

## Production of Bioethanol Via Enzymatic Saccharification of Rice Straw by Cellulase Produced by *Trichoderma Reesei* Under Solid State Fermentation

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**Abstract:** Alternative substrates to produce useful chemicals such as biofuel have been attractive. Rice straw, one of the most abundant lignocellulosic wastes by-products world wide can be used for this purpose. In the present study the production of cellulase by *Trichoderma reesei* F-418 cultivated on alkali treated rice straw using solid state fermentation (SSF) technique. The high cellulase activity was obtained when the fungus was cultivated on substrate with about 75 % (v/w) moisture, pH 4.8 for 5 days incubation at 28±2°C, as it gave 16.2 IU/g substrate. The obtained cellulase of 1.2 IU/ ml culture filtrate was applied for saccharification (5% w/v) of alkali treated rice straw, in 0.1M citrate buffer pH 4.8 in shaker water bath of 100 rpm. Sugary solution of 1.07 % glucose was achieved after 16 hrs. The sugary solution was concentrated to give 10% (w/v) glucose. Ethanol fermentation was conducted by *Saccharomyces cerevisiae* SHF-5 under static condition giving 5.1% (v/v) ethanol after 24 hrs. The fermented mash contained 3.6 g/L yeast cell can be utilized as fooder yeast used for animal feeding. [New York Science Journal. 2010;3(4):72-78]. (ISSN: 1554-0200)

**Key words:** rice straw, *Trichoderma reesei* F-418, cellulases, fermentation, biofuel.

### 1. Introduction

Nowadays, alcohol fuels have been produced on industrial scales by fermentation of sugars derived from wheat, corn, sugar beets, sugar cane etc. The enzymatic hydrolysis of cellulosic materials to produce fermentable sugars has an enormous potential in meeting global bioenergy demand through the biorefinery concept, since agri-food processes generate millions of tones of waste each year (Xeros and Christakopoulos 2009) such as sugar cane baggase, wheat straw and rice straw. In fact rice straw (RS) is one of the most abundant lignocellulosic wastes by-products world wide and provides an alternative substrate to produce useful chemicals such as biofuel (Yao et al., 2007).

RS has traditionally been dried and burned in the fields reducing the local air quality considerably, this have directed a world wide attention to wards utilization of RS for bioethanol. It contains between 25-45 % of cellulose, 20- 30 % hemicellulose and 10-15 % lignin (Sun and Cheng., 2002).

Cellulose is degraded by enzymes known as cellulases that are able to hydrolyse the cellulose polymer to its monomer, the sugar glucose, that is naturally fermented to ethanol by the yeast *Saccharomyces cerevisiae*.

Fungi are able to degrade cellulose, hemicellulose and lignin in decaying plants by a complex set of excreted hydrolytic and oxidative enzymes (Gosh and Gosh, 1992).

Lignocellulose degrading organisms have been used for the conversion of lignocellulosic materials into soluble sugars or solvents in several

biotechnological and industrial applications (Gomes et al., 2006).

Current technology for conversion of lignocellulose to ethanol requires chemical or enzymatic conversion of the substrate to fermentable sugars followed by fermentation by a microorganism. The large amounts of enzymes required for enzymatic conversion of hemicelluloses and cellulose to fermentable sugars impacts severely on the cost effectiveness of this technology (Xeros and Christakopoulos 2009). The physical support and the energy required for a fungus to grow and produce the desired metabolite is primarily provided by a substrate (Pandy et al., 2001).

Considerable research efforts have been made to improve conversion yields of lignocellulosic materials by the insertion of a pre-treatment step prior to the enzymatic hydrolysis. Dilute NaOH is an effective pre-treatment for lignocellulosic materials with relatively low lignin content of 10 to 18% (Bjerre et al., 1996).

A large variety of microorganisms are capable of degrading plant cell wall materials (Carle-Urioste et al., 1997 and Acebal et al., 1986). It was reported by many authors that *Trichoderma reesei* is capable of increase the production of cellulase from substrate like corn straw and similar others. *T. reesei* can be chosen as model fungus as it shows both the challenging rheology and a cellulase complex product that is shear-sensitive (Weber and Agblevor, 2005).

