



Enzymatic hydrolysis of alkali pretreated rice straw for the enhanced production of fermentable sugars

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ABSTRACT

To produce fermentable sugars from the alkali pretreated rice straw using fungus and their enzymes by biodegrading processes, first the fungus was grown on the rice straw by solid state fermentation method. Then enzymes were isolated and applied for the hydrolysis of rice straw. To improve the production of enzymes the growth conditions via initial pH of the growth substrate, moisture content, incubation time and additional nutrients were optimized. The maximum enzyme (221.32±9.62 (U/g) Carboxymethyl-cellulase, 12.21 ±0.29 (U/g) Filter-paperyase, 74.32±1.62 (U/g β-Glucosidase) and 317.32 ±7.98 (U/g) xylanase) activities were obtained at pH 7.0, 90% moisture content, and incubation period of 96 hours of solid state fermentation. The hydrolysis of the rice straw using the fungal culture filtrate (3%) in 10 hours resulted the yields of 33.56g/l reducing sugars. It indicated that the enzyme solution fabricated in the lab from the fungal fermentation is highly effective for the production of fermentable sugars.

Keywords: Rice straw, solid state fermentation (SSF), *Aspergillus tamari*, Cellulase

INTRODUCTION

Depletion of fossil fuels is the biggest problem in the current scenario; energy industries and research scientist are keenly working to develop alternative to the fossil fuels [1]. Bioethanol may be a sound alternative to the existing fuels because of their feed stock abundance in nature [2]. Generally bioethanol is produced by using food crops as a feedstock, for a long term we cannot utilize them, to overcome these hurdles utilization of non edible lignocellulosic biomass is the only option [3]. The non-food biofuel feedstocks like rice straw, wheat straw, and sugarcane baggase are having the great potential to meet the demand [4, 5]. But the cost of conversion processes like pretreatments and enzyme productions are the biggest obstacles. Large scale production of bioethanol is not yet developed due to their production cost [6]. Hydrolysis of lignocelluloses materials into simple sugars by various steps via pretreatments and enzyme hydrolysis are recommended [7, 8]. Number of pretreatment methods are reported to degrade lignocellulosic biomass, including microwave and alkaline treatments irradiation [9], hydrothermal [10], steam explosion [11], fermentation [12]. But the proposed technology does not satisfy the bioenergy industry requirements. Hydrolysis of pretreated bioethanol feed stock by enzymatic hydrolysis might be a viable method for the large scale production process. The mechanism behind enzyme hydrolysis is that the enzymes will degrade the polysaccharides into simple sugars which were useful for the production of bioethanol. Polysaccharide degrading enzymes are mainly produced from microbial source by liquid and solid state fermentation method but the cost effective production is the main bottleneck [13].

Rice straw is the available bulk byproduct of the agro industries. Currently these byproducts are being used as feed stock for the production of bioethanol [14]. Suitability of bioethanol feed stocks via rice and wheat straw, corn stover and bagasse has been well studied and these agro-residues are considered as the major feedstock for the future fuels [15]. Rice straw will be a more suitable feed stock for the production of bioethanol because of its translation nature. Present work focused mainly on the conversion of rice straw into the fermentable sugars as promising renewable feed stocks. The composition of rice straw changes according to rice variety, season, soil condition and harvest time. Generally the rice straw was composed of $39.5 \pm 2.5\%$ w/w cellulose, $30.5 \pm 1.7\%$ w/w hemicellulose, $19 \pm 1.8\%$ w/w lignin, and $11 \pm 1.5\%$ w/w ash, as determined by previously described methods [14, 15]. In this paper, we report a production of cellulolytic enzymes their saccharification effect on rice straw. To produce fermentable sugars from rice straw, initially the fungus was grown on it and then hydrolysed using fungal cellulolytic enzymes. The fermentable sugar formed by the enzyme mediated degradation was examined and compared with other reported methods.

EXPERIMENTAL SECTION

2.1. Microorganism

Aspergillus tamarii (MTCC5152) was isolated from the tannery effluent soil was propagated on potato dextrose agar (PDA) medium (Himedia-India). Slants were grown at 30°C for 7 days and stored at 4°C, and sub-cultured fortnightly.

2.2. Rice straw

Domestically procured rice straw was washed, air dried, size fractioned to 0.5mm and stored at room temperature. The air-dried rice straw was cutting-milled and passed through a 2-mm screen sieve. The rice straw was collected and stored at cold room until use.

