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Research Paper

Bioethanol production from rice straw residues

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Abstract

A rice straw - cellulose utilizing mold was isolated from rotted rice straw residues. The efficient rice straw degrading microorganism was identified as *Trichoderma reesei.* The results showed that different carbon sources in liquid culture such as rice straw, carboxymethyl cellulose, filter paper, sugar cane bagasse, cotton stalk and banana stalk induced *T. reesei* cellulase production whereas glucose or Potato Dextrose repressed the synthesis of cellulase. *T. reesei* cellulase was produced by the solid state culture on rice straw medium. The optimal pH and temperature for *T. reesei* cellulase production were 6 and 25 °C, respectively. Rice straw exhibited different susceptibilities towards cellulase to their conversion to reducing sugars. The present study showed also that, the general trend of rice straw bioconversion with cellulase was more than the general trend by *T. reesei*. This enzyme effectively led to enzymatic conversion of acid, alkali and ultrasonic pretreated cellulose from rice straw into glucose, followed by fermentation into ethanol. The combined method of acid pretreatment with ultrasound and subsequent enzyme treatment resulted the highest conversion of lignocellulose in rice straw to sugar and consequently, highest ethanol concentration after 7 days fermentation with *S. cerevisae* yeast. The ethanol yield in this study was about 10 and 11 g.L^{-1}.

Key words: cellulase, *Trichoderma reesei*, rice straw residues, enzymatic hydrolysis, bioethanol.

Introduction

Ethanol from renewable resources has been of interest in recent decades as an alternative fuel to the current fossil fuels. Lignocellulosic biomass like wood and agricultural crops residues, *e.g.*, straw and sugar beet pulp are potential raw materials for producing several high-value products like fuel ethanol and biodiesel. lignocelluloses contains up to 80% from the polysaccharides (Kaparaju *et al.*, 2009). These renewable raw materials look promising for replacing environmentally unfriendly fossil hydrocarbon raw materials and hence, creating "green" products. In contrast to traditional fuels, bioethanol does not contribute to the greenhouse effect, being a CO_2 neutral resource.

Rice straw is a by-product of rice production and great bioresource. It is one of the abundant lignocellulosic waste materials in the world. It is annually produced about 4 million tons in Egypt. Rice straw can potentially produce 205 billion liter bioethanol per year in the world, which is about 5% of total of consumption. It is the largest amount from a single biomass feedstock. Rice straw predominantly contains cellulose 32-47%, hemicelluloses 19-27%, lignin 5-24% and ashes 18.8%. The pentoses are dominant in hemicelluloses which contains xylose. Xylose is the most important sugar followed by arabinose and hexoses. The carbohydrate of rice straw involves glucose 41-43.4%, xylose 14.8-20.2%, arabinose 2.7-4.5%, mannose 1.8% and galactose 0.4% (Roberto *et al.*, 2003). Changes in how agricultural field residues are managed further complicate farming economies. In the past, disposal of straw by burning was an accepted practice. This practice is now being challenged due to concern over the health effects of smoke from burning fields. In these waste products, the polysaccharides, cellulose and hemicellulose are intimately associated with lignin in the plant cell wall (Ballerini *et al.*, 1994). The lignin component acts as a physical barrier and must be removed to make the carbohydrates available for further hydrolysis processes. Therefore, the pretreatment is a necessary process for utilization of lignocellulosic materials to

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obtain ultimately high degree of fermentable sugars. Bioconversion of cellulosic biomass into fermentable sugar, for production of ethanol using microorganisms, especially cellulose degrading fungi, makes bioethanol production economic, environmental friendly and also renewable. Several pre-treatment processes have been developed for lignocelluloses, which function by an enlargement of the inner surface area. This is accomplished partly by solubilization of the hemicelluloses and partly by degradation of the lignin. The pre-treatments are: Milling and grinding, pyrolysis, high-energy radiation, high pressure steaming, alkaline or acid hydrolysis, hydrogen peroxide treatment, hydrothermal treatment, steam explosion, wet oxidation and biological treatment such as enzyme or microbial conversion: (Fan *et al.*, 1982; McGinnis *et al.*, 1983; Hormeyer *et al.*, 1988; Olson and Hahn-Hagerdahl, 1997; Bollok 1999; Soni *et al.*, 2010).

