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Potential utilization of rice straw for ethanol production by sequential fermentation of cellulose and xylose using *Saccharomyces cerevisiae* and *Pachysolen tannophilus*

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Abstract:

This study presents bioethanol production from alkali (4% NaOH) pretreated rice straw by Separate Hydrolysis and Fermentation (SHF) method using yeast cells, *Saccharomyces cerevisiae* and *Pachysolen tannophilus*. Optimum temperature for both organisms was found to be 30°C and, Optimum pH for *Saccharomyces cerevisiae* and *Pachysolen tannophilus* was investigated as 5.5 and 6 respectively. Rice straw was an attractive lignocellulosic material for bioethanol production since it is one of the most abundant renewable sources in India. The pretreatment of biomass is necessary to remove the lignin matrix prior to enzymatic hydrolysis and subsequent fermentation. The rice straw was pretreated with 4% NaOH for 90 minutes at 80°C to degrade the lignin matrix. Finally, 45.40% of lignin was removed. The structural changes induced by pretreatment were observed by SEM analysis. The optimum enzyme activities estimated for cellulase and xylanase were 20 FPU and 250 IU respectively. Maximum amount of reducing sugars, 287mg per gram of pretreated rice straw, were released on hydrolysis with cellulase, followed by xylanase. The hydrolysate was inoculated with the yeast, *saccharomyces cerevisia,* for cellulose fermentation and it was inactivated by applying heat prior to xylose fermentation by *pachysolen tannophillus*. The amount of ethanol produced from alkali pretreated rice straw by sequential fermentation with *saccharomyces cerevisiae* for 3 days and *pachysolen tannophillus* for 2 days was 24.5% (v/w).

***Index Terms*— Alkali pretreatment, Hydrolysis, Fermentation, Heat inactivation**

I. INTRODUCTION

Exponential growth in population has increased the energy consumption and it urged an alternate way of energy generation. Lignocellulosic biomass, including forestry residue, agricultural residue, yard waste, wood products, animal and human wastes, etc., is a renewable resource that stores energy from sunlight in its chemical bonds [1]. Lignocellulosic biomass typically contains 50%-80% (dry basis) carbohydrates that are polymers of 5C and 6C sugar units. The most common feedstock for the production of ethanol is raw sugar from sugarcane or sugar beet, or starch found in corn, maize, and sorghum [2]. Biofuel production from traditional food crops leads to rise in food price and threat in food security. Biofuel production from non edible plant products such as rice straw, bagasse, corn cob, stover compromise the ongoing food vs fuel debate. Rice straw predominantly contains cellulose 32-47%, hemicelluloses 19-27% and lignin 5-24%, ashes 18.8%. 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% [3]. India is the second largest producer of paddy in the world after China and has 30,000 varieties of paddy crops [3]. The structural complexity of rice straw due to the presence of lignin is a major constraint for enzymatic and microbial attacks, and it is a serious obstacle to biomass based fuels [4]. Production of ethanol from lignocellulosic biomass contains three major processes: they are pretreatment, hydrolysis, and fermentation [5]. The pretreatments are divided into Physical (e.g. mechanical comminution, hydrothermal pretreatment), Physico-chemical (e.g. steam or CO2 explosion, ammonia fiber explosion), Chemical (e.g. ozonolysis, acid or alkaline hydrolysis) and biological processes (with microorganisms such as fungi). These pretreatments make the cellulose content of rice straw more susceptible to subsequent enzymatic hydrolysis. The sugars formed on hydrolysate are fermented into ethanol by most common microorganisms *Saccharomyces cerevisiae* and *Zymomonas mobilis*, which do not metabolize the pentose sugars, especially xylose [6]. One-third of the reducing sugars obtained on hydrolysis are pentoses, primarily xylose [7].Fermentation of xylose to ethanol increases the amount of ethanol production. *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* have been reported for the xylose conversion [6]. The biofuels production can improve rural employment and rural economics; and use of these fuels may increase energy security [8].

This study investigated the conversion of both cellulose and xylose sugars to ethanol by sequential fermentation with *Saccharomyces cerevisiae* and *Pachysolen tannophilus*. We also investigated structural changes and disintegration of polymers by using Scanning electron microscopy (SEM).