*T. reesei* is the most extensively studied and it has served as a model for fungal lignocellulosic

degradation. In solid state cultivation *T. reesei* secrete a complex array of degradative enzymes. The objectives of this study were to the hydrolase enzyme productions by *T. reesei* in solid state cultivation under different concentrations from sodium hydroxide (NaOH), moisture, pH, inocubation period.

## 2. Materials and Methods

### 2.1. Microorganisms

*T. reesei* F-418 identified previously by Khaled (2006) and *S. cerevisiae* SHF-5 identified previously by Fadel (1997) were obtained from Microbial Chemistry Department (NRC). Fungal culture was maintained on PDA and yeast culture was maintained on malt glucose yeast peptone (MGYP) medium (Gurav and Geeta, 2007), both organisms were stored at  $4\pm 1^\circ\text{C}$  and subculture fortnightly.

### 2.2. Preparation of rice straw

Rice straw collected from field used as substrate for enzyme production, it was ground and sieved to 40 mesh, then pretreated with 1%, 5%, 10% sodium hydroxide (10ml/g substrate) for 1 hour in water bath ( $100^\circ\text{C}$ ). The pretreated rice straw was allowed to cool, filtered and washed to neutral pH, and then it was dried at  $60^\circ\text{C}$  in an oven for 12h. the dried RS was kept ready for the further use.

### 2.3. Inoculum preparation

Fungal culture was inoculated onto PDA medium in the Petri dish, after 72 hrs, the spores were harvested using sterilized water with 0.1% Tween 80 (Smith et al., 1996).

### 2.4. Solid-state fermentation (SSF) cultivation systems

Fungus was performed in 250 ml Erlenmeyer flask containing 10 g of pretreated RS. The moistening agent used was Mandel's medium (1976) which added as follows: 20, 30, 40 and 50 ml. The Mandel's medium was prepared with the following composition (g/l) 10.0g; urea, 0.3; peptone, 0.75; yeast extract, 0.25;  $(\text{NH}_4)_2\text{SO}_4$ , 1.4;  $\text{KH}_2\text{PO}_4$ , 2.0;  $\text{CaCl}_2$ , 0.3;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 and trace elements (mg/l):  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 5;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 1.6;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.4 and  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 20.0 (Mandels et al., 1976). The medium and the trace elements were autoclaved separately. The flask was cooled down at room temperature and a known amount of sterilized trace elements was added. The flasks were then inoculated with (5 ml) ( $1 \times 10^6$  spores/ml) of the moistening agent and incubated for 5 days at the ambient temperature ( $28 \pm 1^\circ\text{C}$ ), three replicates were used for each treatment. At the end of fermentation time 0.1 M citrate buffer pH 4.8 was added to give total 50 ml/flask. The flasks were shaken at 150 rpm for 1hr then filtered to obtain clear filtrate for determining enzyme activity.

### 2.5. Effect of pH on production of enzyme

Fifteen ml from liquid Mandel's medium was poured into 100 ml conical flasks containing 5 g of pretreated RS (1%NaOH), adjusted to different pH values ranged from of 3.6 to 6.0 using 0.1M citrate buffer. Flasks were autoclaved at  $121^\circ\text{C}$  for 15 min. The cooled flasks were inoculated with above fungus inoculum. Flasks are incubated at  $28 \pm 2^\circ\text{C}$  for 5 days, 2 replicate were used for each treatment.

### 2.6. Effect of incubation period on production of enzyme

The conical flasks containing 10 gram from pretreated RS (1%NaOH) were moisted with 30 ml media, all flasks autoclaved at  $121^\circ\text{C}$  for 15 min then inoculated with 5 ml from fungal spores and incubated for 2, 3, 5, 7, 10 days at  $30 \pm 2^\circ\text{C}$ . Three replicates were used for each treatment.

### 2.7. Enzyme extraction

Fifty milliliters of 0.1 M citrate buffer pH 4.8 was added to the SSF medium after cultivation. The mixture was vigorously homogenized on a rotary shaker for 30 minutes at 200 rpm. The solid biomass residues were separated from the suspension by filtration through Whatmann filter paper No.1. The filtrate was used for estimation of filter paper activity by the method of Mandel et al. (1976).

