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Utilization of cellulosic wastes by *Rhizopus oryzae* PR 7 Went & Geerlings for the production of extracellular endoglucanase and β-glucosidase

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Rhizopus oryzae Went & Geerlings PR7, MTCC 9642, an endoglucanase producing strain was found to synthesise extra cellular β-glucosidase along with endoglucanase when grown in presence of cellulosic wastes like dried leaves, water hyacinth and coconut shell, as sole carbon source. Out of these waste substrates, coconut shell dust and water hyacinth were acting as the best inducers for the biosynthesis of endoglucanase and β-glucosidase respectively. Highest amount of enzymes were obtained at a range of substrate concentration of 0.25-0.75% (w/v) in all three types of wastes used and within a period of 48 hrs only. Peptone was found to have some enhancing effect on enzyme production. Depending on the type of carbon source used the pH optima varied within a range of 5-8 for endoglucanase and 6-7 for β-glucosidase. Inoculation with two discs of fungal mat supported best production of enzymes. The ability of waste degradation and rapid rate of endoglucanase and β-glucosidase production may enable the strain to be utilized for commercial production of these enzymes.

Key words : Endoglucanase, β-glucosidase, waste utilization. cellulase, *Rhizopus oryzae*

INTRODUCTION

Lignocelluloses constitute a major portion of agricultural and forest wastes and cause serious pollution problem after accumulating in the environment. The huge amount of residual plant biomass, considered "waste", which is generally burnt out, can potentially be converted into different value added products including biofuels, chemicals and cheap carbon sources for fermentation, improved animal feeds and human nutrients by cellulose degrading microorganisms. (Kuhad *et al.,* 2007). The bioconversion of agrowaste based lignocellulosic materials to energy has gained much interest during the recent past (Baig, 2005). The enzymic hydrolysis of cellulose may be achieved by cellulase, a synergistic enzyme comprises of three component enzymes: endoglucanase (E.C.3.2.1.4), exoglucanase (E.C.3.2.1.9) and β-glucosidase (E.C.3.2.1.21) that convert cellulose to glucose. Cellulase production by different organisms in submerged state fermentation has received more attention and is found to be cost prohibitive because of the high cost of process engineering (Singh *et al.,* 2009). As the cost of enzymes constitutes a major part of the total cost of hydrolysis (Dapula *et al.,* 1999), curtailing the cost of enzyme production will surely improve the economics. On the other hand, substrate costs account for the major fraction of the costs of cellulase production as such the use of cheap biomass resources as substrates can help to reduce cellulase prices (Wen *et al.,* 2005). Therefore, utilization of cellulosic wastes in the fermentation media has been suggested as a feasible alternative for the production of low cost enzymes. Although a number of reports are available on endoglucanase production by various fungal strains utilizing agrowastes (Ali *et al.,* 1991, Pothiraj *et al.,* 2006, 2007, Acharya *et al.,* 2008, Omojasola *et al.,* 2008), almost no report is available on the simultaneous production of endoglucanase and β-glucosidase from fermentation of cellulosic wastes.

In the present study, the production of extracellular endoglucanase and β-glucosidase from the fermentation media supplemented with waste material like dried leaf, the most abundant garbage of garden; water hyacinth (*Eichhornia crassipes*), a hazardous aquatic weed and coconut shell, a significant agricultural residue has been reported.

MATERIALS AND METHODS

Organism : *Rhizopus oryzae* Whent & Geerlings PR7 MTCC 9642, (Karmakar and Ray, 2010) was isolated from the decaying vegetaion enriched soil of West Bengal, India.

Preparation of inoculum : The inocula were prepared by making hyphal discs (0.5 cm diameter) from fungal mat grown on 1% PDA plates. Two discs were used to inoculate 20 ml of medium. (Ray & Chakraverty, 1998).

Cultivation of the strain : The strain was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml Basal Medium (BM) composed of (gl^{-1}) : peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4$ 0.1 and carboxymethyl cellusose (CMC) 0.5. (pH : 6) at 37°C for 48 hrs.