II. Materials and Methods

A. *Culture maintenance*

The baker's yeast was purchased from local bakery and stored in refrigerator. Baker's yeast (*Saccharomyces cerevisiae*) was cultivated aerobically for 48hrs at room temperature in yeast peptone dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) [9]. *Pachysolen tannophilus* (MTCC 1077) for xylose fermentation was obtained from Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. *P. tannophilus* was cultured in malt yeast agar medium (3g/L malt extract; 3g/L yeast extract; 5g/L peptone; 10g/L glucose, and 20g/L agar) for 48hrs at room temperature. Both the cultures were subcultured at 30 days interval [10].

B. *Optimization of temperature and pH*

To optimize pH and temperature, 100µl of 24 hrs cultures of *S. cerevisiae* and *P. tannophilus* were inoculated into 100 ml of respective media and incubated at room temperature at different pH, 4.5, 5, 5.5, 6 and pH was optimized based on taking Optical Density at 6 hrs interval for 24 hrs[10]. To optimize the temperature, 100µl of 24 hrs cultures of *S. cerevisiae and P. tannophilus* were inoculated into 100 ml of respective media and incubated at optimized pH at different temperatures, 30°C, 35°C, 40°C, 45°C[10]. Growth was measured by taking Optical Density at 530nm (OD_{530}) using UV–Visible spectrophotometer for every 6 hrs interval for 24 hrs.

C. *Pretreatment*

The rice straw was collected from agricultural fields in Gobicettipalayam, Erode. Tamil nadu, India.100g of rice straw was pretreated with 4% Sodium Hydroxide for 90 minutes at the temperature of 80°C by kept in hot water bath [11].The morphological changes induced in the rice straw due to pretreatment were analysed by Scanning electron microscopy after 24 hrs of drying in hot air oven. The samples of native and pretreated rice straw were prepared by attaching it on a specimen stub. The samples were sputter-coated with Gold and observed at an accelerating voltage, 20kv.The images were taken with magnification of 250X and 1500X for native and pretreated rice straw respectively [12].

D. *Enzymatic Hydrolysis*

The cellulase and xylanase enzymes were purchased from Noor enzymes, Calcutta, India. Cellulase Enzyme activity was determined by Filter paper assay using whatman filter paper as substrate and the activity was expressed in Filter paper units (FPU). The optimal cellulase enzyme load was determined by treating the pretreated rice straw with different Filter Paper Units such as 10, 15, 20, and 25[13]. Xylanase activity was determined by using oat spelt xylan as substrate and the optimal xylanase enzyme load was determined by treating the pretreated rice straw with different International Units (IU) such as 200,250,300 and 350. The hydrolysis time for cellulase is 36 hrs and for xylanase is 24 hrs [14]. These optimizations were done based on maximum amount of reducing sugars released, estimated by Dinitrosalicylic (DNS) method [15]. The sequence of hydrolysis with either cellulase at first and then xylanase or xylanase at first and then cellulase is also determined based on amount of maximum reducing sugars released, estimated by DNS method.

E. *Fermentation*

The 6% (v/v) inoculum of *Saccharomyces cerevisiae*, was prepared at Optical Density value, 0.2 at 620 nm (OD620) [16] and inoculated into 100ml hydrolysate for anaerobic fermentation at 30°C for 3 days. After fermentation, *Saccharomyces cerevisiae* cells were inactivated by kept at 50°C for 6 hrs [18]. Xylose is pentose sugar that cannot be digested by *S. cerevisiae*. Consequently, the hydrolysate was inoculated with 4% (V/V) of 24 hrs *Pachysolen tannophillus* and semi-aerobic fermentation was done for 2 days [16]. The fermentated medium was filtered and centrifuged to remove microbial debris. The supernatant was distilled at 78°C and the ethanol content in the distillate was estimated by chromic acid assay [18].

III. Results and Discussion

A. *Optimum pH and Temperature*

The optimum pH observed for *Pachysolen tannophillus* and *Saccharomyces cerevisiae* was 6 and 5.5 respectively, shown in Fig.1 and Fig.2.The same results for pH were obtained for both organisms by Prabu *et al* [10]. The maximum growth was observed at temperature, 30°C for both organisms *Pachysolen tannophillus* and *Saccharomyces cerevisiae* shown in Fig.3 and Fig.4. Prabu *et al* reported that the optimum temperature for *Pachysolen tannophillus* and *Saccharomyces cerevisiae* was 26 ± 2°C and 30°C respectively [10].

