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Optimized Production of Cellulase by *Aspergillus niger* Using *Ricinus communis* Seed Coat Waste

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A study was conducted to find out the potential of castor seed coat waste for production of cellulase by *Aspergillus niger*. The powdered waste was fermented in a flask containing mineral salt medium. Various process parameters were optimized for enhancing the yield of cellulase. The maximum activity of CMCase (28.14 U/ gram dry substrate) and FPase (18.14 U/ gram dry substrate) were recorded under optimum conditions of inoculum size (9 discs), incubation period (120h), substrate concentration (7g), pH (5.5), temperature (35°C), moisture level (1:3), CMC (0.5%) as carbon source and pepton (0.5%) as nitrogen source. From this study, it can be concluded that *Aspergillus niger* holds the potential of converting castor seed coat waste into cellulose.

Key words: CMCase and FPase activity, agricultural waste, optimization.

Cellulase is an important industrial enzyme produced by a number of microorganisms. Plant biomass containing lignin, hemicelluloses and cellulose in appreciable amount is a rich source of substrate for the production of cellulase.[1,2] Many species of fungi efficiently produces enzymes that enable them to break down these polysaccharides and proteins into sugars and amino acids that can be assimilated easily. Microbial fermentation processes are broadly of two types- solid state fermentation and submerged fermentation. Solid state fermentation is cheaper and its cultural conditions like lower water activities are especially suitable for the growth of fungal species.[3,4] Castor (*Ricinus communis*) is a flowering plant of Euphorbiaceae family, commonly known as oil plant and palm of christ due to use of its oil in perfume base industries and medicinal uses. Present study is an attempt to fermentatively produce cellulase from *Ricinus communis* seed coat waste using *Aspergillus niger* at laboratory level and to optimize the fermentation conditions for its maximum production.

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MATERIALS AND METHODS

Chemicals

Analytical grade chemicals and media from Himedia were used.

Substrate

Ricinus communis (castor) seed coat waste was collected from Village- Sadra, District- Gandhinagar, Gujarat, India. It was then grinded to pass from 3mm sieve and stored in air tight plastic jar for future use.

Isolation and Identification of organism

Five different fungal species were isolated from farm soil of Village- Sadara using CMC agar. Out of five isolates, one, potent cellulose degrading fungi was screened out on the basis of their higher enzyme activity and identified.

Inoculum preparation and Solid State Fermentation (SSF)

Conidial spore of fungi from 7 day old PDA slant was inoculated on potato dextrose agar (PDA) plate and incubated at 30°C for 5-7 days. Discs were prepared by flame sterilized cork borer and used as inoculum. The SSF was carried out in 250 ml Erlenmeyer flask. For it 5g of substrate was wetted with 10ml of mineral salt solution, autoclaved, cooled to room temperature, inoculated with five discs of inoculums from PDA plate and incubated at 30°C for 7 days. After incubation period, the enzymatically hydrolyzed sugars were estimated by adding 50ml of citrate buffer (0.05M, 4.8pH). The extracts were centrifuged at 50 rpm for 15 min at 4°C to remove debris and supernatant was used for enzyme assay.

Optimization parameters for potential degraders (CMCase and FPase)

Optimization for moistening agent, incubation time, inoculums size, substrate concentration, pH, temperature, moisture content, carbon and nitrogen sources was done for potential degrading fungal species to produce higher cellulase activity. All the inoculated flasks were assayed for CMCase and FPase activities and expressed as Units per gram dry substrate (U/gds).

Effect of Moistening agent

In addition to mineral salt medium, Mandels and waber medium, Czapek-dox salt solution, Mandel's and Stenberg medium, Berg's medium were used as moistening agent. 5g substrate was taken in different Erlenmeyer flasks and different moistening agents were added to it. These flasks were autoclaved at 121°C at 15 psi for 15 min and then inoculated. These flasks were incubated for 7 days. After incubation the flasks were assayed for CMCase and FPase activities.

