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Extra Cellular Endoglucanase Production by *Rhizopus oryzae* in Solid and Liquid State Fermentation of Agro Wastes

M. Karmakar and R.R. Ray
Microbiology Laboratory, Department of Zoology, Molecular Biology and Genetics, Presidency College, Kolkata, 700 073, India

Abstract: Extra cellular endoglucanase was found to be produced by *Rhizopus oryzae* PR7 from both liquid and solid state fermentations of various agro wastes. Among the 18 types of wastes tested, orange peel, dried flower and sugar cane bagasse showed promising results. The highest amount of enzyme production was obtained at 48 h of growth at 37°C at static condition. The Liquid State Fermentation (LSF) was found to be better than Solid State Fermentation (SSF) of the wastes for extra cellular endoglucanase synthesis. The pH preferred for enzyme production was found to vary with the nature of cellulosic waste used. The waste substrate at a concentration of 0.75% (w/v) gave the best result, whereas ammonium sulfate was proved to be the most effective nitrogen source for enzyme production. The alkali and acid pretreatment of the agro wastes could not increase enzyme production, while double sterilization enhanced the enzyme production only to a certain extent. The ability to produce high amount of endoglucanase within a very short period of 48 h and the capability of degrading wastes made the strain suitable for commercial production of the enzyme.

Key words: Endoglucanase, *Rhizopus oryzae*, LSF, SSF waste utilization

INTRODUCTION

Cellulose is the major part of plant biomass. Therefore, the wastes generated from forests, agricultural fields and agro industries contain a large amount of unutilized or underutilized cellulose. These wastes generally accumulate in the environment causing pollution problem (Abu *et al*., 2000). The previous negative attitude in which wastes were viewed self consciously as valueless, even offensive and for disposal only, has been replaced in large part by a positive view in which wastes are recognized as raw materials of potential value. Nowadays, these once regarded wastes are regarded as the major renewable natural resource (Acharya *et al*., 2008) and are converted into valuable products such as biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients (Howard *et al*., 2003).

Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose-containing raw materials and waste products that are not exploited efficiently, which include dry or semi dry fruit peels, vegetable jackets, flower petals, husks, seed coats and a large amount of leaves.

The utilization of these wastes for the production of strategic chemicals and fuel requires hydrolysis of all the components which is accomplished by the enzyme cellulase.

Corresponding Author: Rina Rani Ray, Microbiology Laboratory, Department of Zoology, Molecular Biology and Genetics, Presidency College, Kolkata, 700 073, India

Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Chellapandi and Himanshu, 2008) of which endoglucanase (endo-1, 4-β-glucanase or carboxy methyl cellulase) (EC 3.2.1.4), cleaves linkages at random and releases cellobiosyl units from the non reducing end of the cellulose chains (Bhat and Bhat, 1997). Endoglucanase has a wide range of applications in processing of food, animal feed, textile, fuel; chemicals industries, paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Tarek and Nagwa, 2007; Beguin and Auber, 1993; Howard *et al*., 2003).

This study focuses on utilization of various agricultural residues by the present working strain for the production of endoglucanase and evaluation of various fermentation parameters for enzyme production both in solid and submerged conditions.

MATERIALS AND METHODS

Organism

Rhizopus oryzae PR7 MTCC 9642, an endoglucanase producing strain was isolated from the decaying vegetation enriched soil of Eastern India. The strain was identified and deposited to Microbial Type Culture Collection, India. The present study was conducted between early summer to late fall of this year (April-August, 2009).

Chemicals

All chemicals used were of analytical grade.

Preparation of Inoculum

The fungus was grown in 1% PDA plates for 48 h at 28-30°C. The inocula were prepared by making hyphal discs (0.5 cm diameter). Each disc was used to inoculate 10 mL of medium (Ray and Chakraverty, 1998).

Cultivation of the Strain

The strain was cultivated in 100 mL Erlenmeyer flasks each containing 20 mL Basal Medium (BM) composed of (g L^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4.7H_2O$ 0.1 and carboxymethyl cellulose (CMC) 0.5. (pH: 6).