Studies are currently being made on conversion of the residues by microbial and enzymatic degradation to usable products such as enzymes and sugar syrups which uses in different applications (Andren *et al.*, 1976). Cellulolytic enzymes play an important role in natural biodegradation process in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi and bacteria. In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol (Olson and Hahn-Hagerdahl, 1997; Levy *et al.*, 2002; Nunes *et al.*, 2011). Fungal cellulases are inducible enzymes that are usually excreted into the environment (Bhat and Bhat, 1997) and depend on cellulose type (amorphous or crystalline) acting on the organism (Ortega *et al.*, 2001). The role of the fungi *Acremonium* spp., *Chaetomium* spp., *Trichoderma reesei*, *Trichoderma viride*, *Penicillium pinophilum*, *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*), *Fusarium solani*, *Talaromyces emersonii*, *Trichoderma koningii*, *Fusarium oxysporum*, *Aspegillus niger* and *Rhizopus oryzae* in the cellulose degradation process in various environments has been well documented (Toyama *et al.*, 1981; Teeri, and Koivula, 1995; Bhat and Bhat, 1997; Schülein 1997; Murashima *et al.*, 2002; Kovacs *et al.*, 2009).

Therefore, in the present study an attempt has been made for the optimization of the cellulase production from fungal strain and its application in the bioconversion of rice straw residues into glucose for the production of second generation bioethanol.

Materials and Methods

Media

Mineral salt medium (MSL) as described by Drews (1968), Luria Bertani Medium (LB), Trypticase soy broth (TSB) and Potato Dextrose Agar (PDA) were used in the present study.

Sampling and cellulolytic microorganisms isolation

Samples of rotted rice straw were collected from stored rice straw by the farmers from different regions in Kafr EL-Sheikh Governorate, Egypt.

In laboratory, 1 g of rotted - milled rice straw was added to the conical flask containing 99 mL of MSL medium and mixed for 30 min on a rotary shaker (150 rpm) at room temperature. Ten-fold dilutions were prepared and then 100 μL of each dilution were spread on plates containing MSA (mineral salt agar) + carboxymethyl cellulose (10 g/L as a sole source of carbon) (pH 7; for bacteria), (pH 5.5; for fungi) using a glass spreader. Petri plates were then incubated at 25 °C for 7 days. The isolates were maintained on respective media slants. The plates were incubated at 25 °C for 24 h monitored for appearance of clear zone. For observations, plates were stained with 1% Congo red dye (15 min), followed by destaining with 1 M NaCl solution for 20 min. Cellulolytic strains were selected on the basis of the hydrolysis zone surrounding the colonies as described by Teather and Wood (1982), Bradner *et al.* (1999), Peciulyte (2007) and Belal (2008). The cultures were identified based on the cultural, morphological and biochemical characteristic as described by (Rifai 1969; Domsch *et al.*, 1980; Parry *et al.*, 1983; Burgess *et al.*, 1994).

Saccharification of rice straw by the isolated microorganisms in liquid culture

The isolated colonies were then tested for their ability to grow and degrade rice straw. Rice straw was milled and sieved to 40 mesh. One gram of milled rice straw was inserted into each 500 mL Erlenmeyer flask with cotton stopper. One hundred mL of the mineral salt medium was transferred into a 500 mL Erlenmeyer flask, and after autoclaving was inoculated with 3 mL from fungal spores suspension containing 10^6 spores/mL or bacterial cell suspension containing 10^7 cfu/mL. The cultures were incubated at 150 rpm and 25 °C for 14 days. After 14 days of cultivation, culture aliquots were filtered through cheese cloth and centrifuged at 5000 rpm to remove solids and filtered using sterile membrane filter (0.2 mm). The supernatant was used as the crude enzyme solution (Belal and El-Mahrouk, 2010). The protein concentration of enzyme was determined according to Lowry *et al.* (1951). The supernatants were assayed for their enzymatic activity. Cellulase activity was determined by incubating 0.5 mL of the supernatant (at a concentration of 300 μg protein/mL) with 0.5 mL of 0.6 mg of filter paper No.1 in 0.05 M citrate buffer (pH 4.8). It was incubated with agitation at 50 °C for 12 h. After incubation, the reaction was terminated by adding 3 mL of 1% 3,5-dinitrosalicylic acid (DNS) reagent to 1 mL of the reaction mixture and heated for 10 min. In these tests, reducing sugars were estimated calorimetrically according to Miller (1959), using glucose as standards. One unit of cellulase activity is defined as the amount of enzyme

that releases 1 μmol reducing sugars (measured as glucose) per ml per minute.

Effect of different substrates on *Trichoderma reesei* cellulase production in liquid culture

Different substrates such as carboxymethyl cellulose, filter paper, bagasse, rice straw, cotton stalk, banana stalk, glucose were used in mineral salt medium to evaluate their effect on *T. reesei* and cellulase production. Each substrate was dried and milled. One gram of each substrate was inserted into each 500 mL Erlenmeyer flask with cotton stopper. One hundred mL of the mineral salt medium was transferred into a 500 mL Erlenmeyer flask, and after autoclaving was inoculated with 3 mL from fungal spores suspension containing 10^6 spores/mL (one-week-old colonies of fungi grown at 25 °C on PDA plates). Potato dextrose (PD) was used as complex medium and it was carried at the same conditions. Mineral salt medium was inoculated with *T. reesei* in absence of any substrate. The cultures were incubated at 150 rpm and 25 °C for 14 days. The activity of *T. reesei* cellulase and reducing sugars were determined as described above.

