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Bioethanol productions from rice polish by optimization of dilute acid pretreatment and enzymatic hydrolysis

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Lignocellulose materials are abundant renewable resource for the production of biofuel from fermentative organism (*Sacchromyces cervesiae*). Rice polish is cheapest and abundant lignocelluloses resource and has potential to produce bioethanol. The main steps for the conversion of biomass into glucose required dilute acid pretreatment, but it also released inhibitory compounds which reduced the ethanol yield. So, attempt has been made to minimize the effect of inhibitory compounds as well as optimized the condition like glucose recovery, xylose solubilization and lignin degradation during dilute acid pretreatment. Maximum lignin degradation, less glucose loss, better xylose solubilization obtained with dilute sulphuric acid 1.5% at 100°C for 30 min. During enzymatic hydrolysis (Novozyme) with 0.75 mL/2 g, enzymatic loads for 72 h gave 4.27mg/mL glucose while with indigenous enzyme load 1 mL/2 g gave 0.953 mg/mL of glucose.

Key words: Bioethanol, Dilute acid pretreatment, enzymatic hydrolysis, rice polish.

INTRODUCTION

Increase prices of molasses and its limited availability divert the attention toward alternate resource (Raghavendra et al., 2007). A variety of lignocellulosic materials like agricultural residues, municipal and Industrial wastes are being investigated for bioethanol production (Ying et al., 2009). Cellulose is the abundant biopolymer on the earth, and is considered for best renewable energy resource (Farzaneh et al., 2008). The major limitations exist for the production of ethanol from agricultural are compact physical and chemical associations between lignin and polysaccharides of plant cell wall along with cellulose crystallinity. Lignin forms a protective covering around cellulose and hemicellulose, which restrict the enzymatic degradation and as well as reduced the yield of ethanol (Dawson and Boopathy, 2008). So, the recovery of cellulose from biomass by dilute acid treatment increased the cellulolytic activity.

The key or economical important step for the

production of ethanol depends on efficient conversion of cellulose to their monomeric sugars (Takagi et al., 1977).

Rice is an essential and important cash crop in Pakistan. Production of rice was estimated 6883 thousand tons during 2009 to 2010 which almost 1% less than last year (Economic Survey of Pakistan, 2009, 2010). Rice polish is a by-product of rice milling and is the cheapest source of cellulose material which available in bulk quantity in major rice growing areas of the world (Ambreen et al, 2006). The different strains of Thermoanaerobacter and Clostridium were used for the production of ethanol from different lignocellulosic biomass. *S. cerevisiae* or *Zymomonas mobilis* has advantage on other strain due to its efficiency of conversion of glucose and to ethanol tolerance but the *S. cerevisiae* or bacterium *Z. mobilis* cannot utilize multiple sugars to ethanol (Sommer et al., 2004; Badal et al, 2005). Rice polish has potential for the production of ethanol and also there is no proper utilization in Pakistan. There is no major research paper work available for the production of ethanol from rice polish. So, the study was distributed in three phase, In first phase, the dilute acid treatment was optimized on the basis of less glucose loss, better solubilization of

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xylose and maximum degradation of lignin. In the second phase of this study, alternaria alternate and *Aspergillus niger* was screened for the determination of maximum indigenously cellulase yield. The last phase of this study deals with the better recovery of glucose by enzymatic hydrolysis for maximum ethanol yield.

MATERIALS AND METHODS

Sample collection

An analytical grade chemical manufacture by Merck (Germany) was used during research. Rice polish was collected from rice mill of Gujranwala, Pakistan. The sample were chopped, oven dried, and ground to pass 80 mesh size US standard sieve. Dried samples were stored in sealed plastic bags at room temperature.

Dilute acid pretreatment

The dilute sulphuric acid concentration 0.5, 1, 1.5, 2, 2.5 and 3%(w/v) were added in flask containing 5 g sample of rice polish and one flask were kept as control filled with distilled water. The treatment were performed at 100°C in water bath for residence time of 15, 30, 45 min. The pretreated samples were filter and residue was washed with distill water to remove acid. Residue was dry at 40°C in incubator and further used to determine the lignin percentage after each treatment. The filtrate was centri-fuge at 10,000 rpm (Centurion Scientific Ltd, UK) for 10 minutes and used for glucose and xylose analysis.

