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Optimization of Process Parameters for the Production of Cellulases under Solid State Fermentation

Nitesh Jadon[2], Nirmala Devi[1], Shashank Garg[2], Anupam Kumar[2], Yogender Pal Khasa[1] and Manoj Kumar[2]

[1]Department of Microbiology, University of Delhi, South Campus, Benito Juarez Road, New Delhi - 11 021, India.
[2]Department of Biotechnology, Lovely Professional University, Phagwara, Punjab - 144 402, India.

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A cellulolytic fungal strain was successfully isolated from local region and further subjected to solid state fermentation (SSF). Optimization of production parameters were done at shake flask level to apply at production scale. Low cost approach was determined for commercial viability of such industrial enzyme. Basic as well as applied parameters were optimized for better production. The maximum activity were recorded as 2.9 U/mL, 8.3 U/mL and 110 U/mL for FPase, CMCase and β-glucosidase respectively at 30 C and pH 6 with wheat bran as substrate with 20% (w/w) inoculums concentration on 5th day of incubation. These parameters can further be used as benchmark for upscaling of the process for commercial production under SSF conditions.

Key words: Cellulases, Solid State Fermentation, Opimization, FPase, CMCase, β-glucosidase.

Application of cellulases (3.2.1.4) are attributed to a wide range of industries such as textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives as well as in bioethanol production[1]. Cellulases have been commercially available for more than 30 years, and these enzymes have represented a target for both academic as well as industrial research[2,3]. These potentials are due to the capacity of cellulases in saccharification of lignocellulosics to fermentable sugars which can be used for production of bioethanol, lactic acid, and single cell protein[4]. Majority of studies on cellulase production have focused on fungi, with relatively lesser emphasis on bacterial sources[1]. Bacterial sources of cellulases include *Bacillus*, *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetivibrio, and* actinomycetes in particular *Streptomyces* species[5, 6]. Fungal sources of cellulases includes *Aspergillus niger; A. nidulans; A. oryzae; A. terreus; Fusarium solani; F. oxysporum; Humicola insolens; H.grisea; Melanocarpus albomyces; Penicillium brasilianum; P. occitanis; P. decumbans; Trichoderma reesei; T.longibrachiatum; T. harzianum; Chaetomium cellulyticum; C. thermophilum; Neurospora crassa; P. fumigosum;Thermoascus aurantiacus;Mucor circinelloides; P. janthinellum; Paecilomyces inflatus; P. echinulatum; Trichodermaatroviride.* Brown rot fungi *Coniophora puteana; Lanzites trabeum; Poria placenta; Tyromyces palustris; Fomitopsis sp.*White rot fungi *Phanerochaete chrysosporium;*

* To whom all correspondence should be addressed.
Mob.:+91-9855030374
E-mail: manojjnu@gmail.com

Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotusostreatus; Phlebia gigantean[7] . The physical and chemical parameter which greatly affects the production of extracellular cellulase in microorganisms includes temperature, pH, aeration[8], and medium constituents[9]. The effect is generally combinatorial and relationship between these variables has a marked effect on the ultimate production of the cellulase. Various works demonstrates the effect of these parameters on the ultimate production of the cellulases by different bacteria[8, 10] and fungi[11,12]. Solid-state fermentation (SSF) is method alternative to submerged fermentation used for the production of enzymes. Solid-state fermentation involves the cultivation of microorganisms on a solid substrate, such as grains, rice and wheat bran. SSF has many advantages over submerged fermentation. These include, high volumetric productivity, relatively high concentration of product, less effluent generated and simple fermentation equipment[l]. Some important problems associated to SSF are: design for upscaling and control of operations related to unit operations and fermentation parameters. Mass transfer related processes like oxygen and nutrient transfer to biocatalyst are major rate limiting steps in SSF. Diffusion of product across the solid media creates increase in cost of recovery. Nevertheless, high productivity and less contamination motivates the commercial production of enzymes though SSF. Present study is done to screen and isolate fungal species which can produce cellualse and to optimize the parameters for production of cellulase under SSF conditions by "OneVariable at One Time" approach.

MATERIALS AND METHODS

Screening and isolation of cellulase producing fungi: A total of 16 soil samples were collected from locally available garden and wood store and from dead woods and wood components and mycelium growing on living tree. Processed samples were inoculated on Potato Dextrose Agar (PDA) with Carboxy Methyl Cellulose (CMC, 0.5%) and incubated at 30 C for 5 days. Grown fungi were subcultured on PDA+CMC plates and for screening of potent fungi, PDA+CMC agar plates were inoculated with fungal disc (0.8 cm dia) from the growing fresh cultures of respective fungi and incubated at 30 C for 2 days with further screening with Congo Red.

The cellulase producing strains were identified by observing clear zone (halo) of hydrolysis around single colonies.

Production and estimation of extracellular cellulase activity in solid state fermentation

The fungal strains showing significant zone of clearance (CMC hydrolysis) were cultured for cellulolytic enzyme production. The production medium prepared in 250 ml Erlenmeyer flasks, each having 5.0 g of dry wheat bran moistened with mineral salt solution (g/l: $(NH_4)_2SO_4$, 1.4; $CaNO_3$, 0.3; $MgSO_4$, 0.3; $CaCl_2$, 0.3; yeast extract, 0.75; peptone, 0.25 and pH 5.5) to attain the final substrate-to-moisture ratio of 1:4. Autoclaved flasks were inoculated with 4 days old primary inoculums to obtain 5% W/W of fungal dry mass and incubated at 30 C. The fermented wheat bran (mycobran) was aseptically removed from flasks at regular interval, suspended in 50 ml citrate buffer (100 mM, pH 5.0) and shaken gently for 45 min. The extrudates were squeezed through muslin cloth for maximizing the enzyme extraction and centrifuged at 10,000 rpm at 4°C for 10 min. The enzyme solution thus obtained was assayed for various cellulase activities.