Cellulase production in solid state fermentation

Solid state fermentation method was used since it consumes lesser power but produce more concentrated product. Thirty grams of pretreated rice straw were inserted into each 500 mL Erlenmeyer flasks with cotton stopper. Mineral salt liquid medium was used as supplement where 60% (6 mL g^{-1} substrate) of the solution was added into the flask. Each flask was finally inoculated with 10 mL from fungal spores suspension containing 10^6 spores/mL. The spore suspension was obtained from 7 day-old pure culture. After mixing, flasks were incubated at 25 ± 1 °C under static conditions for 32 days (Belal 2003).

Determination of optimal pH and temperature for *T. reesei* cellulase production was investigated in solid state fermentation. The optimum pH for cellulase production was estimated at various pH values 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8 and with appropriate buffer at 25 °C for 32 days. For determination of optimum temperature for cellulase production, the reactions were carried out at 20, 25, 30, 35 and 40 °C at pH 6.5. The mycelia were grown at the test pH or temperature under the conditions mentioned above. Cellulase activity was determined from the culture filtrate. Cellulase extraction was done by adding 300 mL of 0.05 M citrate buffer into each flask and the mixture incubated at 25 °C on an orbital shaker, at 200 rpm min^1 for 1 h. The suspended slurry was filtered through cheese cloth and centrifuged at 5000 rpm for 20 min and filtered using sterile membrane filter (0.2 mm). The supernatant was used as the crude enzyme solution (Belal and El-Mahrouk 2010). The protein concentration of enzyme was determined according to Lowry *et al.* (1951). Cellulase activity was determined as described above.

Bioconversion of rice straw residues into bioethanol using *T. reesei* cellulase

Microorganism

T. reesei as efficient rice degrading microorganism was used for solid state fermentation as described above.

Enzymatic saccharification

One gram pretreated rice straw with 0.495 U/ g rice straw of crude in 0.05 M citrate buffer at pH 4.8 was added into in 100 mL Erlenmeyer flasks with magnetic bar. Final volume was adjusted to 50 mL using citrate buffer. It was incubated at 50 °C for 12 h with agitation. The experiment was performed with 0.3 mg/mL chloramphenicol. After incubation, the reaction was terminated by adding 3 mL of 1% 3,5-dinitrosalicylic acid (DNS) reagent to 1 mL of the reaction mixture and heated for 10 min. In these tests, reducing sugars were estimated calorimetrically according to Miller (1959), using glucose as standards. One unit of cellulase activity is defined as the amount of enzyme that releases 1 μmol reducing sugars (measured as glucose) per mL per min.

Alkali pre-treatment

About 50 g milled dried rice straw were suspended in 5% NaOH in ratio of 1 : 10 (w/v) rice straw and NaOH. After that the samples were incubated in water bath 85 °C for 1 h. (Yoswathana and Phuriphipat, 2010). Finally, pretreated sample was pressed through cheese cloth. The amount of reducing sugar in juice was measured as described above.

Acid pretreatment

About 50 g chopped dried rice straw was suspended in acid solution (1% Sulfuric acid) in ratio of 1: 10 (w/v) rice straw and Sulfuric acid. The mixtures were autoclaved at 121 °C for 15 min. (Yoswathana and Phuriphipat, 2010). After that, the treated sample was pressed through cheese cloth and the amount of reducing sugar in juice was measured as above.

Alkali /enzyme pretreatment

The previous alkali pretreatment condition was carried out for alkali/enzyme pre-treatment. The NaOH treated sample was pressed through cheese cloth. The juice was kept and the remaining pulp was mixed with citrate buffer (1:10 w/v) containing enzyme (0.495 U/ g rice straw). The pH of sample was adjusted at pH 4.8. Sample was incubated in a water bath at 50 °C for 12 h. The reaction was terminated as described above. After that the sample was pressed through cheese cloth and the juice was carried out for sugar content measurement.

Acid /enzyme pretreatment

The previous acid pretreatment condition was carried out for acid/enzyme pre-treatment. For enzymatic hydroly-

sis the sample was treated as described in alkali/enzyme pretreatment (Yoswathana and Phuriphipat, 2010).

Ultrasound pretreatment

Samples of the mixture of acid pre-treated rice straw (1% acid) and citrate buffer (pH 4.8) at a weight ratio of 1:10, was placed in glass beaker, and were subjected to ultrasound pretreatment before the addition of enzyme. The sample was treated with ultrasoound at 40 W for 10 min and the temperature during ultrasonic treatment was 50 °C. After ultrasound treatment the sample was subjected to enzyme (0.495 U/ g rice straw) treatment as described above. The reaction was terminated as described above. After that the sample was pressed through cheese cloth and the amount of reducing sugar was measured as mentioned above. One treatment was subjected to ultrasound without any treatment with acid or enzyme. All treatments were conducted in triplicate (Yoswathana and Phuriphipat, 2010).

