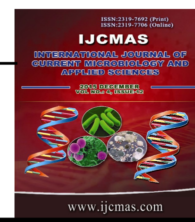
Int.J.Curr.Microbiol.App.Sci (2015) 4(12): 382-401



International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 12 (2015) pp. 382-401**
http://www.ijcmas.com

Original Research Article

Bioconversion of Rice Straw into Ethanol: Fungi and Yeasts are the Backbone Microbiota of the Process

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Keywords

Rice straw, *Aspergillus niger*, *Trichoderma reesei*, *Saccharomyces cerevisiae*, Saccharification, Fermentation, Ethanol

A B S T R A C T

In a series of laboratory experiments, the rice straw was pretreated with sodium chlorite followed by biological treatments for bioethanol production. Both *Aspergillus niger* NRRL-3 and *Trichoderma reesei* NRRL-11460 were successfully grown on basal medium enriched with 1 % (w/v) of untreated rice straw and either cellulose-, hemicellulose- or holocellulose- by products of the straw hydrolysis. Appreciable amounts of extracellular enzymes were produced in these very special cultivation media. Among tested enzymes, xylanase was produced in the highest quantity (40.54 IU ml^{-1}), carboxymethyl cellulase ranked the second (3.35 IU ml^{-1}) while cellobiase and filter paper-ase scantly detected. Holocellulose seemed the pioneer byproduct supporting the enzyme production followed by cellulose, hemicellulose was the inferior in this respect. Apart from substrate and enzyme type, the fungus *T. ressei* over came *A. niger* for enzyme formation. The enzymatic activity of the introduced inocula obviously reflected on the producing sugar pools. Compared to others, *T. reesei* acted more actively, the produced reducing sugars could be arranged in the descending order: 2.62 mg ml^{-1} (*T. ressei*) > 2.52mg ml^{-1} (*A. niger* + *T. ressei*) > 1.10 mg ml^{-1} (*A. niger*). The optimal level of reducing sugars was scored at the 8th day-interval in hollocellulose-amended fungal culture medium, the respective quantities of 22.90, 20.30 and 13.22 were produced in the presence of *T. reesei*, mixed inoculum and *A. niger*. Raising the ammonium sulphate over the recommended level in fungal culture medium significantly stimulated sugar production. Simultaneous saccharification and fermentation (SSF) of cellulose by *Saccharomyces cerevisiae* and cellulases was evaluated in three basal media. Ammonium sulfate-enriched medium was the pionerric compared to others. The impact of some SSF conditions (cellulose concentration, enzyme quantity, incubation period and shaking rate) on ethanol production was discussed.

Introduction

Among cereals, rice is the world's second largest crop after wheat, however, it produces unlimited amounts of residues. According to the Egyptian Environmental

Affairs Agency (EEAA, 2008), more than 2 million acres are cultivated in the country with an average production of *ca*. 6.12 million tons/ year. The processing of rice yields extraordinary quantities of straw as residue. Not less than 20 % is used for paper and fertilizers production as well as fodder and the remaining part is left in the open fields for burning along a period that may extend to > 30 days to get rid of leftover debris. The resulting emission obviously contributes to the air pollution known as the "Black Cloud".

It is well established that, plant cell walls are the most abundant renewable source of fermentable sugars on earth and are the major reservoir of fixed carbon in nature. The main components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant (Yang *et al*., 2007). Cellulase enzymes can hydrolyze cellulose forming glucose and other commodity chemicals. Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry as well as textile industry (Zhou *et al*., 2008). One of the potential applications of cellulases is the production of ethanol fuel from lignocellulosic biomass which is a good substitute for gasoline in internal combustion engines. The most promising technology for the conversion of the lignocellulosic biomass to ethanol is based on the enzymatic breakdown of cellulose by cellulase enzymes (Ahamed and Vermette, 2008).

Many fungal strains secrete higher amounts of cellulases than bacterial ones, *Trichoderma reesei* is a model fungus for studying cellulase production (Kubicek *et al*., 2009) and is a unique industrial source of cellulase. Besides *Trichoderma reesei*, other fungi such as *Aspergillus niger* (Ong *et al*., 2004) have been employed in cellulase production. The application of pure cultures dominates in biological processes, but through adopting mixed cultures, their combined metabolism results in considerable effects on higher enzymes production. Therefore, special attention has been given to mixed cultures used for increasing enzyme production, such as cellulase (Ahmed *et al*., 2010) and xylanase (Tames *et al*., 2003). Fermentation time, substrate concentration and pH of the fermentation medium were all optimized by mixed culture of *Trichoderma viride* and *Aspergillus niger* for the maximum enzyme production (Kavitha and Nagarajan, 2011).

The present work is one of the on-going research attempts to improve enzyme and ethanol production by *Trichoderma reesei*, *Aspergillus niger* and *Saccharomyces cerevisiae* on rice straw and some intermediate products of its pretreatment. Optimization of SSF conditions for ethanol production by *Saccharomyces cerevisiae* and enzymes was among the targets of the study.

Materials and Methods

Rice Straw

Sun-dried rice straw samples collected from private fields at Moshtohor-Kalioubia governorate during the harvesting season of 2012-2013 were used as a source of lignocellulosic biomass. Samples were milled to particles that passed a 40 mesh screen. The chemical profile of the agricultural waste was determined, this encompassed: lignin (Tanaka *et al*., 1985), cellulose (Updegraff, 1969), protein (Lowry *et al*., 1951), fats and waxes (AOAC, 1990), ash (AOAC, 1980), reducing sugars (Miller

et al., 1960), total nitrogen (Black *et al.*, 1965), and glucose by Spanish kits. Hemicellulose was determined by subtracting the sum of the previous constituents weights from that of rice straw.

