A novel approach to produce glucose from the supernatant obtained upon the dilute acid pre-treatment of rice straw and synergistic action of hydrolytic enzymes producing microbes

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Abstract

The present work refers to a process involving the use of dilute nitric acid pretreatment and enzymatic hydrolysis for the transformation of rice straw into simple sugars. Acid pre-treated rice straw was separated into the pulp and supernatant through centrifugation and filtration. The two fractions are then converted into simple sugars by combined action of microbes producing cellulase and laccase enzymes. These microbes were isolated from soil samples which were collected from different locations with varying altitudes, expected to harbour microbes with high-hydrolysing activity. The nitric acid pretreatment was carried out at 30 °C, 200 rpm for 72 h. After 72 h, the culture supernatants were analysed for the presence of glucose with the help of HPLC. The supernatant fraction separated after the acid pre-treated rice straw produced highest amount of glucose (205 mg/g of rice straw) upon subsequent hydrolysis with synergistic action of cellulase and laccase-producing microbes.

Keywords Rice straw · Cellulose · Laccase · Glucose · Biomass transformation

Introduction

Rice is one of the three leading crops; wheat and maize being other two. About 42% of calories consumed by the human population are made up of these three crops. In fact, more than 3.5 billion people depend on rice for more than 20% of their daily calorie intake [1]. India is one of the largest exporters of Basmati rice in the world and in the years 2012–2013; the export of Basmati rice from India was estimated to be worth 1.9 billion approximately [2]. All the data point towards a large amount of rice straw production annually. The production of rice straw sums to approximately 700–800 million tons per year,

globally [3]. In India, the total production of rice straw is approximately 97.17 Mt [4]. It is estimated that 730 billion litres of bioethanol can be produced from rice straw alone [5]. One of the prominent uses of lignocellulosic biomass like rice straw, corn cob, maize straw, sugarcane bagasse etc. is converting them into biofuels. Biofuel conversion is usually done by first saccharification of the biomass into simple sugars and then utilising those simple sugars to produce bioethanol, biobutanol etc. by simple fermentation [6, 7]. But the lignocellulosic structure is quite tough due to the presence of lignin and hemicellulose structure which makes it difficult to transform the cellulose into sugars and eventually biofuels by simple chemical or enzymatic hydrolysis methods. This is why the biomass needs various kinds of pretreatments which range from physical pretreatments like cutting, grinding and milling to reduce the biomass particle size and the crystallinity of cellulose. The chemical pretreatments involve acids, alkali, organosolv agents and or ionic liquid treatments. The use of acids as a method of pretreatment has been increased in the past due to the high amount of sugars it produces without the requirement of any other hydrolysis processes [8, 9]. However, the use of enzymatic hydrolysis along with the acid pretreatment results in higher amount of sugars, which can be used for

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the production of other useful products like toluene, benzene, xylene, lignin monomer molecules, oligosaccharides, xylose and other varieties of sugars like xylose, tagatose and psicose etc. [10, 11]

The use of different enzymes in various combinations has been previously reported for the production of simple sugars and biofuels from lignocellulosic biomass. The use of cellulase and xylanase to produce glucose has been reported. The glucose was then fermented by *Saccharomyces cerevisiae* to produce 0.172 g ethanol/g of biomass [12]. In another study, the production of total reducing sugars of up to 93% from delignified rice straw by the application of commercial cellulase and pectinase was done. Then, *P. guilliermondii* was used to convert resultant sugar to 7.72 g L⁻¹ ethanol [13]. The synergistic action of cellulase with xylanase was used in a study to produce total reducing sugars from corn cob, corn stover and rice straw. The addition of xylanase along with the cellulase enzyme enhanced the conversion rate and resulted in the increase of sugar yield by 133%, 164% and 545% respectively from corn cob, corn stover and rice straw [14]. Another study reported the development of a cellulase and expansin co-expressing strain of *S. cerevisiae*. This strain was used to produce ethanol from phosphoric acid swollen cellulose. The final yield was 3.4 g L⁻¹ ethanol as compared to 2.5 g L⁻¹ produced by the strain only expressing cellulase [15].

