification by Mixture of Several Fungal Semi Purified Cellulose Degrading Enzymes

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ABSTRACT

Twenty eight pre-identified fungal isolates and strains were tested qualitatively for their cellulolytic activities, among which 12 superior producers were quantitatively tested. Three *Aspergillus* spp. namely A5, A6 and A7 were found to be the best cellulolytic fungi, while both *Aspergillus terreus* NRRL260 and *Phanerochaete chrysosporium* NRRL6361 were found to be the best xylanase producers. On solid state fermentation (SSF) using pretreated rice straw (RS), the *Aspergillus* spp. A5, A6 and A7 gave total cellulases (as FPase), exo-cellulases (as CMCase) and cellobiase (as β-glucosidase) activities of ≥ 16, 130 and 21U.g^{-1}ds, respectively. On the other hand, *Aspergillus terreus* NRRL260 showed maximum xylanases activity of 804 U.g^{-1}ds. The recovered crude enzymes forming the crude enzyme mixture (CEM) was concentrated (LCEM), ammonium sulfate fractionated (PPE1), were semi-purified by gel permeation on Sephadex G100 and finally lyophilized to powder form (PPE2). The resulting cellulases specific activities were 0.50, 0.68, 1.01 and 1.47 U.mg^{-1}, respectively. Those purification and concentration steps resulted in purification fold increase by 1.28, 2.08 and 2.78 for LCEM, PPE1 and PPE2, respectively. PPE2 achieved maximum degree of saccharification (DoS) at enzyme load ≥ 10 FPU, substrate concentration ≥ 5% and incubation period after 48 hr to be 85%, 87% and 88%, respectively.

Key words: fungal cellulases; fungal xylanases, *Aspergillus* sp., *Phanerochaete* sp., saccharification, rice straw.

Introduction

Cellulases enzymes of main functional types; Endo cellulases (EC 3.2.1.4), Exocellulases/1,4-β-cellobiosidase (EC 3.2.1.91), Glucosidase/1,4-β-cellobiase (EC 3.2.1.21); are comprising 15% of enzymes produced worldwide and are considered one of the most important enzyme types beside protease (18%) and amylase (25%), as stated by Sajith et *al*., (2016). Their enzymatic hydrolysis of cellulosic biomass generates sugars that serve as raw materials in the production of valuable products such as ethanol (Yinbo et al., 2006; Nathan et *al*., 2014), organic acids (Shen and Xia, 2006; Zhang et *al*., 2007), free sugars, antibiotics and animal feeds (Cao et *al*., 2014). Commercialization of fermentable sugars production from lignocellulosic biomass faces many obstacles like the prohibitive cost of cellulases preparations needed for conversion of lignocellulosic biomass into fermentable sugars (Mahmud and Gomes, 2012). Broda *et al*., (1996) stated that the use of cheaper raw materials and cost effective fermentation strategies like SSF could improve the economics of cellulase production. Pandey (2003) demonstrated that SSF, used at large scale production for fungal bioconversion of lignocellulosic materials into valuable commodities, needed fewer infrastructures and produced more concentrated enzymes via low input of process engineering, considered as economic advantage.

On the other hand, cellulases production by different organisms in submerged fermentation (SmF) has received more attention but considered cost-prohibitive due to its high cost of process engineering (Chandra *et al*., 2007). Recently, several studies were held on cellulase production under solid state fermentation (SSF) in cost effective manner (Gupta et *al*., 2015; Kuila et *al.*, 2015; Mangalanayaki and Madhavan 2015). Although SSF has several advantages for higher cellulase

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production, it has different drawbacks for large scale enzyme production such as it requires large space for enzyme production, less amount of enzyme are extracted after fermentation, purification of enzyme is difficult …,etc. In such a case, submerged fermentation (SmF) is used for production of several industrially important enzymes (cellulase, xylanase, laccase etc.) due to several advantages such as greater control of environmental factors (temperature, pH, .. etc), requires smaller space, higher amount of enzyme can be extracted after fermentation and purification of the enzyme is easier, as stated by Sharma et *al.*, (2016).

The synergistic effect of endo and exo cellulases in hydrolyzing the insoluble crystalline cellulose was determined by the degree of synergistic effect (DSE) in the work of Beldman et *al.*, (1988). They stated that with a specific ratio of endo to exoglucanase best achievement could be accomplished. Nevertheless, saccharification was also affected by the degree of substrate polymerization besides the selective behavior of either exo or endoglucanase in adsorption to the cellulosic substrate. In a similar trend, no single strain was capable of producing enough amounts and perfect ratios of those enzymes collectively. Consequently, several fungi belonging to the genera *Aspergillus* have been selected and used to develop an enzymatic saccharification process for various kinds of cellulosic materials. The performance and productivity of cellulolytic enzymes produced by those fungi were insufficient for perfect saccharification of cellulosic substrates as it was well known that this ability was influenced by the composition of the enzyme components especially FPase, β-glucosidase and xylanase (Yamanobe *et al*., 1987 and Mekala *et al*., 2008). Besides, screening of naturally occurring cellulolytic microorganisms or mutant strains secreting higher levels of cellulases is of immense importance for developing economically competitive bioprocess strategies for commercial applications (Kubicek et al., 2009; Reddy *et al*., 2014).

The present work was conducted to study the utilization of the pretreated rice straw (RS) and sugar cane bagasse (SCB) as sole carbon sources and enzyme inducers by the selected fungal strains, for production of active mixtures of cellulases and xylanases using solid-state fermentation (SSF). Preparation of crude and partial purified active enzyme mixture of cellulases and xylanase for conversion of the pretreated lignocellulosic biomass carbohydrates into fermentable sugars was examined as well.

Materials and Methods

The lignocellulosic materials used in this study included rice straw and sugarcane bagasse from local market. Both lignocellulosic materials were separately chopped, washed in tap water and dried under vacuum at 60°C for 2 days. Then lignocellulosic materials were pretreated with NaOH (2%) and NaClO (1.5%) to ensure the highest delignification degree and increasing their cellulose content, according to Mohy *et al*., (2015). Both rice straw and sugar cane bagasse after treatment were designated as RS and SCB, whose total carbohydrate contents were 75 % and 86 % (W/W), respectively. The chemicals and reagents used in this study include carboxy methyl cellulose (CMC), cellulose powder and salicin (Merk Ltd.), Xylan and egg albumin (Sigma-Aldrich Chemical Co.) and 3,5 di nitro salycilic acid (BDH). Filter paper Whatman no.1 was used in filter paper assay for cellulases (FPase).