Microbiota

Two fungal strains recommended for cellulose-hydrolyzing enzymes production from organic wastes were used. Those are *Aspergillus niger* NRRL-3 and *Trichoderma reesei* NRRL-11460 obtained from the Northern Regional Research Laboratory, Peoria, Illinois, USA. Microorganisms were cultivated and maintained on potato dextrose agar (PDA) medium (Oxoid, 1982). Besides, one *Saccharomyces cerevisiae* NRRL Y-12632 strain characterized by high ethanol production efficiency was obtained from the Northern Regional Research Laboratory, Peoria, Illinois, USA. The candidate was grown on the basal medium of Ooshima *et al.* (1986). Cultures were monthly subcultured, incubated at 30 °C for 48 hr. and 7 days for yeast and fungal strains, respectively and subsequently stored at 4 °C for inocula preparation.

Rice Straw Pretreatment

The pretreatment of rice straw with acidified sodium chlorite was performed in a water bath using sodium chlorite and acetic acid at 70 °C adopting the modified procedure of Hubble and Ragauskas (2010) illustrated in Figure (1).

Enzyme Production

Freshly prepared inocula of *T. reesei* and *A. niger* were obtained by transferring spores from a 7-10 day old culture slant to 50 ml basal medium of Mandels and Weber (1969) containing 2 % glucose (w/v) in 250 ml Erlenmyer flasks. The inoculated flasks were agitated on a rotary shaker (150 rpm) for 24 hr. at 30 °C. The carbon source as an important factor that affects the growth of microorganisms and conspicuously reflects on enzyme production was added as 1% (w/v) of either untreated rice straw, cellulose, hemicellulose or holocellulose as a sole carbon source. The organic substrate-enriched basal medium (20 ml) was placed in 100 ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. The medium was inoculated with 1 ml of the fungal inoculum and the mixture was agitated on a rotary shaker (150 rpm) for 7 days at 30 °C. The supernatant was used for enzyme production and estimation of hydrolytic potential of the produced enzymes. Enzymes examined were carboxymethyl cellulase, cellobiase, filter paper-ase and xylanase using the procedures of Takao *et al*. (1985). One international unit (IU) of enzyme activity is defined as the amount of enzyme releasing 1 µmole of reducing sugar (glucose or xylose) from the substrate per minute.

A 12-day incubation experiment was executed to monitor the enzyme production and hydrolytic potential of the fungal inocula when cultivated on holocellulose-amended (1% w/v) basal medium. The latter was inoculated with the fungal cultures and the mixture was incubated in rotary shaker (150 rpm) at 30 °C for 12 days. At 2-day intervals, supernatant samples were taken and assessed for enzyme production and hydrolytic activity.

A number of organic and inorganic substances (holocellulose, urea, ammonium sulphate and potassium di-hydrogen phosphate) was added to basal medium inoculated with a mixed culture of *A. niger* and *T. reesei*. The respective additive concentrations were 0.75-2.0, 0.1-0.6, 1.2-1.6 and 1.0-6.0g l^{-1}. After incubation at 30°C for 8 days, representative samples were taken and assessed for enzyme and sugar production.

Ethanol Production by Simultaneous Saccharification and Fermentation (SSF)

The SSF experiments were carried out in 250 ml Erlenmeyer flasks, each containing 100 ml of fermentation medium. Flasks were tightly stoppered and CO_2 was trapped to guarantee anaerobic conditions (Fig. 2). Flasks containing cellulose (12%) and the basal medium were sterilized at 121°C for 15 min. The cellulase enzyme solution was sterilized through a micro-filter and added in the concentration of 10 UFP/g cellulose. Yeast inoculum (10 % v/v) was used to start the fermentation process. Flasks were incubated at 30 °C on a rotary incubator shaker at 150 rpm for 72 hr. Optimization of ethanol production by SSF was evaluated in basal medium of different conditions including cellulose concentration, FP-ase enzyme level, incubation period and culture shaking rate. Cellulose concentrations added were 2, 4, 6, 8, 10, 12, 14 and 16 % (w/v). The FP-ase enzyme was added to culture medium as 5, 10, 15, 20 and 25 IU ml^{-1}. The ethanol produced was measured at daily intervals for 4days. Shaking rates examined were 50, 100, 150 and 200 rpm.

Statistical Analysis

Data were analyzed for least significant differences according to Snedicor and Cochran (1980).

Results and Discussion

The raw rice straw and three of the chemically produced derivatives were experimented for the ability of three fungal bioformulations to produce a number of extracellular enzymes. The enzyme amounts conspicuously differed from one substrate to another and fungal inoculum as well (Table, 1). Apart from microbiota (Fig, 3A), holocellulose supported the highest level of carboxymethyl cellulase (CMC) production (6.93 IU ml^{-1}). Cellulose ranked the second among the tested substrates (4.70 IU ml^{-1}). The lowest quantity of the enzyme (0.73 IU ml^{-1}) was detected in culture medium enriched with hemicellulose. Of the examined fungal cultures (Fig, 3B), *Tricoderma reesei* exhibited relatively higher enzyme production pattern approximated 5 IU ml^{-1}. As low as 1.36 IU ml^{-1} of the enzyme was produced by *Aspergillus niger*. The composite inoculum of both accumulated 3.62 IU ml^{-1} in average in the organic matter-amended culture media.

In respect to cellobiase, holocellulose kept its superiority among the other tested materials in activating the enzyme production, an average of 0.3 IU ml^{-1} was accumulated in fungi-treated culture media. Lower enzyme amounts of 0.02-0.08 IU ml^{-1} were obtained in presence of other added materials. On the contrary to CMC enzyme, the fungus *A. niger* behaved more actively than *T. reesei*, the respective cellobiase production levels were 0.16 and 0.02 IU ml^{-1}. The dual culture of both microorganisms resulted in production of 0.17 IU ml^{-1}.