2.3. Inoculum preparation

To 7 days old *Aspergillus tamarii* culture slants, 10ml of 0.1% tween-80 solutions was added and the spores were dislodged using an inoculation needle under sterile conditions. Spores in the solutions were collected in a sterile flask and the suspensions were diluted appropriately for the required spore density. Viable spore density was determined by the serial dilution of the spore suspension and spread plating methods.

2.4. Solid state fermentation (SSF)

Rice straw weighed (each 10g dry weight) and put into 250 ml Erlenmeyer flask, the basal nutrients were added (% of Sucrose-1.0, 2KH₂PO₄- 0.03, CaCl₂.2H₂O- 0.03, MgSO₄.7H₂O- 0.005, FeSO₄.7H₂O-0.002, CoCl₂.6H₂O-0.0016, MnSO₄.7H₂O- 0.001) to improve the fungal growth. Initial pH of the fermentation substrate (6, 7,8,9,10,11 and 12), moisture content (75, 80, 85, 90, 95, and 100 (%)) using distilled water, incubation time (1-7 days) and additional nutrients were optimized. The experimental flasks were sterilized at 121°C for 15 min. SSF experiments were commenced by adding spore suspension (1×10^6 spore/g of rice straw) into each flask. The mash was mixed in the flasks to enable even distribution of the inoculums and then incubated in a static incubator at 28 °C for up to 8 days. To investigate the impact of additional nutrients on the cellulolytic enzyme production, 0.5% cellulose, sucrose, glucose, peptone, beef extract and yeast extract were added independently to the rice straw before autoclaving. All the SSF were carried out in triplicate.

2.5. Extraction of crude enzymes

Rice straw undergoing SSF (1g) were suspended in 40 ml sterile water and mixed well. The suspensions were centrifuged at 3000 rpm at 4°C for 30 min. The supernatant (fungal extract) was used for the experiments as the crude enzyme mix. The protein contents were determined by the modified method of Lowry *et.al* (1951) [16] using Bovine serum albumin as a standard.

2.6. Determination of Carboxymethyl-cellulase (CMCase)

CMCase activity was assayed using a modified method described by Wood and Bhat [17]. The CMCase activity was measured by mixing 0.1 ml of enzyme solution with 0.1 ml of 1.0% (w/v) Carboxymethyl-cellulose in 10 mM sodium phosphate buffer, pH 7.0 at 37°C for 60 min. The reaction was stopped by adding 1.0 ml of 3, 5-dinitro salicylic acid (DNS) reagent. The mixture was boiled for 10 min cooled in ice and its optical density was read at 546 nm. The CMCase activity was calculated by using a calibration curve for glucose. One unit of CMCase was defined as the amount of enzyme that released 1 µmol of glucose per min.

2.7. Determination of Filter-paperase (FPase)

The activity of FPase was assayed according to the method explained by Wood and Bhat [17] with some modifications. Briefly, the methods are similar to the CMCase assay method, but the substrate used was Whatman No.1 filter paper (FP) strip (1x6 cm) soaked in 1.0 ml of 10 mM sodium phosphate buffer pH 7.0 at 37°C for 60 min. The reaction was stopped by adding 1.0 ml of 3, 5-dinitro salicylic acid (DNS) reagent. The mixture was boiled for 10 min cooled in ice and its optical density was read at 546 nm. One unit of FPase was defined as the amount of enzyme that released 1 μ mol of glucose/ min.

2.8. β -Glucosidase

β -Glucosidase activity was measured by the method of Herr (1979) [18]. The reaction mixture contained 1 ml of 2 mM p-nitrophenyl- β -D-glucopyranoside (pNPG) and 0.1 ml of enzyme solution. This reaction was carried out at 50°C for 5 min. The reaction was stopped by adding 2 ml of 1 M sodium carbonate (Na_2CO_3) solution. The amount of p-nitrophenol was determined by absorbance at 405 nm using a spectrophotometer.

2.9. Xylanase

Xylanase activity was determined by the modified method of Bailey et al. (1992) [19]. 1% beech wood xylan was dissolved in 0.05 M citrate buffer, at pH 7.0. The reaction mixture contained 0.5 ml of 1% beech wood xylan and 0.5 ml of enzyme solution. The reaction was carried out at 50°C for 5 min. It was stopped by adding 3 ml of DNS solution and heating the tube in a boiling water bath for 15 min. The amount of sugar released was measured by absorbance at 540 nm using a spectrophotometer. Unit of enzyme activity was defined as the amount of enzyme required to release 1 μ mol of xylose from the substrate per min under given assay conditions.