In the present study, a process has been developed to achieve the maximum production of simple sugars through dilute acid pretreatment and the use of mixed microbial cultures as the source of enzymes to hydrolyse the rice straw. The supernatant separated after the dilute acid pretreatment of the rice straw was used for the enzymatic hydrolysis and glucose produced was analysed through high-performance liquid chromatography.

Materials and methods

Materials

Rice straw, a lignocellulosic biomass used in this study was purchased from local famers, Mauli Baidwan, Mohali, Punjab, India. The industrial grinder of Electrom instruments (CO, USA), the domestic mill cum pulveriser (Natraj Premium mill) of Yash Marketing, Tamilnadu, India, were used for physical treatment. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louise, MO, USA). The solvents and acids were obtained from TCI Chemicals (India) Pvt. Ltd.

Isolation of hydrolytic enzyme-producing microbes

To isolate the hydrolytic enzyme-producing microbes, sieved soil samples from different locations of Palampur district, Kangra, Himachal Pradesh, India, were collected. They were used to make dilutions up to 10⁻⁴ and were spread on 1%

carboxymethylcellulose nutrient agar and 2 mM guaiacol nutrient agar plates and incubated at 30 °C overnight [16, 17]. The resultant microbes were screened for their cellulase and laccase activity. The positive isolates were picked up and re-streaked on nutrient media until pure cultures were obtained. The isolates were again checked for their enzyme activity to confirm and saved at 4 °C for further studies. The cultures with maximum hydrolytic activities were identified by 16S rRNA-sequencing method and screened [18] and BLASTn [19] analyses. The sequences were submitted to NCBI [20] for the acquisition of the accession number.

Physical pretreatment of rice straw

Thoroughly washed and oven-dried rice straw was subjected to physical pretreatment by cutting the rice straw into smaller pieces by a simple paddy cutter which was then grounded into further smaller pieces by an industrial grinder. The final product was then grounded into fine powder by domestic mill cum pulveriser.

Chemical pretreatment of the rice straw

Physically pre-treated rice straw was subjected to chemical pretreatment. The pulverised rice straw was treated or incubated with dilute nitric acid for 2 h at 50 °C and atmospheric pressure. Different concentrations of acid, ranging from 0.5 to 5%, were used to check the effect of dilute acid on the production of sugars from rice straw. After that, the pulp and the supernatant were separated through filtration using muslin cloth and then separated the smaller particles by centrifugation at 12,000 rpm for 20 min. The pulp and supernatant part were then enriched with nutrient media to support the growth of microbial cultures. The separated fractions of pre-treated rice straw, still containing acid were subjected to autoclaving for 15 min. Final pH of the separated fractions were in the range of 4.5–5.5.

Enzymatic hydrolysis of pre-treated rice straw

The pre-treated rice straw was subjected to enzymatic hydrolysis using synergistic action of the microbial cultures producing hydrolytic proteins, cellulase and laccase. The enriched pre-treated rice straw pulp and supernatant were inoculated with 1% microbial cultures. The cultures were then grown for 72 h at 30 °C, 200 rpm. The culture supernatants were eventually analysed for the presence of simple sugars like glucose through HPLC.

Estimation of simple sugar in pulp of dilute acid pre-treated and enzyme-hydrolysed rice straw

For the initial study, the pulp of acid pre-treated and enzyme-hydrolysed rice straw was used to produce simple sugars like glucose. This was done by subjecting the rice straw with different concentrations of acid pretreatment (0.1%, 0.5% and 1%). The pulp collected after the pretreatment was then enriched with nutrient broth autoclaved and then hydrolysed by the combined action of microbial cultures producing hydrolytic proteins, cellulase and laccase.

Estimation of total reducing sugars after chemical and enzymatic hydrolysis of rice straw pulp

DNS method was used to determine the total reducing sugars (TRS) produced from the rice straw pulp and the supernatant. This was done as an initial step so as to determine how much total sugar is produced after the 0.5% acid pretreatment alone and synergistic action of microbial cultures producing cellulase and laccase. The supernatant of cultures grown for 72 h were used for sugar estimation.