Again, holocellulose deemed the most effective in case of filter paper-ase enzyme production, an average amount of 0.3 IU ml^{-1} was determined in the substrate-enriched fungal culture. This was followed by cellulose (0.12 IU ml^{-1}). As low as 0.04 IU ml^{-1} was estimated in presence of either rice straw or hemicellulose. No significant differences were recorded between *T. reesei* and the dual culture. *A. niger* was the inferior in this respect.

Compared to the above mentioned enzyme systems, extraordinary amounts of xylanase were present in fungal cultures supplemented with the various organic

amendments. Based on their effects on the enzyme production, the incorporated materials into growth media could be arranged in the descending order: hemicellulose (60.35 mg ml^{-1}) > holocellulose (57.42) > rice straw (29.85) > cellulose (14.55). *T. reesei* seemed the pioneeric formulation in xylanase production (average of 52 mg ml^{-1}) followed by mixed culture (40.2) and *A. niger* (29.40).

The ability of fungal inocula to ferment the organic wastes to sugars was substrate-and fungi-dependent (Table, 2). The highest quantities of reducing sugars (2.33-6.87 mg ml^{-1}) were produced from holocellulose (Fig, 4A). Cellulose, as well, was successfully fermented with the production of up to 2.85 mg ml^{-1}reducing sugars. Almost similar amounts were formed in culture media enriched with either hemicellulose (average of 0.67 mg ml^{-1}) or rice straw (0.69 mg ml^{-1}). Among the fungal strains (Fig, 4B), *T. reesei* acted more actively compared to others: the produced reducing sugar quantities could be arranged as: 2.62 mg ml^{-1} (*T. reesei*) > 2.52mg ml^{-1} (*A. niger* + *T. reesei*) > 1.10 mg ml^{-1} (*A. niger*).

Irrespective of substrate and microbiota, the amounts of glucose detected in culture media ranged from 0.28 to 4.67 mg ml^{-1}, dual fungal preparation on holocellulose was the superior while *A. niger* on hemicellulose was the inferior.

Based on the previous results that indicated the high decomposability of holocellulose over the other tested wastes, the substrate was selected for further studies.

Actually, the time course of decomposition of a given organic substrate for enzyme and sugar formation is among the criteria that govern the priority for selecting the proper material. All the enzymes produced in holocellulose-received basal medium attained their peaks at the 8th day of incubation, thereafter, the production rate gradually decreased (Table, 3). At that date and irrespective of the introduced inocula, xylanase was produced in the highest level of 66.46 IU ml^{-1} in average (Fig, 5A), followed by CMC ase (8.07 IU ml^{-1}). Very low quantities of either cellobiase or filter paper-ase not exceeding 0.37 IU ml^{-1} were scored. Among the fungal biopreparations (Fig, 5B), the 8-day old *T. reesei* produced an average enzyme mixture pool of 22.9 IU ml^{-1}, an estimate that represents 12.8 and 73.2 % increases over than the dual culture and *A. niger* alone, respectively.

Although the highest quantities of reducing sugars were produced at the 8-day period (Table, 4), the rate of production varied from strain to another (Fig, 6B). Related to reduced sugars formed after 2 days, the amounts harvested at the 8th day represented 151.5, 135.3 and 94.7 % for *T. reesei*, dual inoculum and *A. niger* respectively.

Proportional increases in glucose amounts were recorded up to the 8th day of fungal inoculation, thereafter the levels decreased gradually (Table, 4 and Fig, 6A). Contradicting to reducing sugars, the behavior of fungi was completely different in case of glucose formation. In this respect, *A. niger* was the pioneer (ca. 218 %), respective lower increase percentages of 152 and 135 were estimated for holocellulose fermented by *T. reesei* and mixed culture.

The synthetic medium modifications for better fungal performance were among the targets of the present study. Some necessary ingredients represented by ammonium sulphate, urea and potassium phosphate were added in different levels to culture

medium inoculated with both *A. niger* and *T. reesei*. This is beside the enrichment of the medium with holocellulose in different rates.

As shown in Table (5), reducing the ingredients from the recommended levels negatively reflected on the enzyme production. On the other hand, raising the quantities obviously supported the production of the enzymes, a phenomenon that was quantity-dependent. Nine out of 42 cases of increased ingredients reduced the enzyme production, those represented *ca.* 21 % of the total estimates.

Interestingly, the incorporation into the chemically-synthetic fungal medium of the intermediate waste holocellulose activated the enzyme production levels that further increased with increasing the waste amount up to 100% except in case of filter paper-ase enzyme. In general, the rate of increase depended upon the enzyme tested.

As reported with enzyme production, both reducing sugars and glucose formation in culture medium inoculated with fungal dual inoculum decreased as the level of recommended additives decreased (Table, 6). Such reduction was more conspicuous in case of reducing sugars (average of 28.86 %) compared to 30.49% for glucose. Again, increasing the additive amounts supported higher sugar production.

Raising urea concentration 2 times and potassium phosphate 3 times the recommended doses decreased the production by 15.9% for either. A reduction of 3.3 % was recorded for either reducing sugars or glucose due to increasing urea level. For potassium phosphate, the respective estimate of 3.3 was scored. Akin to enzyme production, sugar produced in culture medium was supported due to all the added levels of holocellulose.

A number of short-term incubation experiments was designed to optimize ethanol production from cellulose by cellulases and *S. cerevisiae* grown in basal medium enriched with cellulose (SFF). Data in Table (7) indicate that the capacity of the yeast strain to produce ethanol was cellulose quantity-, FP-ase enzyme level-, incubation period- or shaking rate-dependent.

In 150 rpm-shacked culture medium received 10 UFP-ase enzyme and incubated at 30 °C for 72 hrs., raising the cellulose quantity from 2 to 4% increased ethanol produced by > 300 %. Proportional increases were recorded with increasing cellulose concentration. High level of ethanol was formed (6.0 %) in 16 % cellulose-amended culture medium.