Effect of Incubation time

The optimized medium was autoclaved and inoculated with 7 days old PDA culture. The flasks were incubated at 30°C temperature. After 24 hr interval, starting from 72hr to 168hr of incubation, the flasks were assayed to check the maximum enzyme activity.

Effect of Inoculum size

All flasks were inoculated with 7 days old PDA plate containing various number of spore such as 3, 5, 7, 9, 11 and 13 discs of 0.5 cm diameter and incubated at 30^0C for optimized incubation time.

Effect of substrate concentration

In our study various substrate concentration ranged 3g, 4g, 5g, 6g, 7g and 8g were used. All the flasks were moistened with optimized concentration of moistening agent and autoclaved. The flasks were then inoculated and incubated at 30°C temperature for optimized incubation time.

Effect of pH

Enzyme production is very sensitive to pH of the fermentation medium which was determined by adjusting the pH of culture medium at different levels like, 4.0, 4.5, 5.0, 5.5., 6.0 and 6.5.

Effect of Temperature

In order to determine the optimized temperature, inoculated fermented medium containing flasks were kept at 25°C, 30°C, 35°C, 40°C and 45°C.

Effect of Moisture level

Fungi prefer moist environment for their growth. All the flasks containing substrate moistened with various ratio of substrate to moistening agent such as 1:1, 1:2. 1:3 and 1:4.

Effect of Carbon source

0.1% of different carbon sources such as sucrose, carboxymethyl cellulose, xylose, wheat flour, and cellobiose were added in optimized moistening agent to check their effect on the synthesis of cellulase.

Effect of Nitrogen source

Various nitrogen sources such as ammonium nitrate $[(NH_4)_2NO_3]$ potassium nitrate $[KNO_3]$, yeast extract, protease peptone, urea at 0.5% concentrations were added to optimized moistening agent. All the flasks were autoclaved and inoculated and incubated at optimized parameters.

Enzyme assay (CMCase and FPase)

Cellulase activity (CMCase and FPase) was determined at 50°C by using carboxymethyl cellulose sodium salt (1mL) and whatman no.1 filter paper (50 mg) as a substrate in 1 mL 0.1 M citrate buffer (pH 4.8) and 0.5 ml of enzyme extract respectively. The mixture was incubated at 50°C for 30 min. The reducing sugars released were measured using 3,5-dinitrosalicyclic acid (DNSA). Control is prepared with 10 min boiling of enzyme extract and then immediately kept at 0°C. One unit of endoglucanase activity was expressed as the amount of enzyme required to release 1 µmol

reducing sugars per ml under the above assay condition using glucose as a standard.[5]

RESULTS AND DISCCUSION

Screening, Isolation and Identification

Five different types of fungi were isolated using CMC agar and named FCAS-1, FCAS-2, FCAS-3, FCAS-4, and FCAS-5. Their CMCase activity is shown in Table 1. FCAS-1 fungal species showing highest CMCase acticity was chosen for further study and identified as *Aspergillus niger* at Agharkar Research Institute, Pune, India.

Optimization study

Optimization is an important aspect in order to find out suitable growth conditions for maximum microbial biosynthesis of cellulolytic enzyme. It induces parameters such as moistening agent, incubation time, inoculum size, substrate concentration, pH, temperature, moisture level, carbon source and nitrogen source.

Effect of moistening agent

To find out the best fermentation medium supporting maximum biosynthesis of cellulase, Mineral Salt Solution (MSS), Czapek Dox Medium (CDM), Mandel's and Sternberg salt Medium (MSM), Berg's Medium (BM), and Mandel's and Waber Medium (MWM) were used for this study. Data (Table-2) show that the maximum CMCase (5.83 U/gds) and FPase (3.79 U/gds) activities with *Aspergillus niger* were recorded in Mineral Salt Solution. Maximum cellulase production in mineral salt solution has also been reported.[6,7] The higher activity might be due to the capacity of this medium to supply all the required nutrients and minerals to the fungal isolate for its maximum activity.