Twenty eight fungal candidates were generously offered by the Department of Microbiology, SWERI, ARC, Egypt. Twenty four fungal isolates belonging to four filamentous fungal genera namely *Trichoderma* (11 isolates), *Aspergillus* (11 isolates), *Acremonium* (one isolate) and *Pencillium* (one isolate) as confirmed by Mycology Research and Plant Diseases Survey Department at Plant Pathology Research Institute, ARC, Egypt. Four fungal strains namely *Aspergillus terreus* (NRRL260), *Phanerochaete chrysosporium* (NRRL6361), *Pleurotus ostreatus* (NRRL2366) and *Trichoderma viride* (NRRL 1698) were included in this work.

Microbiological media used in the present study were all adjusted at initial pH=5 and then sterilized at 121°C/20 min. Potato-dextrose agar (PDA) medium was used for fungal maintenance (Booth, 1971). CMC agar medium was used for screening cellulytic fungi (Florencio *et al*., 2012). Standard mineral based medium was used for production of cellulases and xylanases in SmF (Copa-Patino *et al*., 1993). Modified mineral salt medium was used for production of cellulases and xylanases in SSF (Mandels and Weber, 1969). Inoculum growth medium containing (gl^{-1}): Glucose, 10.0; $(NH_4)_2SO_4$, 1.0; Peptone, 1.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.3 and $CoCl_2$, 0.3 (Fadel; 1994).

Required spore suspension of 10^6-10^7 spores / ml was inoculated into inoculum growth medium at a ratio of 2:50 (V/V) and incubated on orbital shaker incubator at 35°C / 200 rpm for 36 hours, as recommended by Fadel (1994). The culture was used as inoculum for enzyme production at the rate of 5% (v/v) in SmF and 2 ml/ 2 g substrate in SSF. SmF enzyme production was conducted in 250 ml Erlenmeyer flask containing 100 ml of modified mineral salt medium supplemented with 1 % crude RS or SCB. The medium was inoculated with spore suspension, incubated in a shaking incubator at 35°C / 200 rpm for a suitable period. It was followed by the separation of fungal mass by filtration. SSF was carried out by autoclaving 2 g of the substrate at 121°C for 30 min and moistened to 80 % with mineral salt medium (Mandels and Weber, 1969). It was inoculated with spore suspension and incubated at 35°C for a suitable period. To harvest crude enzyme preparation, 50 ml of the sodium citrate buffer (0.05 M, pH 4.8) were added to fermented substrate and agitated to 150 rpm for 2 h. The slurry was filtered through four layers of muslin cloth and Whatman # 1 filter-paper, followed by centrifugation at 4000g at 4°C for 20 min.

Crude enzyme separation and partial purification: The resultant cell-free supernatants from chosen fungal cultures were used as crude enzyme preparation (CEM) and concentrated 2-Folds by lyophilization under vacuum at -40°C using Edwards Modulyo Tray freeze dryer (model EF4-174) to be considered as new crude enzyme mixture LCEM. As described by Scopes (1993), partial purification of LCEM was done by precipitation using ammonium sulfate with subsequent concentrations of 20, 40, 60 and 80%. After each step the precipitate was removed by centrifugation at 4000 rpm and 4°C for 15 minutes and dried using small cold acetone portions. The precipitated proteins were collected by centrifugation at 5000 rpm at 4°C for 20min after each precipitation step. Each fraction was dissolved in 10ml 50 mM Tris-HCl buffer of pH 7.5 and then dialyzed at 5°C against the same buffer in semi permeable tubing having cut-off 12 kDa (Sigma-Aldrich). The partially purified highly active fractions (having high FPase activity) were mixed and designated PPE1. The whole PPE1 was applied to a Sephadex G-100 column (2.5 by 50 cm; Pharmacia Biotech Inc.) equilibrated with Tris-HCl buffer (50 mM, pH 7.5) buffer. The rate of flow was adjusted at 10ml/15min. The protein in the collected fractions was monitored at 280 nm (UV) spectrophotometricaly, while cellulolytic (FPase) and xylanolytic activities corresponding to allocated protein fractions were determined as described before (Scopes, 1993). Those active fractions of FPase activities ≥ 10U were recombined, lyophilized to powder form and designated as PPE2.

Enzyme assay: Filter paper cellulolytic enzyme activity (FPase) was measured to estimate the total synergistic cellulolytic activity (exoglucanase, endoglucanase and β-glucosidase), while carboxy methyl cellulose for cellulolytic enzyme activity (CMCase) used to estimate endoglucanase activity. Both assays were done according to the standard procedure recommended by the commission of Biotechnology IUPAC (Ghose, 1987). β-glucosidase activity was determined according to Saddler (1982). One unit of cellulolytic activity (FPase, CMCase or β-glucosidase) was defined as the amount of enzyme required for liberating 1µM of reducing sugars as glucose and was expressed in filtrate as $U.ml^{-1}.min^{-1}$ or $U.g^{-1}ds$ (ds: dry substrate). Xylanase activity was determined as described by Mahmoud and Gomes (2012). One unit of xylanase activity was defined as the amount of enzyme required for liberating 1µM of reducing sugars as xylose. All timed reactions were stopped by the addition of 3,5 di nitro salycilic acid (DNS) reagent for determination of the released reducing sugars . The resulting reducing sugars were determined by (DNS) method described by Miller (1959). Protein determination was done according to Bradford (1976) using bovine serum albumin as a standard.

Optimizing saccharification conditions for the pretreated RS: Enzymatic hydrolysis was carried out using the prepared enzyme mixture and a set of experiments were conducted to study the influence of time (6-48 hours), substrate concentration (2.5 -10%), enzyme load of 5-20 $U.g^{-1}$ substrate (FPU) on enzymatic hydrolysis of RS. Samples were withdrawn, centrifuged at 4000 rpm for 15 min and the supernatant was analyzed for reducing sugars content.

Calculations: The enzymatic index (EI), FPase yield, FPase fold and the degree of saccharification (DOS) were calculated as follows (Mohy *et al*., 2015):

$$\text{Enzymatic index(EI)} = \frac{\text{cellulolytic hydrolysis zone(mm)}}{\text{fungal growth zone(mm)}}$$

$$\text{FPase yeild\%} = \frac{\text{Purification step Fpase}}{\text{Crude SUP FPase}} \times 100$$

$$\text{FPase fold} = \frac{\text{Purification step Specific FPase}}{\text{Crude SUP Specific FPase}}$$

Degree of saccharification or Saccharification % (DoS)

$$\text{DoS} = \frac{\text{total released reducing sugars (mg)}}{\text{total carbohydrates in substrate}} \times 0.9 \times 100$$

Statistical analysis: All results data were accomplished in triplicates and statistically evaluated by least significant differences (LSD) in one way completely randomized analysis of variance (ANOVA) at significance 5% calculated using CoStat (6.311) software (Maruthai *et al*., 2012).