Increasing the FP-ase enzyme quantity in culture medium obviously stimulated ethanol production. The amount of 15 IU ml^{-1} cellulose was the optimal for ethanol formation (5.95 %).

Prolonged incubation resulted in the accumulation of increased quantities of ethanol. The maximum level of 5.9 % was recorded at the 72 hr-incubation interval.

Shaking rate of *S. cerevisiae* culture medium was among the factors that govern the conversion of cellulose into ethanol. As high as 5.9 % ethanol was produced when the yeast culture was shaked at 150 rpm. Faster shaking resulted in no more ethanol formation.

In the time of rising fuel prices, utilization of abundantly available biomass as an alternative resource of untraditional energy is one of the thrust area of research. Currently, scientists focus on the extensive use of renewable and economically available biomass for the production of useful

products. Rice straw biomass can be used as renewable energy adopting some suitable technologies. The idea is to remove or alter the hemicellulose or lignin, decrease the crystallinity of cellulose, increase the surface area (Mosier *et al*., 2005) and increase the material digestibility through enzymatic hydrolysis to liberate mono saccharaides that are subsequently converted to different valuable products such as ethanol (Jorgensen *et al*., 2007). The production of a number of substantial industrial enzymes secreted by some microorganisms in submerged cultures with rice straw and its by-products have received much attention as well.

Lignocellulose, the most abundant renewable biomass produced by plants from photosynthesis, varies in its composition depending on plant species, plant parts, growth conditions and its structure is rigid and low degradable for cellulose enzymes (Ding and Himmel, 2006). Therefore, the pretreatment must be implemented prior to enzymatic saccharification and required as well to facilitate a morphogenesis as the initial stage in the enzymatic hydrolysis of cellulose (Teeri *et al*., 1992).

In the present piece of work, the pretreatment process for enzymatic hydrolysis of rice straw has been demonstrated by combining the delignification by acidified sodium chlorite with the disintegration of cellulose structure and the alteration of crystalline structure by hot water and sodium hydroxide. Klein-marcuschamer *et al*. (2012) evaluated the synergistic effect of delignification and swelling on the enzymatic saccharification of rice straw and concluded that the removal of total lignins, including the surface and the internal, is an indispensable pretreatment for achieving high efficient enzymatic saccharification of rice straw.

Indeed, the pretreatment process is indispensable to remove lignin and to disintegrate the cellulose structure with loss (degradation) of cellulose and hemicellulose fractions. It is worth to mention that pretreatment with sodium chlorite acidified by acetic acid (acidified sodium chlorite) perhaps meets the requirement of delignification and effectively solubilizes lignin at moderate temperatures (Kahar, 2013).

It is an interesting observation that, the one-time acidified sodium chlorite pretreatment of rice straw, in the present study, seemed appropriate to remove as much as possible the lignin fraction from the waste. Here, the study of Hubbell and Ragauskas (2010) emphasized that the acidified sodium chlorite treatment should be sufficient to remove lignin from cellulose samples with lignin content below 30% (w/w) when the reaction was repeated two times, and it should be performed at least three times for higher lignin contents. This means that the applied process in the present work might remove the surface lignin, but it was not clear whether the implemented procedure was effective for removal of integrated lignin including the internal one. According to the study of Kahar (2013), three times repeated delignification was not enough, resulting in *ca*. 80 % (w/w) removal unless the swelling by sodium bicarbonate was applied. In this context, the swelling deems to act significantly not only to remove surface lignin but in the removal of integrated lignin as well.

A vast array of literature proved the superiority of both *Aspergillus niger* and*Trichoderma reesei* as potential cellulases-producing microbiota that convert lignocelluloses into fermentable sugars and fuel ethanol (Muthvelayudham and Viruthagiri, 2006; Bharathiraja and

Jayamuthunagai, 2008; Kavitha and Nagarajan, 2011). Therefore, those fungal members were experimented for the degradation of the waste materials in the present study.

Irrespective of substrate nature and enzymes estimated, the fungus *T. reesei* overcame *A. niger* in its activity (Fig, 7). Numerous investigations proved the superiority of the former over the other fungal species in waste degradation and enzyme production (Sukumaran *et al*., 2005; Devendra *et al*., 2012; Kim *et al*., 2014).

Based on the ecological concept "the environment selects", the fungal mixed culture of *A. niger* together with *T. reesei* was the inoculum that was experimented in the further studies, this is to guarantee the performance of either or both.

Different fermentation conditions were optimized for enzyme production by fungal strains in an attempt to minimize production costs to commercialize the enzyme production technology. This encompassed the alteration of a number of critical factors that have profound influence on the production of the end products. Among those, the incubation time or the time course for enzyme production is the most important (Ghori *et al*., 2011). For all the enzymes produced (carboxymethyl cellulase, cellobiase, filter paper-ase and xylanase) by fungal inocula grown on holocellulose-enriched culture medium, the maximum production levels were achieved at the 8th day interval and further incubation markedly reduced the production. Actually, this optimum time is longer than that observed by some other investigators (Ahmed *et al*., 2005; Kirchner *et al*., 2005; Dedavid *et al*., 2009). In this respect, Ogel *et al*. (2001) mentioned that the time course which is required to reach the maximum levels of enzymes might be affected by several factors including the presence of different ratios of amorphous to crystalline components.

Replacement or changing the concentration of nitrogen source in the growth medium causes an extent of disturbance for protein synthesis as well as the product formation (Ahmed *et al*., 2010). Results of this study confirmed that increasing the level of urea supported the maximum enzyme production compared to $(NH_4)_2SO_4$, similar results were obtained by Ghori *et al.* (2011). Raising potassium phosphate concentration enhanced enzyme production as well. In all cases, reducing the mineral contents below the recommended levels in culture medium seriously decreased the quantities of enzymes produced.

