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PRODUCTION OF CELLULASE BY *ASPERGILLUS NIGER* UNDER SUBMERGED AND SOLID STATE FERMENTATION USING COIR WASTE AS A SUBSTRATE

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ABSTRACT

Aspergillus niger was used for cellulase production in submerged (SmF) and solid state fermentation (SSF). The maximum production of cellulase was obtained after 72 h of incubation in SSF and 96 h in Smf. The CMCase and FPase activities recorded in SSF were 8.89 and 3.56 U per g of dry mycelial bran (DBM), respectively. Where as in Smf the CMase & FPase activities were found to be 3.29 and 2.3 U per ml culture broth, respectively. The productivity of extracellular cellulase in SSF was 14.6 fold higher than in SmF. The physical and nutritional parameters of fermentation like pH, temperature, substrate, carbon and nitrogen sources were optimized. The optimal conditions for maximum biosynthesis of cellulase by *A. niger* were shown to be at pH 6, temperature 30 ºC. The additives like lactose, peptone and coir waste as substrate increased the productivity both in SmF and SSF. The moisture ratio of 1:2 (w/v) was observed for optimum production of cellulase in SSF.

Key words: *Aspergillus niger*, coir waste, cellulase, submerged fermentation, solid-state fermentation.

INTRODUCTION

Complete cellulose hydrolysis to glucose demands the action of exoglucanases, endoglucanases and β-glucosidases. Exoglucanases (1,4-β-D-glucancellobiohydrolase, EC 3.2.1.91) are usually active on crystalline cellulose and cleave disaccharide units either from non-reducing or reducing end. Endoglucanases (1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) are more active against the amorphous regions of cellulose and they can also hydrolyze substituted celluloses, such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC) internally. β-Glucosidases (EC 3.2.1.21) cleave cellobiose and other soluble oligosaccharides to glucose (10). These enzymes find potential applications in the production of food, animal feed, textile, fuel, chemical, pharmaceutical industries and in waste management (9, 40).

The production of cellulase has been reported from a wide variety of bacteria (22) and fungi (4, 34). However, filamentous fungi are preferred for commercial enzyme production, because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria (8). Almost all fungi of genus *Aspergillus* synthesize cellulase, therefore this genus has the potential to dominate the enzyme industry. *Aspergillus* and *Trichoderma* spp. are well known

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efficient production of cellulases (33).

Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. However, solid state fermentation (SSF) technique can improve the yield and reduces the cost of enzyme production (16, 21). Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water contents (30). There are several reports describing use of agro industrial residues for the production of cellulose such as wheat straw, wheat bran and rice straw as substrates (11, 35, 2, 19). The other advantages of SSF include superior productivity, simple technique; low capital investment, low energy requirement and less water output (42, 38), better product recovery and lack of foam build up and reported to be most appropriate process for developing countries (39).

The cellulase production by filamentous fungi in SmF and SSF has been studied extensively (15, 1, 38). However, there is no report on comparison of the cellulase production by SmF and SSF conditions. From this point of view, the organism was isolated and demonstrated for their improved efficiency in SmF and SSF for the production of cellulase using agro-industrial waste as raw material. The influence of various parameters was evaluated under two different fermentation conditions. In the present study, we describe the comparison of cellulase production by *Aspergillus niger* in SmF and SSF systems.

MATERIALS AND METHODS

Microorganism

The fungus was isolated from coir retting ground area of Karimangalam, Dharmapuri Dist, Tamil Nadu, India. The isolate was grown on CMC agar medium and screened according to Wood and Bhat (41). The culture was identified based on colony morphology and microscopic examination (Devenathan *et al*. 2007). The isolated fungal colony was subcultured and maintained on Czapek-Dox-agar slants and stored at 4 ℃ in a refrigerator, until needed.