Results and discussion

Qualitative screening for cellulolytic activity of fungi:

Fungal candidates were grown on CMC agar medium for 7 days to evaluate their ability to produce cellulytic enzymes of considerable magnitude through clear zone test confirmed by Gram's iodine stain test, as presented in Table (1).

Table 1: Initial screening for cellulolytic activity of 28 fungal candidates.

Fungi		H	G	EI	Fungi		H	G	EI
Trichoderma spp.	T1	14.6	12.2	1.20^{jk}	*Aspergillus* spp.	A5	32.1	17.1	1.88^{ab}
	T2	18.2	13.1	1.39^{fghijk}		A6	31.4	17.00	1.85^{abc}
	T3	16.9	12.8	1.32^{ghijk}		A7	32.7	16.8	1.95^{a}
	T10	16.7	12.4	1.35^{ghijk}		A8	32.1	16.9	1.90^{ab}
	T11	17.9	12.8	1.40^{fghijk}		A9	27.0	15.9	1.70^{abcde}
	T12	13.7	10.2	1.34^{ghijk}		A17gr	18.6	13.8	1.35^{ghijk}
	T12gr	12.4	10.8	1.15^{k}		A19y	19.6	14.0	1.40^{fghijk}
	T13	13.3	11.8	1.13^{k}		A19B	20.0	13.8	1.45^{efghij}
	T14	24.3	15.4	1.58^{cdefg}		A20	32.5	16.8	1.94^{ab}
	T15	14.9	12.0	1.24^{ijk}		A25	28.1	17.0	1.65^{bcdef}
	T16	17.3	13.1	1.32^{ghijk}		A30	32.4	16.9	1.92^{ab}
Acremonium sp.	Acr26	26.5	15.2	1.77^{abcd}	*Penicillium* sp.	P4	15.9	12.5	1.27^{hijk}
A. terreus	NRRL 260	26.9	15.8	1.70^{abcde}	*Pl. ostreatus*	NRRL 2366	23.0	15.3	1.50^{defghi}
Ph. chrysosporium	NRRL6361	22.0	14.2	1.55^{defgh}	*T. viride*	NRRL 1698	30.2	16.4	1.50^{defghi}

H: hydrolysis diameter (cm), G: growth diameter (cm), EI: Enzyme Index = H /G,
EI values descending arrangements designated as superscript small letters at LSD=0.295.

All the 28 fungal candidates showed signs of growth on CMC agar medium and demonstrated positive results in the Gram's iodine test. Fungi including *Aspergillus* spp A5, A6, A7, A8, A20 and A30 were significantly the most efficient among all fungal candidates exhibiting enzyme indices ranging from 1.9 to 2. Following, *Acremonium* sp. Acr26, *Aspergillus* spp. *(A9 and A25)*, *A. terreus* NRRL260, *Ph. chrysosporium* NRRL6361 and *T. viride* T14 exhibited enzyme indices ranging from 1.5 to 1.8. Otherwise, fungi exhibiting enzyme indices lower than 1.5 were neglected.

On the same trend, Bhat and Bhat (1997) demonstrated that nearly all the fungi that had been reported for the production of cellulases were mesophilic fungi and the best known cellulase producers included species of *Trichoderma, Aspergillus, Acremonium, Penicillium, Rhizopus* and *Fusarium.* Also, many of filamentous fungi such as *Aspergillus* and *Trichoderma* spp. are well known as efficient producers of cellulases (Milala et *al.*, 2009; Immanuel *et al*., 2006 and Cianchetta *et al.*, 2010). Recently, Reddy *et al*. (2014) isolated and identified fungal isolates having cellulolytic activities including *A. niger*, *A. flavus*, *Trichoderma* sp., *Penicillium* sp., *Acremonium* sp. and *Fusarium oxysporium*.

On the other hand, Ruegger and Tauk-Tornisielo (2004) stated that the enzyme index could be used as a simple and rapid criterion to select strain that had potential for enzyme production. On these basics, Florencio *et al*. (2012) evaluated the cellulolytic potential of 49 strains of *Trichoderma*. They found that 10 strains (equivalent of 22.4% of the total number of fungal strains) displayed greater EI values than 1.5 and were selected for submerged fermentation (SmF) for further quantitative evaluation.

Considerably in the present work, 12 fungal candidates showing an enzyme index greater than 1.5 were considered to be potential producer of cellulases and were subjected to further quantitative screening for both cellulases and xylanase production in SmF.

Quantitative screening for cellulases and xylanase production by selected fungi:

The selected 12 cellulolytic fungi (having greater EI than 1.5) were further tested quantitatively for their cellulases and xylanases production magnitude on standard mineral basal medium on rice straw (RS) and sugar cane bagasse (SCB) individually in SmF. The growth process was carried out in flasks for 5 days. Activities of total cellulases (FPase), endoglucanase (CMCase), β-glucosidase and xylanase determined in the culture filtrates of the 12 fungi are illustrated with error bars in Figures (1) and (2), with the best results being designated as small letters (a, b, c) based on their specific LSD.

Fungi produced cellulases and xylanase in different magnitudes according to the type of carbon source. It was obviously clear that most of the fungal candidates exerted greater FPase and xylanase activities on RS than on SCB. The highest FPase activities were recorded on RS (LSD= 0.050) by *Aspergillus* spp. A5, A6 and A7 (0.32-0.35 $U.ml^{-1}$) being slightly higher than the activities recorded on SCB (LSD=0.045) for *Aspergillus* spp. A5 and A6 (0.29 $U.ml^{-1}$). Xylanase maximum activities were recorded for *A. terreus* NRRL260 and *Ph. chrysosporium* NRRL6361, being 16.5 and 15.9 $U.ml^{-1}$ on RS (LSD=0.509), while recorded 12.75 and 10.62 $U.ml^{-1}$ on SCB (LSD=0.349), respectively. On the other hand, mostly each fungal candidate grown on either sole carbon sources produced CMCase and β-glucosidase nearly of similar magnitude, individually. Remarkably, *Aspergillus* spp. A5, A6 and A7 proved to be the best CMCase and β-Glucosidase producers on both RS and SCB, as their CMCase ranged 6.3-7.9 $U.ml^{-1}$ on RS (LSD=0.412) and 5.7-6.3 $U.ml^{-1}$ on SCB (LSD=0.317), while their β-glucosidase ranged 2.0-2.9 $U.ml^{-1}$ on both RS (LSD=0.111) and SCB (LSD=0.098), respectively.