Of the intermediates formed via decomposition of rice straw by *A. niger* and/or *T. reesei,* holocellulose was added into synthetic culture medium in different concentrations. Increasing its level up to 2 g l^{-1} steadily increased the enzyme productivity. These findings are in conformity with those of Yang *et al*. (2006) and Zheng *et al*. (2010) who reported that cellulases production by *A. niger* was significantly increased when the organism was grown in corn stover-supplied culture medium.

Several other microorganisms capable of ethanol production effectively have also been identified including *Saccharomyces cerevisiae* (Sridee *et al*., 2011; Yamada *et al*., 2011; Laopaiboon and Laopaiboon, 2012). In the present study, production of ethanol from cellulose using cellulases and *S. cerevisiae* (SSF) was investigated. The used *Saccharomyces cerevisiae* strain successfully produced ethanol from cellulose.

Table.1 Enzyme Production ($IUml^{-1}$) by Fungal Candidates in Growth Medium Amended with Various Carbon Sources

Fungal strains \ Carbon sources	Rice straw	Cellulose	Hemicellulose	Holocellulose
	Carboxymethyl cellulase			
A. niger NRRL-3	0.44	1.99	0.31	2.71
T. reesei NRRL-11460	1.65	7.41	1.16	10.08
Mixed culture	1.05	4.70	0.73	8.00
LSD (0.05)	6.15			
	Cellobiase			
A. niger NRRL-3	0.10	0.15	0.02	0.38
T. reesei NRRL-11460	0.01	0.02	0.02	0.04
Mixed culture	0.05	0.08	0.02	0.52
LSD (0.05)	0.04			
	Filter paper-ase			
A. niger NRRL-3	0.02	0.07	0.02	0.14
T. reesei NRRL-11460	0.06	0.17	0.06	0.32
Mixed culture	0.04	0.12	0.04	0.40
LSD (0.05)	0.10			
	Xylanase			
A. niger NRRL-3	22.80	11.10	39.50	44.20
T. reesei NRRL-11460	36.90	18.00	81.20	72.00
Mixed culture	29.85	14.55	60.35	56.06
LSD (0.05)	0.33			

Table.2 Fungal Hydrolytic Potential ($mg\ ml^{-1}$) of Organic Materials Introduced into Synthetic Growth Medium

Fungal strains \ Carbon sources	Rice straw	Cellulose	Hemicellulose	Holocellulose
	Reducing sugars			
A. niger NRRL-3	0.42	1.24	0.41	2.33
T. reesei NRRL-11460	0.96	2.85	0.94	5.37
Mixed culture	0.69	2.04	0.67	6.87
LSD (0.05)	3.61			
	Glucose			
A. niger NRRL-3	0.28	0.84	0.28	1.59
T. reesei NRRL-11460	0.65	1.94	0.64	3.65
Mixed culture	0.47	1.39	0.46	4.67
LSD (0.05)	0.04			

Table.3 Time Course of Enzyme Production (IU ml^{-1}) by Fungal Strains in Culture Medium Supplemented with Holocellulose

Fungal strains	Incubation period (days)					
	2	4	6	8	10	12
	Carboxymethyl cellulase					
A. niger **NRRL-3**	**1.54**	**1.93**	**2.41**	**3.01**	**2.26**	**1.69**
T. reesei**NRRL-11460**	**3.41**	**8.61**	**10.00**	**11.20**	**8.96**	**7.17**
Mixed culture	**3.04**	**7.68**	**8.93**	**10.00**	**9.48**	**9.23**
LSD (0.05)	**0.18**					
	Cellobiase					
A. niger **NRRL-3**	**0.13**	**0.27**	**0.33**	**0.42**	**0.38**	**0.31**
T. reesei **NRRL-11460**	**0.01**	**0.01**	**0.04**	**0.05**	**0.05**	**0.05**
Mixed culture	**0.17**	**0.34**	**0.43**	**0.65**	**0.63**	**0.65**
LSD (0.05)	**0.04**					
	Filter paper-ase					
A. niger **NRRL-3**	**0.08**	**0.10**	**0.12**	**0.15**	**0.11**	**0.09**
T. reesei **NRRL-11460**	**0.14**	**0.27**	**0.33**	**0.35**	**0.28**	**0.22**
Mixed culture	**0.21**	**0.41**	**0.46**	**0.50**	**0.40**	**0.32**
LSD (0.05)	**0.05**					
	Xylanase					
A. niger **NRRL-3**	**25.29**	**31.46**	**39.25**	**49.30**	**36.98**	**27.57**
T. reesei **NRRL-11460**	**24.36**	**61.51**	**71.43**	**80.00**	**63.99**	**51.19**
Mixed culture	**29.25**	**50.84**	**68.31**	**70.08**	**56.06**	**54.33**
LSD (0.05)	**0.16**					

Table.4 Hydrolysis Pattern of Holocellulose (mg ml^{-1}) by Fungal Members with Prolonged Incubation

Fungal members	Incubation period (days)					
	2	4	6	8	10	12
	Reducing sugars					
A. niger **NRRL-3**	**1.33**	**1.66**	**2.06**	**2.59**	**1.95**	**1.45**
T. reesei **NRRL-11460**	**2.37**	**4.55**	**5.64**	**5.96**	**4.77**	**3.82**
Mixed culture	**3.65**	**7.03**	**7.78**	**8.59**	**6.87**	**5.49**
LSD (0.05)	**0.18**					
	Glucose					
A. niger **NRRL-3**	**0.56**	**1.13**	**1.41**	**1.78**	**1.61**	**1.29**
T. reesei **NRRL-11460**	**1.61**	**3.09**	**3.83**	**4.06**	**3.24**	**2.60**
Mixed culture	**2.48**	**4.74**	**5.29**	**5.84**	**4.67**	**3.73**
LSD (0.05)	**0.05**					

Table.5 Changes in Enzyme Production (IU ml^{-1}) in Synthetic Medium Inoculated with Mixed Fungal Culture in Presence of some Additives (related to controls)