Cultivation in Solid State Fermentation Medium

Rhizopus oryzae was cultured in 100 mL Erlenmeyer flasks containing totally dried substrates and salts (based on 10 mL medium) moistened with 0.5 mL of distilled water at 37°C. Cultures were picked up at different time intervals, sterile water was added to make up its final volume to make it equivalent to that of 10 mL LSF media, followed by a thorough cyclomixing.

Measurement of Growth

The growth was measured by turbidometric method at 650 nm (Noisommit-Rizzi *et al*., 1996).

Enzyme Assay

In both LSF and SSF, the grown culture was filtered through filter paper (Whatman No. 1) and filtrate was used centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. To measure 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 5 min. The reducing

sugar released was measured by the dinitrosalicylic acid method (Bernfeld, 1955) taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of endoglucanase was defined as that amount of enzyme that liberated 1 milli mole of glucose per milliliter per minute of reaction.

Cellulosic Materials

Various cellulosic wastes were collected from roadside dumps, market effluents, agricultural fields and temple wastes. Those were dried, pulverized and sieved as 40 mesh particle size before using in fermentation media in place of pure carboxy methyl cellulose.

Pretreatment Procedures

Waste celluloses namely dusts of orange peel, dried flower and sugar cane bagasse were individually treated with 1(N) NaOH, 1(N) HCl and incubated at room temperature for 120 min. The mixtures were washed with distilled water to neutralize. The wastes were also treated by sterilizing at 121°C for 15 min for two successive times. These pretreated wastes were used as carbon sources in fermentation media.

Optimization of other Parameters

The inoculum size was optimized by cultivating the strain with varying the number of hyphal discs (1, 2 or 3) prepared from PDA plate containing *R. oryzae*. Agro waste supplemented cultures were incubated at 28°-30°C and 37°C to check the preferred temperature for endoglucanase production. The concentrations of agro wastes used as sole carbon source were varied to optimize the substrate concentration of submerged culture of *R. oryzae* PR7. Similarly, the effects of various nitrogen sources namely peptone, tryptone, urea and ammonium sulfate 0.09% (w/v) were tested. The optimum pH was determined by adjusting the pH of the agro waste supplemented liquid fermentation media at a range from 4.0-9.0. Fermentation time was optimized by putting various flasks each containing agro waste supplemented medium at 37°C for 24-96 h both at LSF and SSF of *Rhizopus oryzae* PR7. Each experiment was carried out in triplicate and their values were averaged.

RESULTS AND DISCUSSION

Effect of Agro Waste as Inducer of Endoglucanase Synthesis

R. oryzae PR7 was found to degrade various cellulosic agro wastes both in liquid as well as solid state fermentation conditions (Table 1), of which orange (*Citrus sinensis*) peel, dried flower (Marigold-*Calendula officinalis*) and sugar cane bagasse showed very promising results both in LSF and SSF, whereas dust from coconut shell showed good endoglucanase production only in LSF (Table 2). Therefore, further experiments were carried out with orange peel, dried flower and sugar cane bagasse only.

Effect of Temperature

Production of extra cellular endoglucanase by *R. oryzae* PR7 was preferred at 37°C, where under the influence of thermal dimorphism it took up the yeast like morphology instead of conventional filamentous form (Morrrow and Fraser, 2009). Although inoculum was prepared from mycelial form, enzyme production was found to be better by the yeast forms appeared at 37°C in all three types of agro waste supplemented fermentation media (Fig. 1).

Effect of Sate of Fermentation

Although, in other fungal strains, rate of production was higher in SSF than in submerged condition (Da-Silva *et al*., 2005), in the present strain, LSF of various agro wastes

Table 1: Effect of different agro wastes on endoglucanase production by *R. oryzae*

	Enzyme activity (U mL^{-1})	
Cellulose source	LSF	SSF
Carboxy methyl cellulose (Pure)	2.1	2.0
Orange peel	2.6	2.2
Sugar cane bagasse	2.1	1.8
Dried flower	2.5	2.1
Coconut shell	2.1	0.4
Water hyacinth	1.3	1.3
Rice straw	1.3	0.8
Coir	1.0	0.4
Potato jacket	0.9	0.6
Dried grass	1.2	0.5
Cotton seed	1.5	0.3
Jute stick	1.0	0.3
Jute fibre	1.0	0.2
Cotton	1.0	0.1
Neem leaf	1.1	0.9
Saw dust	1.3	0.7
Dried leaf	1.8	0.8
Rice husk	1.8	0.5
Betel nut shell	1.0	0.2