Estimation of simple sugar (glucose) after acid pretreatment (0.5–5%) and enzymatic hydrolysis of rice straw

The acid-treated (0.5–5%) rice straw in pulp and the supernatant forms were hydrolysed by the microbial cultures producing cellulase and laccase. Acid-treated pulp and supernatant samples, without any enzyme hydrolysis, served as controls. After 72 h, the cultures were centrifuged at 12,000 rpm for 10 min at room temperature and filtered through 0.22 μm filter. The clear filtrates were used for the HPLC analysis (RID) (Model: 1260, Agilent technologies, CA, USA) for the quantification of glucose. The HiPlex-ca column was used for the analysis of sugars.

Fig. 1 Isolation of potential cellulase- and laccase-producing bacterial strains: **a** CYPE agar plate stained with Congo red showing cellulose producing isolate. **b** Nutrient agar guaiacol plates showing red coloured positive colonies for laccase-producing isolate. Final positive isolates are shown in inset

Analytical methods

Analysis of morphological changes of rice straw after pretreatment and enzymatic hydrolysis

The scanning electron microscope was used to analyse the surface of the rice straw before and after the pretreatments and enzymatic hydrolysis. The samples of rice straw pulp after the treatments were taken out and air dried for 24 h before processing. The microscopic analyses were done by benchtop scanning electron microscope (JEOL NEOSCOPE JCM-600, Tokyo, Japan). The air-dried samples were placed on carbon tape and coated with Au-Pt particles at 220 V for 1 min using JEOL Smart Coater, USA. The images were taken at a resolution of 200 μm .

Determination of reflectance spectra of treated and untreated rice straw

Fourier transform infrared (FTIR) spectrometer (Agilent technologies, Cary 600 series, CA, US) was used at a wave number range of 400–4000 cm^{-1} with 4 cm^{-1} resolution to determine the changes in the rice straw chemical structure. The spectrometer was equipped with universal ATR (attenuated total reflection) accessory (GladiATR, PIKE technologies, WI, US). Air-dried samples were used for the analysis, and data was analysed according to the literature present on pretreated rice straw FTIR studies.

Results

Isolation and identification of hydrolytic enzyme-producing microbes

The isolated microbes producing hydrolytic enzymes cellulase and laccase were identified as *Bacillus* sp. (accession no. KY910423) and *Microbacterium* sp. (KY953018) (Fig. 1).

Physical, chemical and enzymatic pretreatment of the rice straw

Physical or mechanical pretreatment was done to reduce particle size. The size of the particles depends upon the process undertaken for the transformation of lignocellulosic biomass [21]. The final size of the biomass particles was kept 2 mm, which showed a reduction in the recalcitrance of the biomass. Chemical pretreatment with different concentrations of nitric acid was found to be most suitable to produce maximum amount of simple sugars. Out of all the microbial cultures showing cellulase and laccase activity, the combination of two cultures *Bacillus* sp. and *Microbacterium* sp. were found to be the best for optimal production of simple sugars from biomass. The pH neutralisation with a base was avoided as it resulted in salt formation which could later interfere with microbial growth and hence the sugar production.

Simple sugar in pulp of dilute acid pre-treated and enzyme-hydrolysed rice straw

The synergistic action of cellulase- and laccase-producing microbes was found to be an effective method to achieve a high degree of saccharification of lignocellulosic biomass. The HPLC results after 72 h of enzymatic hydrolysis of acid-treated rice straw pulp are presented in Table 1. As seen from the table, the pulp did not produce a significant amount of glucose. Therefore, both the pulp and supernatant fractions were used separately for the enzymatic hydrolysis.