### 2.8. Bioassay of cellulase (FPase)

Bioassay of cellulase (FPase) was carried out according to Mandel et al. (1976) as follow: 0.5 ml crude enzyme and 1.5 ml (0.05 M) citrate buffer pH 4.8 were added to test tubes containing 0.6 mg of filter paper No.1. The test tubes were incubated in water bath adjusted at  $50^\circ\text{C}$  for 1 h., 3 ml of 3,5-Dinitrosalicylic acid. Tubes were boiling at water bath for 15 minutes, then cold, volume of test tubes was increased up to 10 ml by adding distilled water, then measured colorimetry at 540 nm against blank of sugar free sample. Standard curve was performed with glucose solution.

### 2.9. Enzymatic Saccharification of RS

Enzymatic saccharification of alkali treated rice straw was carried out in reaction mixture containing 5 g treated rice straw in 100 ml 0.1 M citrate buffer pH 4.8 with 1.2 IU/ml. The reaction mixture was incubated on a water bath rotary shaker adjusted to  $50^\circ\text{C}$  and 75 rpm. The samples were withdrawn at intervals 4, 8, 16 and 24 hrs. for glucose and total sugars were determination.

### 2.10. Ethanol production

#### 2.10.1. preparation of sugar solution

The containers involved reaction mixture were filtered to obtain sugar

solution produced from enzyme action. The sugars solution was concentrated in a rotary vacuum to give 10% glucose (w/v).

### 2.10.2. Medium of ethanol production

The medium used for ethanolic fermentation was composed of (g/L): 100 glucose (sugars solution obtained from enzymatic saccharification of rice straw), 0.2 g yeast extract and 5.0 g peptone, pH was adjusted to pH 4.5. The medium is introduced in 250ml capacity flasks containing 100 ml of the fermentation medium. The flasks were autoclaved for sterilization.

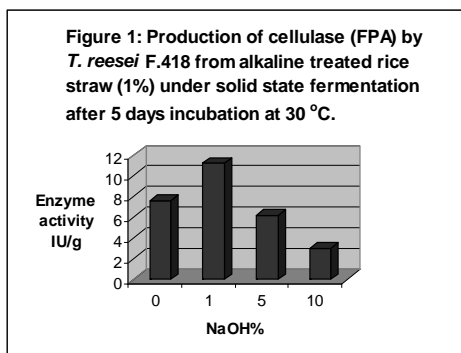
### 2.10.3. Ethanol production

The sterilized flasks were inoculated with a loop of *Saccharomyces cerevisiae* F.25, and then incubated at 34°C under static conditions. Samples are withdrawn after 12, 24, 36 and 48 hrs for ethanol and residual glucose and sugars determination.

## 3. Results

### 3.1. Effect of alkaline pretreatment of rice straw on the release of sugar

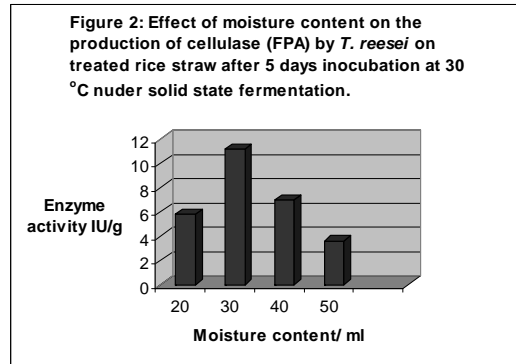
The effect of different concentration of sodium hydroxide on enzyme releases from alkali treated rice straw fermented with fungus is presented in Figure 1. The highest enzyme activity was achieved using RS pretreated with 1% NaOH (11.17 IU/g) followed by 5% NaOH (6.17 IU/g) and 10% NaOH presented very low value (2.99 IU/g). In the other side untreated RS with NaOH presented suitable value from release sugar (7.59 IU/g).



### 3.2. Effect of moisture content on enzyme production

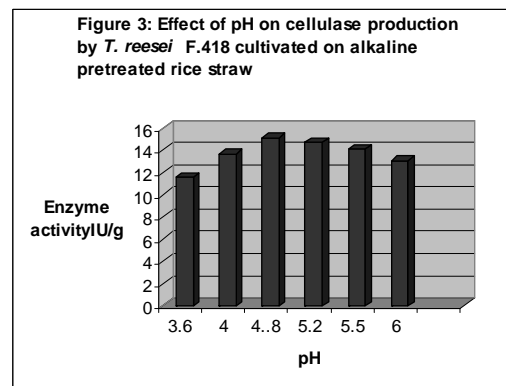
Figure 2 shows that the enzyme production with varying moisture levels. Ratio 1:2, 1:3, 1:4 and

1:5 (solid : liquid) using moistening solution composed of above Mandles medium in the cultivation system at different volumes 20, 30, 40 and 50 ml. The cellulase production was optimum using the lower addition at 30 ml as the production was 11.17 IU/g substrate followed by addition at 40 ml, since the production was 6.96 IU/g. Lower or higher moisture levels yielded lower cellulase activity.



