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Saccharification of agro wastes by the Endoglucanase of *Rhizopus oryzae*

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ABSTRACT

Extracellular high activity endo-glucanase produced by Rhizopus oryzae PR 7 was used for the bioconversion of various agro wastes like orange peel, sugarcane bagasse, dried flower, water hyacinth and coconut shell. Among the agro wastes tested, saccharification percentage was highest in orange peel followed by sugar cane bagasse. The maximum amount of bioconversion was accomplished within 30-45 minutes of incubation. The optimum pH and temperature for such bioconversion was 7.0 and 33°C respectively. Highest rate of saccharification was found at a substrate concentration of 5mg/ml and there was a positive correlation between enzyme concentration and saccharification. The amount of glucose production was enhanced in presence of Mn^{2+} and after pre treatment with deionized water.

Key words: saccharification, cellulase, agro wastes, *Rhizopus oryzae*.

INTRODUCTION

Cellulose being the principal constituent of the cell wall of most terrestrial plant is the most abundant and renewable resource for the production of food, fuel and chemicals [1]. Large quantities of cellulosic wastes, generated from agricultural residues, forests and agro industrial practices generally accumulate in the environment and cause pollution problem [3]. Active efforts are being made to convert waste cellulose resources into either glucose or alcohol, and use this either as fuel or as a valuable starting material for chemical synthesis[4]. Biodegradation of cellulosic wastes is accomplished by cellulolytic enzymes and cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds [5,6] of which endoglucanase act internally on the chain of cellulose cleaving β-linked bonds liberating non-reducing ends, and exoglucanases act removing cellobiose from this non-reducing

end of cellulose chain. Finally, β-glucosidase completes the saccharification by splitting cellobiose and small cello-oligossaccharides into glucose molecule [7] .

Saccharification of cellulose to glucose by microbial cellulases has attracted the attention of the researchers, as this is the first step of bioconversion of cellulose material into valuable products such as sugar, fine chemicals and biofuels [8] . As the cost of cellulosic substrates play the central role in determining the economy of the saccharification process, lot of emphasis had been given to the usage of low price substrates and therefore screening of the agricultural wastes for release of sugars as Organic wastes from renewable forest and agricultural residues are rich sources of cellulose [9] .The saccharification of different agro wastes has been reported by other workers employing enzymes from different organisms [10,11,12,13] .

In India, orange peel, sugar cane bagasse, dried flower and coconut shell after usage are left and dumped unattended for natural degradation, which causes generation of obnoxious odour with consequent environmental pollution. Water hyacinth on the other hand causes serious problem in water bodies by acting as a nursery for malarial parasites and are considered as harmful garbage.
In the present paper, the saccharification of these potent wastes by endoglucanase from *Rhizopus oryzae* PR7 was studied and various parameters affecting saccharification process were evaluated.

MATERIALS AND METHODS

Enzyme source: A strain of *Rhizopus oryzae* PR7 MTCC 9642 [14], isolated from eastern India was grown in basal medium composed of (gl^{-1}): peptone 0.9;$(NH_4)_2HPO_4$ 0.4; KCl 0.1;$MgSO_4.7H_2O$ 0.1and carboxymethyl cellulose (CMC) 0.5. (pH 7) at 37°C for 48 hours. The culture broth was centrifuged at 5,000 g for 5 minutes and the supernatant was used as enzyme source.

Enzyme assay: Endoglucanse activity was measured by incubating the assay mixture (1ml) containing an equal volume of enzyme and 1 %(w/v) CM-cellulose 0.1M phosphate buffer (pH 7) was incubated at 33°C for 5 minutes. The reducing sugar released was measured by the dinitrosalicylic acid method [15] taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of endoglucanase was defined as the amount of enzyme that liberated 1µ mol of glucose per ml per minute of reaction.

Saccharification of agro waste substrates: The agro wastes were collected from market dumps and temple effluents, washed thoroughly with water, air dried, pulverized and sieved to 40 mesh particle size, before using as substrate for saccharification.