Pre-treatment of substrates

The procured cellulosic substrates such as rice husk, rice bran, coir waste, wheat bran and saw dust were ground to fine powder and the substrates were individually treated with 1% (w/v) NaOH solution in the ratio of 1:10 (substrate: solution) for 1h and was brought to neutral pH by washing thoroughly with distilled water and dried at room temperature. The treated substrates were autoclaved at 121℃ for 1 h (18).

Preparation of inoculum

The inoculum was prepared by growing the organism in 250 ml Erlenmeyer flask with 100 ml of Czapek-Dox broth containing g/l of sucrose, 30; sodium nitrate, 3; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, trace; agar, 15. The medium was inoculated from the Czapek-Dox agar slants and incubated at 30 °C for 3 days in a shaker (200 rpm) before it was used for the fermentation process.

Submerged fermentation (SmF)

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium. The composition of the medium contained the following g/l of distilled water. L-Glutamic acid, 0.3; NH_4NO_2, 1.4; K_2HPO_4, 2.0; $CaCl_2$, 2.0; $MgSO_4$, 0.3; protease peptone, 7.5; $FeSO_4$, 5.0; $MnSO_4$, 1.6; $ZnSO_4$, 1.4; tween 80, 20 % (v/v) ; coir waste, 30. The medium was sterilized by autoclaving at 121℃ for 15 min. Each flask was inoculated with 1ml of the above said inoculum. The cultures were incubated on a rotary shaker (120 rpm) at 30℃ for 72 h.

Solid state fermentation (SSF)

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks that contained 10 g of coir waste and 15 ml of distilled water (moistening agent). The flasks were sterilized

at 121℃ for 15 min and cooled to room temperature. About 1ml of inoculum was added, mixed well and incubated at 30℃ in a humidified incubator for 96 h. The flasks were periodically mixed by gentle shaking.

Enzyme extraction

At the end of the fermentation the culture broth from submerged fermentation was centrifuged at 6000 rpm for 15 min and the supernatant was used as a source of extracellular enzyme. In solid state fermentation (SSF) the enzyme was extracted from the coir waste by mixing homogenously the entire waste with (1:10 w/v) distilled water and agitated on a rotary shaker (120 rpm) at 30 ℃ with a contact time of 1h. Dampened cheese cloth was used to filter the extract and pooled extracts were centrifuged at 6000 rpm for 15min and the clear supernatant was used as a source of extracellular enzyme.

Enzyme assay

Cellulase [(filter paperase (FPase) and carboxymethyl cellulase (CMCase)] activities were assayed according to the method described by Ghose (17).One unit of enzyme activity (CMCase and FPase) is defined as the amount of enzyme which releases 1 µmole of reducing sugars per min with glucose as standard, under the assay condition described above. The values of enzymatic activity were expressed as U/ml for SmF and U/g of dry mycelial bran (DMB) for SSF.

Optimization of process parameters in SmF and SSF

Evaluation of optimized culture conditions in SSF and SmF using *Aspergillus niger:* The protocol adopted for the standardization of fermentation parameters was to evaluate the effect of an individual parameter. The parameters optimized were: substrates (rice husk, rice bran, coir waste, wheat bran, saw dust), temperature (20 to 40 ℃), pH (4.5 to 8), fermentation period [(24 to 192 h in SmF) and (24 to 120 h in SSF)].

Effect of supplements (carbon & nitrogen sources)

Smf: Various carbon and nitrogen sources at concentration of 5% w/v were supplemented as individual components to the fermentation medium containing coir waste as substrate. The medium was inoculated and incubated at 30 ºC for 3 days in an orbital shaker incubator (120 rpm). The broth was centrifuged and the enzyme assay was carried out. Care was taken to see that monosaccharides and disaccharides were prepared as 10 X solution and sterilized separately by autoclaving at 10 lbs for 10 min. and required concentration was added to the medium before inoculation.