Detoxification

The pretreated samples with acid, ultrasound and enzyme were mixed with wood activated charcoal (20:1 w/w sample: Charcoal) and then agitate for 2 days on magnetic stirrer at room temperature. The samples were filtered through filter paper No. 5 (Whatman, Germany) after charcoal treatment to remove the charcoal (Yoswathana and Phuriphipat, 2010). The filtrate was subjected for sugar measurement as described above.

Fermentation

Bioethanol fermentation was conducted in liquid state fermentation. The yeast *Saccharomyces cerevisae* was used for fermentation. The yeast inoculum was prepared in YEPD broth. A loopful of twenty four hours old culture was inoculated and incubated at 30 °C on rotary shaker (200 rpm) for twenty four hours. The initial yeast count in fermentation sample was $8x10^8$ cfu /mL.

The production medium used for ethanol fermentation was composed of: glucose (sugars solution obtained from saccharified rice straw), 0.1% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.05% $MgSO_4.7H_2O$ and 0.1% yeast extract, pH was adjusted to pH 5. The medium is introduced in 250 mL capacity flasks containing 100 mL of the fermentation medium. The pH of the medium was adjusted to 5. This inoculum was used at 10 percent to inoculate saccharified rice straw from the pretreated samples. All experiments were incubated at 30 °C on rotary shaker (200 rpm) for 7days. The ethanol content was measured after 7days fermentation (Sandhu *et al.*, 1998; Patel *et al.*, 2007).

Ethanol estimation

One mL of the fermented wash was taken in 500 mL pyrex distillation flask containing 30 mL of distilled water. The distillate was collected in 50 mL flask containing 25 mL of potassium dichromate solution (33.76 g of $K_2Cr_2O_7$ dissolved in 400 mL of distilled water with 325 mL of sulphuric acid and volume raised to 1 litre). About 20 mL of distillate was collected in each sample and the flasks were kept in a water bath maintained at 62.5 °C for 20 min.

The flasks were coaled to room temperature and the volume raised to 50 mL. Five mL of this was diluted with 5 mL of distilled water for measuring the optical density at 600 nm using spectrophotometer (Caputi *et al.*, 1968). A standard curve was prepared under similar set of conditions by using standard solution of ethanol containing 2 to 14% (v/v) ethanol in distilled water and then ethanol content of each sample was estimated (Yoswathana and Phuriphipat, 2010).

Results and Discussion

There are several important aspects that should be considered for the development of any bioprocess in solid state fermentation. These include selection of suitable microorganism and substrate, optimization of process parameters and separation of the product. In order to achieve high enzyme yield, efforts are made to develop a suitable medium and to work out the favourable environmental conditions for the proper growth and maximum secretion of enzyme. Development of such medium requires using the right selection of cheaper and readily available components.

Rotted rice straw residues were used as source for isolation of the cellulolytic microorganisms in the present work. Eight microorganisms were isolated by using clear zone formation on MSA (mineral salt agar) containing carboxymethyl cellulose as a sole source of carbon. A preliminary classification based on cultural and morphological characteristics of the isolates revealed that the rice straw residues - degrading microorganisms belong to the group of fungi as well as to the group of bacteria. Among 8 isolated strains, seven fungal strains were identified as *Trichoderma reesei, T. viride*, *Penicillium* sp., *Phanerochete chrysosporium*, *Pacilomyces* sp., *Aspergillus niger* and *Myrothecium* sp. (Table 1).

One out of 8 rice straw - degrading microorganisms was gram -positive, motile, rod shaped bacterium and spore former. Results of identification (Table 2) showed that, the rice straw degrading bacterial strain was identified as *Bacillus pumilus*.

Fungi are well-known agents of decomposition of organic matter in general and cellulose substrates in particular (Lynd *et al.*, 2002; Soni *et al.*, 2010).

Obviously, fungi play an outstanding role in degrading rice straw, since the majority of strains belong to this group. It is known that many genera of fungi play an important role in degradation of anthropogenic substrates. The bacterial isolate were also routinely streaked onto plates of nutrient agar or nutrient agar for bacterial strains but the fungal strains were further purified by using acidic complex medium (PDA).

Table 1 - Colony and morphological characteristics of fungal strains.