2.10. Alkaline pretreatments of rice straw

Rice straw was mixed with 2.0% NaOH solution, at a solid to liquid ratio of 1:10 (w/v), autoclaved at 121°C for 30 min. The autoclaved rice straw was adjusted to pH 7.0 by HCl and then rinsed with distilled water to remove alkali residues. Collected biomass was dried in an oven at 40°C until the weight was constant.

2.11. Hydrolysis of rice straw

To examine the potentiality of the enzymes in hydrolysis of rice straw three different concentrations of enzymes solutions 1% (30U/g), 2% (60U/g), 3% (90U/g), 4% (120U/g) and two different substrates were used. Hydrolysis substrate-1: Alkali pretreated and fungal fermented rice straw. Hydrolysis substrate-2: Alkali pretreated and autoclaved rice straw. The hydrolysis was carried out by adding 2g (dry weight) substrate into the enzyme solution, and the samples were shaken in a water bath at 150 rpm, 50°C and for 3 days. Samples were collected and centrifuged at 13,000 rpm for 10 minutes; the reducing sugar concentrations in the supernatant were analyzed by dinitrosalicylic acid (DNS) method.

2.13. Determination of reducing sugar

The sugar content following hydrolysis of the agro wastes was determined using the dinitrosalicylic acid (DNS) colorimetric method [20] and the sample measured at 491 nm using UV-VIS spectrophotometer. The reducing sugar content was subsequently determined by making reference to a standard curve of known glucose concentrations.

RESULTS AND DISCUSSION

3.1. Effect of initial medium pH on cellulase production

Effect of initial culture medium pH (Fig-1) shows that *A. tamarii* was able to secrete all the main components of cellulolytic enzymes over a broad pH, ranging from 5.0 to 8.0. Significant activity of cellulases were detected at initial culture pH ranging from 6 and 8.0; and the highest activities of all the three component of cellulase (FPase, CMCase and β -glucosidase) and xylanases were obtained at pH 7.0. The optimum pH obtained from this study was in the sort of those reported for cellulase production by *T. reesei* [21]. Cellulase production by *Aspergillus niger* MS82 was maximal when the initial culture pH was adjusted to 6.0 or 7.0 [22]. On the other hand, Juhasz et al. [23] stated that the maximum cellulase production was obtained at pH ranging from 3.0 to 5.0. Normally, the pH of the culture increased during the first two days of cellulase fermentation by fungi due to utilization of nutrients, hemicellulose and amorphous cellulose from lignocellulosic materials for growth. After a dynamic growth, the culture pH decreased due to the formation of carboxylic groups and carbonic acids from lignin [24]. At this stage, the fungus started to exploit the crystalline portion of cellulose and starts secreting cellulase. During the fermentation, the culture pH was reduced to acidic when cellulose was consumed by the fungi. Reduction in culture pH was due to

the assimilation of ammonium ions by the fungal mycelium [25]. After cellulose has been fully consumed, the culture pH was increased possibly due to the utilization of organic acids accumulated in the culture during the earlier stages of fermentation. In cellulase fermentation by *T. reesei*, organic acid is produced in direct relation to the amount of cellulose consumed [26]. Under favorable conditions, *T. reesei* might produce large quantities of cellulase, while the presence of acids would cause a severe decrease in culture pH from 5.5 to 3.0 [26]. At culture pH of below 5.0, inhibition of growth and inactivation of cellulases occurred. Therefore, appropriate pH control is necessary for the enhancement of cellulase fabrication.

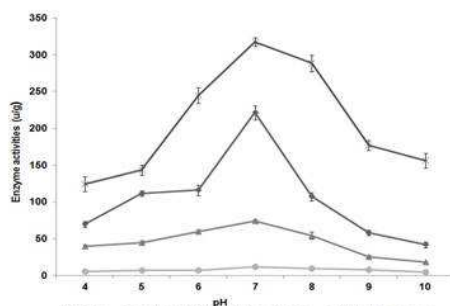


Fig.1. The impact of initial medium pH on cellulolytic enzyme production using rice straw. (■) carboxymethyl-cellulase; (●) Filter-paperase; (▲) β -Glucosidase; (□) Xylanase.