Media

Baker Yeast (*S. cervesiae*) were grown in complete YPD medium (1% w/v yeast extract, 2% w/v peptone, 5% w/v glucose) at 30°C and stored 4°C. This strain was further used in bioethanol production.

Cellulase production

The cellulase producing fungus *A. niger* was collected from University of Agriculture Faisalabad while *Alternaria alternata* were selected for production of cellulase collected from fungal bank's stock cultures of Institute of Plant Pathology and Mycology, Punjab University, Lahore. After incubation very fine spores of both strains were stained with lactophenol blue and observed under microscope to identify pure strain and further sub-cultured the strain and stored at 4°C.

Inoculum preparation

The compositions of the fermentation media (in g/L) were: glucose, 70; yeast extract, 10, peptone, 50 as described by Yu et al. (2007). The 100 mL inoculum medium were prepared in 500 ml Erlen-meyer flasks closed with cotton plug and mediums were adjusted to pH 5 and autoclaved at 115°C for 15 min before use. The 100 mL inoculum flask placed in rotary shaker (Heidolph unimax 1010 model) 120 rpm at 28 ± 0.5°C for *S. cervesiae* while shaking for *A. niger and A. alternate* was adjusted at 180 rpm. A cell count of about 2.1×10 cell ml was obtained after 72 h and used as inoculum for future experiments. The cellulase fermentation carried out inoculated with 3 ml sporulation medium (containing 10^7) cells/mL of *A. niger* and *A. alternata*.

Cellulase fermentation

Cellulase production was carried out in duplicate Erlenmeyer flasks (containing 5 g of rice polish, moistened with 10 ml distilled water) substrates with one control (deprived of *A. niger* inoculum). The flasks were cotton plugged and autoclaved at 121°C for 15 min and incubation at 30°C for 1-7 days (Krishna, 1999; Pandey et al., 2001). The optimum incubation period for *A. niger* and *A. alternata* was optimized for the production of maximum cellulase (FPase activity).

Culture harvesting/ Isolation of crude cellulase enzyme

Enzyme was harvested by adding 100 ml distilled water in each Erlenmeyer flask and shaking at 180 rpm in orbital shaker incubator for 1 h. The flasks were filtered through muslin cloth then separately in filtration assembly to collect crude cellulase enzyme. Enzyme filtrates were centrifuged at 10,000 rpm for 10 min. The supernatants were collected carefully filtered through Millipore filter to avoid spore. The filtrate was used for enzyme assay and further crude enzyme was used for biomass hydrolysis.

Enzymatic hydrolysis

The enzyme Celluclast was gifted by Novo Nordisk (Bagsvaerd, Denmark). Enzymatic activities (FPU) were measured according to the methods described by Ghose. Enzyme activity of Celluclast (cellulase) was determined as 74 FPU/mL. The enzymatic hydrolysis was carried out in 250 mL of blue capped reagents bottle containing 50 mM citrate buffer (pH 4.8) and two gram substrate. The varying cellulase enzyme (Novozyme and Indigenous) concentration that is 0.25, 0.5, 0.75, 1 mL /2 g were loaded at 50°C containing 25 mg/mL tetracycline and 62.5 mg/mL augmentum. The 1mL samples were collected at 0, 3, 6, 24, 48, 72 h and heat in water bath for 10 min then cool and centrifuge at 10,000 rpm for five minutes. The glucose concentration was estimated after sample collection. The maximum glucose yield after treatment utilized for fermentation process.

Bioethanol fermentation

Fermentation was carried out in duplicate by loading 2 mL of yeast from inoculum containing 10^7 cells/mL of fermentation medium in 250 mL Erlenmeyer flasks. The samples were collected after 24, 36, 48 and 72 h.

Biomass determination

Cell density was measured on spectrophotometer (Humas Think HS 3300, Korea) 600 nm absorbance (Summer and Somers, 1944).