In this regard, Milala *et al*. (2009) demonstrated that filamentous fungi particularly *Aspergillus* spp. were well known as efficient producer of cellulases and reported *A. niger* as higher producer upon growth on cellulosic substrates. Immanuel *et al*. (2006) selected *A. niger* and *A. fumigatus* as efficient cellulase producers. Sorensen *et al.* (2011) selected 5 among 25 cellulolytic fungal isolates for synergy studies with commercial enzymes where two isolates identified as *A. niger* and *A. saccharolyticus* were found as promising candidates for enzyme production. Mahamud and Gomes (2012) demonstrated that among a large number of lignocellulosic debriss decomposing fungi, 4 fungal isolates were identified of which *A. niger* was found to be potent in the case of CMCase, FPase and xylanase activity using references medium supplied with 2% pretreated SCB. Also, Reddy *et al*. (2014) quantitatively evaluated 9 cellulolytic fungal isolates for their potentiality to produce cellulases in submerged culture and found that *A. niger* and *A. flavus* strains exhibited the highest cellulolytic activity. Also, Kolet (2015) stated that *A. niger* was tested among 7 fungal isolates and found to be an exo-cellulase and endo-cellulase producers.

According to the obtained results, it could be concluded that the three fungal candidates including *Aspergillus* spp A5, A6 and A7 had appreciable cellulolytic activities, while the two fungal candidates *A. terreus* NRRL 260 and *Ph. Chrysosporium* NRRL 6361 had the best xylanolytic activities. Consequently, those five fungi were chosen in further studies for production of active enzyme mixtures that could be applied in enzymatic saccharification of pretreated RS and SCB.

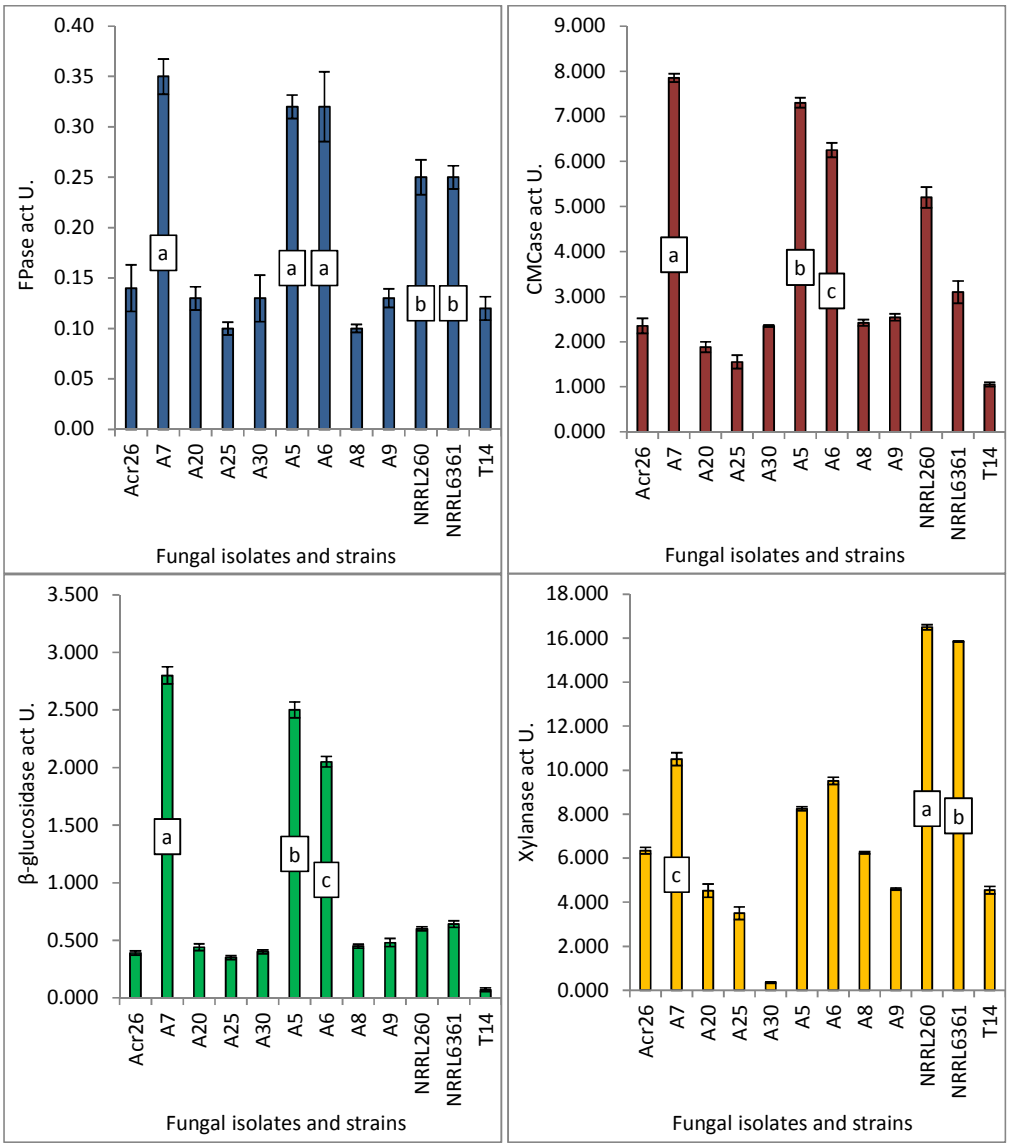

Fig. 1: Quantitative screening for cellulolytic and xylanolytic activities of the selected 12 cellulolytic fungi grown in SmF process on pretreated rice straw (RS). Best results being designated as small letters (a, b, c) based on their specific LSD.