Fungal strains	Colony and morphological characteristics
Trichoderma reesei	Colonies growing rapidly (5.5-7 cm). Conidiation tardy and scattered in minute tufts, pale yellow-green. Conidiophores typical of the section, rarely rebranched. Phialides cylindrical, or slightly inflated, mostly 5.5.8 x 2.0-3.7 µm. Conidia pale green, ellipsoid, (3.0-)3.5 x 4.5 x 2.3-3.0 µm.
T. viride	Colonies fast-growing (5-9 cm). Conidiation forming compact tufts or more effuse, glaucous to dark bluish-green. Reverse typically uncoloured, less often pale yellowish. Odour usually distinctly aromatic, as of coconut. Conidiophores usually not extensively branched and having a relatively loose arrangement, branches most often paired, or single or 3-verticillate, often appearing flexuous. Phialides frequently paired, or arising singly or 3-verticillate, narrowly lageniform, 8-14 x 2.4-3.0 µm. Conidia globose to ellipsoidal, usually conspicuously warted, bluishgreen to dark green, 4.0-4.8 x 3.5-4.0 µm.
Penicillium sp.	Colonies are usually fast growing, in shades of green, Conidiophores arising from the mycelium singly or less often in synnemata, branched near the apex , penicillate, ending in phialides, conidia (phialopores) hyaline or brightly colored in mass, 1-celled, mostly globose or ovoid, in dry basipetal chains.
Phanerochete chrysosporium	Colony is white. The white fruiting body is very flat, broadly effuse, moist, somewhat attached, with a jagged outline. The hyphal system is monomitic, simple septate, branched, with hyaline crystals and cylindrical cystidia that range from 3-9 µm in diameter. *Phanerochaete chrysoporium* has smooth basidiocarp usually less than 0.25 µm thick. Basidiospores are less than 8 µm long. Cystidia smooth or with occasional scattered granules. Cystidia mostly over 100 µm long. *Phanerochaete chrysosporium* displays both homothallic and heterothallic-bipolar sexuality.
Paecilomyces sp.	Growth rate is rapid and colonies are flat, powdery or velvety in texture and mature within three days, The surface colony color is initially white becoming yellow, yellow - green, yellow - brown, olive - brown, pink, or violet, depending on the species while reverse is dirty white, buff or brown; and A sweet aromatic odor may be observed with older cultures. Septate hyaline hyphae, conidiophores, phialides, conidia, and chlamydospores are present; Conidiophores are often branched and carry the phialides at their tips, and with size ranging from 3-4 x 400-600 µm; Phialides are thin, swollen at their bases, elongated at their tips, and are usually grouped in brush - like structures at the ends of the conidiophores; Conidia are oval to fusoid in shape, unicellular, hyaline to darkly colored, smooth or rough, and appear in long chains; and Chlamydospores are sometimes present.
Aspergillus niger	colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Conidial heads are large (up to 3 mm x 15-20 um in diameter), globose, dark brown, becoming radiate and tending to split into several loose columns with age. Conidiophores are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne on brown, often septate metulae. Conidia are globose to subglobose (3.5-5.0 um in diameter), dark brown to black and rough-walled.
Myrothecium sp.	Slow growing species whose thallus is velvety- white with the formation of glowing black pustules upon ageing. The reverse is yellowish, cream to pinkish at the periphery. The phialides are cylindrical, aggregated in tight brush-like parallel clusters of two or six. The conidia are olivaceous, unicellular, and elliptical with a truncate extremity, the other one bearing a fan-shaped appendix of 6.5-8 x 2-5 µm. The conidia are borne by a short and wide foot surrounded by white mycelium.

Results in Figure 1 showed that the strains were tested for their growth ability on MSL supplemented with rice straw as a sole source of carbon. Among 8 isolated strains, one strain was identified as *T. reesei* exhibited higher cellulase and reducing sugars productivity from rice straw than the other isolated strains. It indicates that this strain have the highest degradability for rice straw than the other strains. *Trichoderma* is known as a very good producer of cellulases, perhaps due to the different adaptability of fungi to the anthropogenic substrates and different resistance to the factors affecting fungal populations during the recycling procedures. Our results are in agreement with previous findings reported by Teather and Wood (1982), Bradner *et al.* (1999) and Peciulyte (2007).

Therefore, *Trichoderma reesei* as efficient for productivity of extracellular cellulase and reducing sugars was selected for the further studies.

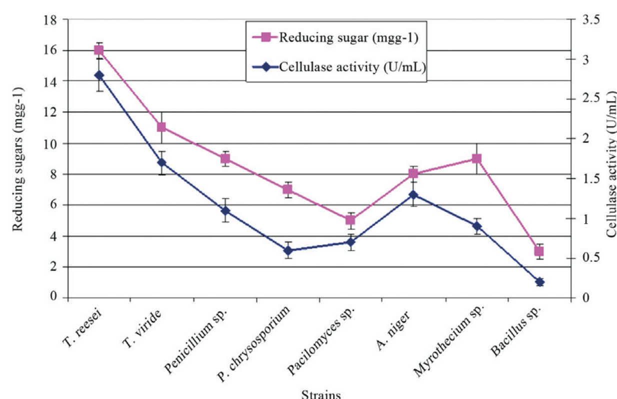

Figure 1 - Saccharification of rice straw by the isolated microorganisms.