Reducing sugars

The reducing sugar concentration in the medium was determined using 3,5-dinitrosalicylic acid reagent (Miller, 1959). Xylose concentrations were determined by Miller (1959), which is dev-eloped by El-Enshasy (1998) in PhD thesis. The 50 µL of sample was taken from filtrate add 350 µL Citrate buffer (pH.6.5) then add 600µL of DNS to stop reaction and boil for 5 minute. The absorbance was taken at 540 nm on spectrophotometer (Humas

Table 1. Composition (%) of unpreteated sample of rice polish.

Composition	Percentage
Dry weight	91.4±0.1
Moisture	8.6±0.1
Crude protein	14±0.6
Ether Extract	19.6±0.4
Cellulose	21.4±0.7
Lignin	27±0.5

Think HS 3300, Korea).

Lignin estimation

The lignin content in treated and untreated samples was first treated with 1.25% H_2SO_4 for two hours and 72% H_2SO_4 hydrolysis for four hours. The residues was filtered and washed with distilled water to neutralized sulphuric acid and oven dried at 105°C for constant weight. The lignin was expressed by using the formula (Milagres, 1994).

$$\text{Ligin \%} = \frac{\text{Ligin weight}}{\text{Biomass}} \times 100$$

Proximate analysis

Crude protein, ether extract, crude ash and moisture content were analyzed by using AOAC methods (Helrich, 1990).

The determination of raw cellulose

Raw cellulose determined by Weendize method, under the name of Henneberg-Stohmann method (1975).

Determination of ethanol content by dichromate method

Acid dichromate solution (0.1 M $Cr_2O_7^{2-}$ in 5 M H_2SO_4) was prepared by dissolving 7.5 g of potassium dichromate in dilute sulfuric acid and the final volume was adjusted to 250 mL with deionized water. Standard curve was made by adding 300 µL of ethanol solutions were filled into small plastic caps and placed into beakers containing 3 mL of acid dichromate. The beakers were covered and sealed with parafilm and kept at room temperature for 30 min. The absorbance was recorded at 590 nm on spectrophotometer (Humas Think HS 3300, Korea) (Bennett, 1971).

RESULTS AND DISCUSSION

The rice polish composition of pretreated sample are represented in Table 1 which showed that the cellulose content was 21.4% and the lignin content was 27% with some crude protein and oil.

Dilute acid pretreatment

Dilute acid pretreatment of rice polish influenced on

releasing glucose but there was also released of some inhibitory compound like furfural, hydroxymethyl furfural and phenolic compound which affect the hydrolysis process. To avoid inhibitory compound, the pretreated sample was filtered and dried at 50°C for 12 h and placed in sealed bag. The dilute acid pretreatment was selected on the basis of less degradation of glucose and maximum loss of xylose as well as lignin degradation.

There was a minimum quantity of glucose loss occurred at 0.5% acidic treatment for 15 min but other parameters like xylose and lignin degradation contribute non significant effects on lignin loss (Figure 1). The good amount of glucose loss was examined at 2% acidic treatment after 45 min.

The results indicate that there was maximal xylose loss at 2% dilute acid treatment for 45 min at 100°C but there was also maximum glucose loss at this treatment and less lignin degradation so 45 min treatment was relatively better as compared to other treatments (Figure 2).

Figure 3 shows that there was maximum amount of reduction of lignin at 1.5% of dilute sulphuric acid prêtreatment for 30 min as compared to other treatments. These results were further selected for enzymatic hydrolysis.

Incubation period for *Aspergillus niger*

Incubation period for cellulase production by *Aspergillus niger* from rice polish are shown in Table 2. The maximum cellulase activity (21.97 ± 0.80 µg/ml) was obtained after 72 h at 35°C with 120 rpm. After 72 h, the cellulase activity trends shifted toward descending order.

Incubation period for *Alternaria alternata*

Incubation period of *A. alternate* during solid state fermentation of rice polish showed ascending trend from 1st day to 4th day and then decline after 4th day (Table 3). The optimum yield of cellulase from rice polish was obtained after four days of incubation at 11.81 ± 0.05 µg/mL/min at 30°C for 120 rpm. This high yield enzyme was further used in enzymatic saccharification.

Enzymatic hydrolysis

The hydrolysis of cellulose in pretreated sample and enhanced quantity of glucose release after enzymatic hydrolysis were the key steps towards bioethanol production. The varying concentration like temperature and time were adjusted and trialed in triplicate on rice polish. Cellulase in rice polish buffer medium had significant ($p<0.05$) impact on the conversion of cellulose to sugars.