SSF: Various carbon and nitrogen sources at concentration of 4% w/v were supplemented as individual components to the flasks that contain 10 g of coir waste and moistened with 15 ml of distilled water (moistening agent). The flasks were sterilized and cooled to room temperature. About 1 ml of inoculum was added, mixed well and incubated at 30ºC in a humidifying incubator for 4 days. At the end of fermentation the contents from each flask were extracted with distilled water and the supernatant was used for enzyme assay.

The effect of moisture content in SSF was studied by varying the ratio of coir waste to distilled water (2.5 to 12.5).

The optimum conditions for enhanced cellulase production were verified by performing the experiments using the selected parameters at their respective optimum conditions.

Comparative evaluation of SmF and SSF systems for enzyme production

The enzyme yield produced in SmF was compared with the yield obtained in SSF using coir waste as substrate according to Solis Pereira *et al* (36).

RESULTS AND DISCUSSION

Isolation and screening of cellulolytic organism

The soil sample collected from coir retting zone area was serially diluted in saline and was spread on to the

carboxymethyl cellulose (CMC) agar plates. The plates were incubated at 30ºC for 3-5 days. Cellulase producing fungal colonies were selected after flooding the plates with congo red (0.1% w/v), followed by destaining with NaCl (0.1 M). The colony that showed largest halo forming zone was selected for the present study.

Identification of fungal isolates

Colony morphology: The selected strain was subcultured on CMC agar medium. Initially the colonies were white and changed to black, as culture matured. When immature the colonies were covered with white fluffy aerial mycelia, while mature colonies showed salt and pepper effect which was covered with black spores and reverse of the colony was buff colored.

Microscopic examination of the isolates

The matured colonies were subjected to lacto phenol cotton blue staining and microscopic examination was made. From the microscopic observation it was clear that the colony showed the hyaline septate hyphae. The conidial head was large and appeared black to brownish black. The conidiophores were hyaline or brownish near the vesicle. Each vesicle appeared globose in shape and cover with brownish sterigmata on the entire surface in two series. Based on the colony morphology and microscopic observation the strain was confirmed as *Aspergillus niger* (12).

Optimization of fermentation parameters in SmF and SSF

Substrates: Among the 5 substrates screened, coir waste gave the maximum cellulase production when fermented with *Aspergillus niger* under SmF and SSF (Table 1). Considerable amount of enzyme production was observed on wheat bran and rice bran. Comparatively less enzyme production was observed with the rest of the substrates in both SmF and SSF. Since coir waste showed maximum production of cellulase, it was selected for further optimization studies for SmF and SSF systems. In a study conducted by Ojumu *et al* (29) on *Aspergillus flavus* reported that the saw dust, corncobs and bagasse were found to be the best substrates for the production of cellulase. Oberoi *et al* (28) reported kinnow pulp as the best substrate for the production of cellulase in SSF. Sugarcane bagasse, tea production waste, coconut coir pith, rice husk, wheat bran, rice bran *etc*., have been employed for production of cellulase using a variety of microorganisms such as *Trichoderma, Aspergillus, Penicillium, Botrytis, Neurospora etc*., (31).

Temperature

Incubation temperature plays an important role in the metabolic activities of a microorganism. In the present study the optimum temperature for maximum enzyme production was recorded at 30ºC under SmF and SSF (Table 1). About 83 % of cellulase production was observed at 35ºC. Whereas more than 50 % of cellulase was produced when grown at 20, 25 and 40ºC in SmF. 55 and 68% of cellulase production was recorded when the organism was grown at 40 and 20ºC, respectively in SSF. The results of SmF process in the present study confirm the findings of Devanathan *et al* (12) and Kathiresan and Manivannan (23) for *Aspergillus* sp. Asquieri and Park (6) found that the optimum temperature for production of CMCase from thermostable *Aspergillus* sp. was 37ºC, whereas the maximum cellulase production was observed at 40ºC for *Aspergillus terreus* QTC 828 (3). In general the temperature maintained in SSF system is in the range of 25 to 35ºC and depends on the growth kinetics of the microorganism employed rather than on the enzyme produced (25). Ali *et al.* (3) reported maximum yield of cellulase from *Aspergillus niger* Z10 strain and *A. terreus* at 40ºC, respectively in SSF.

pH

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed

during the growth of microbes also affects product stability in the medium (20). Optimum pH for maximum production of cellulase was 6.0 when grown in SmF and SSF (Table 1). Similar observation was made for cellulase production by *A. terreus* QTC 828 in SmF by Ali *et al* (3) and *Trichoderma reesei* in SSF by Doppelbauer *et al* (13), whereas pH 7 was reported by Krishna (24) for the production of bacterial cellulases by using banana wastes in SSF.