Additive concentration (gl^{-1})	Carboxymethyl cellulase	Cellobiase	Filter paper-ase	Xylanase
	Ammonium sulphate			
1.4(control)	10.00	0.65	0.50	70.08
1.2	-3.88	-0.29	-0.15	-23.22
1.3	-2.36	-0.13	-0.06	-14.02
1.5	+2.65	+0.20	+0.13	+11.56
1.6	+0.12	+0.044	+0.07	-3.89
LSD (0.05)	0.07			
	Urea			
0.3 (control)	12.65	0.85	0.63	81.64
0.1	-7.17	-0.47	-0.29	-29.57
0.2	-5.87	-0.29	-0.23	-15.31
0.4	+0.38	+0.03	+0.02	+2.45
0.5	+1.95	+0.16	+0.17	+28.01
0.6	-3.55	-0.21	-0.02	-5.00
LSD (0.05)	1.52			
	Potassium di hydrogen phosphate			
2.0 (control)	14.60	1.01	0.80	109.65
1.0	-5.15	-0.02	-0.10	-7.20
3.0	+0.15	+0.01	0.00	+1.10
4.0	+0.49	+0.03	+0.02	+3.32
5.0	+1.95	+0.10	+0.05	+15.88
6.0	-3.18	-0.01	-0.13	+7.20
LSD (0.05)	15.00			
	Holocellulose			
1.0 (control)	16.55	1.11	0.85	125.53
0.75	-5.35	-0.32	-0.25	-24.33
1.25	+0.97	+0.09	+0.28	+14.92
1.50	+3.38	+0.17	+0.10	+43.37
1.75	+4.95	+0.21	+0.11	+46.47
2.0	+0.70	+0.11	-0.04	+10.27
LSD (0.05)	0.08			

Table.6 Changes in Reducing Sugars and Glucose Production in Synthetic Medium Inoculated with Fungal Dual Culture in Presence of some Additives (related to controls)

Additive concentration (gl^{-1})	Reducing sugars	Glucose
	Ammonium sulphate	
1.4 (control)	8.59	5.84
1.2	-2.66	-1.81
1.3	-1.17	-0.79
1.5	+2.13	+1.45
1.6	+1.19	+0.81
LSD (0.05)	0.19	0.05
	Urea	
0.3 (control)	10.72	7.29
0.1	-4.66	-3.17
0.2	-3.90	-2.65
0.4	+0.36	+0.24
0.5	+2.84	+1.93
0.6	-0.35	-0.24
LSD (0.05)	0.17	0.03
	Potassium di hydrogen phosphate	
2.0 (control)	13.56	9.22
1.0	-3.85	-2.62
3.0	+0.12	+0.08
4.0	+0.40	+0.27
5.0	+0.98	+0.66
6.0	-2.16	-1.47
LSD (0.05)	0.20	0.05
	Holocellulose	
1.0 (control)	14.54	9.88
0.75	-4.39	-2.98
1.25	+0.44	+0.31
1.50	+1.74	+1.19
1.75	+1.79	+1.23
2.0	+0.69	+0.48
LSD (0.05)	0.18	0.04

Table.7 Ethanol Production by *S. cerevisiae* under Various Cultivation Conditions

Levels	Ethanol yield (%)										
Cellulose concentration (%)[(a)]	**0.00**	**0.48**	**1.94**	**2.92**	**3.10**	**3.25**	**3.90**	**4.88**	**5.90**	**5.95**	**6.0**
2		*									
4			*								
6				*							
8							*				
10								*			
12									*		
14										*	
16											*
Enzyme quantity UFP(IU ml^{-1})[(b)]											
5	*										
10									*		
15										*	
20										*	
25										*	
Incubation period (hr.)[(c)]											
24	*										
48					*						
72									*		
96									*		
Shaking rate (rpm)[(d)]											
Static	*										
50	*										
100						*					
150									*		
200									*		

Fermentation conditionsare: (a) enzyme concentration, 10 UFP-ase g^{-1} cellulose;incubation period, 72 hr; shaking, 150 rpm, (b) cellulose concentration, 12%; incubation period, 72 hr;shaking, 150 rpm, (c) cellulose concentration, 12%; enzyme concentration, 10 UFP-ase g^{-1} cellulose;shaking, 150 rpm, (d) cellulose concentration, 12%; enzyme concentration, 10 UFP-ase g^{-1} cellulose; incubation period, 72 hr.

Figure.1 Process Flow Diagram of Entire Set of the Experiment

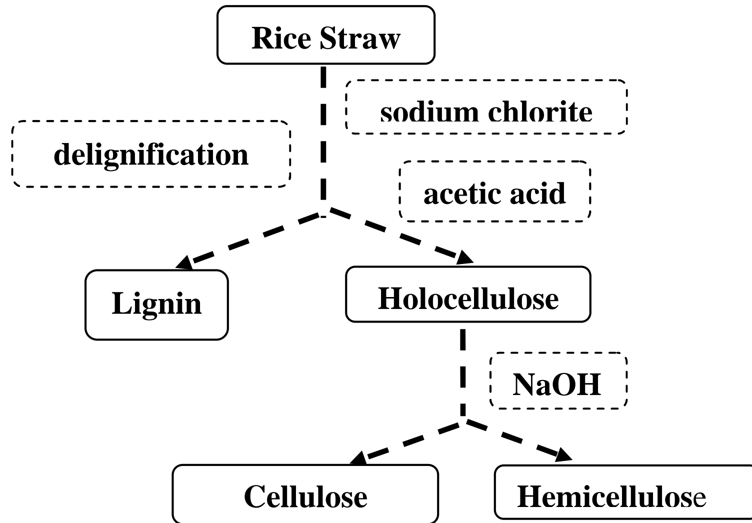


Figure.2 Simultaneous Saccharification and Fermentation of Cellulose by *Saccharomyces cerevisiae* and Cellulases