Cultivation time: 72 h

Table 2: Effect of pretreatment of agrowastes on the production of endoglucanase by *R. oryzae* PR7

	Endoglucanase activity (U mL^{-1})					
	Orange peel		Dried flower		Sugarcane bagasse	
Treatments	LSF	SSF	LSF	SSF	LSF	SSF
Untreated	1.7	2.0	1.4	2.5	0.4	0.1
1(N) NaOH	0.7	0.2	0.1	0.5	0.3	ND
1(N) HCl	1.0	0.9	0.7	2.0	0.3	0.2
Twice autoclaving	1.3	2.0	1.9	1.7	0.5	0.6

Cultivation time: 85 h

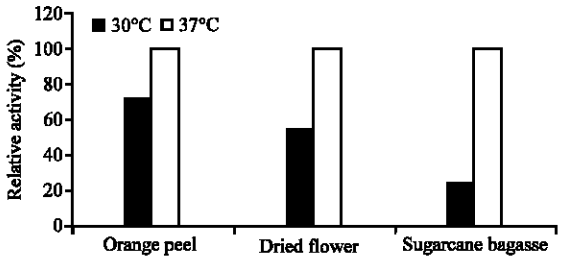

Fig. 1: Effect of cultivation temperature in agro waste supplemented culture media for the production of extra cellular endoglucanase by *R. oryzae* PR7

showed better results than that of their respective solid state cultures except in water hyacinth. This might be due to the fact that the yeast forms prefer to grow and synthesize enzymes in submerged condition as growth is restricted only to the surface of the solid matrix of SSF.

Effect of pH

Optimization of pH on enzyme production indicated that various source of cellulosic waste required various pHs for enzyme production, which might be due to their respective

lignin content. As for example the optimum pH for orange peel and sugar cane bagasse was 8.0, whereas it was 6.0 for dried flower (Fig. 2). The observation was comparable to other strains *Rhizopus* oryzae that can thrive in a pH range, of 5.0-7.0. (Murashima *et al*., 2002).

Effect of Substrate Concentration

All three agro waste substrates could best induce the growth and endoglucanase production at a concentration of 0.75% (w/v) (Fig. 3). Higher concentration of orange peel could restore the amount of enzyme production, but higher concentration of dried flower and sugar cane bagasse in the fermentation media reduced the enzyme production probably due to the adverse effect of higher concentration of nutrient supplements present in these substrates on enzyme production (Omojasola *et al*., 2008) or as a result of hindrance of mass transfer of oxygen by higher amount of solid substrate.

Effect of Nitrogen Sources

Among the nitrogen sources tested, ammonium sulfate was proved to be the best nitrogen source for enzyme production (Fig. 4) an observation similar to that of Kashem *et al*. (2004). But enzyme production was remarkably decreased in presence of urea, a report contrary to that of by *Aspergillus niger* (Acharya *et al*., 2008).

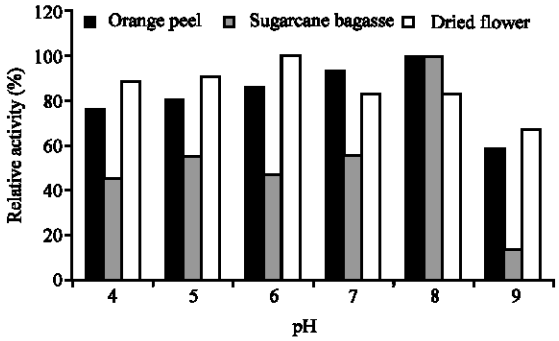

Fig. 2: Effect of pH of the agro waste supplemented culture media for the production of extra cellular endoglucanase by *R. oryzae* PR7