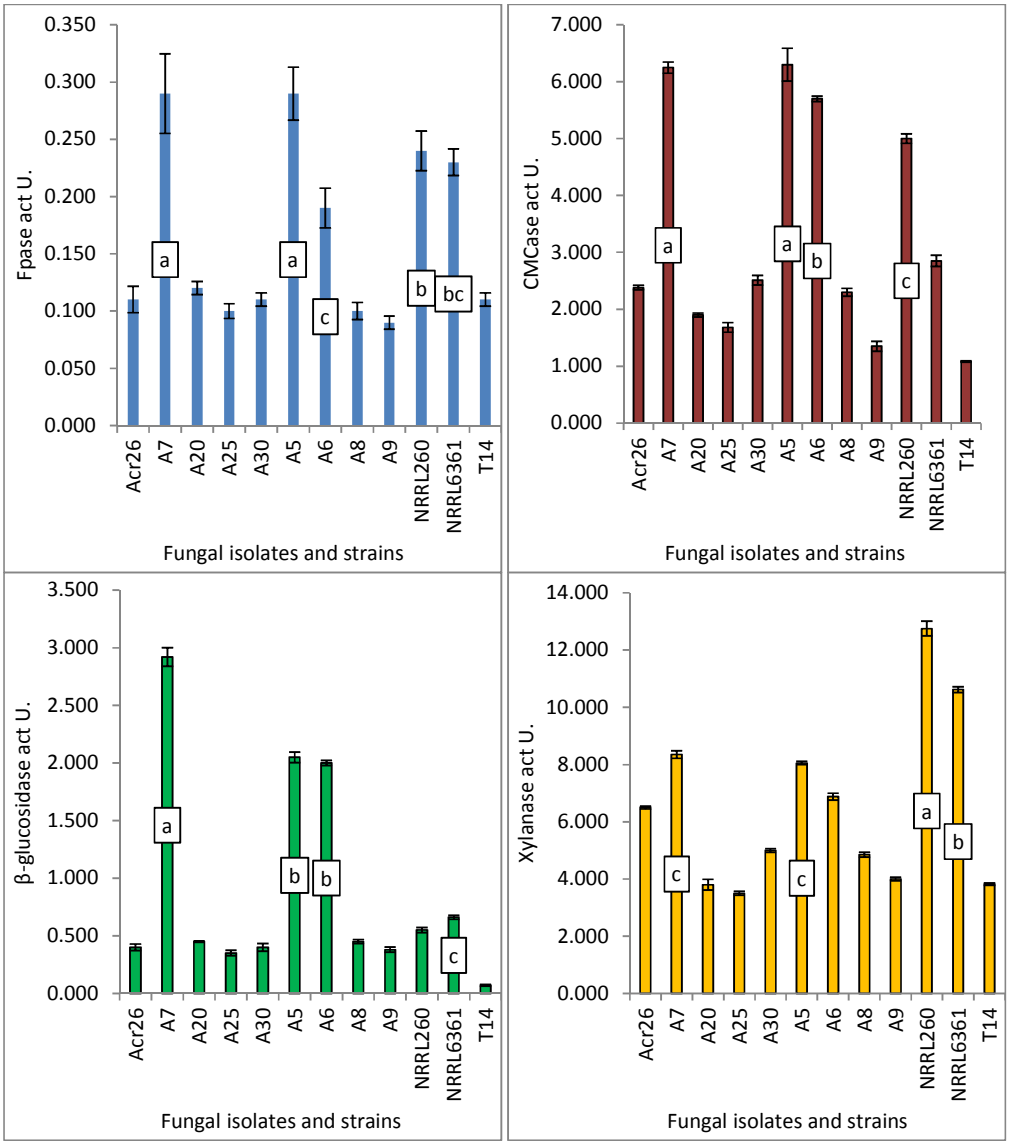

Fig. 2: Quantitative screening for cellulolytic and xylanolytic activities of the selected 12 cellulolytic fungi grown in SmF process on sugar cane bagasse (SCB). Best results being designated as small letters (a, b, c) based on their specific LSD.

Cellulases and xylanases production using solid-state fermentation (SSF)

Yamanobe *et al*. (1987) found that several strains were needed collectively for producing enough amounts of highly effective mixtures of cellulolytic and xylanolytic enzymes. With this concept, the present work aimed to choose the most active cellulases and xylanass producers among the five potent fungal candidates (*Aspergillus* spp. A5, A6 and A7, *A. terreus* NRRL260 and *Ph. chryososporium* NRRL6361) for production of an effective cellulases and xylanase mixture. This was achieved by growing them individually on SSF using the same mineral salt medium of Mandels and Weber (1969). The produced enzymes were then recovered individually from SSF and assayed for FPase, CMCase, β-glucosidase and xylanase enzyme activities. Enzyme activities were expressed as $U.g^{-1}$ dry substrate ($U.g^{-1}ds$), as illustrated with error bars in Figures (3) and (4), with the best results being designated as small letters (a, b, c) based on their specific LSD.

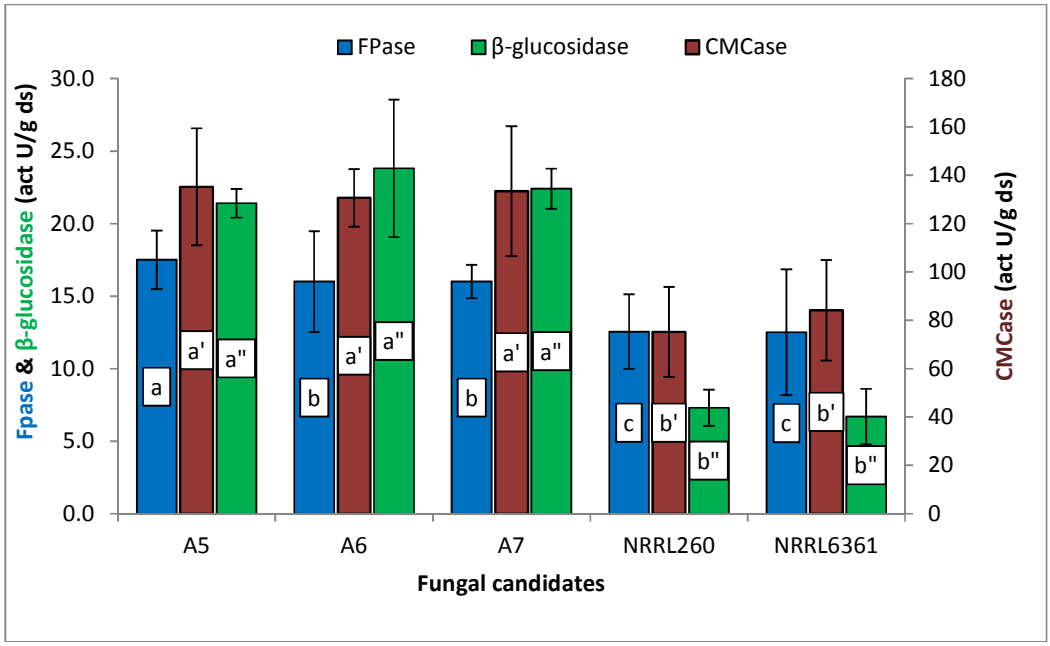

Fig. 3: Crude enzyme activities of the five cellulolytic fungi grown on SSF using pretreated RS as the sole carbon source. Highest results being designated as small letters (a, b, c) based on their specific LSD.

