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SACCHARIFICATION AND ALCOHOL FERMENTATION OF STEAM-EXPLODED RICE STRAW

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Abstract

Pretreatment of rice straw by steam explosion prior to enzymatic saccharification has been investigated. The study sought the optimum conditions of steam pressure and steaming time. Rice straw was exploded at various steam pressures (2·55, 3·04, 3·53 and 4·02 MPa) for steaming times ranging from 0·5 to 10 min. The susceptibility of the pretreated substrate to cellulase enzymes was greatly influenced by the steam pressure and steaming time of pretreatment. It was found that high steam pressure (3·53 MPa) for a short steaming time (2 min) effectively enhanced enzymatic saccharification and alcohol fermentation of rice straw. The results demonstrated that no additional treatment, such as extraction with water or chemicals, is required to hydrolyze the steam-exploded sample to a greater extent if it is pretreated under an optimized set of conditions.

Key words: Saccharification, alcohol fermentation, steam explosion, rice straw.

NOMENCLATURE

C_C	concentration of cellobiose [g/l]
C_E	concentration of ethanol [g/l]
C_G	concentration of glucose [g/l]
C_R	concentration of reducing sugar [g/l]
C_X	concentration of xylose [g/l]
O.D.	optical density of cells
P	steam pressure [MPa]
t	steaming time [min]
t_f	fermentation time [h]
t_s	saccharification time [h]

INTRODUCTION

Hydrolysis in the native state of lignocellulosics is slow, mainly because the interassociation of lignin, hemicellulose and cellulose forms a barrier against enzymatic attack. Hence, a pretreatment stage to break the tight structure of lignocellulosics and to make cellulose amenable to enzymatic degradation is necessary. A number of different pretreatment methods, namely physical, chemical and biological types, have been developed in an effort to enhance the rate and extent of hydrolytic degradation. Very few of these can be regarded as technologies, most are at the stage of laboratory practice. Also, many physical and chemical pretreatment methods are, unfortunately, economically unfeasible. A pretreatment method of current interest, autohydrolysis-explosion, based on a combination of physical and chemical effects under high pressure and temperature has attracted attention (Jurasek, 1979; Moniruzzaman *et al.*, 1992).

Puri and Mamers (1983) used steam explosion to pretreat wheat straw, bagasse and eucalyptus wood chips. The temperature for pretreatment was 200°C, with cooking times ranging from 0 to 60 min. Additional pressure was added to the reaction chamber by injecting pressurized carbon dioxide gas. Maximum glucose conversions of 81, 78 and 75%, respectively, were obtained for the three substrates studied. Treatment times to gain these conversions were 5 min for wheat straw and bagasse and 15 min for eucalyptus chips.

Mes-Hartree *et al.* (1983) pretreated five different agricultural residues by steam explosion: barley straw, wheat straw, corn stover, corn stalks and alfalfa stalks. The pretreatment condition for all these substrates was kept constant at 560 psi for 60 s exposure time. Reducing sugars produced by subsequent enzymatic hydrolysis from the pretreated samples varied depending on the nature of the substrate.

It is thus obvious from the above studies that the optimum pretreatment conditions need to be determined for each of the lignocellulosic residues before enzymatic hydrolysis, in order to achieve maximum sugar production.

This paper concerns the application of steam explosion as a pretreatment method for rice straw. The study attempted to find the optimum conditions for obtaining fermentable sugars at maximum yield. The effects of steam pressure and steaming time on

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the enzymatic saccharification and subsequent fermentation of pretreated samples were studied.

METHODS

Substrate

Air-dried rice straw (obtained locally) was reduced to about 10 cm length by chopping and used as raw material.

Steam-explosion apparatus

The steam-explosion apparatus (Fig. 1) consisted of a steam generator, a pressurized reactor, a receiver and a condenser with a silencer. The reactor was insulated to maintain constant temperature. The capacity of the reactor was 1·2 l, the maximum working pressure and temperature were 6 MPa and 275°C, respectively. Approximately 100 g of straw was introduced into the reactor, which was then steam heated (the reactor heat-up time varied from 10 to 20 s, depending on the pretreatment temperature used). After the desired steaming time (the heat-up time not included), a ball valve at the bottom of the reactor was suddenly opened to bring the reactor rapidly to atmospheric pressure. The solid and liquid products were explosively released into a collection device. The gaseous products were passed from the top of the receiver into the condenser.