Total reducing sugars in rice straw pulp after chemical and enzymatic hydrolysis

As seen from Fig. 2, cellulase enzyme treatment was found to produce 8.19 mg/ml TRS compared to 1.29 mg/ml in acid untreated rice straw. Similarly, cellulase treatment was also observed to produce higher TRS in pulp obtained after 0.5% acid treatment of rice straw. Whereas, in supernatant fraction obtained after 0.5% acid treatment of rice straw, the TRS

Fig. 2 Total reducing sugars estimated by DNS assay method in: untreated rice straw (Controlu), untreated rice straw hydrolysed with cellulase (Cu), laccase (Lu) and cocktail of cellulase- and laccase-producing microbial cultures (CLu); acid-treated rice straw pulp (Controlp), acid- and cellulase-producing microbial culture-treated pulp fraction (Cp), acid- and laccase-producing microbial culture-treated pulp fraction (Lp), acid and cocktail of cellulase- and laccase-producing microbial cultures-treated pulp fraction (CLp); acid-treated rice straw supernatant (Controls), acid- and cellulase-producing microbial culture-treated supernatant fraction (Cs), acid- and laccase-producing microbial culture-treated supernatant fraction (Ls), acid and cocktail of cellulase- and laccase-producing microbial cultures-treated supernatant (CLs). The acid treatment was 0.5% nitric acid only

produced was highest upon treatment of mixed cultures producing cellulase and laccase. Hence, mixed microbial cultures producing cellulase and laccase were found to be efficiently utilising crystalline cellulose present in the supernatant fraction as compared to the pulp fraction.

Simple sugar glucose in rice straw after acid pretreatment (0.5–5%) and enzymatic hydrolysis

As per Table 2, the amount of glucose produced was found higher in the acid-treated supernatant fraction hydrolysed with mixed cultures producing cellulase and laccase. The amount of glucose produced in the supernatant of the 0.5%, 1%, 2% and 5% nitric acid-treated rice straw was 39, 90, 124 and 174 mg/g respectively. The acid-treated supernatant fractions which were hydrolysed with cellulase- and laccase-producing

Table 1 Estimation of glucose in culture supernatant

Pulp (g)	Microbial culture producing hydrolytic enzyme	Nitric acid pretreatment (%)	Glucose (mg/ml)
1	Cellulase	0.1	0.07
1	Laccase	0.1	0.02
1	Cellulase + laccase	0.1	0.07
1	Cellulase	0.5	0.02
1	Laccase	0.5	Undetectable
1	Cellulase + laccase	0.5	Undetectable
1	Cellulase	1	0.01
1	Laccase	1	Undetectable
1	Cellulase + laccase	1	Undetectable

Table 2 Estimated glucose concentration (mg/g) in pulp and supernatant of 0.5–5% of nitric acid pre-treated and enzymatically hydrolysed rice straw

Nitric acid treatment (%)	Culture	Concentration (mg/g of rice straw)			
		Pulp		Supernatant	
		Sample	Control	Sample	Control
0.5%	Cellulase	25	NIL	32	39
0.5%	Laccase	15		30	
0.5%	Cellulase + laccase	—		50	
1%	Cellulase	—	NIL	88.5	90
1%	Laccase	—		80	
1%	Cellulase + laccase	—		127	
2%	Cellulase	—		135	124
2%	Laccase	—	NIL	137	
2%	Cellulase + laccase	—		140	
5%	Cellulase	—	NIL	200	174
5%	Laccase	—		182	
5%	Cellulase + laccase	—		205	

microbial cultures produced 30–205 mg glucose/g of rice straw. Also, the increase in glucose amount in the supernatant fraction was approximately 22%, 29.13% 18.5% and 15.12% as compared to the control samples without the enzyme hydrolysis, with 0.5%, 1%, 2% and 5% acid treatment. The higher amount of glucose was obtained from rice straw on 5% nitric acid pretreatment and mixed microbial cultures producing cellulase and laccase. The conversion rate of rice straw cellulose to glucose was approximately 50% with this combination. Various steps of the process has been illustrated in Fig. 3.

Fig. 3 Schematic diagram showing steps followed for the production of simple sugars from rice straw

Morphological changes in rice straw pulp on pretreatment and enzymatic hydrolysis

Figure 4 shows the effect of chemical pretreatment and enzymatic hydrolysis on the morphology of rice straw pulp. After the acid pretreatment, the rice straw was observed to be discoloured which increased further after the enzymatic hydrolysis (Fig. 4a–c). SEM was used to analyse the morphological changes happened after various treatments. Lignocellulosic biomass of untreated rice straw show chapped and irregular structure (Fig. 4d). On acid treatment, the rice straw particles become more smooth, regular and uniform in structure (Fig. 4e). Lignocellulosic biomass became more and more smooth and more refined fibre structure on subsequent autoclaving (Fig. 4f), cellulose hydrolysis (Fig. 4g), laccase hydrolysis (Fig. 4h) and combined hydrolytic action of cellulose and laccase (Fig. 4i).