Effect of Incubation time

In our experiment, enzyme production was carried out after 72 hr of incubation upto 168hr. This study shows maximum CMCase (8.79 U/gds)

Table 1. CMCase activities of various fungal isolates

Fungal Isolates	CMCase activities (U/gds)
FCAS-1	4.53
FCAS-2	3.33
FCAS-3	0.55
FCAS-4	1.48
FCAS-5	2.87

Table 2. Effect of different moistening agent on cellulase activity

Moistening agent	Cellulase activity (U/gds)	
	CMCase	FPase
Mineral Salt Solution	5.83	3.79
Czapek-dox Medium	5.09	2.59
Mandel's and Stenberg medium	3.88	2.49
Berg's medium	1.29	1.29
Mandels and Waber medium	2.49	2.49

Table 3. Effect of different incubation time on cellulase activity

Incubation Time(hour)	Cellulase activity (U/gds)	
	CMCase	FPase
72	7.69	4.07
96	7.96	4.90
120	8.79	5.84
144	5.64	5.74
168	5.55	5.55

Table 4. Cellulase activity at different inoculum size

Number of discs	Cellulase activity (U/gds)	
	CMCase	FPase
3	6.66	4.44
5	7.59	4.53
7	10.36	4.90
9	12.77	5.83
11	9.44	3.88
13	8.51	3.79

Table 5. Cellulase activities at different substrate concentration

Substrate Concentration (%)	Cellulase activity (U/gds)	
	CMCase	FPase
3	9.07	4.44
4	14.07	5.55
5	15.36	5.92
6	15.73	7.59
7	16.11	8.51
8	14.07	5.92

and FPase (5.84 U/gds) activity at 120h (**Table 3)**. Result obtained are in agreement with studies in which the enzyme activity increased progressively with the incubation from 0 to 120h and reached the maximum (2.3 U/gds) at 120h.[8] After 120h, the decreased enzyme activity was also observed.[9-11] This decrease in enzyme activity may be due to depletion of nutrients in the medium causing stress on the fungal physiology and inactivation of enzymes secretion.[9] The decrease in enzyme activity may also be due to accumulation of harmful byproducts in the culture medium and proteolysis of enzyme[12].

Effect of Inoculum size

Inoculum size also affects the maximum cellulase enzyme production.[13] In our study, fermentation medium was inoculated with 3, 5, 7, 9, 11 and 13 discs of 0.5 cm diameter each. The obtained results showed that maximum CMCase activity (12.77 U/gds) and FPase activity (5.83 U/gds) was recorded with 9 discs (**Table 4)**. In our study, the enzyme activity increase progressively with the inoculums size from 3 to 9 discs, reached the maximum at 9 discs and decreased thereafter. It can be explained on the basis of fact that the lower inoculums size required longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme whereas the higher inoculum size may cause nutrient depletion and reduced metabolic activity. Therefore, a balance between the proliferating biomass and available nutrient would yield an optimum at which the enzyme synthesis would be maximum.[8]

Effect of Substrate concentration

The sufficient substrate concentration is one of the important factor in SSF. In our experiment 3 to 8% of castor seed coat were used in flask. Our results show maximum CMCase activity (16.11 U/gds) and FPase activity (8.51 U/gds) at 7% substrate concentration (**Table 5).** Higher substrate concentration may reduce the enzyme production.[6]

Effect of pH

The effect of H^+ ion concentration has a marked effect on microbial activities. Maximum CMCase activity (22.49 U/gds) and FPase activity (9.44 U/gds) were observed at 5.5 pH (**Table 6**). Maximum activities of CMCase and FPase at 5.5 pH has been observed by many workers.[1,14-17] The instability of these enzymes is very low and high pH values is due to fact that they are proteins which are generally denatured at extreme pH values.[10] Higher growth and enzyme biosynthesis by fungal cultures at pH of 5.5 was reported by.[18]