It was clearly analyzed that the enzymatic load (Novozyme) of 0.75 ml/2 g gave high glucose recovery.
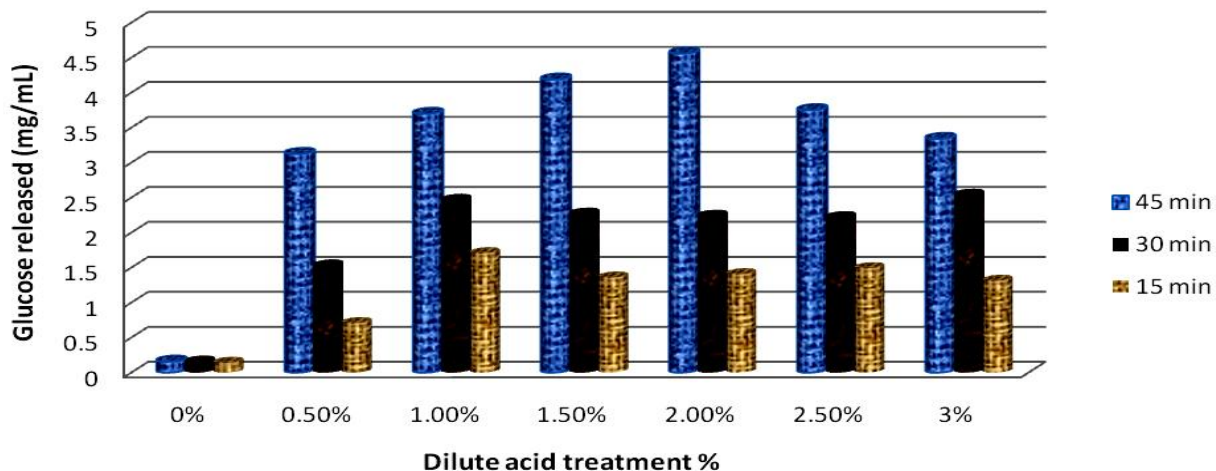
Figure 1. Glucose released by varying the residence time and dilute acid treatment at 100°C. The average l.s.d. ($p < 0.05$).
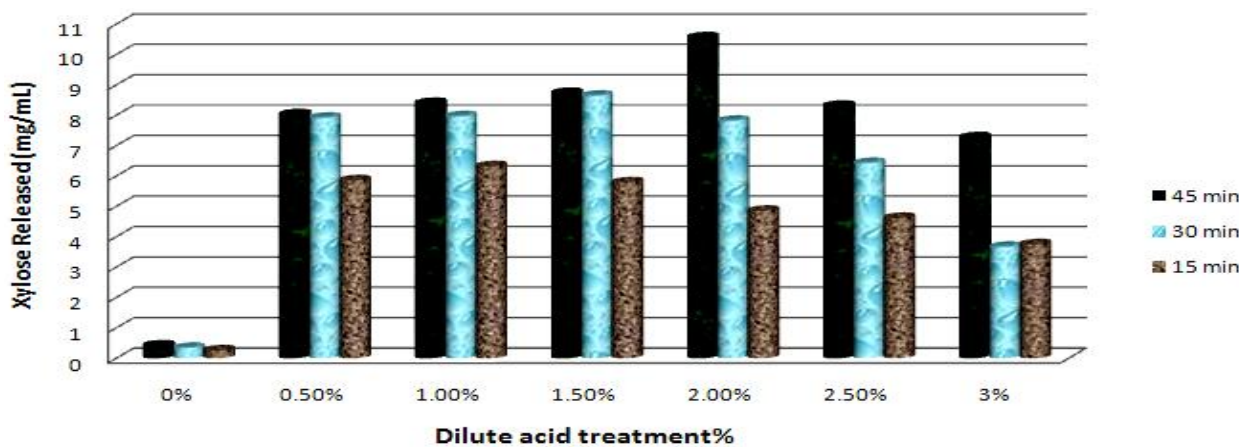