The operating variables examined in this study were the steaming time (0·5, 1, 2, 3, 5 and 10 min) and the steam pressure (2·55, 3·04, 3·53 and 4·02 MPa). The reaction temperatures for these pressures were 225, 235, 243 and 251°C, respectively.

Enzyme

Meicelase, a commercial preparation of *Trichoderma resei* cellulase (Meiji Seika Co. Ltd, Tokyo, Japan) was used. This cellulase contains the following enzyme activities (in units/mg) (Dekker & Wallis, 1983): FPU, 0·53; CM-cellulase, 7·90; β-glucosidase, 3·28; cellobiase, 2·27; xylanase, 0·31; and β-xylosidase, 0·02.

Enzymatic saccharification

The exploded samples of rice straw were freeze-dried and then used in the saccharification experiment (unless otherwise stated). The standard saccharification was carried out in a 300 ml Erlenmeyer flask in a thermostated incubator at 50°C for 120 h agitated at 150 rpm. The pH of the suspension was adjusted to 5·0 with 0·5 M phosphate buffer. The substrate and enzyme concentrations were 2% (w/v) and 0·2% (w/v), respectively, in a total volume of 100 ml. Samples (2 ml) were taken periodically, centrifuged and the supernatants were analyzed for reducing sugars, glucose, cellobiose and xylose concentrations. The percent saccharification and glucose yield were calculated using the following equations:

$$\text{Saccharification}(\%) = \frac{\text{reducing sugars formed (g/l)} \times 0{\cdot}9}{\text{carbohydrates} \times 100 \text{ straw (g/l)}}$$

$$\text{Percent glucose yield} = \frac{\text{glucose formed (g/l)} \times 0{\cdot}9}{\text{cellulose in straw (g/l)}} \times 100$$

Water extraction and alkali treatment

Some of the exploded samples were further extracted with water at room temperature for 2 h and/or treated with sodium hydroxide (0·1 g/g substrate). For the sodium hydroxide treatment, 25 g of the exploded sample was taken into a 1 l flask containing 2·5 g sodium hydroxide in 500 ml distilled water. The flask was then heated in a boiling water bath for 1 h. The alkali-treated sample was filtered through a nylon cloth (double layer) and washed in running tap water until free of alkali.

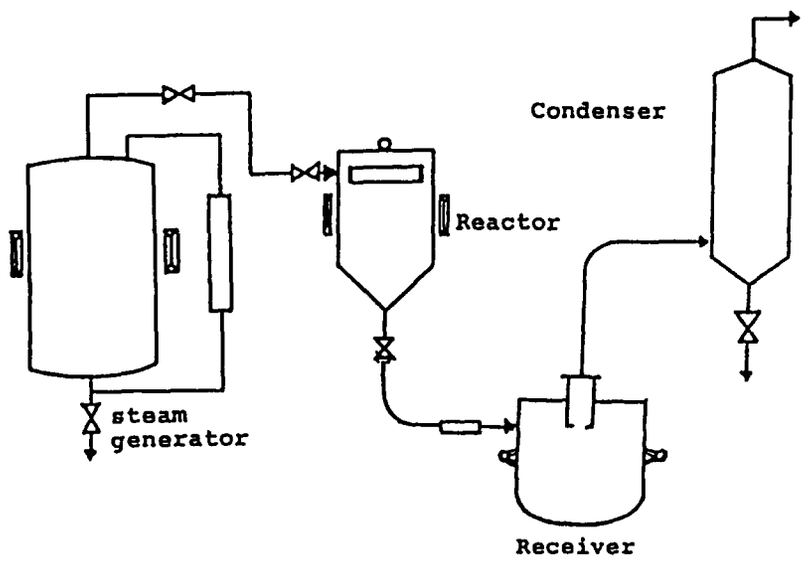

Fig. 1. Steam-explosion apparatus.

Microorganisms

Saccharomyces cerevisiae ATCC 26603 was grown at 30°C and maintained at 5°C in a medium which contained, per litre: glucose 20 g, peptone 20 g, yeast extract 10 g and agar 18 g.

Growth medium

The media for growth contained, per litre: 13 g KH_2PO_4; 0·7 g K_2HPO_4; 2·0 g NH_4Cl; 0·1 g $MgSO_4 \cdot 7H_2O$; 1·0 g yeast extract; and 10 g glucose. The sugar solutions were autoclaved separately in order to prevent reactions with other ingredients. The pH was adjusted to 5·0 and temperature was kept at 30°C.