Changes in the chemical structure of rice straw before and after pretreatment

The FTIR spectra of untreated, acid pre-treated and enzymatically hydrolysed rice straw pulp fraction is shown in Fig. 5. Significant changes in the bands present at 1422 cm^{-1} , 3333 cm^{-1} , 1034 cm^{-1} and 2925 cm^{-1} were observed. The transmittance band at 1422 cm^{-1} present in untreated rice straw powder has disappeared from the acid pre-treated and enzymatically hydrolysed rice straw spectra. From Fig. 5, it is clear that the band at 898 cm^{-1} has disappeared. A substantial decrease in the band peaks at 3333 cm^{-1} (hydroxyl group O-H stretching), 2925 cm^{-1} (methyl and methylene group C-H stretching), 1633 cm^{-1} (conjugated aryl ketone C=O

Fig. 4 Structural changes in rice straw after pretreatment and enzymatic hydrolysis: **(a)** untreated rice straw, **(b)** rice straw after acid treatment and **(c)** rice straw after enzymatic hydrolysis. SEM images of **(d)** untreated rice straw, **(e)** acid pre-treated rice straw, **(f)** rice straw after autoclaving, **(g)** rice straw after hydrolysis by cellulase-producing microbial culture, **(h)** rice straw after hydrolysis by laccase-producing microbial culture and **(i)** rice straw after hydrolysis by cellulase- and laccase-producing microbial cultures. The inset in every figure shows the zoomed-in image of the rice straw structure

stretching) and 1034 cm^{-1} (plane deformation of aromatic C-H) was also observed.

Discussion

Lignocellulosic biomass has proved to be one of the most valuable and renewable resources present nowadays.

Biomass like rice straw, corn cob, wheat straw, maize straw and sugarcane bagasse have been used frequently to produce a variety of useful products like simple sugars, oligosaccharides, organic acids and alcohols including biofuels like bioethanol, biobutanol etc. Since its utilisation started in 1980s to transform it produces biofuel, lignocellulosic biomass has been used and has been increased worldwide [7]. As rice is the staple diet of half

Fig. 5 FTIR spectra of untreated rice straw, acid-treated rice straw and enzymatically hydrolysed (hydrolysis by cellulase-producing microbe, laccase-producing microbe and combined hydrolysis by cellulase- and laccase-producing microbes)

of the human population, rice straw is understandably one of the most prominently produced agricultural wastes there is [8]. It is high in cellulose and hemicellulose. It contains cellulose 32–47%, hemicelluloses 19–27%, lignin 5–24% and ashes 18.8%. The carbohydrate content of rice straw mainly involves glucose 41–43%, xylose 14–20%, arabinose 2.7–4.5%, mannose 1.8% and galactose 0.4%. But this valuable resource has been wasted by using it as a feedstock or burning in the fields due to lack of knowledge regarding its usefulness. In addition, the practice of burning is hazardous to the environment [22]. Approximately, 730 billion litres of bioethanol can be produced from rice straw alone [5]. Therefore, looking for the prospects of utilising the rice straw, various methods have been developed to saccharify the biomass to produce biofuels. However, the conversion of rice straw cellulose to sugars is difficult due to the presence of a tough lignin wall protecting the cellulose from any kind of enzymatic or chemical treatment. This problem is avoided by various kinds of chemical, heat or pressure pretreatments depending upon the desired product. Chemical pretreatments