Enzyme assay : The grown culture was filtered through filter paper (Whatmann No 1) followed by the centrifugation of the filtrate at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. To determine the activity of endoglucanase, the assay mixture (1 ml) containing an equal volume of enzyme and 1% (w/v) CM-cellulose dissolved in 0.1(M) phosphate buffer (pH-6) was incubated at 33°C for 10 min. To measure the activity of β-glucosidase, the assay mixture (1 ml) containing an equal volume of enzyme and 0.5% (w/v) salicin dissolved in 0.1(M) phosphate buffer (pH-6) was incubated at 37°C for 30 minutes. The reducing sugar released in either case was measured by the dinitrosalicylic acid method (Bernfeld, 1955) taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of endoglucanase and β-glucosidase was defined as that amount of enzyme that liberated 1μ mole of glucose per ml per minute of reaction from CM cellulose and salicin respectively.

Cellulosic waste materials : The cellulosic wastes, namely dried leaves, water hyacinth and coconut shell, were collected from garden and roadside dumps, market effluents and pond wastes. Those were dried, pulverized and sieved as 40 mesh particle size before using in fermentation media in place of pure carboxymethyl cellulose.

Optimization of other parameters : Effect of various factors like cultivation time, waste substrate concentration, cultivation temperature, pH, inoculum size, nitrogen sources on enzyme production were tested by varying the determining factors under otherwise optimal conditions.

Each experiment was carried out in triplicate and their values were averaged.

RESULTS AND DISCUSSION

The strain was found to have a relatively high amount of extracellular endoglucanase and a moderate amount of β-glucosidase synthesizing ability. Highest production of endoglucanase and β-glucosidase was achieved within 48 hrs of growth in all types of fermentation media except in water hyacinth supplemented media, where it took 96 hrs to reach the peak. (Fig 1). The strain showed best enzyme production at 37°C at static condition (data not shown).

In the present strain, substrate concentration was found to play a significant role in enzyme production. As evident from Table 1, highest amount of enzymes were obtained at a range of substrate concentration of 0.25-0.75% (w/v) in all three types of wastes used, beyond which enzyme synthesis became reduced.

A pH of 8 was suitable for endoglucanase production from dried leaf and coconut shell, whereas acidic pH (pH 5) was preferred for same enzyme production from water hyacinth (Table 2). This variation of pH indicates the compositional difference of the waste substrate. The range of preferred pH for β-glucosidase production (Table 3) as highest production was obtained after inoculation with 2 discs of 0.5 cm diameter. Increase in inoculum size reduced the enzyme yield, as higher

Table 1 : Effect of substrate concentration on enzyme production

Wastes used	Enzymes (U/ml)	Substrate concentration (% w/v)			
		0.25	0.5	0.75	1.0
Dried leaf	Endoglucanase	150	350	300	300
	β-glucosidase	67	34	17	16
Water hyacinth	Endoglucanase	250	300	600	350
	β-glucosidase	17	50	87	67
Coconut shell	Edoglucanase	750	600	450	150
	β-glucosidase	18.5	66.7	50	50

pH : 6, inoculum size : 2 disc.

Table 2 : Effect of pH on enzyme production

Wastes used	Enzymes (U/ml)	pH				
		5	6	7	8	9
Dried leaf	Endoglucanase	349	351	499	550	450
	β-glucosidase	23.4	34.5	56.7	53.4	51
Water hyacinth	Endoglucanase	487	302	250	198	101
	β-glucosidase	20	48.5	42	33.5	33.5
Coconut shell	Edoglucanase	500	600	702	799	694
	β-glucosidase	50	66.7	50.1	17	ND

Substrate concentration : 0.5%(w/v)

Table 3 : Effect of inoculum size on enzyme production

Wastes used	Enzymes (U/ml)	Inoculum size (No of disc)		
		1	2	3
Dried leaf	Endoglucanase	200	350	340
	β-glucosidase	16.7	33	16.7
Water hyacinth	Endoglucanase	250	300	270
	β-glucosidase	33.44	50.1	33.4
Coconut shell	Edoglucanase	150	600	400
	β-glucosidase	18.3	66	33.4