Preparation of inoculum

Inoculum was prepared by transferring organisms by loop from a 48 h old culture on agar slants to a 300 ml Erlenmeyer flask containing 100 ml of growth medium. Cultures were incubated for 18–24 h. Cells were harvested by centrifugation (6000 *g*, 5 min) and resuspended in fermentation broth to give a final $O.D._{660}$ of approximately 0·5. The inoculum used in the fermentation experiment was 10% (v/v).

Fermentation of enzymatic hydrolyzate

The hydrolyzate liquid after sterile centrifugation was subjected to fermentation in a 300 ml flask with the addition of nutritional salts, traces of antibiotic and yeast inoculum in a total volume of 100 ml. The flask was then incubated in a rotary shaker at 150 rpm for 40 h. Samples (2·5 ml) were taken periodically and assayed for cell concentration, glucose and ethanol concentrations.

Analytical methods

Reducing sugars were measured by the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1945). Glucose was measured specifically by the glucose-oxidase method (Lloyd & Whelan, 1969). Xylose and cellobiose concentrations were determined by high-performance liquid chromatography (Shimadzu LC-9A) equipped with an Asahipak NH2P-50 column. Chemical composition of the pretreated sample was analyzed by the method of Chua and Wayman (1979). The concentration of cells was assayed by measuring the absorbance of a sample at 660 nm. Ethanol concentration was assayed by a gas chromatograph equipped with a Porapak Q column.

RESULTS AND DISCUSSION

In Fig. 2(a) and (b) the hydrolysis curves at the steam pressure of 2·55 MPa are shown. It was difficult to hydrolyze the pretreated material at this steam pressure. However, it was quite clear that the longer the pretreatment steaming time, the higher the sugar concentration. A concentration of 11 g/l reducing sugar [Fig. 2(a)] containing 6·8 g/l glucose [Fig. 2(b)] was thus obtained after steaming the straw for 10 min. The reducing sugar and glucose formation rates were dramatically increased when a pretreatment steam pressure of 3·53 MPa was used. Even for short times, the yield was higher than that previously achieved at 2·55 MPa. The hydrolysis curves for this steam pressure are shown in Fig. 3(a) and (b). The concentration reached about 15 g/l reducing sugar [Fig. 3(a)] and 8 g/l glucose [Fig. 3(b)] for the sample which was steamed for 2 min. More than 2 min steaming time at this steam pressure decreased the saccharification efficiency.

These observations clearly indicate that the susceptibility of the pretreated substrate to cellulase enzyme was greatly influenced by the steam pressure and steaming time of pretreatment.

Figure 4(a) and (b) summarizes the effects of steam pressure and steaming time on reducing sugar and glucose production by enzymatic saccharification. As can be seen [Fig. 4(a)], at 3·53 MPa the concentration of reducing sugar reached a maximum

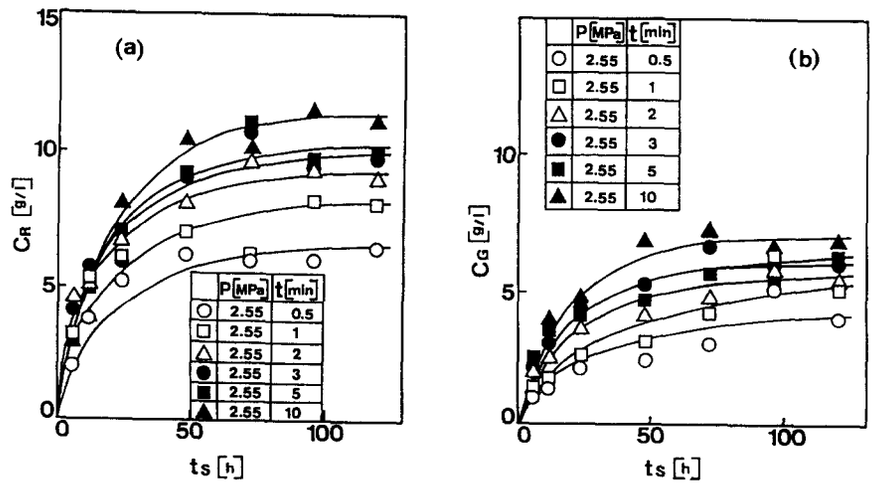

Fig. 2. Enzymatic saccharification of exploded rice straw at 2·55 MPa: (a) reducing sugar concentration; (b) glucose concentration.