utilising acids, alkali, and organic solvents enable enzymes to reach hydrolytic enzymes to the amorphous cellulose. But all these pre-treatments are usually very expensive and the by-products formed afterwards are difficult to process and pose serious threat to the environment. Use of high concentrations of acids or alkali also slows down the conversion process as the by-products like HMF and furfural inhibit the cellulase enzymes and destroy the equipment carrying out the reaction process [8]. Therefore, alternative methods of pretreatments are being searched for the transformation of biomass. Dilute acid pretreatment is one such technique which utilises minimum amount of acid and produced optimum amount of simple sugars. In addition, the uses of hydrolytic enzymes bring the maximum amount to conversion. The present work focuses on the use of dilute acid as the pretreatment method and enzymatic hydrolysis of the rice straw by the combined action of microbial cultures producing two different hydrolytic enzymes. Also, the final amount of glucose was stable and no by-products such as HMF or furfural peaks were detected suggesting the commercial viability of the

process for industrial application (Supplementary Fig. 1). The approach of utilising synergistic action of two or more enzymes to hydrolyse the biomass for the efficient production of sugars from rice straw has been used in the past [14].

The hydrolytic enzymes used in the present report are cellulase and laccase. The pretreatment steps involved physical and chemical processes. Pretreatments are one of the essential and most expensive steps in the transformation of lignocellulosic biomass. Previous reports suggest that the average size of biomass particles affect the total sugar yield and the smaller particle size (5 mm or less) helps to produce more sugar than bigger-sized particles [21]. The physical pretreatments also reduce the crystallinity of the cellulose present in the biomass which is usually not accessible to the enzymes in the hydrolysis process. Also, the physical treatment increases the surface area of the biomass, ensuring enzymatic hydrolysis. The particle size was kept around 2 mm which is the standard biomass size used to carry out enzymatic hydrolysis [23].

In the present work, dilute nitric acid has been used as the method of a two-step chemical pretreatment process. Chemical pretreatment includes the use of any acid, alkali, organic solvents and ionic liquids etc. at different temperature and pressure to break the hemicellulose and lignin wall to reduce the recalcitrance of the biomass [24]. The use of concentrated acids as a method of pretreatment has been used widely in the past. However, the concentrated acid results in the formation of many inhibitors and is corrosive. A study used the dilute sulphuric acid pre-treated rice straw and found the optimum conditions for the enzymatic hydrolysis to produce maximum amount of fermentable sugars. The authors used 0.5% sulphuric acid at 121 °C for 60 min on rice straw and found through HPTLC that maximum amount of sugars that were obtained was glucose and xylose [25]. A different approach to produce sugars was done in a study where authors carried out dilute acid pretreatment of corn stover at the concentrations of 2%, 4% and 6% at a temperature of 80, 100 and 120 °C. They found that pretreatment of 2% sulphuric acid at 120 °C for 43 min is optimum for the maximum production of xylose [26]. Therefore, keeping in mind the necessity to reduce the concentration of acids being used in the process as well as the time, this process was developed utilising a minimum amount of acid for a lesser amount of time to avoid the formation of by-products.

The two-step process allows us to separate the supernatant and the pulp which are hydrolysed separately by the microbial cultures producing hydrolysing enzymes. Usually, the supernatant is either again added to the biomass or is fermented separately for the production of biofuels [27]. The present method allows us to utilise the crystalline cellulose present in the supernatant to produce simple sugars like glucose.

In the present report, it was found that a combination of cellulase- and laccase-producing microbes with acid pre-