Cellulase production with various carbon sources

T. reesei was grown in mineral salt medium with various carbon sources such as carboxymethyl cellulose, filter paper, bagasse, rice straw, cotton stalk, banana stalk, glucose at 25 °C for 14 days with shaking at 150 rpm. PD was used as complex medium.

The obtained results in Figure 2 indicate that the extracellular cellulase was produced only during growth of *T. reesei* on carboxymethyl cellulose, filter paper and all

Table 2 - Morphological characterstics and biochemical activities of rice straw degrading bacterial strains.

Test	Isolate No. 1E
Shape of cell	Rods
Sporulation, Spore shape	+
Motility	Motile
Gram reaction	+
Anaerobic growth	-
Lecithinase reaction (Lv reaction)	-
Citrate utilization	+
V.P. reaction	+
Nitrate reduction	-
Indole production	-
Growth in 7% NaCl	+
Starch hydrolysis	-
Casein hydrolysis	+
Gelatin hydrolysis	+
Catalase reaction	+
Urease activity	-

+: positive; -: negative.

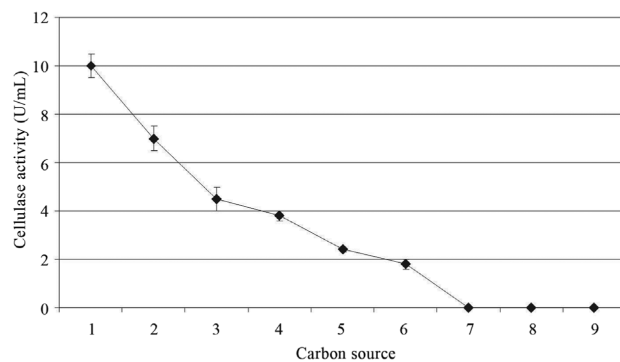

Figure 2 - Effect of different carbon sources on *Trichoderma reesei* cellulase production, where: 1 -carboxymethyl cellulose, 2-filter paper, 3-bagasse, 4-rice straw, 5-cotton stalk, 6-banana stalk, 7-glucose, 8-PD as complex medium, 9-MSL medium plus *T. reesei* without any carbon source.

agricultural residues (bagasse, rice straw, cotton stalk, and banana stalks) in MSL medium as carbon sources. The results demonstrated that a maximum cellulase activity was obtained when carboxymethyl cellulose followed by filter paper, bagasse, rice straw, cotton stalk were used as substrate. On the other hand in PD as a complex medium or in MSL + glucose, enzyme secretion is not induced. According to Schlegel (1992) most enzymes systems involved in substrate degradation are inductive enzymes. In this study i found that when glucose was used as a carbon source, no activity was detected whereas higher amounts were produced when the carboxymethyl cellulose, filter paper and all agricultural residues (bagasse, rice straw, cotton stalk, and banana stalks) were used as a carbon source. PD was also suppressed *T. reesei* cellulase production. The choice of an appropriate substrate is of great importance for the successful production of cellulase. The substrate not only serves as a carbon source but also produces the necessary inducing compounds for the microorganism. This results are in agreement with my previous findings and other investigators while secretion of Poly(ε-caprolactone) - hydrolase was only induced in the culture supernatant with Poly(ε-caprolactone) as aliphatic homopolyester or BTA 45:55 (Ecoflex) as copolyester as substrates but was not induced on glucose or GYM as complex medium (Lin and Kolattukudy, 1978; Oda *et al.*, 1995; Murphy *et al.*, 1996; Belal 2003; Belal 2008).

Effect of pH and temperature on cellulase production in solid state fermentation

Environmental factors do not only influence the rice straw residues to be degraded, they also have a crucial influence on the microbial population and on the activity of the different microorganisms themselves and also the amount of the enzyme production depends on the biomass. Factors such as temperature and pH (Karpouzas and Walker, 2000), have important effects on the microbial degradation of rice straw and so these conditions must be considered when the biodegradability of rice straw is tested. Belal (2008) found that *Trichoderma viride* cellulase affected by pH and temperature. Studies were performed in solid state fermentation to optimize different fermentation conditions (pH and temperature) for cellulase production from *T. resei*. The production of cellulase by *T. reesei* F-418 was studied in solid-state fermentation (SSF). Solid state fermentation has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, low capital investment, low energy requirement and less wastewater output and better product recovery (Asgher *et al.*, 2006).