Figure 2. Xylose solublizations after varying dilute acid treatment and varying the residence time at 100°C. The average l.s.d. ($p < 0.05$).
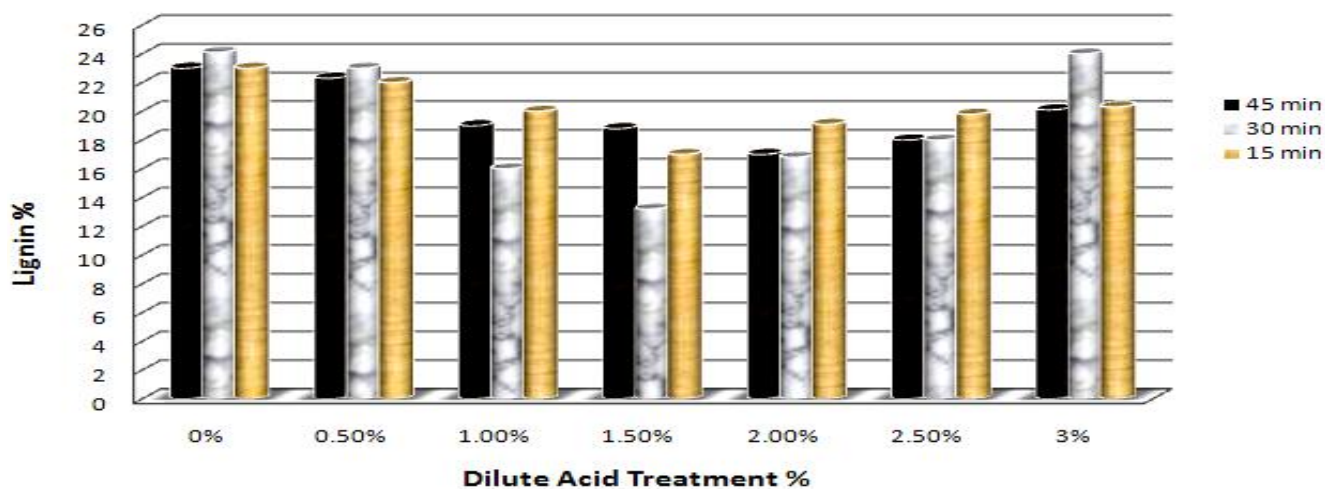

Figure 3. Lignin content (%) after varying dilute acid pretreatment and varying the residence time at 100°C. The average l.s.d. ($p < 0.05$).

Table 2. Incubation period for cellulase production by *Aspergillus niger* under SSF.

Substrate	1 Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
Rice polish	2.11 ± 0.03	2.39 ± 0.04	6.71 ± 0.03	4.23±0.01	2.11±0.05	1.84±0.02	1.06 ± 0.02

The results are presented as Mean ± S.D (n=2).

Table 3. Incubation period for cellulase production by *Alternaria alternata*.

Substrate	1 Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
Rice polish	4.44 ± 0.03	8.51± 0.05	9.52 ± 0.04	11.81 ± 0.05	6.71 ± 0.02	6.55 ± 0.01	5.52 ± 0.01

