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RESEARCH ARTICLE

MICROBIOLOGY

PRETREATMENT OF RICEBRAN FOR EFFECTIVE PRODUCTION OF CELLULASE BY ASPERGILLUS NIGER

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ABSTRACT

Rice bran a chiefly available agricultural lignocellulosic waste was pretreated with both acid (HCl) and alkaline (NaOH) at concentrations from 1N to 3N and 1N to 15N respectively, for a residence time from 10 to 30 min at 121°C for delignification of lignin from lignocellulose. The pretreatments were evaluated based on percentage of total lignin removal from lignocelluloses. Up to 73.26% and 54.50% lignin removal were observed in 10N NaOH and 1N HCl respectively. The optimum amount of sugars (4.973 mg g^{-1}) and protein (4.760 mg g^{-1}) were observed with 5N Sodium hydroxide treatment with 10 min of steaming. Acidic pre-treatment of rice bran resulted in peak concentrations of sugars (3.410 mg g^{-1}) with 1N HCl and protein (3.3 mg g^{-1}) with 2N HCl with steaming period of 10 min. The pretreated lignocellulose with different concentrations from 0.5% to 2% were used as substrate for cellulase production by fungal culture *Aspergillus niger*. The yield of the enzyme was reached maximum with Fpase, CMCase and β-glucosidase were 9.3, 8.5 and 1.14U/ml respectively.

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KEYWORDS

Rice bran, pretreatment, *Aspergillus niger*, cellulase

INTRODUCTION

Enzymes are being utilized in a large number of processes in the areas of industrial, environmental and food technology. Filamentous fungi are widely important for commercially important enzyme productions, as the level of enzymes produced by these cultures is higher than those obtained from yeast and bacteria[1]. Cellulase is one of such enzymes to hydrolyse cellulose from agricultural wastes. Cellulose is the major constituent of lignocelluloses available in large quantities and is the most important reservoir of carbon for the production of glucose, fuel and chemical stock. Utilization of this natural resource for production of useful materials through saccharification process by enzymatic means, cellulase is a challenging task in the field of biotechnology.

Lignocellulose is the major structural component of plant biomass such as woody and nonwoody plants and represents major part of renewable organic matter and substrate available for conversion to fuels. It is inexpensive, plentiful and renewable. It is a complex polymer consisting of cellulosic fibrous bundles encased in a polymer of matrix of lignin and hemicellulose and occurs abundantly in the nature. In view of its cheap source and abundance, this material can be used for microbial production of cellulase. But untreated lignocelluloses are slowly degraded by microorganisms because of the compact and stringent structure of β -1,4 linkages in cellulose and due to the close association of lignin with cellulose and hemicellulose leaving very few reactive sites available for enzyme attachment[2]. The recalcitrant nature of the lignocellulosic substrate, in fact, becomes impediment to its commercial exploitation. Pretreatments aim at increasing the surface area of cellulose by removing the lignin seal, solubilizing hemicellulose, disrupting crystallinity, and increasing pore volume and then makes it amenable for enzymatic hydrolysis or microbial biodegradation with higher enzyme production. A number of pretreatment processes like milling and grinding, pyrolysis, oxidation with H_2O_2 and Para acetic acid , high energy radiation, high-pressure steaming, alkaline or acid hydrolysis, gas treatment(chlorinedioxide, nitrogen dioxide, sulphurdioxide, ozone), hydrogen peroxide treatment, organic solvent treatment , steam explosion, wet oxidation and biological treatment are being employed to remove the lignin and hemicellulose from lignocelluloses and open the crystalline structure in cellulose. In view of this background, this study aimed at isolating *A.niger* from the effluents of fruit pulp industry and evaluating its cellulolytic activity on untreated and pretreated ricebran.

MATERIALS AND METHODS

(i) ***Microorganism***

The present strain was isolated from the effluents of fruit pulp industry and identified as *A.niger* by morphological and biochemical characterization. The culture was maintained on potato dextrose agar (PDA) slants and used for further studies.

(ii) ***Substrate***

Rice bran is a low cost source of cellulose, abundantly available in coastal parts of Andhra Pradesh, India and other parts of the world where rice is being cultivated as a staple food crop. Rice bran was pre-treated by

Sodium hydroxide and hydrochloric acid and was used as substrate to cellulolytic attack.

(iii) ***Experimental procedure***

Collected rice bran was cut into smaller fragments of 3- 6cm size. About 10 g of finely cut rice bran was mixed with 20 ml of acid/ alkali solution (Sodium hydroxide and Hydrochloric acid of various normalities) and autoclaved for various time periods at 121^0C and 15 psi. This was allowed to cool and then filtered using a muslin cloth. The residue was washed with water until neutral pH was obtained. It was then dried at 60 °C overnight and used as substrate for further studies.

The release of total sugar[3] and extracellular protein[4] contents were estimated before and after the pre-treatment. SSF was performed and the production of cellulase was estimated in terms of percentage of solubilization[5].