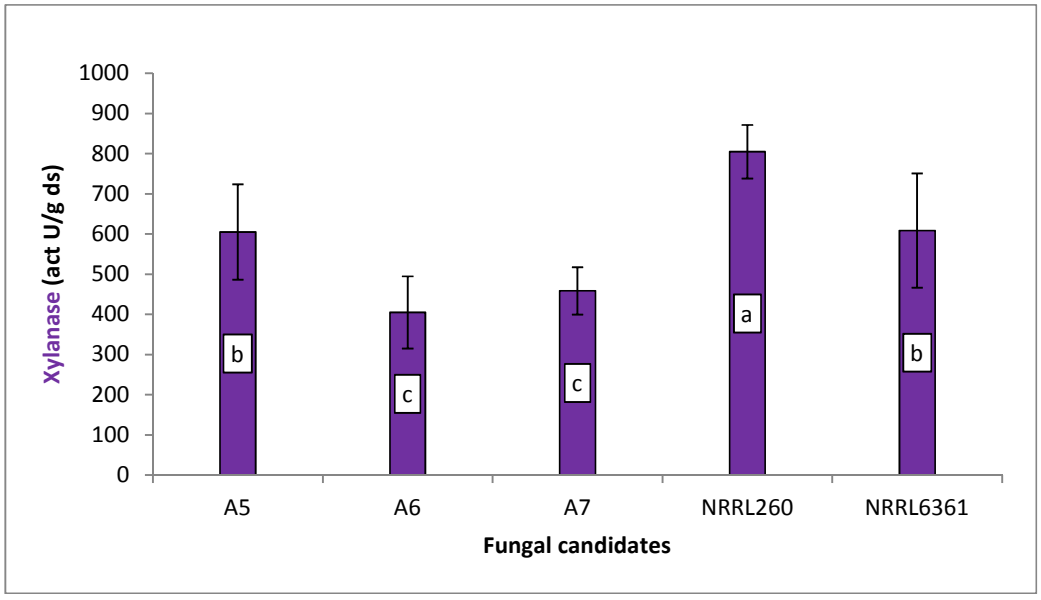

Fig. 4: Crude enzyme activities of the five xylanolytic fungi grown on SSF using pretreated RS as the sole carbon source. Highest results being designated as small letters (a, b, c) based on their specific LSD.

The crude enzymes individually recovered from SSF of *Aspergillus* spp. A5, A6 and A7 had nearly similar and maximum FPase (LSD=0.347), CMCase (LSD=13.505) and β-glucosidase (LSD=6.765) activities recording ranges of 16-17, 130 -135 and 21-23U.g^{-1}ds for each enzyme type, respectively. Meanwhile, the crude enzymes recovered from SSF of *A. terreus* NRRL260 had the highest xylanase activity of 804 U. g^{-1}ds (LSD=143.181). Lower enzymatic activities were recorded in the crude enzyme extract recovered from SSF of *Ph. chrysosporium* NRRL6361.

In this regard, Lakshmikant (1990) studied the biodegradation of cellulose powder, SCB and wheat straw by 5 cellulolytic fungi belonging to 4 genera (*Aspergillus*, *Chaetomium*, *Scopulariopsis*, *Trichoderma* and *Trichothecium*) in solid state culture fermentation (SSF)He found that all the tested fungi produced cellulolytic enzymes, of which *Chaetomium globosum* exhibited the highest performance followed by *Trichoderma koningii*.

On the other hand, the xylanase produced by *A. terreus* NRRL260 recording 804 U. g^{-1}ds in the present work was better than that produced by *A. terreus* isolate grown on wheat bran through SSF recording 116 U. g^{-1}ds (Kaushik and Malik, 2016) and also better than that produced by *A. fumigates* strain SCB4 grown on a mixture of sugarcane bagasse and wheat bran (1:1) through SSF recording 574 U. g^{-1}ds (Gomes et *al.*, 2016).

Partially purified active enzyme mixture

Depending on the previous data, a mixture was constructed from equal volumes of crude enzyme extract in citrate buffer gathered from A5, A6 and A7 SSF cultures (of higher cellulolytic activities) and *A. terreus* NRRL260 SSF culture (of higher xylanolytic activity). The mixture designated as crude enzyme mixture (CEM) was subjected to concentration and successive partial purification steps to give LCEM, PPE1 and finally PPE2. The fractionated LCEM was assayed for FPase total activity (U_T), as shown in Figure (5). The only active fractions including 40%, 60% and 80% gave 307, 422 and 281U_T, respectively. There mixture (PPE1) gave 904 U_T.

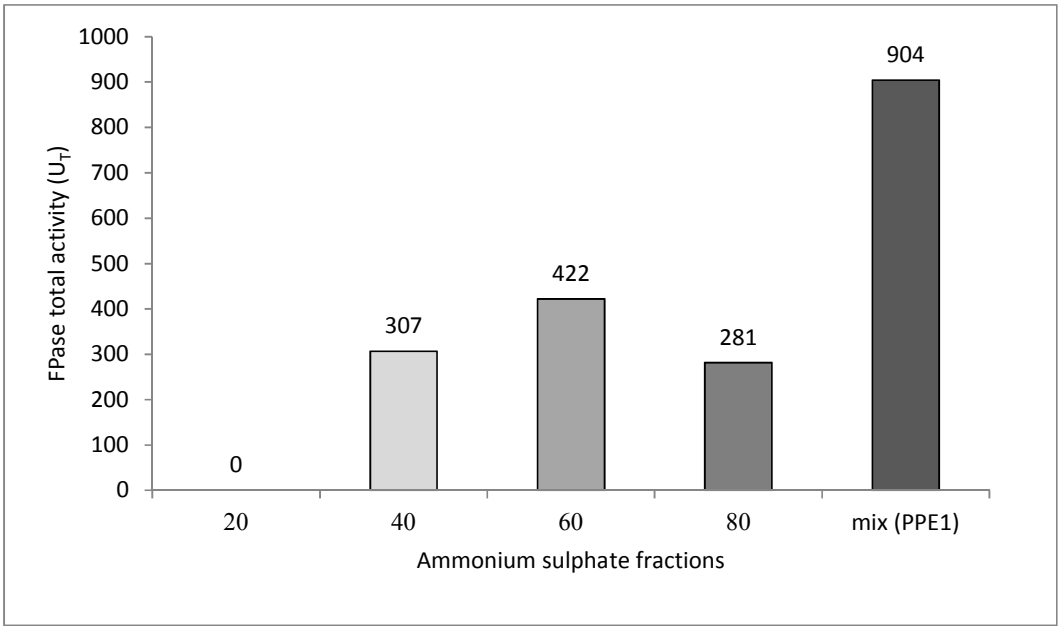

Fig. 5: LCEM partial purification by ammonium sulfate precipitation method

Consequently, column gel permeation separation for the PPE1 on Sephadex G100 was carried out to relocate active FPase fractions. Those active fractions whose FPase exceeded 10U were remixed and subjected to lyophilization at -40°C until being in a powder form (PPE2). The PPE2 was assayed after lyophilization for FPase, Xylanase and protein content, guaranteeing to embed all possible cellulolytic and xylanolytic activities as shown in Figure (6).