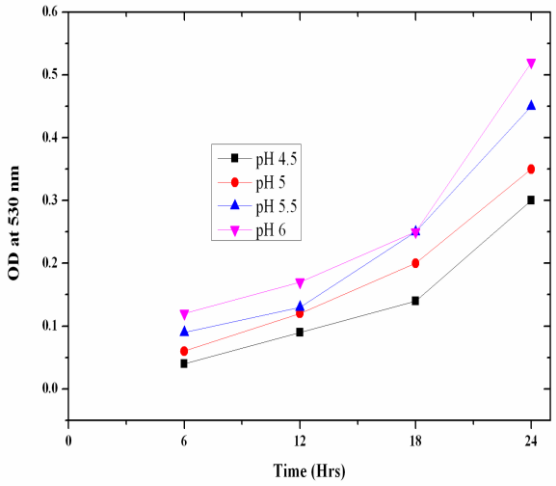

Fig.1. Optimum pH for growth of *Pachysolen tannophillus*

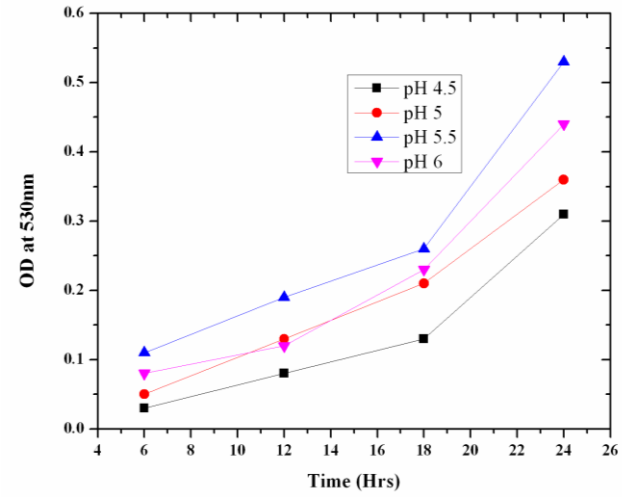

Fig.2. Optimum pH for growth of *Saccharomyces cerevisiae*

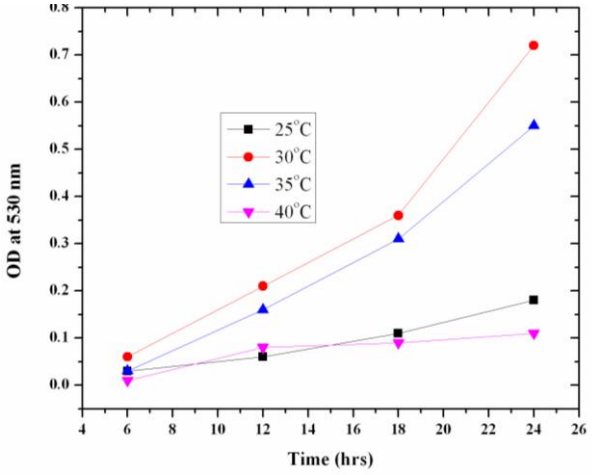

Fig.3. Optimum temperature for growth of *Pachysolen tannophillus*

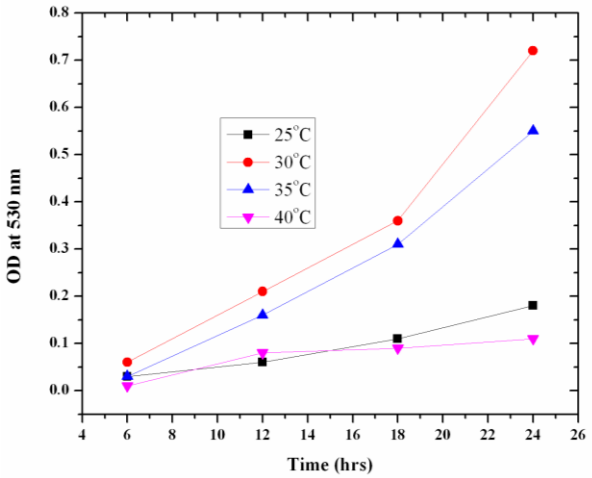

Fig.4. Optimum temperature for growth of *Saccharomyces cerevisiae*

B. *Pretreatment*

Alkali pretreatment partially removed the lignin and it reduced the crystallinity of cellulose in rice straw. So the rice straw became more susceptible to subsequent Enzymatic Hydrolysis. The amount of lignin present in untreated and pretreated rice straw was estimated as 18.50 %.and 10.10% respectively. The percentage of lignin removed was calculated as 45.40 %. Kim *et al* obtained 52.60% of lignin removal for the same pretreatment conditions [11]. The structure of native rice straw, and morphological changes induced due to alkali pretreatment were analysed by scanning electron microscopy (SEM), shown in Fig.5 and Fig.6. Partial degradation of lignin matrix that surrounds cellulose was observed in SEM images. The alkali pretreatment destroyed the surface and formed cracks, breaks, pores on the surface [12]. The SEM images clearly demonstrated that the pretreatment altered the structure, increased the porosity and so the sugars are more accessible to the enzymes.

C. *Enzymatic hydrolysis*

The optimum enzyme activity of cellulase and xylanase was 20 FPU (Fig.7) and 250 IU (Fig.8) respectively based on