Optimum pH

The influence of pH on biomass yield of *T. reesei* cellulase and reducing sugars production is shown in Figure 3. Generally, the optimum pH was 6 for *Trichoderma reesei*. The maximum *T. reesei* cellulase and reducing sugars production were recorded at pH6. *Trichoderma reesei* grew at quite wide pH range (from 4 to 8). This variation is very useful to use these isolates in degradation test in different environments at different pH. Therefore, it can expect that this strain can tolerate the pH change during the degradation process thereby increase the degradation potential for this strain. The optimal pH for fungal cellulases varies from species to species, though in most cases the optimum pH ranges from 3.0 to 6.0 (Garg and Neelakantan, 1981; Niranjane *et al.*, 2007).

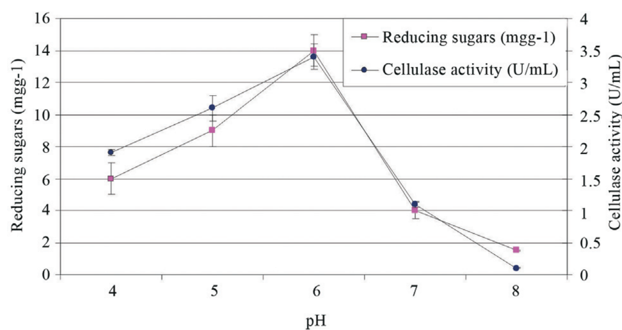

Figure 3 - Effect of pH on *T. reesei* cellulase production.

Optimum temperature

The temperature for cellulase production of *T. reesei* was optimized. The effect of different temperatures on *T. reesei* cellulase production is shown in Figure 4. The optimum temperature for maximal cellulase production was found to be 25 °C at pH 6. Further increase in temperature resulted in decrease in cellulase production. A temperature 25 °C appears to be the optimum for *T. reesei* cellulase production.

Hence optimum pH 6 and optimum temperature 25 ?C were used in all the subsequent experiments

Production of bioethanol from rice straw

Figure 5 illustrates the obtained reducing sugars resulted from different treatment with *T. reesei*, *T. reesei* cellulase, Acid 1%, Alkali 5% and ultrasound for rice straw. The individual treatment and the combination of the treatments resulted in high yield of reducing sugars. This results show the positive effect of *T. reesei*, *T. reesei* cellulase, Acid 1%, Alkali 5% and ultrasound treatment during pretreatment of rice straw on polysaccharide conversion into sugar. In case of the treatment with Acid 1%, Alkali 5% and ultrasound and subsequent with *T. reesei* cellulase were found to be effective. Autoclaving for sterilization has affected and resulted in increase in sugar content.

The production of cellulase is a key factor in the hydrolysis of cellulosic material and it is essential to make the process economically viable. Reduction in the cost of cellulase production can be achieved by the use of cheap and easily available substrates. *T. reesei* cellulase was produced by the solid state culture. This enzyme effectively led to enzymatic conversion of acid, alkali and ultrasonic pretreated cellulose from rice straw into glucose, followed by fermentation into ethanol. As shown in Figure 5, a maximum increase in glucose concentration was achieved after acid pre-treatment or ultrasound and subsequent enzyme. The combined method of acid pretreatment with ultrasound and subsequent enzyme treatment resulted the highest conversion of rice straw to sugar and consequently, highest ethanol concentration after 7 days fermentation with *S. cerevisae*. The ethanol yield was also observed to be the highest in these treatments. The ethanol yield in this study was 11 $g.L^{-1}$.

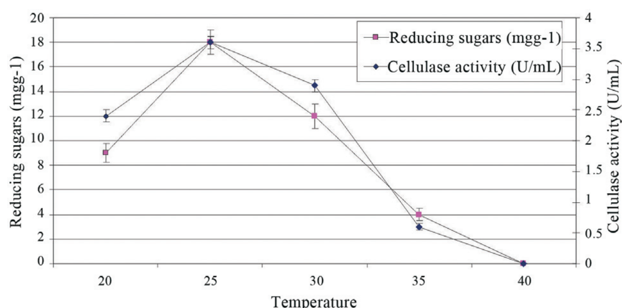

Figure 4 - Effect of temperature (°C) on *T. reesei* cellulase production.

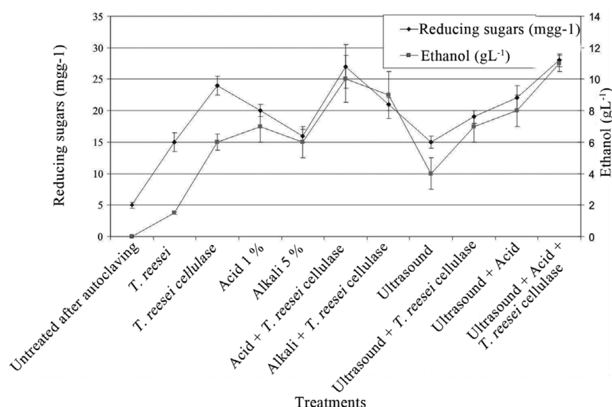

Figure 5 - Bioethanol production from rice straw hydrolyzate fermented by *S. cerevisae*.