A suspension of substrate (5mg/ml) in 0.1M phosphate buffer (pH 7) was incubated with endoglucanase in a screw capped tube for 30 minutes at 33°C. The resultant supernatant was centrifuged at 2000 g for 2 minutes was analyzed by DNSA method [15] using glucose as standard.

The percentage of saccharification was calculated [10] as:

$$\text{Saccharification (\%)} = \frac{\text{Glucose (mg/ml)}}{\text{Substrate (mg/ml)}} \text{X } 100$$

Effect of various factors on saccharification: To determine the effect of incubation time, saccarification was carried out under the conditions cited above. At specific time intervals aliquots (1ml) were removed and the amount of reducing sugar was estimated [16] . Effect of pH on saccharification was determined by the varying the pH of assay mixture from 4 to 9. The effect of increasing substrate concentration and enzyme concentration were estimated by changing the substrate and enzyme concentrations respectively keeping the other factors unchanged. The role of metal ions, thiol compounds and thiol inhibitors on sugar production were checked by adding 10mM of each one in saccharification mixture.

Pretreatment of agrowaste substrates: The substrates were treated with 0.1N NaOH or 0.1N HCl for 60 minutes followed by washing, neutralization or were simply treated with deionized water for 60 minutes. All these pretreated substrates were oven dried at 55°C and were used as substrate in saccharification mixture,

Determination of end product of saccharification: The end products of saccharification of agro wastes by endoglucanase was analysed by TLC on a pre coated TLC plate (Merck) using a solvent system of butanol: acetic acid: water (3:3:1v/v), developing it with 0.1% methanolic orcinol in 10% H_2SO_4 followed by heating the plate at 110°C[17] .

RESULTS AND DISCUSSION

In order to reduce the cost of saccharification, various wastes collected from agricultural fields and domestic effluents were used as substrates of which orange peel showed highest tendency in cellulose bioconversion followed by sugar cane bagasse. The differential rates of saccharification can be explained by the nature and complexity of the substrate; the higher the crystallinity and /or structural complexity, the lower the hydrolysis rate [18] .

The maximum bioconversion was accomplished within 30-45 minutes of incubation, except for sugarcane bagasse and water hyacinth and orange peel (Fig 1). It was followed by a slow rate of increase in sugar production, probably due to substrate and / or enzyme limitation [19] or as a result of product accumulation and consequent product inhibition [20] .

Initially, the concentration of substrate was directly correlated with the rate of saccharification (Fig 2) as it showed a sharp increase in sugar production when the substrate concentration increased from 2.5mg/ml to 5mg/ml. Highest rate of bioconversion was achieved in presence of 5mg/ml i.e.0.5% (w/v) of substrate in all types of agro wastes used. But the sugar production did not increase at the same pace with further increase in substrate concentration .As a result, rate of saccharification gradually decreased. This could be due to an enzyme limited reaction.