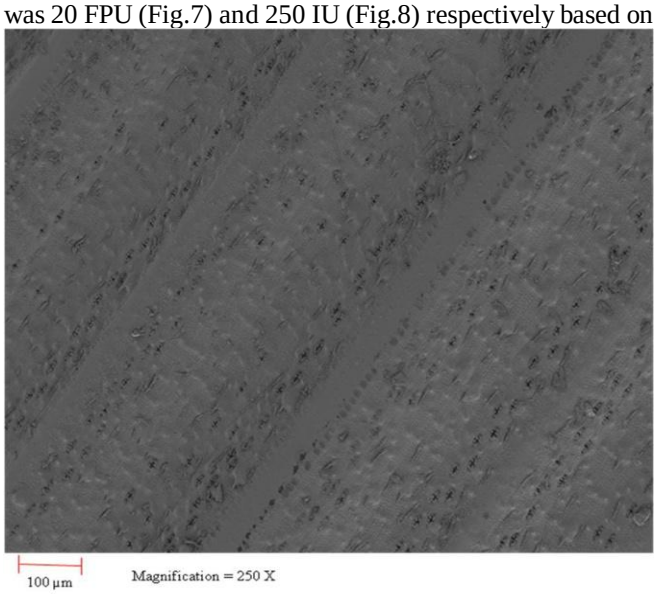

Fig.5. SEM image of native rice straw

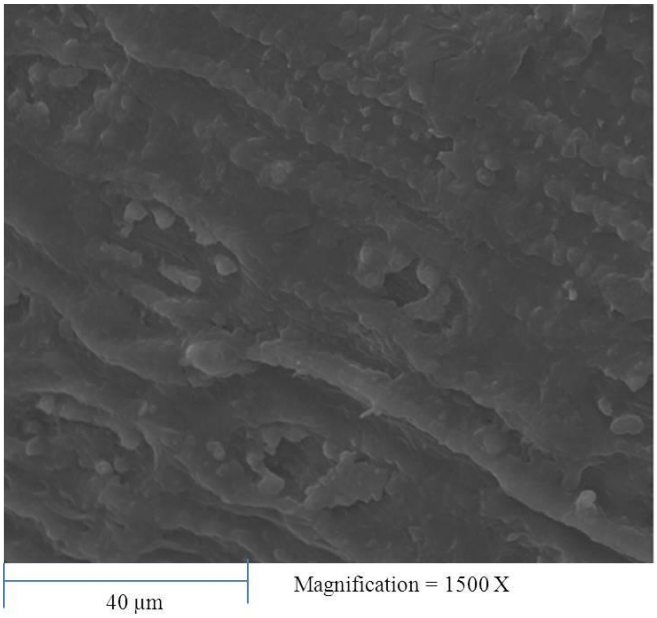

Fig.6. SEM image of alkali pretreated rice straw

maximum amount of reducing sugars obtained from 1g gram of pretreated rice straw, estimated by DNS method. The total amount of reducing sugars obtained by hydrolysis with cellulase and subsequently with xylanase was 287.00 mg per gram of pretreated rice straw. The total amount of reducing sugars obtained with xylanase hydrolysis, followed by cellulase was 238.00 mg per gram of pretreated rice straw. Hydrolysis with cellulase enzyme followed by xylanase gave higher sugar yield than hydrolysis with xylanase followed by cellulase. It indicates that the cellulase enzyme might improve digestibility of xylan in subsequent hydrolysis with xylanase. The sequence of hydrolysis should be cellulase at first and then xylanase. Kim *et al* reported that maximum yield of glucose was 252.60 mg per gram of rice straw with cellulase enzyme alone [11]. The excess amount of reducing sugars obtained in our study might be xylose.

D. *Ethanol Estimation*

The volume of ethanol present in 43 ml of distillate was 24.50ml, estimated by chromic acid assay [18]. The amount

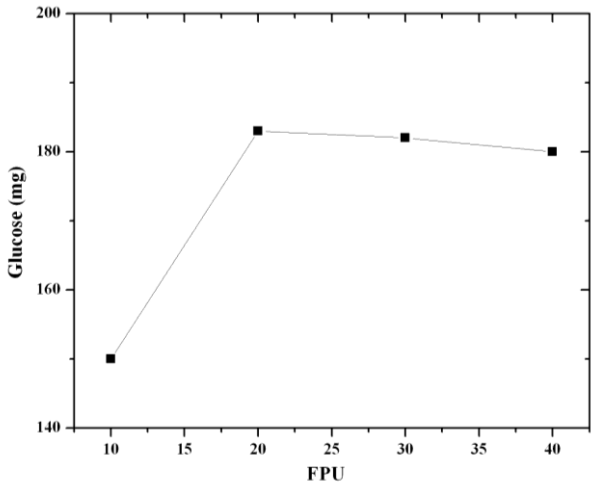

Fig.7.Optimum cellulase activity

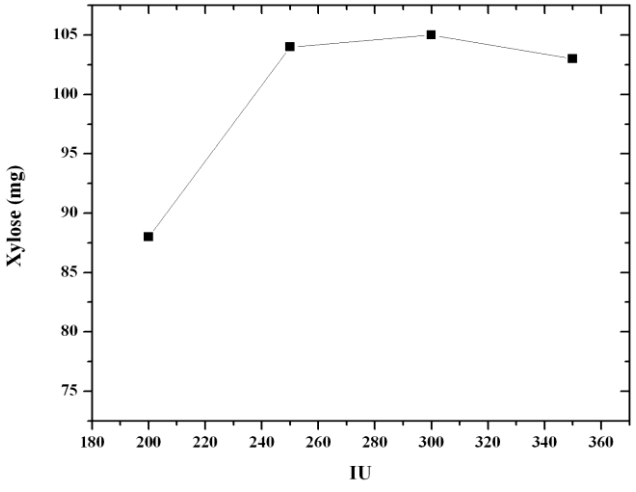