The production of ethanol from any lignocellulosic biomass generally involves four process - feedstock pretreatment, enzymatic saccharification, fermentation and ethanol recovery. Lignocellulosic residues including rice straw, sugarcane bagasse, wheat straw, corn stover, spruce and municipal solid waste have been researched by several workers for microbial and enzymatic bioconversion with commercial or in-house produced cellulase into glucose employing various pretreatment protocols including acid, alkali and steam (Li *et al.*, 2007; Patel *et al.*, 2007; Kovacs *et al.*, 2009; Rabelo *et al.*, 2009; Yoswathana and Phuriphipat, 2010). Following pretreatment, plant cell wall polysaccharides are more susceptible to enzymatic hydrolysis that breaks them into monomeric (single) sugars that can be fermented into ethanol (Lynd *et al.*, 1999).

Cellulase production from rice straw with fungi like *Trichoderma reesei* through solid state fermentation is important because in this way production of cellulase can be increased, which further help to produce cellulose. This an important enzyme required for breakdown of polysaccharides into monosaccharide, those can further converted into ethanol and other alcohols through fermentation process. Cellulase has a lot of industrial applications including pro-

duction of food and medicines and help to breakdown the waste plants materials to clean up the environment.

The positive effect of acid pre-treatment or ultrasonic and subsequent enzyme treatment could be explained as better access of enzyme to lignocellulose materials in rice straw.

The ultrasound pre-treatment and subsequent enzyme treatment resulted in yield of reducing sugars. This phenomenon was attributed to the effect of ultrasound on the structure of cellulose and its crystalline arrangement. Namely, cellulose granules contain both ordered crystalline regions and amorphous regions, in which polymer chains are less well ordered and more susceptible to attack by cellulase action. Thereby, the ultrasound pretreatment may be effective.

The acid pre-treatment or ultrasound and subsequent enzyme treatment yielded high amount of reducing sugar which were fermented to yield ethanol. The final glucose concentration decreased, due to enzyme inhibition by glucose accumulation. Similarly, Kolusheva and Marinova (1991) concluded that elevated concentration of glucose significantly decreased the starch hydrolysis rate and affected the enzyme inhibition. Vlasenko *et al.* (1997) have found that after acid pretreatment (10% solid mater, 0.8% acid and 160 °C) followed by enzyme treatment the yield of glucose was 43 g L^{-1} or 43% conversion of cellulose to glucose. The results in present study are low than (Vlasenko *et al.*, 1997) reported. This is may be due to lower temperature (121 °C compare to 160 °C) during acid treatment or because of different conditions during enzyme treatment. The decrease of sugar content in acid treated samples is may be to convert of monomeric sugars (xylose, glucose) to furfural and hydroxymethyl furfural. These substances are toxic substances for yeast and can inhibit the yeast growth. During detoxification of samples part of sugar could be adsorbed on activated charcoal leading reduction of total sugar in sample.

In the present study, Alkali rice straw was used for production of cellulase by *T. reesei* F-418. The purpose of the alkaline pretreatment was delignification, the removal of lignin is necessary for cellulose to become readily available for the enzymes, which permit the yeast to convert the glucose into ethanol (Wyman 1994). Combined acid pretreatment and ultrasonic followed by enzyme treatment resulted highest sugar yield. This results show the effectiveness of combination of physical and chemical pre-treatment prior to enzymatic hydrolysis of lignocellulose material to sugar. Abedinfar *et al.* (2009) have investigated the fermentation of rice straw (pretreated with diluted acid and subsequent enzyme treatment) rice straw using *Mucor indicus* and *Rhizopus oryzae*. They have found an ethanol yield of 0.36-0.43 g g^{-1} using *Mucor indicus* which was comparable with the corresponding yield by *S. cerevisae* (0.37-0.45). *Rhizopus oryzae* produce 0.33-0.41 g/L ethanol. The ethanol yield in this study was about 0.42 g/L. That is in accordance with ethanol yield reported from literature (Abedinfar *et al.*, 2009). The remaining sugar of 35-45% w/v is may be xylose. Xylose is pentose sugar that can not be digested by *S. cerevisae*. Bioconversion offers a cheap and safe method of not only disposing the agricultural residues by solid substrate fermentation (SSF) of rice straw under optimum conditions, but also it has the potential to convert lignocellulosic wastes into usable forms such as enzymes and reducing sugars that could be used for ethanol production. This enzyme system effectively led to enzymatic conversion of ultrasonic and acid pretreated cellulose from rice straw into glucose, followed by into ethanol. These results help to reduce the environmental pollution which caused by agricultural residues.

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