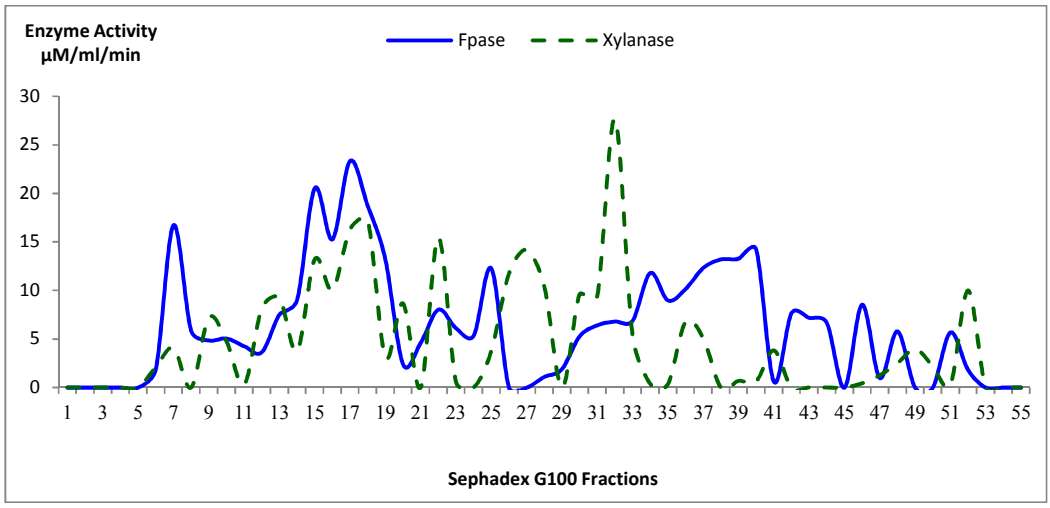

Fig. 6: Cellulolytic and xylanolytic activities of the separated fractions from partially purified enzyme mixture PPE1 on Sephadex G100 column (2.5 by 50 cm; Pharmacia Biotech Inc.) equilibrated with Tris-HCl buffer (50 mM, pH 7.5) buffer with flow rate of 10ml/15min.

The previous CEM1, LCEM2, PPE1 and PPE2 were assayed stepwise for FPase according to Yamanobe *et al*., (1987) and their soluble protein contents were determined according to Bradford method (1976), as presented in Table (2).

Table 2: Total protein contents, total FPase specific activities, yields % and purification folds corresponding to crude enzyme mixture successive concentration and purification steps.

Purification steps	Protein Total soluble (mg)	FPase Total activity (U_T)	Yield (%)	Specific activity ($U.mg^{-1}$)	Purification Fold
CEM	2340	1230	100	0.53	1.00
LCEM	1575	1068	86.83	0.68	1.28
PPE1	900	904	73.50	1.01	2.08
PPE2	360	528	42.93	1.47	2.78

Similar technique was employed for purification of cellulolytic enzymes produced by *T. reesei* but with specific activity of 0.63 $U.mg^{-1}$ proteins and purification fold of 1.65 (Nasr, 1994). The relatively high value found in the present study (1.47 $U.mg^{-1}$ protein and 2.78 fold) could be explained by the fact that those values represent the activity of total cellulolytic enzymes excreted by the four selected fungal strains as other reports showed (Copa-Patino, 1993).

Others produced from different individual fungi and were separated on Sephadex-G100 as from *A. terreus* M11 (Gao et al., 2008), *A. glaucus* XC9 (Tao et al., 2010) and *T. viride* (Iqbal *et al*., 2011) gave yields %-purification folds of 14%-18, 22.3%-21.5 and 2.1%-2.3, respectively , while on Sephadex-G75 *A. niger* 322 (Peshin and Mathur, 1999) and on Sephadex-G50 *T. harzianum* (Ahmed *et al*., 2009) gave 33%-6 and 10.3%-21.9, respectively.

The present study findings were in line with those illustrated by Chen *et al*. (2008) who investigated enzymatic hydrolysis of maize straw polysaccharides for production of fermentable sugars. The alkaline pretreated maize straw was hydrolyzed by *T. reesei* cellulases and the resulting DoS at 48h recorded 65.9%. Supplementing cellobiase from *A. niger* greatly reduced the inhibitory effect caused by accumulated cellobiose and the DoS at 48h was improved up to 81.2%. Thus the collective synergistic action of cellulases from more than fungal source proved to be practical application for an efficient saccharification process.

Also, according to Asztalos et *al.* (2012), the synergistic effect gathering the actions of endo-cellulases, exo-cellulases and β-glucosidases led to higher performance on cellulose than that individual action of each enzyme as well as spatial factors such as enzyme crowding and substrate (cellulose) surface spatial heterogeneity.

Those facts could explain in the present work the reason for the increase in cellulases performance (FPase activity) of the PPE1 (from A, A6 and A7 filtrates) which was higher than the individual FPase activity of each fungal source as they might differed in their types (endo-cellulases, exo-cellulases and β-glucosidases) and ratios, as shown in Figure (5).

Enzymatic saccharification of alkaline/hypochlorite pretreated rice straw

Cellulolytic enzymes mixture PPE2 of total cellulolytic and xylanolytic activities of 1.056 U_t and 1.504 $U_t.mg^{-1}$, respectively, was used in saccharification of RS. Several experiments were conducted to characterize the PPE2 saccharification behavior on RS under different parameters as follows:

1- Time course:

The whole saccharification experiments were conducted for 48h and reducing sugars were estimated at various time intervals. Initially, the reaction mixture contained 2.5% w/v of RS, while the enzyme load was adjusted to 10 $U.g^{-1}$ of substrate (10 FPU) in acetate buffer (0.05M pH=4.8) and incubated at 50°C / 150 rpm. The DoS was measured against time (4 hr time intervals) to evaluate time-dependent enzymatic hydrolysis of RS as shown in Table (3). Time course of enzymatic hydrolysis of RS showed a rapid initial increase of reducing sugars up to 12h and the rate of this increase was substantially reduced at later stages. The substrate was saccharified up to 42% within 12h hydrolysis time. After 24h the DoS increased to about 62%. Further increase of the hydrolysis time from 24h to 48h led to the increase in DoS up to 88%.

Table 3: Enzymatic saccharification time course of RS using 10 FPU of PPE2.

Time (h)	Released reducing sugars		DoS (%)	Saccharification rate (DoS/h)
	$mg.ml^{-1}$	$mg.g^{-1}$ substrate		
4	4.01	160	19.27	4.82^{a}
8	6.77	271	32.53	4.07^{b}
12	8.85	354	42.53	3.54^{b}
24	12.92	517	62.08	2.59^{c}
36	16.25	650	78.08	2.17^{c}
48	18.39	736	88.37	1.84^{c}

Values descending arrangements designated as superscript small letters at LSD=0.736.