Fig.8. Optimum xylanase activity

of ethanol produced from 100g of rice straw was 24.50ml (19.30g) after 5 days of fermentation with *Saccharomyces cerevisiae* and *Pachysolen tannophillus*. The percentage of ethanol produced was 24.50% (v/w) or 19.30% (w/w). Li *et al* reported that 19.10 g of ethanol produced from 100g of rice straw by sequential inoculation of *Saccharomyces cerevisiae* and *Pichia stipitis* after inactivation of *Saccharomyces cerevisiae* [18].The total amount of ethanol produced was approximately equal from fermentation with *Saccharomyces cerevisiae* and consequently, with either *Pachysolen tannophillus* or *Pichia stipitis*.

IV.CONCLUSIONS

The maximum growth was observed at optimum pH and temperature for both *Pachysolen tannophillus* and *Saccharomyces cerevisiae*. The SEM observations showed that partial degradation of the lignin seal with alkali pretreatment. Hydrolysis with cellulase, followed by xylanase was more efficient for release of maximum reducing sugars. Fermentation of both cellulose and xylose after heat inactivation of *Saccharomyces cerevisiae* prior to xylose fermentation resulted in increased amount of ethanol production. A further study is to optimize the conditions for balanced co-fermentation of cellulose and xylose to reduce the time frame for fermentation.

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