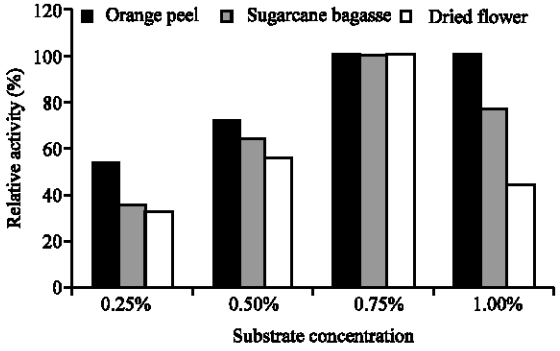

Fig. 3: Effect of concentration of cellulosic agro wastes in the culture media for the production of extra cellular endoglucanase by *R. oryzae* PR7

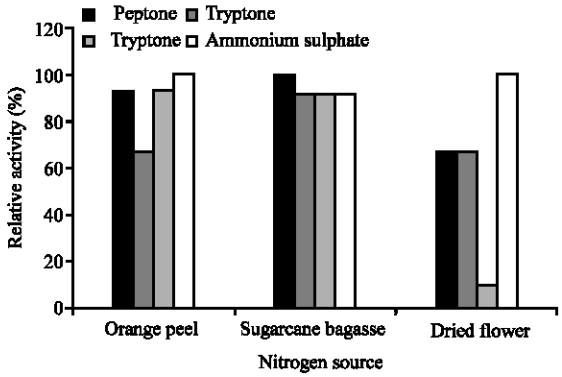

Fig. 4: Effect of various nitrogen sources in agro waste supplemented culture media for the production of extra cellular endoglucanase by *R. oryzae* PR7

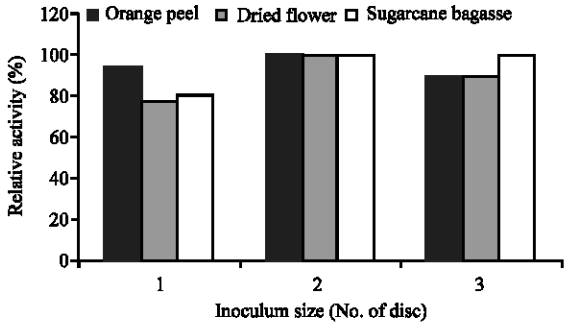

Fig. 5: Effect of inoculum size in agrowaste supplemented culture media for the production of extra cellular endoglucanase by *R. oryzae* PR7

Effect of Inoculum Size

Inoculum size also affected the cellulase production, as highest enzyme production was achieved (Fig. 5) when the culture media supplemented with all three types of agro wastes were inoculated with 2 discs of 0.5 cm diameter. Presence of higher load of fungal mass reduced the enzyme production (Acharya *et al*., 2008).

Effect of Agitation

Agitation caused remarkable loss of enzyme production although the fungal growth was significantly increased (data not shown), a report contrary to that from *Aspergillus niger* (Acharya *et al*., 2008).

Effect of Substrate Pretreatment

Pretreatment showed adverse effect on enzyme production in orange peel supplemented culture media, both in LSF and SSF. The alkali and acid pretreatment showed absolutely no effect in all types of waste supplemented media, whereas double sterilization brought about 1.4 times increase in LSF of dried flower. The most remarkable effect was seen in sugar cane bagasse containing media, with an increase of 1.25 and 6 times in LSF and SSF, respectively. Various pretreatment measures of cellulosic biomass were observed to enhance cellulase production (Milala *et al*., 2009; Acharya *et al*., 2008; Youssef and Betrkaa, 2009),

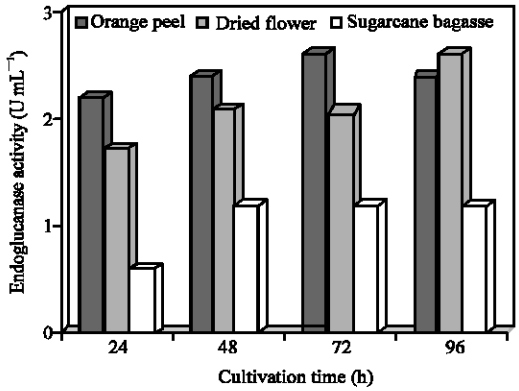

Fig. 6: Time course of extra cellular endoglucanase production by *R. oryzae* PR7 in LSF of various cellulosic agro wastes