(iv) ***Effect of alkali-treated rice bran concentration on cellulase production***

Another experiment was conducted to determine effect of treated rice bran supplementation at different levels ranging from 0.5 to 2.0%(W/V) concentration to the medium and incubated at various time periods on cellulase yields by *A. niger.*

(v) ***Enzyme assays[6]***

Activities of individual enzyme components of cellulase system secreted into the culture medium of *A. niger*, were estimated in accordance with methods listed.

(a) ***Filter paper assay[5]***

Filter paper activity indicates total cellulolytic activity resulting from combined action of different enzyme components present in the culture filtrate. Filter paper activity of the culture filtrate of *A. niger* was determined according to the method.

(b) ***Endoglucanase assay[7]***

Activity of endoglucanase in the culture filtrate was quantified by carboxymethyl cellulase method.

(c) ***β-D-Glucosidase assay[8]***

Activity of β -glucosidase activity in the culture filtrate was based on the method.

RESULTS

Agro industrial residues are generally considered as best substrates for the cultivation of microorganisms to produce enzymes. In this study, after screening several such substrates, ricebran was found to be best substrate for maximum enzyme production. Utilization of rice bran as a potential low cost carbohydrate source was done by other researchers[9,10]. Steaming and mechanical treatment is the basic pre-treatment of lignocellulosic substrates had done in several studies at various levels[11, 12,13,14,15]. To obtain high amount of fermentable sugars in this study, the combined effect of milling, alkali, acid treatment along with steaming was employed and the effect was examined by estimating the percentage of delignification. The percentage loss of rice bran with Sodium hydroxide treatment (Table. 1) was 63-72%and that of Hydrochloric acid (Table. 2) was 34-54%.

Table 1
Pre-treatment of rice bran with sodium hydroxide

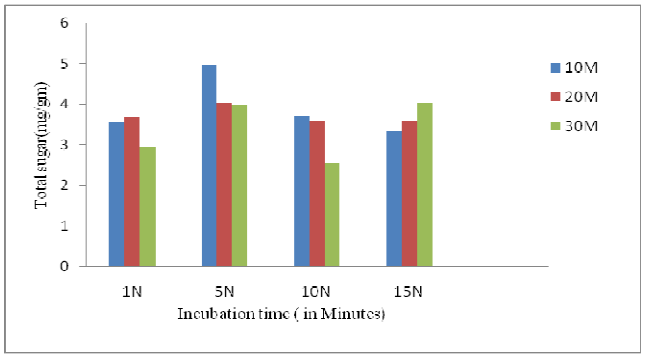
Normality of Sodium hydroxide (N)	Time period of steaming (min)	Percentage of delignification
1	10	67.40
5		71.00
10		73.26
15		70.44
1	20	63.00
5		64.25
10		70.23
15		73.13
1	30	65.00
5		64.22
10		70.34
15		72.11

Table 2
Pre-treatment of rice bran with hydrochloric acid

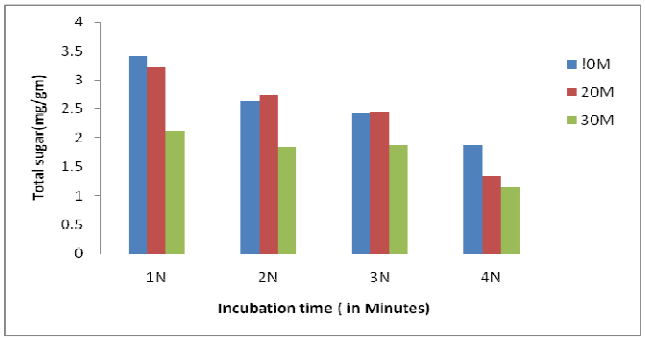
Normality of Hydrochloric acid (N)	Time period of steaming (min)	Percentage of delignification
1	10	54.50
2		44.16
3		35.37
1	20	41.10
2		46.26
3		36.27
1	30	41.31
2		34.67
3		35.34

The release of total soluble sugar and extracellular protein were varied with different normalities of Sodium hydroxide treatment. The optimum amount of sugars (4.973 mg/g) and protein (4.760 mg/g) were observed with 5N Sodium hydroxide treatment with 10 min of steaming (Graph 1 and Graph 2). Acidic pre-treatment of rice bran resulted in peak concentrations of sugars (3.410 mg g^{-1}) with 1N HCl and protein (3.3 mg g^{-1}) with 2N HCl with steaming period of 10 min.(Graph 3 and Graph 4). It shows alkaline treatment was effective to oxidative treatment.