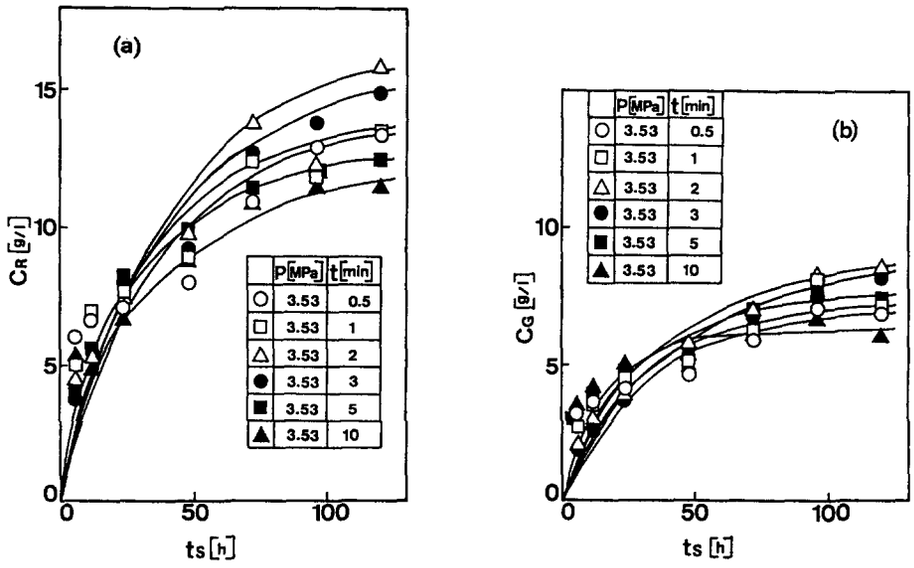

Fig. 3. Enzymatic saccharification of exploded rice straw at 3·53 MPa: (a) reducing sugar concentration; (b) glucose concentration.

at 2 min then decreased considerably with increasing duration of steaming. When the sample was treated at 4·02 MPa the yield of reducing sugar was drastically reduced, even for a shorter steaming time of 0·5 min. This indicates that the substrate was to some extent decomposed and destroyed as the severity of the pretreatment increased. In contrast, at 3·04 or 2·55 MPa, a comparatively small amount of reducing sugars (maximum 12 and 11 g/l, respectively) was obtained although the samples were pretreated for 5 and 10 min, respectively. This implies that the rice straw at comparatively low steam pressure of explosion was not disrupted enough (as found at 3·53 MPa for 2 min steaming time) for enzymatic attack, even using longer steaming times.

The results in Fig. 4(b) also exhibit the same effects of explosion conditions on glucose production by enzymatic saccharification. The samples exploded at 2·55 MPa for 10 min, at 3·04 MPa for 5 min, at 3·53 MPa for 2 min and at 4·02 MPa for 0·5 min produced 6·8 g/l, 7·0 g/l, 8·0 g/l and 4·0 g/l glucose, respectively. However, it is evident from Fig. 4(a) and (b) that the amount of reducing sugar and glucose liberated during enzymatic hydrolysis of various exploded samples attained their maximum values at 3·53 MPa for 2 min steaming time. Thus, it appears that this explosion condition is adequate for producing a material that is highly susceptible to attack by cellulolytic enzymes.

Figure 5(a)–(d) shows glucose, cellobiose and xylose concentration profiles for selected pretreated