Fermentation period

Table 1 shows the effect of fermentation period on the production of the cellulase in SmF and SSF. In the present study cellulase activity increased steadily and reached maximum at 96 h of incubation when grown in SmF. These results are in agreement with the reports made by Devanathan *et al* (12) and Acharya *et al* (1). However, further increase in the incubation time reduced the enzyme production. It might be due to the depletion of macro and micronutrients in the fermentation medium with the lapse in time, which stressed the fungal physiology resulting in the inactivation of secreting machinery of the enzymes (27). Incubation time necessary for optimal production was observed at 72 h in SSF. Short incubation period for enzyme production offers the potential for inexpensive production of enzyme (37). Muniswaran and Charyulu (26) have also reported similar trend in cellulase production using *Trichoderma viride*.

Table 1. Effect of physical parameters on cellulase production in SmF and SSF by *A. niger*

Parameter	SmF (U/ml)		SSF (U/g DMB)	
	CMCase	**FPase**	**CMCase**	**FPase**
Substrates				
Rice husk	0.31	0.25	0.49	0.47
Rice bran	0.51	0.22	1.1	0.5
Coir waste	0.8	0.51	3.42	1.77
Wheat bran	0.72	0.43	1.77	0.8
Saw dust	0.18	0.12	1.2	0.52
Temperature (^{O}C)				
20	1.5	0.8	3	1.51
25	1.5	1.3	3.6	2
30	3.4	1.7	4.44	2.51
35	3.1	1.3	3.57	2.45
37	1.9	1.2	3.51	1.46
40	1.7	1.1	2.5	1.3
pH				
4.5	0.6	0.3	8.2	3.3
5.0	0.81	0.61	9.2	4.1
5.5	1.5	0.82	9.6	4.3
6.0	2	0.91	9.8	4.6
6.5	1.81	0.82	9	2.9
7.0	1.71	0.81	7.3	2.5
7.5	1.3	1.26	6.8	2.2
8.0	0.9	0.5	6.6	2.1
Fermentation period (h)				
24	2.46	1.37	4.5	2.4
48	2.51	1.46	5.3	2.5
72	3	1.53	9	3.6
96	3.46	0.88	7.1	2.3
120	2.2	1	6.9	2.1
144	1.5	0.51	–	–
168	0.52	0.49	–	–
192	0.5	0.47	–	–

Moisture content

Moisture content is a critical factor in SSF processes because this variable has influence on growth, biosynthesis and secretion of enzyme. In the present study the maximum enzyme production was obtained at coir waste to distilled water ratio of 1:2. (Fig.1). Any further increase and decrease in the ratio resulted in decreased enzyme activity. Similar observation was made by Anto *et al* (5) for production of α-amylase by *Bacillus cereus*, whereas Babu and Satyanaryana (7) reported maximum α-amylase enzyme production by thermophilic *Bacillus coagulans* at a high level of substrate moisture ratio of 1: 2.5. According to Lonsane *et al* (25), lower moisture content causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension. On the other hand, higher moisture levels can cause a reduction in enzyme yield due to steric hindrance of the growth of the producer strain by reduction in porosity (interparticle spaces) of the solid matrix, thus interfering oxygen transfer.