Figure.3 Enzyme Production (IU ml^{-1}) Patterns as Affected by Organic Substrates Incorporated into Basal Medium in Presence of Fungal Bioformulations. Effects Attributed to Substrates (A) and Fungal Inocula (B)

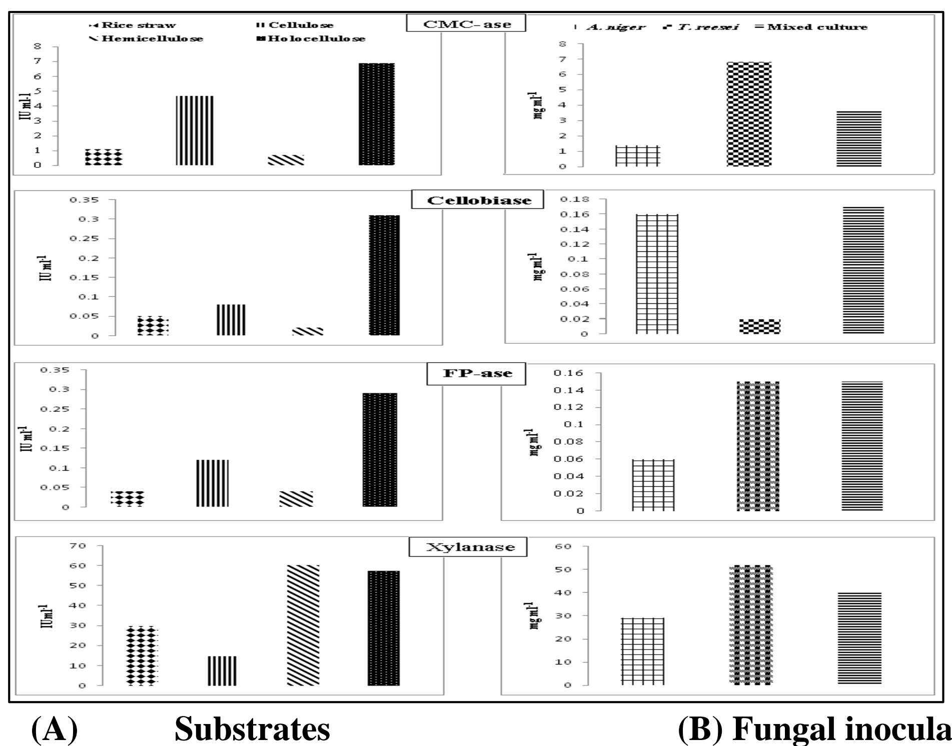


Figure.4 Reducing Sugar and Glucose Production Yields (mg ml^{-1}) due to Substrate (A)- and Fungal Culture (B)-Dependent Effects

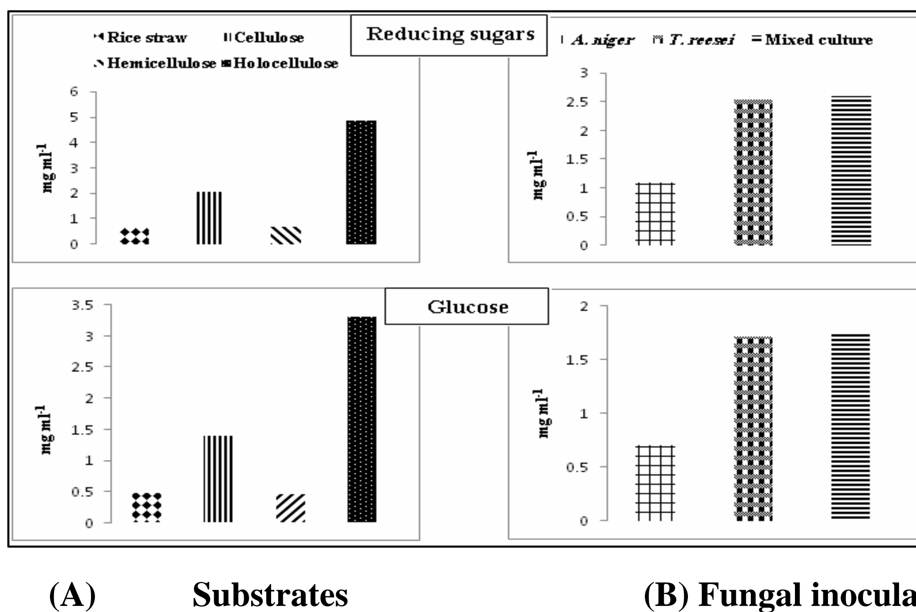
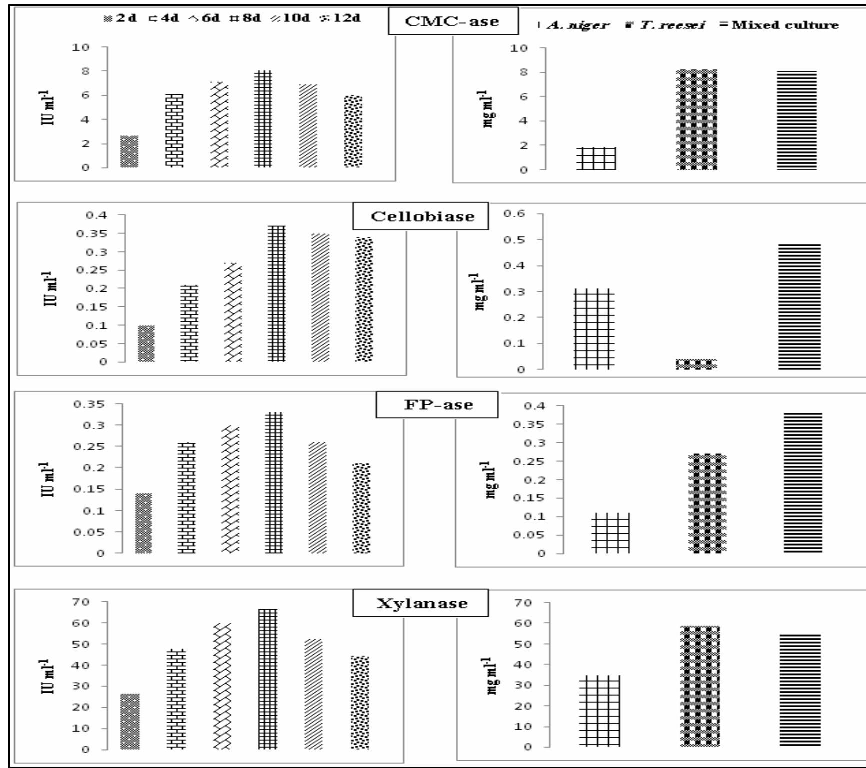
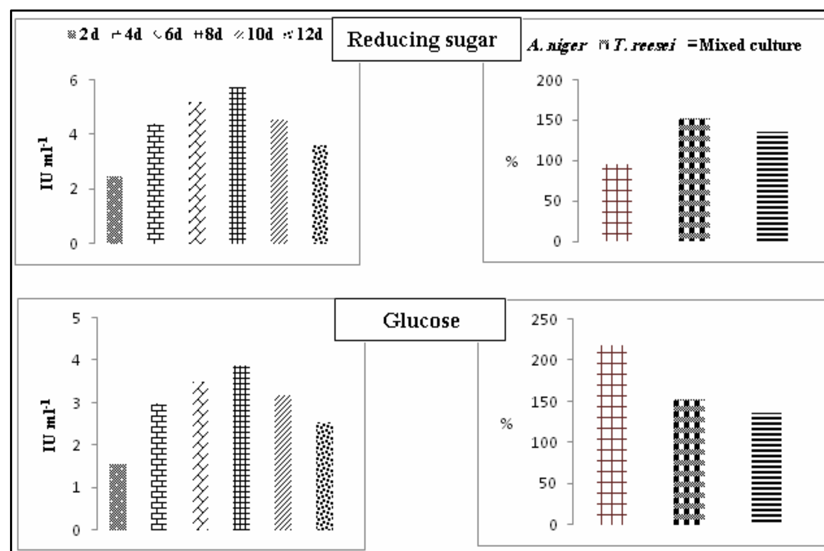