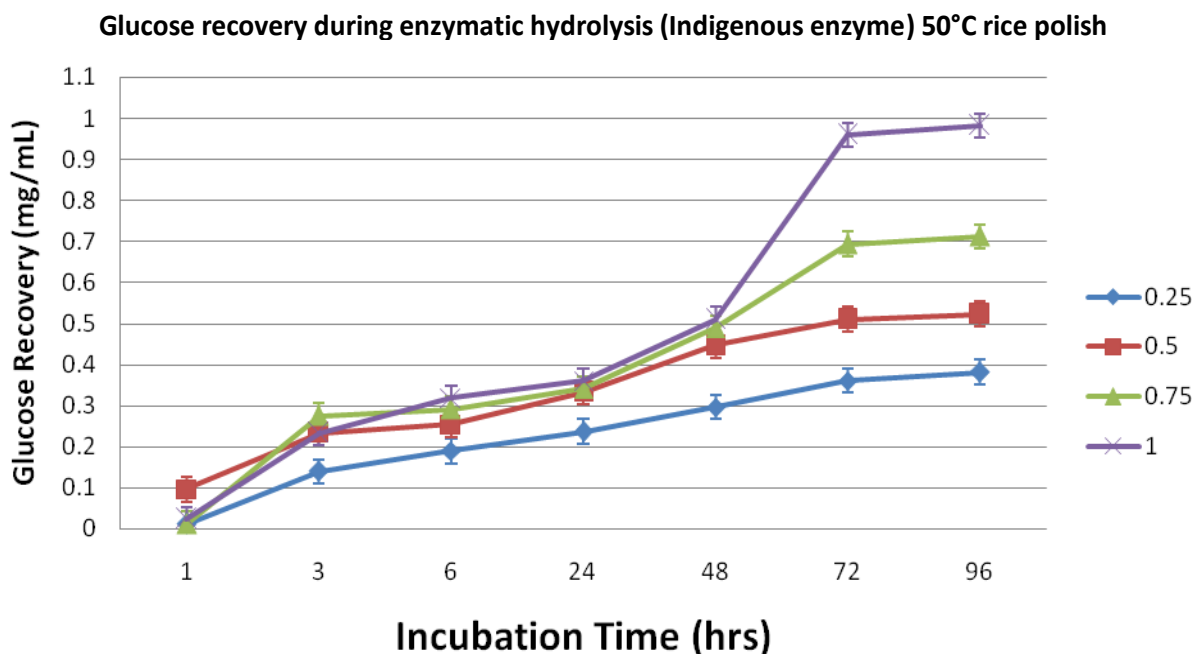The results are represented as Mean ± S.D (n=2).

Figure 4. Optimizing the concentration level of Cellulase (Novozyme) load on 2 g pretreated rice polish as substrate. Glucose recovery is presented as mg/mL.

The glucose released after 0.75 mL cellulase load was 4.27 mg/mL, while 2.16, 3.31and 3.40 mg/mL from 0.25, 0.5 and 1 mL respectively (Figure 4). The 0.75 mL enzymatic load is best for the conversion of cellulose into glucose. While indigenous produced gives maximum yield at 1mL of enzyme load. Results indicate that the drying method after dilute acid pretreatment enhanced enzymatic saccharification and this trend increased with the passage of time.

The glucose recovery after treatment with cellulosic enzyme showed that maximum amount of glucose was achieved after 0.75 mL/2 g load of Cellulase enzyme and this trend increased from 48 to 72 h (Figure 5). It had been estimated that there was an increasing trend of biomass for ethanol production but reduction could be observed after 36 to 48 h time frame (Figure 6).

The results after experiments showed that ethanol produced from biomass was observes maximum after 0.75 mL enzyme load as compared to other treatments (Figure 7). The study showed that the ethanol production from dry pretreated sample gave better results as compared to previous work.