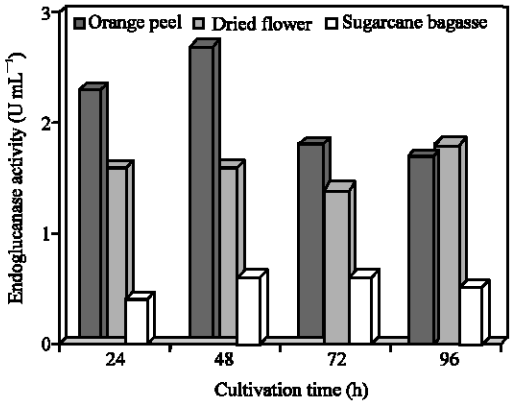

Fig. 7: Time course of extra cellular endoglucanase production by *R. oryzae* PR7 in SSF of various cellulosic agro wastes

which were probably due to the removal of lignin from lignocellulosic substrates (Pan *et al*., 2005) making them more susceptible to the enzymatic hydrolysis. In the present study, no such increase was found by acid and alkali pretreatment, an observation similar to that of Gharpuray *et al*. (1983).

Effect of Cultivation Time

Highest endoglucanase production could be achieved within 48 h of cultivation in case of LSF of dried flower and sugar cane bagasse (Fig. 6). This rapid rate of growth confers with the similar growth rate of *Rhizopus stolonifer* (Pothiraj *et al*., 2006). But orange peel containing LSF medium took more time (72 h) to attain highest enzyme synthesis. Enzyme production did not further increase after 48 h of growth in SSF (Fig. 7).

CONCLUSION

Fungi are the main cellulase producing micro organism and *Aspergillus* and *Trichoderma* are the main fungal genera that were used for commercial production of

Table 3: Spectrum of fungal strains producing cellulase from wastes

Strain	Wastes used	Fermentation conditions	References
A. niger	Saw dust	SSF	Acharya *et al.* (2008)
A. niger	Sorghum bran	LSF	Abu *et al.* (2000)
A. terreus	Cassava waste	SSF	Pothiraj *et al.* (2006)
Rhizopus stolonifer			
A. terreus	Bagasse	LSF	Yousssef and Betrkaa (2009)
A. terreus	Water hyacinth		Ali *et al.* (1991)
A. niger, A. fumigatus	Coir waste, saw dust	LSF	Immanuel *et al.* (2007)
A. heteromorphus	Wheat straw	LSF	Singh *et al.* (2009)
A. nidulans	Rice husk, Millet straw,	LSF	Milala *et al.* (2005)
A. candidus	guinea corn straw, saw dust		Milala *et al.* (2009)
Trichoderma harzianum	Sugarcane bagasse	SSF	Rezende *et al.* (2002)
A. niger	Pineapple waste	LSF	Omojasola *et al.* (2008)
T. longibrachiatum	Orange waste	SSF	Omojasola and Jilani (2008)
Saccharomyces cerevisiae			
T. ressei	Corn cob	SSF	Kassim *et al.* (2004)
			Liming and Xueliang (2004)
T. harzianum	Oil palm biomass	SSF	Alam *et al* (2005)
T. lignorum	Banana wastes		Baig *et al.* (2004)
Thermoascus aurantiacus	Wheat bran, sugarcane bagasse, orange bagasse, corncob, green grass, dried grass, sawdust and corn straw	SSF	Da-Silva *et al.* (2005)
Rhizopus oryzae	Dried flower, orange peel, sugar cane bagassse and others	LSF and SSF	This study

cellulase (Milala *et al.*, 2009; Person *et al.*, 1991). So far the literature study is concerned, most of the studies regarding cellulase production by agro waste degradation have been carried out with these two genera (Table 3), whereas similar report with *Rhizopus* sp., is almost rare. On the other hand, a number of work have been done on wastes like rice and wheat straw (Kocher *et al.*, 2008; Singh *et al.*, 2009) sugar cane bagasse (Rezende *et al.*, 2002) and orange waste (Omojasola and Jilani, 2008), no work has been reported from fermentation of dried flower (Table 3). Therefore the present strain of *Rhizopus oryzae* showing the ability to synthesize high amount of extra cellular endoglucanase within a relatively short period of time, utilizing agro wastes like dried flower, orange peel and sugar cane bagasse, that would otherwise cause environmental pollution, could be used for rapid and commercial production of cellulase.

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