Substrate concentration : 0.5%(w/v), pH:6

Table 4 : Effect of substrate concentration on enzyme production

Wastes used	Enzymes (U/ml)	Nitrogen source			
		Peptone	Tryptone	Ammonium sulphat	Urea
Dried leaf	Endoglucanase	350	350	300	350
	β-glucosidase	33	33	16.7	ND
Water hyacinth	Endoglucanase	300	200	100	200
	β-glucosidase	66.7	17	66.7	66.7
Coconut shell	Edoglucanase	600	660	540	600
	β-glucosidase	66	43.4	22	22

Inculum size : 2 discs pH: 6, substrate concentration: 0.5%(w/v)

load of fungal mass reduced the enzyme production (Acharya *et al.*, 2008).

Among the nitrogen sources tested, peptone proved to be the best nitrogen source for enzyme production (Table 4). But enzyme production decreased in presence of ammonium sulphate, a report contrary to that from Kasherm *et al.* (2004).

From the present study it became evident that the coconut shell dust and water hyacinth were acting as the best inducers for the biosynthesis of endoglucanase and β-glucosidase respectively. Moreover, the rapid rate of production increased the competence of the strain to be employed in commercial production of both the enzymes from these wastes.

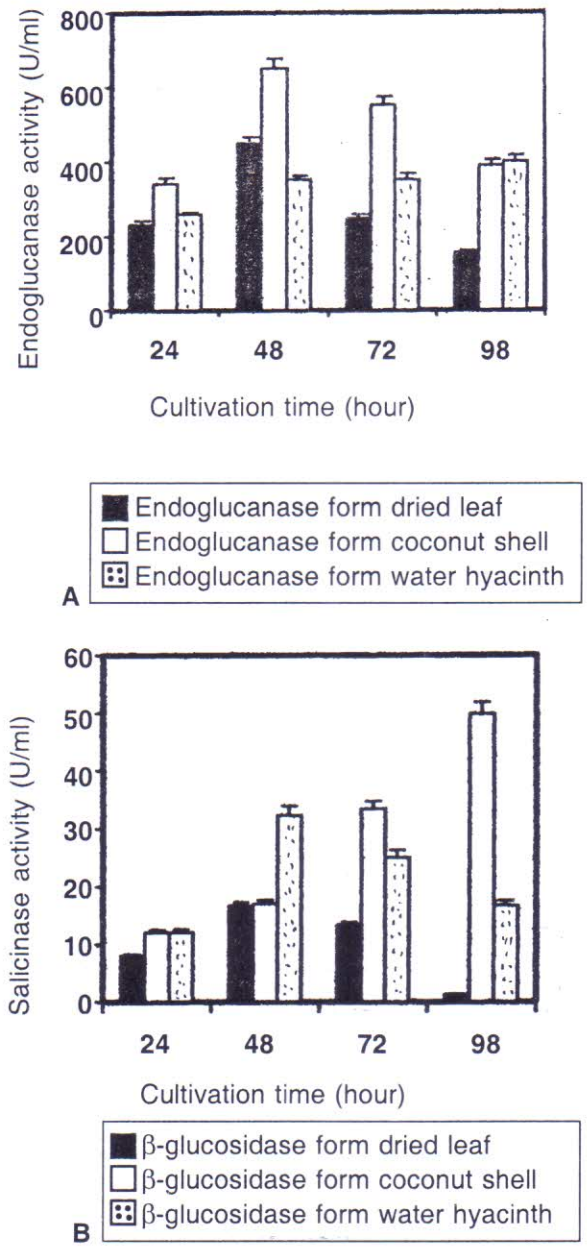

Fig 1. Effect of cultivation time on Endoglucanase (A) β-glucosidase (B) production in *Rhizopus oryzae*.

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