Nearly, similar attitude is found in results obtained by Ahmed *et al*. (2012). They studied the time course of enzymatic saccharification of alkaline treated sugar cane bagasse (SCB) using crude enzyme preparation of *T. viride* and reported rapid initial increase of reducing sugars concentration up to 8h after which the rate decreased at later stages. Gupta *et al*., (2009) explained in their work that cellulases first attacked the easily accessible amorphous regions of the substrate which was translated to the higher initial rate of hydrolysis. Furthermore, it was difficult to hydrolyze crystalline regions of cellulose giving resistance to the enzymatic hydrolysis. The decline in hydrolysis rate beyond that time point could be due to the increasing resistance of the substrate during the course of hydrolysis. The same fact was insisted on by Asztalos et *al.*, (2012), stating that as cellulytic hydrolysis proceeded by multi cellulytic system the cellulose substrate became more heterogeneous and the whole degradation slowed down.

2- Substrate concentration

Substrate concentration is an important factor affecting the enzymatic hydrolysis reaction velocity. Therefore, different weights of RS as substrate (2.5 – 10% w/v) were suspended in 10 ml acetate buffer pH 4.8 with a PPE2 enzyme load of 10 FPU. The mixture was incubated for 48h at 50°C and 150 rpm and the released reducing sugars were determined in the supernatant as shown in Table (4). Worthy to notice that reducing sugars increased as substrate concentration increased up to 7.5%, where higher concentration did not exhibit any appreciable increase in reducing sugars yield (a plateau was reached). With respect to saccharification degree, it increased as substrate concentration

increased up to 7.5%. This could be due to the plateau of enzyme active centers with substrate, as more substrate facilitated more surface action and binding sites for enzymatic action.

Table 4: Effect of RS substrate concentration on enzymatic saccharification by PPE2 (10 FPU).

Substrate concentration (%)	Released reducing sugars		DoS (%)
	$mg.ml^{-1}$	$mg.g^{-1}$ substrate	
2.5	17.45	698	83.98^{b}
5	36.09	722	86.85^{a}
7.5	54.50	727	87.43^{a}
10	72.50	725	87.23^{a}

Values descending arrangements designated as superscript small letters at LSD=1.018

Nasr (1994) found that 10% (W/V) acid/Na-Chlorite pretreated RS was the optimum concentration for saccharification using crude enzyme produced by *T. reesei* NRRL 3653. On the other hand, Valsenko *et al*. (1997) reported that the use of higher substrate concentration up to 15% gave hydrolysate with a higher reducing sugars concentration. These differences could be due to difference in pretreatment, enzyme loading and source. Chang and Holtzapples (2000) reasoned that substrate crystallinity was responsible for the effectiveness of enzyme, as reduction in crystallinity and particle size lead to higher sugar yields even with low cellulases loading.

Depending on cellulase source type, Jeya *et al*. (2009) found that 2.5% (w/v) alkali pretreated rice straw was the optimum substrate concentration used with *Trametes hirsuta* cellulolytic crude enzyme of 20 FPU. Referring to Asztalos et *al.*, (2012) work, they insisted on the fact that increasing substrate concentration allowed more binding sites for all types of cellulases to a limit when it came to the inhibitory effect. The inhibition elevated from more reducing ends generated from the action of exocellulases and the released cellobiose accumulated from the synergistic action of both endocellulases and exocellulases that exceeded the capacity of β-glucosidases to act on releasing more reducing sugars.

3- Enzyme load

Substrate concentration of 2.5 % (w/v) in 10ml acetate buffer pH 4.8 containing individually different enzyme load (5-20 FPU). The mixture was incubated at 50°C and 150 rpm for 48h then centrifuged and the released reducing sugars were determined in the supernatant. Results indicated the increase in DoS from 328 to 730 $mg.g^{-1}$ substrate with increasing enzyme load from 2.5-20 FPU. Maximum DoS was achieved at enzyme load was 10 FPU and became nearly stable with further increase in enzyme load, as shown in Table (5).

Soto et *al*. (1994) stated that in case of the high enzyme load an unexpected resulting steric hindrance on the cellulose surface might had happened, thereby causing inefficiency in the process. This finding could be recognized in the present work, as moderate to no increase was recorded in DoS after using enzyme loads exceeding 10 FPU.

Table 5: Effect of enzyme load on enzymatic saccharification of RS (2.5% w/v) using PPE2.

Enzyme load (FPU)	Released reducing sugars		DoS (%)
	$mg.ml^{-1}$ hydrolysate	$mg.g^{-1}$ substrate	
2.5	8.2	328	39.5^{d}
5.0	13.5	540	65.0^{c}
10.0	17.85	714	85.9^{b}
15.0	18.1	724	87.1^{a}
20.0	18.25	730	87.8^{a}

Values descending arrangements designated as superscript small letters at LSD=0.931

The results in the present work were superior to those obtained by Narra *et al*. (2012). They stated that saccharification of alkaline pretreated rice straw by crude enzyme produced in SSF of *A. terreus* culture gave 676 $mg.g^{-1}$ substrate. The high DoS of 730 $mg.g^{-1}$ substrate in the present work might be due to the used active enzyme mixture composition as it contained more efficient enzymes

than those produced individually by a single fungal strain, same condition with *A. terreus* used by Narra *et al* (2012), in addition to the differences in applied pretreatment technique.

Moreover, Jeya *et al*. (2009) studied the optimization of enzymatic hydrolysis parameters for enhancing saccharification of alkaline pretreated rice straw. They reported that enzyme concentration was found to be the limiting factor for saccharification as the enzymatic hydrolysis of pretreated RS with 20 FPU resulted in a DoS of 70% after 48h of hydrolysis. After optimization, maximum DoS of 88% achieved with enzyme load of 37.5 FPU. Comparatively, the present work results indicated that PPE2 enzyme load of 15 to 20 FPU was enough to achieve a nearby DoS result of 87.1% to 87.8%.

Nevertheless, Asztalos et *al.*, (2012), during their model studying on action of multiple cellulases on cellulose, they referred to enzyme crowding and substrate (cellulose) surface spatial availability to cellulytic action that explained in our present work why no appreciable increase happened in cellulytic activity when enzyme load was above 10 FPU.

Conclusion

According to the obtained results, partial purified enzyme mixture of cellulases, β-glucosidase and xylanase (produced in SSF by the selected fungal strains) proved to be a suitable choice for the industrial saccharification of lignocellulosic biomass. Also the present study established the possibility of using alkaline/hypochlorite pretreated rice straw for the production of fermentable sugars with high efficiency, which can be further utilized for production of biofuel and other valuable commodities via industrial fermentation. It is not only a process exclusively for bioconversion of RS for production of valuable products, but also to be reluctant to accumulate agricultural residues with their undesirable environmental impacts.

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