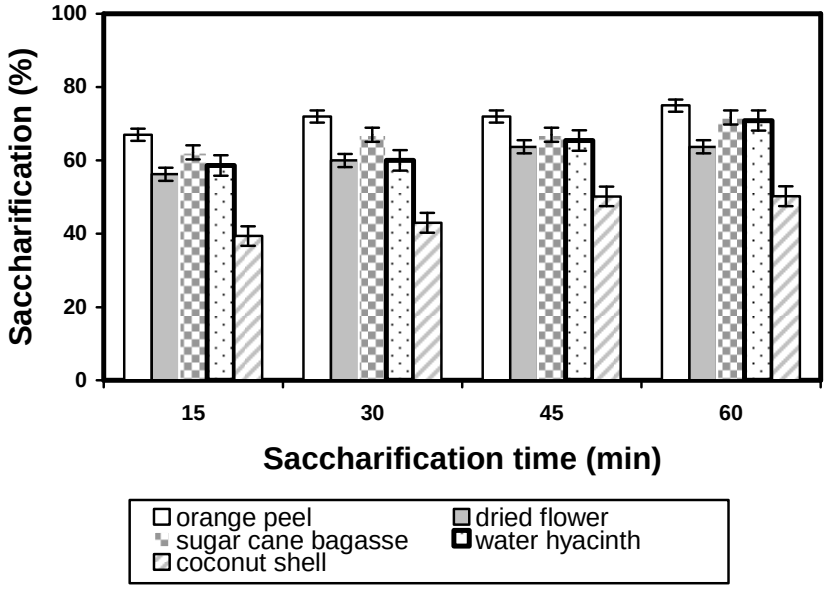

Figure 1. Effect of time on saccharification of agro wastes by endoglucanase of *R.oryzae*.

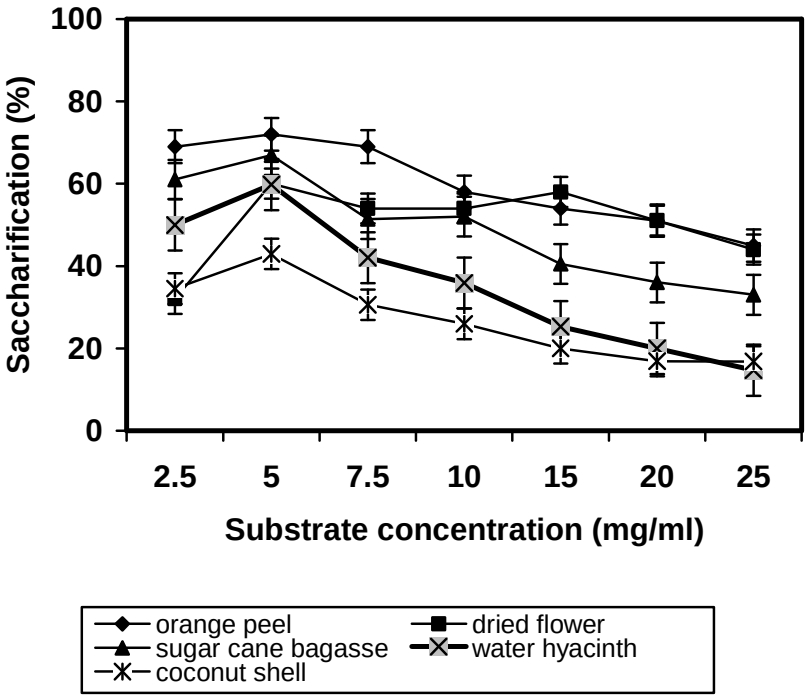

Figure. 2 Effect of substrate concentration on saccharification of agro wastes by endoglucanase of *R.oryzae*.

The existence of enzyme limitation was further confirmed by the positive correlation between the enzyme concentration and rate of saccharification (Fig 3).Similar mode of reaction was also found in the saccharification of lignocellulosics [21.

The temperature and pH optima for saccharification were found to be at 33°C (data not shown), and 7 (Fig 4) respectively. The pH optimum was higher than the acidic pH optima of CMC ase from *Trichoderma lignorum* [10] and *Trichoderma viridae* [22] but similar to that reported from *Bacillus subtilis* [23] and *Humicola* sp. [22].

Amongst the metal ions tested (Table 1), only Mn^{2+} brought remarkable increase in sugar production by all types of agro waste substrates. The bioconversion was reduced in presence of heavy metals due to inactivation of the working enzyme. Although both were thiol compounds, reduced glutathione (GSH) showed a tendency to increase the sugar production whereas rate of bioconversion was remarkably slashed down in presence of β-mercaptoethanol (β-ME). These apparent antagonistic effects of thiol compounds are yet to be explained. However, pCMB, a thiol inhibitor reduced glucose production probably due to denaturation of the active site of the enzyme.