Figure.5 Enzyme Production (IU ml^{-1}) Profiles in Holocellulose-Enriched Basal Medium as Affected by Time Course (A) and Fungal Cultures (B)



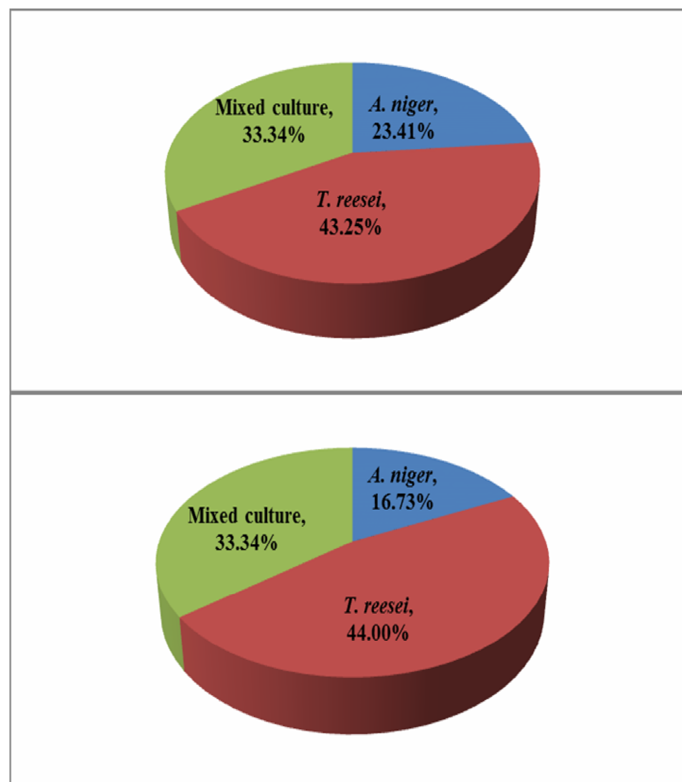
(A) Time course (B) Microbiota

Figure.6 Reducing Sugar and Glucose Production (mg ml^{-1}) due to Incubation Period (A) and Microbiota (B)



(A) Time course **(B) Microbiota**

Figure.7 Comparative Enzyme Production (A) and Hydrolytic Potential (B) Percentages among the used Fungal Preparations



The quantity of the former in cellulose-amended culture medium proportionally increased as the latter concentration increased. Prolonged incubation as well as increasing cellulose content, enzyme level and shaking speed of the culture resulted in significant increases in ethanol formation. Similar results were obtained by Chandel *et al*. (2009) and Yu *et al*. (2010).

In conclusion, it should be realized that, the major bottle neck for comprehensive application of cellulosic wastes in biofuel industry is the high cost of the enzyme production. This needs extensive research to enhance the enzyme activity so that less enzyme is required for the complete hydrolysis of biomass. Here, the biological aspects of cellulosic biomass processing become the crux of future researches involving cellulases and cellulolytic microorganisms. The problems which warrant attention is not limited to cellulose production alone, but a concerted effort in understanding the basic physiology of cellulolytic microbes for better processing and utilization of the most abundant natural resources. Such aspects open the consideration of technologies for pre-treatment of cellulosic materials for microbial attack, processes for cost effective production of cellulases, treatment of biomass for production of hydrolytic products, which can then serve as substrates for valuable metabolites formation. This is beside organisms development by metabolic engineering and finally protein engineering to improve the properties of enzymes to magnify their specific activities, process tolerance and stability.

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