Table 6. Cellulase activity at different pH

pH	Cellulase activity (U/gds)	
	CMCase	FPase
4.0	16.94	7.77
4.5	18.33	8.33
5.0	19.72	8.61
5.5	22.49	9.44
6.0	18.88	8.33
6.5	17.49	8.61

Table 7. Cellulase activity at different temperature

Temperature	Cellulase activity (U/gds)	
(°C)	CMCase	FPase
25	23.05	4.72
30	25.55	11.38
35	26.38	11.66
40	19.72	5.55
45	0.00	0.00

Table 8. Cellulase activity at different moisture level

Moisture	Cellulase activity (U/gds)	
Level	CMCase	FPase
1:1	19.16	9.44
1:2	19.72	10.55
1:3	26.94	12.77
1:4	11.94	5.27

Table 9. Effect of different carbon sources on cellulase activity

Carbon	Cellulase activity (U/gds)	
source	CMCase	FPase
Sucrose	24.81	11.48
Xylose	26.29	15.55
Cellobiose	25.96	16.26
CMC (Carboxy methyl cellulose)	27.03	17.77
Wheat flour	26.29	16.66

Effect of Temperature

Results show that 35°C is the optimum temperature to obtain maximum activities of CMCase and FPase (**Table 7**). High temperature can change membrane composition and can cause the protein catabolism and inhibition of fungal growth.[19] Since enzyme is a secondary metabolite producing during exponential growth phase, the incubation at high temperature could lead to poor growth and reduction in enzyme yield.[8] Our results are in agreement with researchers who found optimum enzyme activity at 35°C. [20-24]

Effect of moisture level

The initial moisture level is a crucial factor affecting the formation of products through solid state fermentation. Optimum initial moisture level for maximum CMCase and FPase activities by *Aspergillus niger* was found as 1:3(w/v) (**Table 8**). Reduced activity at lower moisture level was due to lower degree of swelling and higher water tension which reduces the solubility of nutrients. Similarly, higher moisture level decrease porosity, changes particle structure, promotes development of stickiness, decreases diffusion, lower oxygen transfer or increase formation of aerial hyphae which ultimately resulted in lower activity.[8] Higher enzyme activity at 1:3(w/v) of substrate moisture level has also been reported which supports our results.[11]

Effect of carbon source

Carbon sources play a vital role in the cell metabolism and synthesis of cellulase.[9] In our study CMC was found as best carbon source for growth and maximum cellulase activity of *Aspergillus niger* (**Table 9**).[25-27]

Effect of nitrogen source

Replacement of one nitrogen source with another in the medium causes a change in protein synthesis as well as product formation.[2] In our investigation peptone was observed as best nitrogen source for maximum CMCase activity (28.14 U/gds) and FPase activity (18.14 U/gds) (**Table 10**). Highest cellulase activity with peptone as nitrogen source in SSF has also been reported by many workers.[15,22,28-30]

Table 10. Effect of different nitrogen sources on cellulase activity

Nitrogen source	Cellulase activity (U/gds)	
	CMCase	FPase
Ammonium nitrate	26.29	15.92
Peptone	28.14	18.14
Potassium nitrate	27.03	16.66
Yeast extract	25.92	15.18
Urea	25.55	14.44

CONCLUSION

The present work has been taken up with a view of exploring the possibilities of using castor seed coat waste as a substrate and *Aspergillus niger* as a microbial source for the production of cellulase. The study proved that *Aspergillus niger* was the potent culture for synthesis of cellulase under solid state fermentation with mineral salt solution. In accordance with the results, taking all the influence factors and the results into consideration, the optimal cultural process was considered as follows: 120h incubation period, 9 discs inoculum size, 7g substrate concentration, 5.5 pH, 35°C temperature, 1:3 moisture level, 0.5% CMC as carbon source and 0.5% peptone as nitrogen source.

The result suggest the suitability of using cheap and abundantly available castor seed coat waste for production of cellulase in SSF system under optimized condition, the production and cellulase increase by 4.8 fold compared to unoptimized conditions.

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