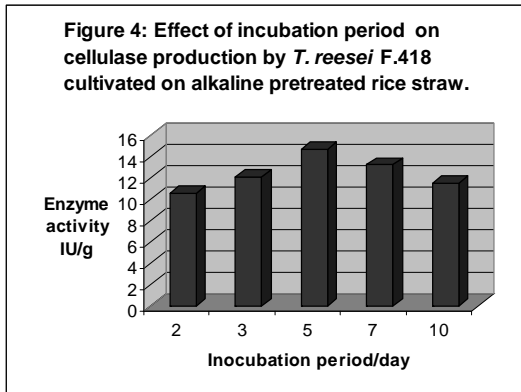
### 3.3. Effect of pH on production of enzymes activity

Figure 3 shows that low and high pH is not suitable for enzyme (FPase) produced by *T. reesei*. Data illustrated that the enzyme can be released optimally in pH 4.8 and the activity was 15.10 IU/g R.S.



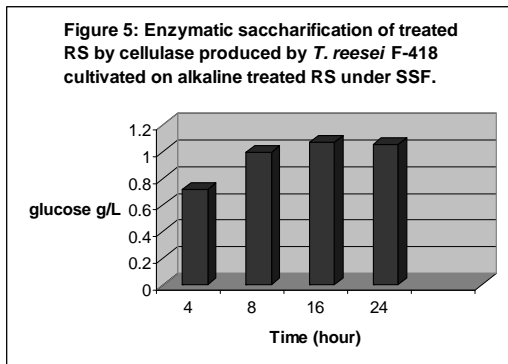
### 3.4. Effect of incubation period

Enzyme production was affected by incubation time, at short time cultivation period and long time, the enzyme production was reduced. Figure 4 shows that a maximum activity of cellulase was released after 5 days incubation then with elongation of cultivation time the yield of enzyme had decreasing trend.



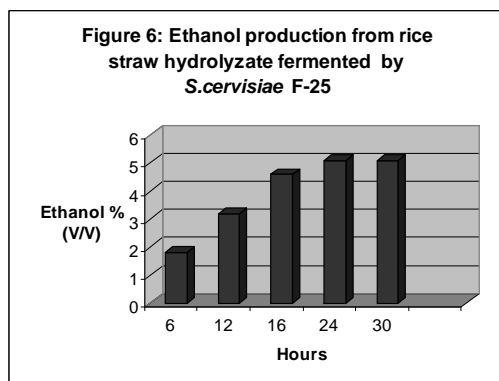
### 3.5. Enzymatic hydrolysis of rice straw by cellulase of *T. reesei*

Figure 5 illustrates the obtained glucose resulted from the action of enzyme on alkali treated rice straw. Maximum glucose yield was obtained after 16 hrs and it was found 1.07%.



### 3.6. Ethanol production

Figure 6 shows the data obtained from glucose fermentation in sugar solution obtained from enzymatic hydrolysis of rice straw by *S. cerevisiae* SHF-5. Data show that about 5.1% (v/v) in the fermented solution was achieved after 24 hrs. The fermented mash contains about 3.6 g yeast can be used as animal feed.



## 4. Discussion

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).

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, 1996). Investigated the effect of concentration of NaOH on production of cellulase, the results revealed to low concentration from sodium hydroxide (NaOH 1%) was the best treatment for maximum value from cellulase (11.17 IU/g), this agreement with Bjerre et al., (1996) who found that dilute NaOH was an effective pre-treatment for lignocellulosic materials with relatively low lignin content of 10 to 18% . in another study by Damisa et al.(2008) the pretreating of substrate with sodium hydroxide may have resulted in the swelling of the particles causing easy removal of the lignin and cellulose depolymerization occasioned by the separation of hydrogen bonds of the cellulose. Generally, the alkali treated residues with low concentration of sodium hydroxide showed higher cellulase yield than the untreated treated residues.