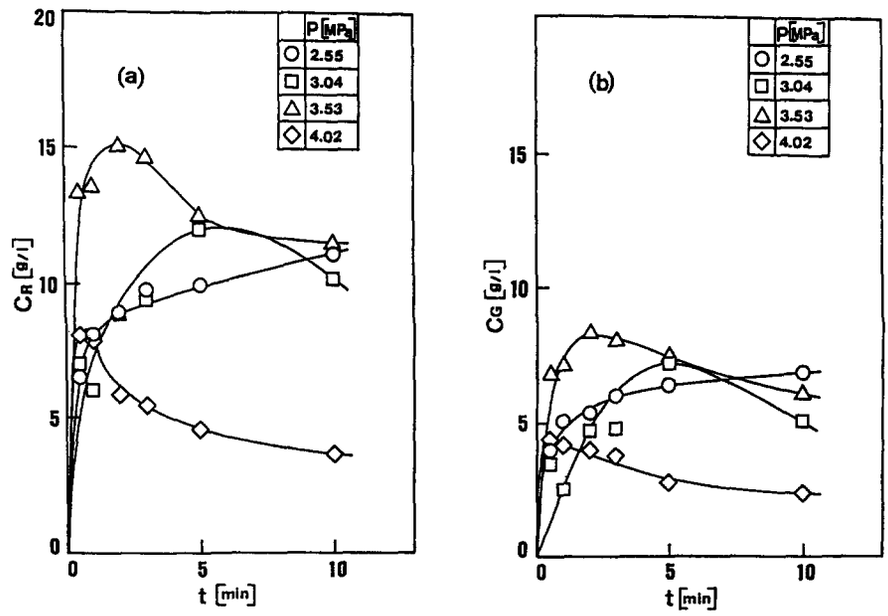

Fig. 4. Effects of steam pressure and steaming time of explosion on enzymatic saccharification of rice straw: (a) reducing sugar concentration; (b) glucose concentration.

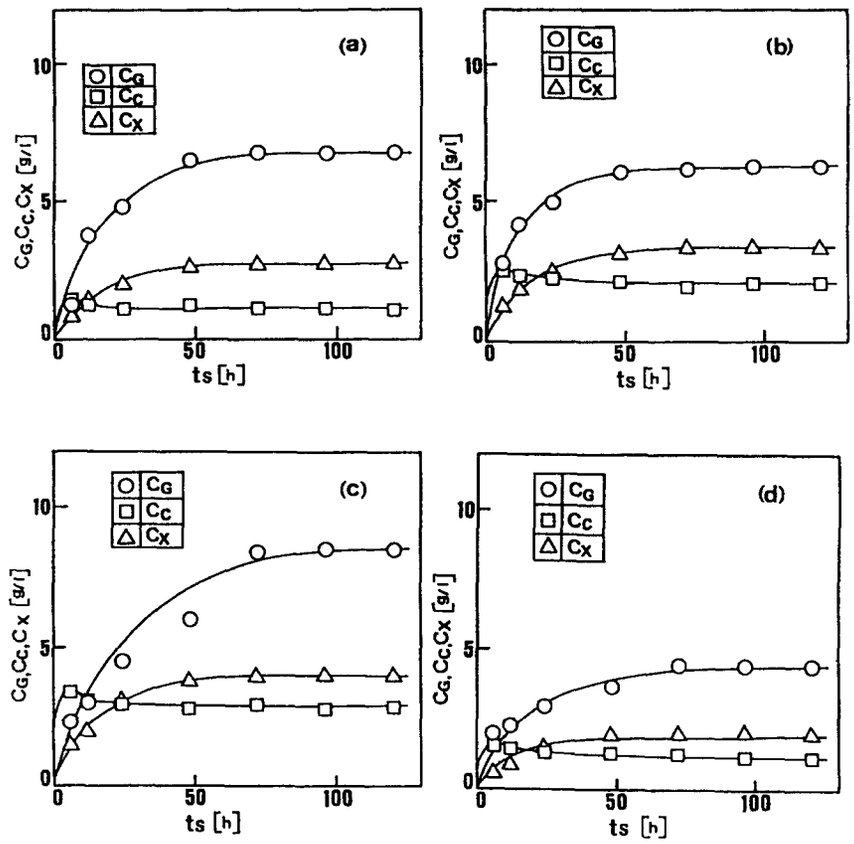

Fig. 5. Glucose, cellobiose and xylose concentrations in enzymatic hydrolyzate: (a) 2·55 MPa for 10 min; (b) 3·04 MPa for 5 min; (c) 3·53 MPa for 2 min; (d) 4·02 MPa for 0·5 min.

samples. Accumulation of cellobiose in the enzymatic hydrolyzate was evident for all the samples tested. This is thought to be due to the end-product inhibition of cellulase enzyme during saccharification (Howell & Mangat, 1978; Wald *et al.*, 1984). However, it is obvious that the pretreatment at 3·53 MPa for the 2 min sample gave the highest yields of sugars: about 8 g/l glucose, 2·5 g/l cellobiose and 4·0 g/l xylose [Fig. 5(c)]. The steam-exploded product can be separated into two fractions if desired: the liquid fraction (rich in xylose monomers and oligomers) and the pulp fraction (consisting of cellulose and lignin). In this study, the two fractions have not been separated. The entire set of exploded products was collected, freeze-dried to form a composite sample and then used in the saccharification experiment. The significant amount of xylose (4 g/l), in addition to glucose and cellobiose, found in the enzymatic hydrolyzate is not surprising.