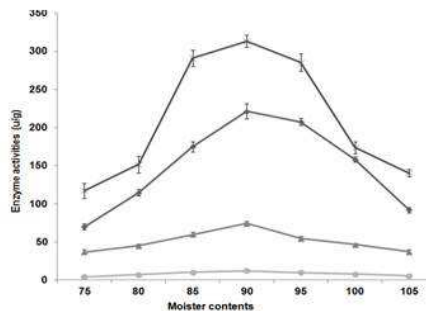


Fig.2. The effect of moisture content on cellulolytic enzyme production using rice straw. (■) carboxymethyl-cellulase; (●) Filter-paperase; (▲) β -Glucosidase; (□) Xylanase.

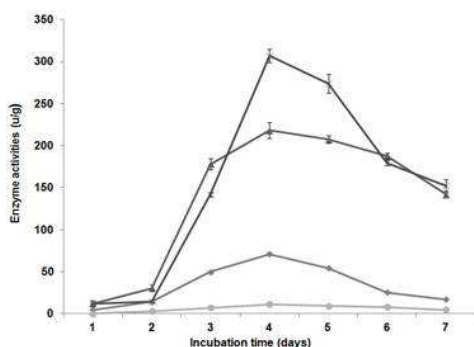


Fig.3. The effect of incubation time on cellulolytic enzyme production using rice straw. (■) carboxymethyl-cellulase; (●) Filter-paperase; (▲) β -Glucosidase; (□) Xylanase.

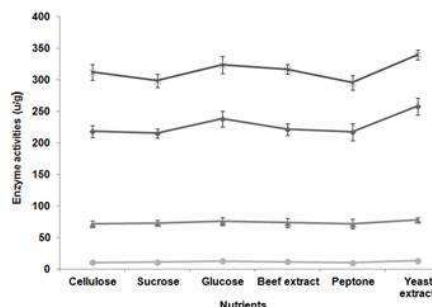


Fig.4. The effect of additional nutrients (0.5%) on cellulolytic enzyme production using rice straw. (■) carboxymethyl-cellulase; (●) Filter-paperase; (▲) β -Glucosidase; (□) Xylanase.

3.2. Impact of moisture content on enzyme production

From the results (Fig-2) various moisture levels of the substrates influenced the production of enzymes. Moisture content (90%) of solid substrates gave the optimum production of Carboxymethyl-cellulase 221.32 ± 9.62 (U/g), Filter-paperase 12.33 ± 1.29 (U/g), β -glucosidase 74.32 ± 2.62 (U/g) and xylanase 313.33 ± 17.98 (U/g). These results agree with results using *A.niger* on wheat straw, sugarcane bagasse and soybean bran [27, 28]. The availability of water content in the solid substrate are thus plays an important role in the production of enzymes. The importance of water in SSF has been widely studied under quantitative aspects, the recommended water content in SSF ranges from 30% to almost 90%, depending on the material.

3.3. Effect of incubation time on enzyme production

Production of biocatalyst is closely related to maximum growth of the organism on the substrates [29] and therefore there is an association between incubation time and enzyme production. The results (Fig-3) showed that during the early growth phase of 1 to 2 days, the cellulolytic enzyme production proceeded at a slower rate after which it increased sharply reaching a high value at 72 hours in alkali treated substrates. The maximum productions was obtained at 96 hours of incubation carboxymethyl-cellulase (218.32 ± 9.62), filter-paperase (11.31 ± 0.29), β -glucosidase (71.12 ± 1.32) and xylanase (307.12 ± 7.91) U/g. Further incubation resulted in a quick decline in the enzyme productions. This decline might be due to cessation of enzyme synthesis together with autolysis. Similar

findings were also reported by some workers [30], in which maximum enzyme production was observed at 96 hours of growth.

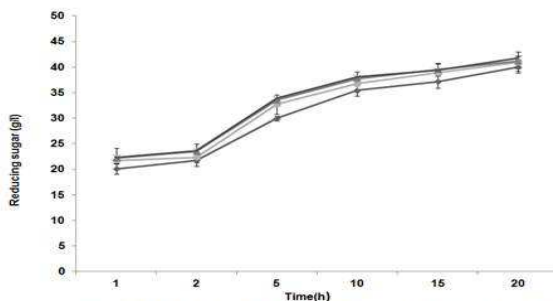


Fig.5. Reducing sugar yield (g/l) from the hydrolysate of alkali treated and fungal fermented rice straw after the treatment of various concentrations of fungal extracts. (•) 1%; (◐) 2%; (▲) 3%; (X) 4%.