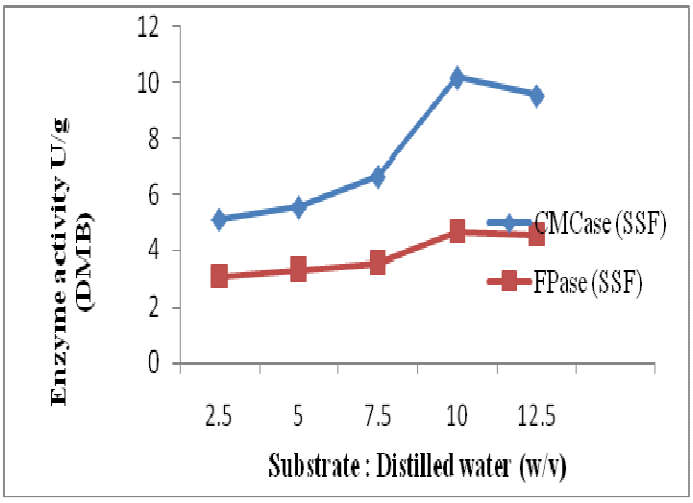

Figure 1. Effect of moistening agent on cellulase production by *A. niger* in solid state fermentation with coir waste as substrate

Inoculum size

In the present study there is a significant increase in cellulase production with an increase in inoculum concentration from 5 to 15 % and found to be maximum at 15%. Higher or lower inoculum size resulted in a significant decrease in enzyme production (Fig.2). Similar findings have been reported for the production of bacterial cellulases by solid state bioprocessing of banana wastes (24). In contrast 10% of inoculum was reported to be optimum for the production of cellulase by *Aspergillus niger* under SSF (15).

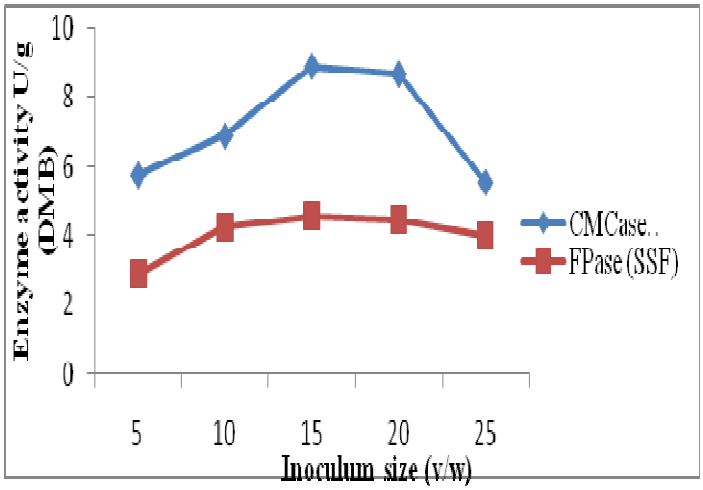

Figure 2. Effect of inoculum size on cellulase production by *A. niger* in solid state fermentation with coir waste as substrate

Carbon sources

Cellulase production was found to be dependent upon the nature of the carbon source used in the culture media. To evaluate the carbohydrates to cause induction or repression of cellulase, the organism was grown on monosaccharide and disaccharides. In general, enhanced production of enzyme was observed with all the tested sugars when compared to the control. Among the carbon sources examined, lactose was found to be the best inducer in SmF and SSF (Table 2). This study substantiates the work of Kathiresan and Manivannan (23) and Devanathan (12) who demonstrated lactose as best inducer of *Aspergillus* sp. Nochure *et al* (27) identified fructose as the best inducer of cellulase in *Clostridium*

thermocellum. In another study dextrin was found to enhance the production of cellulase by *Trichoderma* sp. in SSF (32). Increase in enzyme production with additional carbon sources have been demonstrated by both the SmF and SSF systems as a result of good growth (36).