treated rice straw supernatant produced highest amount of glucose. The cellulase is used to hydrolyse amorphous cellulose present in the biomass to simple sugars whereas, the laccase is used to breakdown lignin walls which are tough to remove and interfere with the hydrolysis of cellulose. Recent studies have shown that laccase enzymes are essential for the biodegradation of lignocellulosic biomass [28]. Also, the cellulose hydrolysis has been shown to improve along with delignification of the biomass [29]. Therefore, it was hypothesised that synergistic action of cellulase and laccase can improve the sugar production after dilute acid pretreatment. There have been studies reporting the use of acid pre-treated supernatant for the production of biofuel as it contained a high amount of fermentable sugars in it. An invention described the use of 25–90% of sulphuric acid in a two-step hydrolysis process to produce sugars from lignocellulosic biomass and the hydrolysate from both the steps were mixed and used to separate sugars and acid [30]. In another invention, the lignocellulosic biomass (rice straw, wheat straw, maize straw etc.) was treated with 0.1–10% of sulphuric acid at 100–150 °C for 20–120 min, and resultant hydrolysate or supernatant was neutralised to obtain pentose which was fermented to produce ethanol. In yet another report, the corn stover was pre-treated with ionic liquid and the supernatant was removed after washing. The residual biomass was treated with commercial cellulase and the supernatant of that hydrolysis was fermented with yeast to produce ethanol [31]. In above all the reports, the supernatant obtained after the pretreatment is usually either discarded or used to isolate simple sugars, or is directly used to ferment simple sugars present in them to produce ethanol. The two microbial cultures used in the process were able to grow and produce enzymes in the pH range of 4–10. Acid hydrolysed biomass have been suggested to be hydrolysed by enzymes active at lower pH or microbes producing hydrolytic enzymes efficiently viable at low pH [32, 33]. The enzymatic hydrolysis was carried out for 72 h at 30 °C, 200 rpm. To our knowledge, this is the first study where the flow through or the supernatant of the acid-treated biomass is being hydrolysed by enzymes to produce simple sugars. It is hypothesised that the cellulase is utilising the amorphous cellulose present in the supernatant fraction whereas, the laccase is aiding in the biodegradation of any lignin present in the supernatant fraction serving the cellulase enzyme to utilise cellulose more efficiently [27, 28]. The highest amount of sugar (glucose) obtained after the cellulase-laccase hydrolysis process of the acid pre-treated rice straw supernatant was 205 mg/g of rice straw. Therefore, it was hypothesised that synergistic action of cellulase and laccase can improve the sugar production after dilute acid pretreatment.

SEM analysis has suggested no difference in the structure of pulp remnants of rice straw upon various treatments, which could be due to the inability of enzymes to hydrolyse the

remaining cellulose in the rice straw pulp. Enzyme hydrolytic action on the lignocellulosic structure seen through SEM analysis has supported their action towards simple sugar production. While no change in the structure of final pulp fraction seen through SEM analysis documented the resistance in hydrolysis of rice straw beyond certain limit.

Changes in the chemical structure of the rice straw before and after the pretreatment was analysed by FTIR spectrometer. Microstructural changes in the rice straw occurred after the pretreatment has been believed to influence the enzymatic hydrolysis to a significant extent [34]. One of the major effects of the acid pretreatment on the rice straw is the removal of crystalline cellulose fraction which was shown by the transmittance band at 1422 cm^{-1} . And the band at 898 cm^{-1} [1] is allotted to amorphous cellulose [35]. Therefore, the fact that rice straw pulp fraction did not produce significant amount of glucose can be explained by this observation that the amorphous cellulose which was converted from crystalline form by acid pretreatment of the rice straw was present in the supernatant fraction. Since amorphous cellulose is more receptive to enzymatic hydrolysis, the supernatant fraction was found to produce higher amount of glucose.

Also, the considerable decrease in the band peaks at 3333 cm^{-1} (hydroxyl group O-H stretching), 2925 cm^{-1} (methyl and methylene group C-H stretching), 1633 cm^{-1} (conjugated aryl ketone C=O stretching) and 1034 cm^{-1} (plane deformation of aromatic C-H) suggested the breakdown of lignin and hemicellulose upon acid pretreatment and enzymatic hydrolysis [34]. The purpose of using dilute acid is to avoid the environmental hazard caused by the end products and acid left after the process. Therefore, the process is environment friendly and also is economical as the end products formed are much less and the sugar produced is high. The present work can be used at an industrial scale for the production of high amount of simple sugars from lignocellulosic biomass.

Conclusion

A simple novel and energy-efficient process involving the use of dilute nitric acid pretreatment and enzymatic hydrolysis for the transformation of rice straw into simple sugars has been developed. For the process, two microbial strains producing cellulase and laccase enzymes were isolated from novel niches. The supernatant fraction obtained upon acid pretreatment of rice straw has produced 205 mg glucose/g of rice straw upon hydrolysis with combined action of cellulase and laccase-producing microbes. Further, the possible mechanism of higher glucose production in supernatant fraction compared to pulp fraction has been elucidated through SEM and FTIR analysis.

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