A number of reports are available on pretreatment of lignocelluosic wastes to remove lignin for enhancing enzyme and sugar production, where treatment of with 2-3% aqueous solution of NaOH only , NaOH / urea and phosphoric acid, and liquid HCl [24] improved saccharification of cotton balls [3];chaff, bagasse, rice hulls [25]; and cellulosic wastes of paper and pulp industries[24] respectively.

But in the present study, treatment with alkali and acid reduced the rate of saccharification in almost all types of agro wastes tested .Similar decreasing trends in saccharification was reported from bacterial cellulase after alkali treatment [23]. Again, in *T. reesei* Cel7A, treatment with acidified sodium chlorite resulted in a dramatic reduction in cellulose digestibility [26] and the authors suggested that near complete removal of xylan and lignin may cause aggregation of the cellulose microfibrils resulting in decreased cellulase accessibility. But amazingly, after treating the pulverized water hyacinth and coconut shell with distilled water at room temperature their bioconversion were increased to 42.8% and 47.6% respectively.

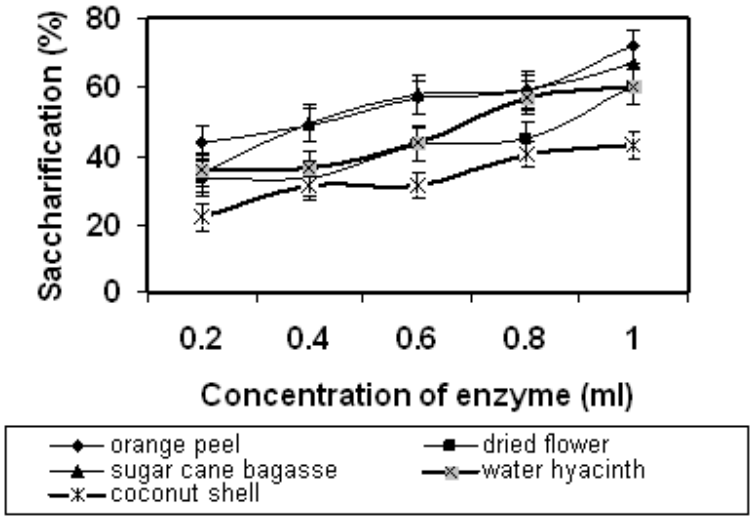

Figure 3. Effect of enzyme concentration on saccharification of agro wastes by endoglucanase of *R.oryzae.*(1ml =500U)

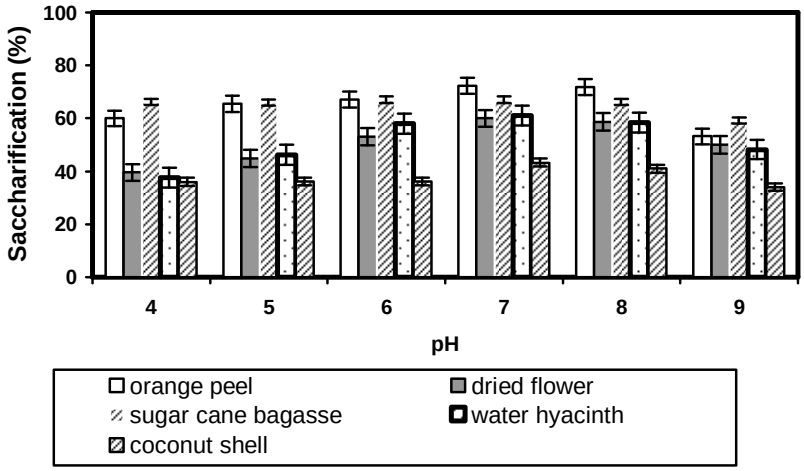

Figure 4. Effect of pH on saccharification of agro wastes by endoglucanase of *R.oryzae.* (1ml =500U)