Conclusion

This research was designed to utilized rice polish which was by product of rice mills in country like Pakistan. The enzymatic hydrolysis of dry pretreated samples was given better glucose recovery level. The removal of inhibitory compounds like furfural and hydroxyl-methylfurfural and phenolic compounds by filtration, pretreatment of biomass and drying of biomass were the key steps to generate good results for ethanol production.
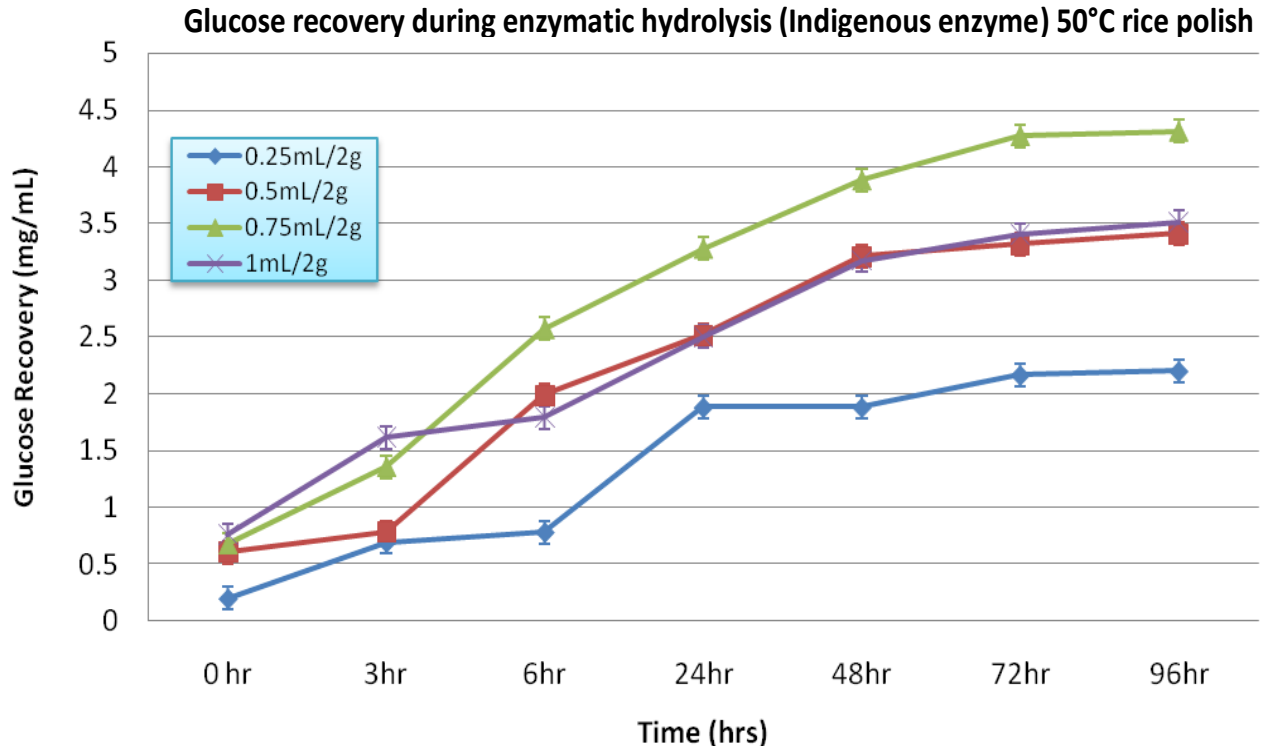
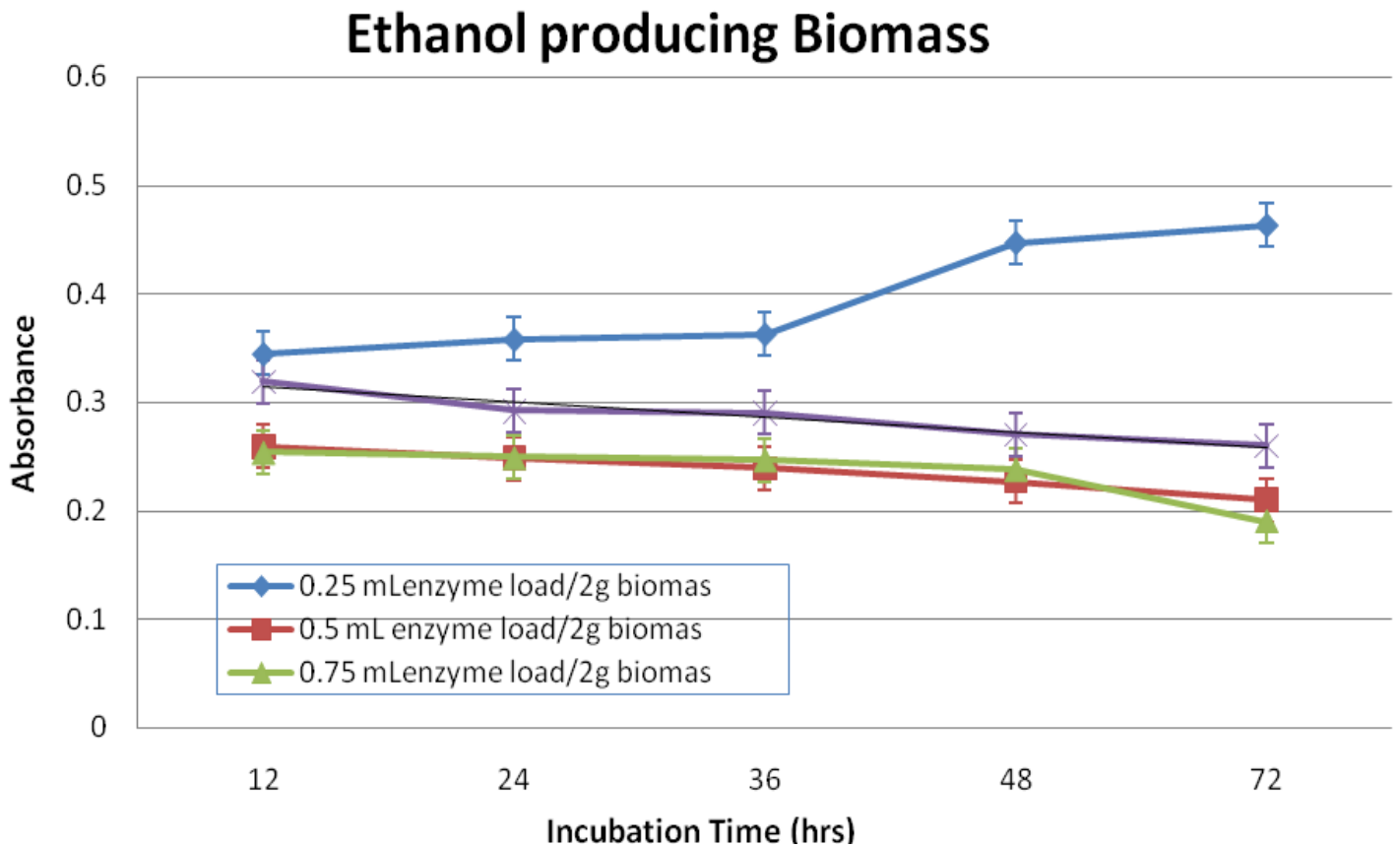**Figure 5**. Optimizing the indigenous Cellulase load on 2 g pretreated rice polish for maximum Glucose recovery.