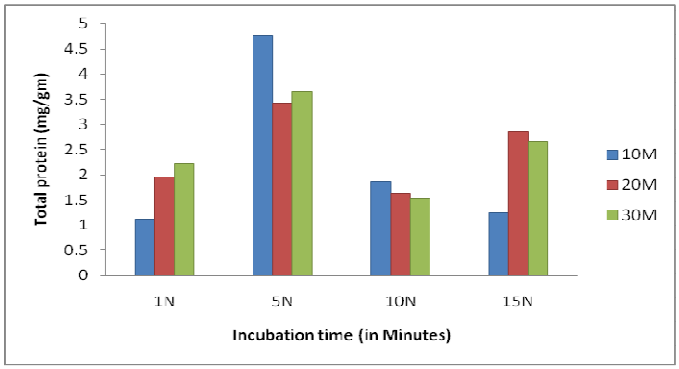
Graph 1
Total sugar content in alkali (5N) pre-treated rice bran



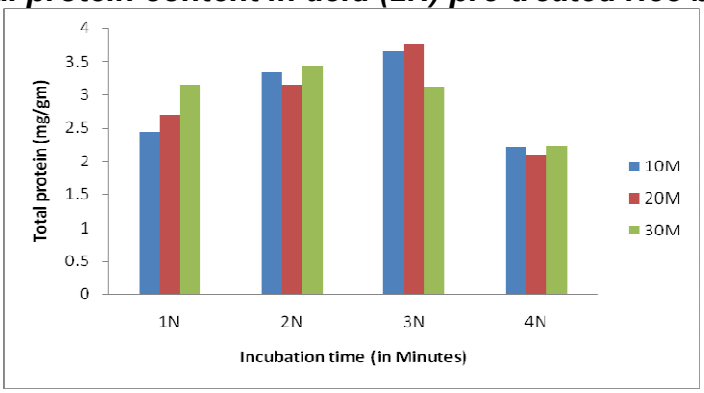
Graph 2
Total sugar content in acid (1N) pre-treated rice bran



Graph 3
Total protein content in alkali (5N) pre-treated rice bran



Graph 4
Total protein content in acid (2N) pre-treated rice bran



In order to optimize the substrate concentration, *A. niger* was grown on Czapek-Dox medium amended with 0.5 - 2% alkali-treated rice bran. Activity of individual enzyme components of cellulase in the culture filtrate of *Aspergillus niger* was estimated in terms of Filterpaperase (Fpase), Carboxymethylcellulase (CMCase) and β-glucosidase. Maximum production of all enzymes except β-glucosidase occurred on 5-day of incubation at 1.0% level and thereafter declined (Table 3). The production of β-glucosidase followed a different pattern. Activity of β-glucosidase reached maximum on 7 day of incubation at 0.5% concentration. Under these conditions, yields of production of three enzymes - Fpase, CMCase and β-glucosidase were 9.3, 8.5 and 1.14U/ml respectively. Further increase in substrate concentration above 1% was limiting factor for cellulase activity. It may be due to high substrate concentration in the reaction medium led to unfavorable for production of enzyme (Table 1). Similar results had also been employed for *Streptomyces sps*[16] and *Aspergillus niger*[17]. According to Narasimha et al[18] maximum cellulase were observed at 1% substrate concentration with *Aspergillus niger* isolated from soil contaminated with cotton ginning mill effluents.

. **Table 3**
Effect of substrate concentrations on cellulase production

Table 3.1
Effect of rice bran concentration on FPase production by Aspergillus niger

Concentration of Pretreated ricebran (W/V)	FPase **(U/ml)**						
	1st Day	**2nd Day**	**3rd Day**	**4th Day**	**5th Day**	**6th Day**	**7th Day**
0.5 %	0.3	1.4	3.2	5.2	7.3	6.1	4.0
1 %	0.2	1.5	3.5	6.8	9.3	6.4	3.9
1.5%	0.4	0.6	3.8	5.7	6.4	5.2	2.6
2%	0.1	0.2	2.7	2.8	3.6	2.8	1.5

Table 3.2

Effect of rice bran concentration on CMCase production by Aspergillus niger

Concentration of Pretreated ricebran (W/V)	CMCase (U/ml)						
	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
0.5 %	0.5	0.9	2.2	4.1	6.7	5.4	3.9
1 %	0.3	1.1	2.4	4.7	8.5	6.5	4.2
1.5%	0.3	0.8	2.6	4.9	6.6	6.1	3.8
2%	0.2	0.3	2.1	3.4	4.9	3.7	2.4

Table 3.3

Effect of rice bran concentration on β-glucosidase production by Aspergillus niger

Concentration of Pretreated ricebran (W/V)	β-glucosidase (U/ml)						
	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
0.5 %	0.00	0.05	0.08	0.54	0.87	1.14	0.89
1 %	0.00	0.01	0.01	0.37	0.55	0.83	0.52
1.5%	0.00	0.00	0.05	0.19	0.43	0.41	0.23
2%	0.00	0.01	0.04	0.14	0.39	0.17	0.14

Values represented in the Table are averages of results of two experiments.

a. Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.
b. Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.
c. One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1µmole of p-nitro phenol/ml/min.

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