The moisture level of the medium is regarded as a fundamental parameter for microbial growth and metabolite formation. Lower moisture level leads to sub-optimal growth, a lower degree of substrate swelling and high surface tension, whereas higher moisture level decrease porosity, which would cause lower oxygen transfer and heat dissipation and enhanced formation of aerial mycelium (Lonsane et al., 1985). This results agreement with our results where the cellulase production was optimum using the lower moisture addition at 30 ml as the production was 11.17 IU/g, and differs from that of Kotwal et al., (1998), who reported that maximum enzyme activity was obtained when the initial moisture content was 86%.

Samia (2008) and Sodhi et al., (2005) reported that moisture content of the substrate is one of the critical factors influencing the outcome of SSF, lower moisture content cause a reduction in solubility of nutrients provided to organism by SSF, a lower degree of swelling and higher water tension. On the other side, reduction in enzyme production at high moisture may be due to the reduction in substrate porosity, changes in the structure of substrate particles, reduction of gas volume and decreasing in microbial growth (Baysal et al., 2003). (Pandey, 1992) concluded that the degree of hydrate one of the

substrate plays an important role on the growth of the fungi and subsequently the enzyme production. Water causes the swelling of the substrate and facilitates good utilization of substrates by the microorganisms. Increasing moisture level is believed to have reduced the porosity of substrate, thus limiting the oxygen transfer into the substrate (Raimbault and Alazard, 1980). The fact that the fungus grows and produces maximum enzyme activity at lower water adding.

Investigated effect of pH of the medium revealed that the optimum value from enzyme production was obtained at pH 4.8. Optimal pH is very important for growth of the microorganism and its metabolic activities. As the metabolic activities of the microorganism are very sensitive to changes in pH, cellulase production by *T.reesei* was affected by varying pH of the medium. Our findings are comparable to previously reported results from literature. Gomes et al. (2006) found that cellulases from *T.reesei* work better in more acidic (4.5-5.0), also Yang et al. (2004) found that maximum production of cellulase at pH 4.5

This corroborates the results of Tenborg et al. (2001) at which the optimum glucose yields were obtained at pH 4.8. pH is among most important factors for any fermentation process and depended upon microorganisms because each microorganism possesses a pH range for its growth and activity (Lonsane et al.,1985).Increasing and decreasing in pH on either side of the optimum value resulted in decrease in growth product fermentation (kokab et al.,2003).

Studying the incubation time revealed that the maximum cellulase production was obtained when fungi cultivated at 120 hrs. With longer cultivation time the yield of cellulase had decreasing trend. Melo et al. (2007) reported that the enzyme level declined with prolonged incubation, this could be due to loss of moisture or denaturation of the enzyme resulting from variation in pH during fermentation. And also Singh et al. Singh et al. (2009) reported that the decrease of enzyme activities may be due to the accumulative effect of cellulobiose. The time of the highest cellulase activity depends upon the substrate and fungus (Ojumu et al.,2003 and Alam et al. 2005).

Effect of enzyme loading on the enzymatic hydrolysis of the pretreated RS, the cellulase enzyme was assayed by measuring the amount of glucose released from the substrates following the secretion of cellulase by organism. Detergents like Tween 80, SDS etc, have been reported to enhance cellulase activities by increasing availability of nutrients (El-Hawary and Mostafa, 2001) and (Gashel, 1992). Pei-jun et al (2004) reported that negative effect of Tween 80 on cellulase activities. Kocher et al. (2008) reported that cellulase can be used to saccharify pretreated rice straw for bioethanol production, and they also tested 4 concentrations of Tween 80 in their

study, and they found a concentration of 0.10% was enhance cellulase activities. Many authors used microbial enzymes for hydrolysis of lignocellulose materials. Fadel (2001) used cellulase and B-glucosidase for wheat straw saccharification for ethanol production. Also cellulase from *T. Reesei* and B- glucosidase from *A. niger* were used for hydrolysis of pine parks (Parajo et al.,1988).

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

Krishna et al., (2001) reported that the maximum yield of ethanol was 5.3, 4.8 and 6.12 from wheat straw, bagasse and  $\alpha$ -cellulose respectively ca 6.4 % produced from glucose, these values are agreement with our results. Rao et al., (1995) could hydrolyze sugarcane bagasse for alcohol production by *S. cerevisiae*. They obtained 40-45 conversion of sugars to alcohol.

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3/1/2010