Figure 6. Biomass growth at different time intervals.
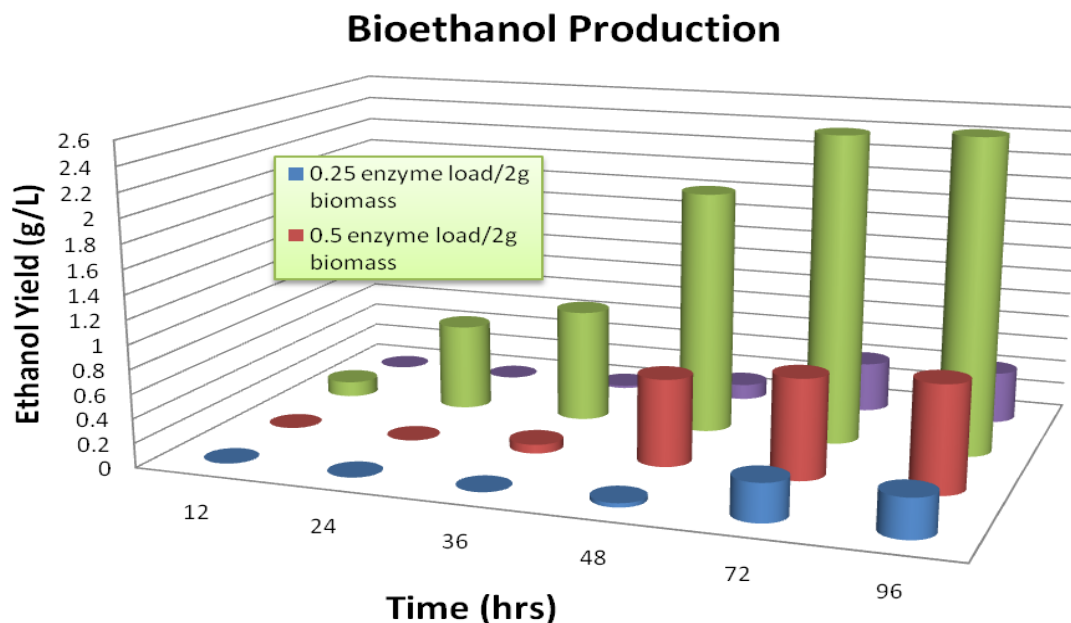
Bioethanol Production

Figure 7. Ethanol yield after different Incubation time.

This research will also be helpful in near future to utilize the rice polish for ethanol production still no pilot scale production available in this regard.

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