Nitrogen sources

The effect of supplementation of nitrogen sources in SmF and SSF system on the enzyme production is shown in Table 2. All the nitrogen sources enhanced cellulase production when compared to control. Among them peptone supported maximum enzyme production followed by beef extract, groundnut oilcake, yeast extract and casein. These results are in agreement with the reports of Kathiresan and Manivannan (23) and Devanathan *et al.* (12) for production of cellulase by *Penicillium fellutanum* and *Aspergillus niger*, respectively in SmF. Enari *et al.* (14) reported that good cellulase production can be obtained with peptone as the organic nitrogen source in SSF.

Table 2. Effect of carbon and nitrogen sources on cellulase production in SmF and SSF by *A. niger*

Supplement	SmF (U/ml)		SSF (U/g DMB)	
	CMCase	FPase	CMCase	FPase
Carbon sources (5 % w/v in SmF and 4 % w/w in SSF)				
Control	0.7	0.4	3.7	2
Glucose	2.52	0.54	12.1	6.5
Xylose	2.2	1.42	15.7	6.6
Lactose	3	1.71	18	10.9
Maltose	2.51	1.5	17.5	6.3
Sucrose	2.54	1.51	13.7	6.2
Nitrogen sources (5 % w/v in SmF and 4 % w/w in SSF)				
Control	0.56	0.4	3.8	2
Yeast extract	1.1	0.6	8.2	4.2
Beef extract	1.53	0.9	11	4.5
Peptone	2.1	1.36	13.8	6.2
Groundnut oil	1.61	1.1	8.1	4.3
Casein	0.53	0.47	4.2	3.8
Sodium nitrate	–	–	4	3.6

Enzyme yields

Under the optimum conditions, the strain produced 2.04 units of cellulase per ml of culture broth (Fig. 3) in SmF and 29.11units of cellulase per gram of dry mycelial bran in SSF.

Comparative evaluation of SmF and SSF system for enzyme titres

The method adopted for comparison of submerged and solid state fermentation is the same as reported by Solis-Pereira *et al.* (36) for pectinase production from *Aspergillus niger* by dividing the yield obtained from SSF in U/g DMB with the yield from SmF in U/ml culture broth. With this method, cellulase production by *A. niger* in SSF and SmF using coir waste as substrate, were compared in terms of their extracellular enzyme production in U/g DMB and U/ml, respectively (Fig. 3). When comparison made between SmF and SSF, production of total cellulase by SSF was 14.6 fold higher than that of SmF.

In conclusion the result of the present study clearly indicates the potential of *Aspergillus niger* can be successfully

cultivated under SSF conditions for the production of cellulase using coir waste as substrate. The coir waste is abundantly available in India as it is one of the major producers of coconut. Having considered the means of reducing disposal problem, therefore it can be effectively utilized by a potential strain like *Aspergillus niger* for production of cellulase which is commercially important.

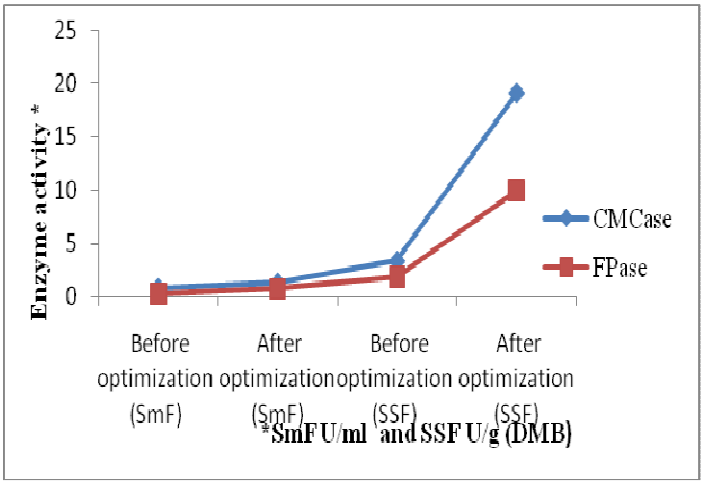

Figure 3. comparative evaluation of cellullase production in SmF and SSF using *Aspergillus niger.*

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