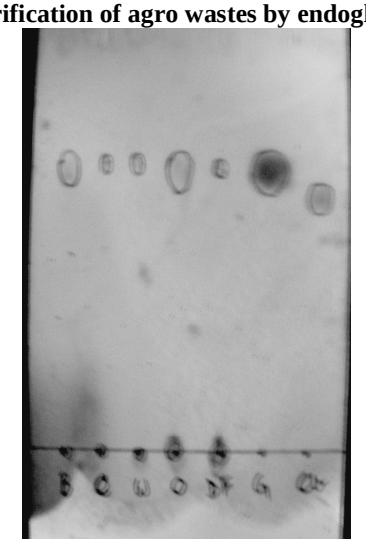

Figure 5. Thin layer chromatographic analysis of the end products of saccharification of the agro wastes by endoglucanase of *R.oryzae.*

(1ml =500U) B: sugarcane bagasse, O: orange Peel, C: coconut shell, DF: dried flower, W: water hyacinth G: glucose Cb: Cellobiose Substrate: 5mg/ml, temperature :33°C,time: 30 min, pH: 7

This might be due to the penetration of water molecules into capillary spaces and consequent breaking of hydrogen bonds inside cellulose molecules that made the enzyme more accessible to the substrates [27,28]. This differential effect of pretreatment on sugar release was probably due to the relative cellulose, hemicellulose and pectin content of the substrate treated.

Glucose was found to be the final product of bioconversion of all types of agro wastes tested (Fig 5), a result similar to that reported from hydrolysis by cellulases of *Thermomonospora* sp[29] and *Trichoderma viridae* ITCC 1433 [30]. Cellobiose was not detected as end product as reported after hydrolysis of avicel, the microcrystalline cellulose [31].

Thus it can be concluded that cellulosic wastes could be easily and rapidly converted into glucose with the help of endoglucanase secreted from *R. oryzae* without the requirement of alkali or acid pretreatments. This might reduce the practice of bioconversion of cellulose to glucose by

chemical degradation namely by mineral acid hydrolysis[20]. As the enzyme used was of high activity and substrates used were from waste material, the entire process might add economy in sugar production.

Table 1. Effect of various additives on sugar production from agro wastes

Additives (10mM)	Glucose (mg/ml)				
	Orange peel	Dried flower	Sugarcane bagasse	Water hyacinth	Coconut shell
None	3.6	3.0	3.3	2.8	2.1
Na^{+}	3.9	3.1	2.4	3.1	1.7
K^{+}	3.3	2.8	2.5	2.4	1.5
Cu^{2+}	1.1	1.4	1.7	1.8	0.9
Mn^{2+}	4.8	4.4	3.4	4.2	1.9
Ca^{2+}	3.3	2.3	3.3	3.0	1.9
Hg^{2+}	0.3	0.4	0.8	0.4	0.3
Ba^{2+}	2.7	3.7	2.2	3.0	1.7
Sn^{2+}	2.9	2.9	2.9	3.0	1.0
Sr^{2+}	3.5	2.6	2.8	2.8	1.1
DTT	3.1	2.6	3.3	3.2	2.3
GSH	3.3	2.8	4.5	3.6	4.7
β-ME	ND	ND	0.5	0.9	0.1
pCMB	0.4	0.9	1.2	0.3	0.7

Substrate concentration = 5mg/ml, Enzyme concentration = 200 U/ml

Table 2. Effect of pre treatments on sugar production from agro wastes.

Substrate (5mg/ml)	Glucose (mg/ml)			
	Untreated	Treated		
		Treated with distilled water	Acid treatment	Alkali treatment
Orange peel	3.6	3.6	2.9	2.8
Dried flower	3.0	3.3	3.0	2.7
Sugar cane bagasse	3.3	3.7	2.3	1.5
Water hyacinth	2.8	4.0	2.5	2.8
Coconut shell	2.1	3.1	2.4	2.1

Substrate concentration = 5mg/ml, Enzyme concentration = 200 U/ml

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