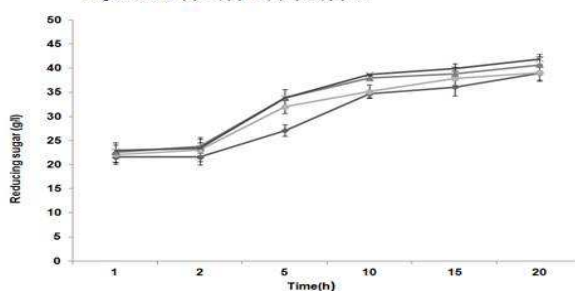


Fig.6. Reducing sugar yield (g/l) from the hydrolysate of alkali treated and autoclaved rice straw after the treatment of various concentrations of fungal extracts. (•) 1%; (◐) 2%; (▲) 3%; (X) 4%.

3.4. Impact of additional nutrients on enzyme production

For the improved synthesis of fungal enzymes, various nutrients were supplemented with rice straw. As shown in Fig-4, the addition of 0.5% nutrient supplementation induces the enzyme production. More promisingly, when a 0.5% yeast extract was added, the Carboxymethyl-cellulase (257.91 ± 13.54), Filter-paperase (12.73 ± 0.62), β -glucosidase (77.71 ± 3.54) and xylanase (339.76 ± 7.88) U/g activities were improved compare to other nutritional supplements. This is in agreement with various studies that the addition of nutrients could improve cellulase production [27, 31]. Supplementation of 0.5% yeast extract stimulates the enzyme production in solid state fermentation [32]. However, *A. niger* FGSCA733 cultured on a *Jatropha curcas*-based substrate found that the addition of nitrogen sources did not enhance cellulase production [33]. *Aspergillus sp* was found to grow on a medium containing yeast extract and minerals (0.5%, in a liquid culture), improved the cellulase activity [27].

3.5. Rice straw hydrolysis

Fungal culture extract with different concentrations (from the SSF using alkali treated rice straw) was tested in the hydrolysis experiments. Alkali pretreated and fermented, alkali pretreated and autoclaved rice straws were used as the substrates for the hydrolyzing experiments. The reducing sugars released from fungal extract hydrolyzed using alkali pretreated and fermented rice straw increased significantly (Fig.5-6) up to 10 hours (37.75 ± 3.67 and 37.93 ± 2.45 g/l). After that the reducing sugar generation was increased, at 20 hours of hydrolysis showed around 41.11 ± 3.07 and 40.67 ± 3.69 g/L. These results suggested that though the fungal extract had a lower detected cellulase activity the original activities must be high, and that it showed better performance when compared to commercial enzyme hydrolysis. This might be due to the fresh preparation of fungal extract from *A. tamaritii* grown on rice straw. The reducing sugar yield from alkali treated cum fermented rice straw was uniformly good in comparison with that obtained from alkali treated and autoclaved rice straw. Feedstock pretreatment is essential to maximize the enzymatic conversion of cellulosic material into fermentable sugars. The study on the saccharification of alkali pretreated and fermented rice straw with *A.tamaritii* cellulase showed high amounts of reducing sugar production. Alkali treatment has been reported to remove lignin and hemicellulose [34]. The high amount of reducing sugars produced from combined alkali-pretreated and fermented substrate could be due to the removal of lignin, and hemicelluloses from cellulose, which enhances the accessibility of the substrate to the enzymes. Removal

of lignin and hemicelluloses also increases the cellulose concentration per gram of treated rice straw. After the enzyme hydrolysis, a significant amount of solid residue remained, indicating that the substrate hydrolysis could be improved further.

CONCLUSION

A novel solid-state fungal fermentation-based biorefining strategy was developed to convert rice straw into a fermentable sugar. *Aspergillus tamarii* was firstly cultured on the rice straw for production of enzymes, followed by the hydrolysis of fermented rice straw using the fungal extract. Alkali modification of the rice straw showed an improved cellulase production which was further increased by adding glucose. Rice straw hydrolysis using the fungal extract resulted in the maximum release of reducing sugars (41.11 and 40.67g/L). This showed that the freshly-prepared fungal extract released higher reducing sugars.

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