To see if removal of lignin by a chemical treatment would aid enzymatic hydrolysis, samples which had been exploded at 2·55, 3·04, 3·53 and 4·02 MPa for 2 min were delignified by treatment with sodium hydroxide, as described previously. The effect of this chemical treatment on enzymatic saccharification is shown in Fig. 6(a) and (b). A very high concentration, approximately 17·5 g/l of reducing sugars [Fig. 6(a)] containing 15 g/l glucose [Fig. 6(b)], was obtained after delignification of the sample which was steam-exploded at 3·53 MPa for 2 min. However, these high concentrations of sugars could be attributed to the higher cellulose content (92%, Table 1) in this sample. On this basis (after alkali treatment) saccharification and glucose yields were 86 and 73%, respectively.

Table 1 shows the percentage saccharification and glucose yield, based on the carbohydrate content in the sample after explosion at 3·53 MPa for 2 min and subsequent treatment with water and alkali. It is evident that extraction of the exploded sample with water or alkali resulted in somewhat lower saccharification and glucose yields than the unextracted sample (enzymically hydrolyzed under similar conditions). It is thought that treatment with water or alkali following steam explosion removed amorphous and smaller cellulose particles (due to extensive washing) which were more susceptible to hydrolysis, while leaving the larger particles of highly ordered, crystalline cellulose which were less degradable.

It has been reported previously by other workers that there was a significant reduction in cellulase activity when culture filtrates were added to lignin-containing substrates, such as steam-treated birch (Puls *et al.*, 1985) and mixed hardwood (Sinitsyn *et al.*, 1983). Mes-Hartree *et al.* (1984) also reported that when the steam-exploded substrates, such as wheat and barley straw, were not water-extracted, the percentage conversion of cellulose to glucose was extremely low.

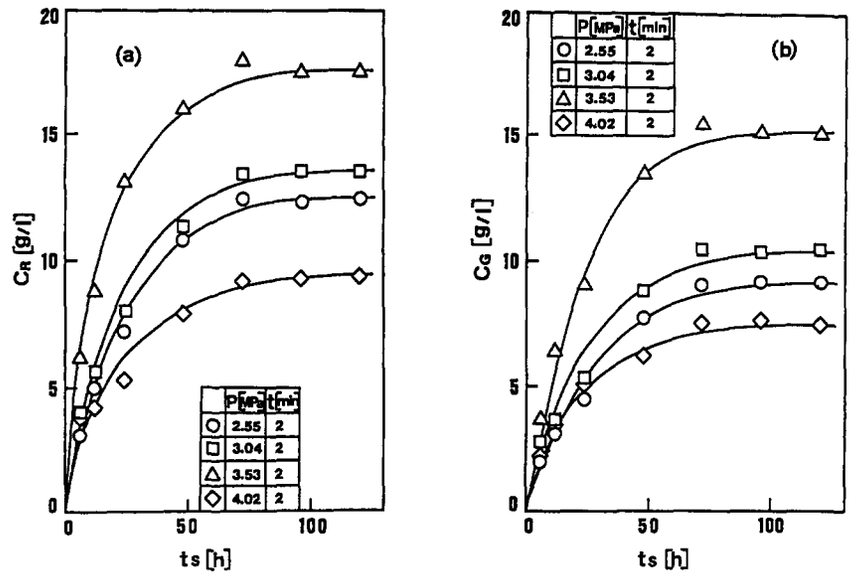

Fig. 6. Enzymatic saccharification of steam-exploded, alkali-treated rice straw: (a) reducing sugar concentration; (b) glucose concentration.

The results presented in Table 1 differ from the above studies and demonstrate that no additional treatment is required to hydrolyze the steam-exploded sample to a greater extent if it is pretreated under optimized conditions. As can be observed, the steam-exploded sample (unextracted) was easily hydrolyzed by the cellulolytic enzyme system with a saccharification efficiency of 92% and a glucose yield of 76%. Treatment with water following steam explosion did not increase hydrolysis efficiency further. It is also evident that the presence of large amounts of lignin in the unextracted (27% total lignin) and water-extracted (36% total lignin) samples did not appear to shield the cellulose from attack by the cellulolytic enzyme. Furthermore, no increase in saccharification was observed after lignin removal (Table 1).

The results presented in Table 1 demonstrate that sufficient hydrolysis (more than 90%) can be achieved only by the steam explosion of rice straw at 3·53 MPa for 2 min of steaming time. Additional treatment with water or chemicals is unnecessary. Furthermore, the extensive loss of material during the extraction step also diminishes its practical feasibility.

Figure 7 shows the time-courses of cell mass concentration, ethanol concentration and glucose concentration in the alcohol fermentation of enzymatic hydrolyzate of exploded rice straw by *S. cerevisiae*. The enzymatic hydrolyzate was obtained after 120 h of hydrolysis of the rice-straw sample, which was exploded at 3·53 MPa for 2 min. An alcohol fermentation was also performed separately with the same amount of reagent-grade glucose at the same fermentation conditions, to see if the enzymatic hydrolyzate of the exploded sample would inhibit the yeast cells' growth and alcohol production, since the presence of growth-inhibitory substance in steam-exploded samples has been reported previously by other workers (Saddler *et al.*, 1983; Mes-Hartree *et al.*, 1984). In the present study, however, a negligible difference was observed in the cell growth and alcohol production from the enzymatic hydrolyzate of the exploded sample as compared with the pure glucose fermentation at an ethanol concentration of 3·5 g/l.

Table 1. Percent saccharification and glucose yield. Sample pretreated at 3·53 MPa for 2 min and subsequently extracted with water and alkali

Sample	Percentage composition based on sample dry wt.				Reducing sugar conc. (g/l) after 120 h	Glucose conc. (g/l) after 120 h	Percentage saccharification	Percentage glucose yield
	Cellulose	Hemicellulose	Soluble lignin	Klason lignin				
Steam-exploded	47	26	17	10	15	8	92	76
Steam-exploded, water-extracted	64	—	22	14	13	9	91	63
Steam-exploded, alkali-treated	92	—	2	6	17·5	15	86	73

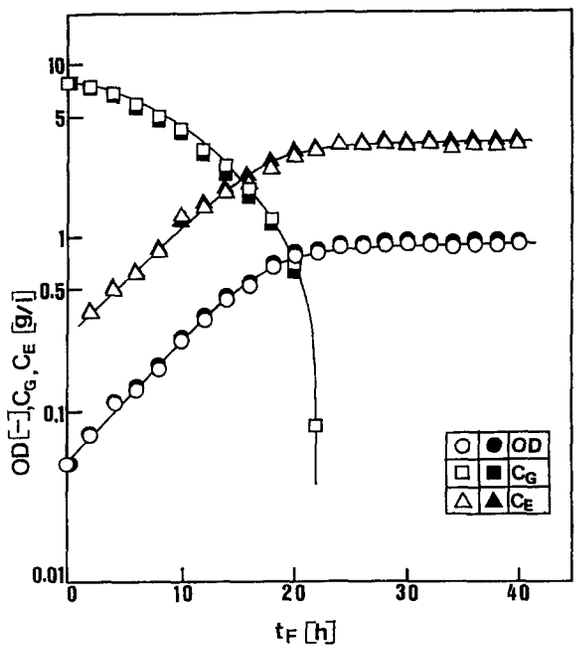

Fig. 7. Comparison of the fermentability of the enzymatic hydrolyzate of exploded rice straw (open symbols) and reagent grade glucose (closed sybols).

CONCLUSIONS

Pretreatment of rice straw by steam explosion prior to enzymatic saccharification has been investigated. The susceptibility of the pretreated substrate to cellulase enzyme was greatly influenced by the steam pressure and steaming time. Pretreatment at 3·53 MPa for 2 min steaming time was optimal for producing a material that is highly susceptible to attack by cellulolytic enzymes. Additional treatment of exploded products with water or chemicals was unnecessary. No inhibition in cell growth and alcohol production was observed during fermentation of the enzymatic hydrolyzate of the sample exploded at 3·53 MPa for 2 min.

The effects of steam explosion on the physicochemical properties of rice straw have been reported in a separate publication (Moniruzzaman, 1995). However, from an economical and technological points of view, it is necessary to optimize the enzymatic saccharification process in order to either lower enzyme loadings or to recycle the enzymes at high substrate concentrations. It is also essential to improve traditional fermentation processes by providing genetically engineered microorganisms that ferment mixed sugars effectively, so that glucose fermentation need not carry the entire processing costs. Future work will be focused on these directions.

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