Assay of enzyme activity of cellulases

The obtained extract was assayed for activities of various cellulolytic enzymes and concentration of extracellular protein viz. exoglucanase, endoglucanase, β-glucosidase.

Exoglucanase (FPase) assay

The total exoglucanase (filter paper cellulase, FPcellulase, Fpase) activity was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman No. 1 filter paper (1.0 cm × 6.0 cm ~ 50.0 mg) as substrate in 1ml sodium citrate buffer (50mM, pH 4.8) at 50°C for 60 min. Reducing sugar were assayed by dinitrosalicyclic acid (DNSA) method of Miller (1959) at 540 nm.

Endoglucanase (CMCase) assay

Endoglucanase (Carboxymethylcellulase, CMCase) activity was assayed by measuring the release of reducing sugars in a reaction mixture containing 0.5 ml of crude enzyme and 0.5 ml of 2% (w/v) CMC solution prepared in 50mM sodium citrate buffer (pH 4.8) incubated at 50°C for 30 min.

Reducing sugars were assayed by dinitrosalicyclic acid (DNSA) method of Miller (1959) at 540 nm.

β-glucosidase assay

β-glucosidase activity was determined by the release of p-nitrophenol (*p*NP) at 430 nm from a reaction mixture containing 1 ml p-nitrophenyl glucopyranoside (*p*NPG) (1 mM), 1.8 ml acetate buffer and 0.2 ml suitably diluted enzyme, incubated at 50°C for 30 min (Wood et al., 1969).

One unit of enzyme activity was defined as the amount of enzyme required to liberate lìmole of glucose or *p*-nitrophenol, from the appropriate substrate, per ml per minute under the assay conditions.

Inoculum preparation

Erlenmeyer flask (250 ml) containing 50 ml of PDB was inoculated with four mycelia discs (0.8 cm dia each) of the respective fungal strain and incubated at 30°C under static cultivation conditions for 2 days. The mycelial mat thus obtained was homogenized with pestle and mortar under sterile conditions and used as primary inoculum for further experiments.

Activity of cellulases produced by numerous isolates under SSF conditions

To screen the most potent islolate solid state fermentation with all isolates was carried out separately in 250 ml Erlenmeyer flasks as per the procedure state above. The samples were collected every 3rd and 5th day and assayed for cellulolytic activity.

Optimization of cellulase production under SSF

The effect of various factors namely time of incubation (1, 2, 3, 4,5 ,6 days), initial pH of solid media (3, 4, 5, 6, 7, 8, 9), incubation temperature (20, 25, 30, 37, 45 C), substrate to moisture ratio (1:1, 1:2, 1:3, 1:4, 1:5, 1:6), inoculum size (10, 20, 40, 60, 80, 100% w/v), were tested. In addition to this the effect of different lignocellulosic substrate (corn-cob, rice straw, wheat bran, cotton stem part, wheat straw, sugarcane baggase) on enzyme production was also determined. Each factor examined for optimization was incorporated further in subsequent experiments. All other experimental conditions were kept constant unless otherwise stated.

Cellulase production from *under* optimized parameters

The fungal strain YPK 2011 was cultured in process conditions optimized with OVAT approach and the activities of cellulases were measured every 24 hours for 10 days.

RESULTS

Screening and isolation of *cellulase* producing fungi

A total of 8 out of 16 fungi isolated from different soil samples were found to produce *cellualses* (Table 1). Table 1 shows the activities of *FPase, CMCase* and *β Glucosidase* in the 8

Table 1. Secondary screening results of different fungi isolated by primary screening

S.No.	Strain Number	Incubation time (Days)	Zone of hydrolysis
1	NN1	2	+
2	NN2	2	++
3	NN3	2	-
4	NN4	3	++
5	NN5	3	++
6	NN6	4	++
7	NN7	4	++
8	NN8	7	+
9	NN9	3	-
10	NN10	3	-
11	NN11	3	-
12	NN12	7	-
13	NN13	3	-
14	NN14	3	-
15	NN15	3	-
16	YPK2011	2	++++

Table 2. Enzyme activities of 8 isolates obtained after secondary conditions under SSF conditions

S.No.	Strain	Incubation time (Days)	Enzyme Activity (U/ml)		
			FPase	CMCase	β-glucosidase
1	NN1	3rd	0.395	1.670	0.547
		5th	0.701	3.212	6.547
2	NN2	3rd	0.461	2.611	4.601
		5th	0.417	1.22	6.854
3	NN4	3rd	0.532	1.464	7.582
		5th	0.221	3.111	8.421
4	NN5	3rd	0.321	2.32	8.861
		5th	0.221	1.627	5.401
5	NN6	3rd	0.623	1.213	5.011
		5th	0.432	0.422	8.541
6	NN7	3rd	0.556	1.401	9.114
		5th	0.251	2.953	9.561
7	NN8	3rd	0.225	1.011	16.891
		5th	0.124	2.121	2.654
8	YPK2011	3rd	1.098	4.085	64.692
		5th	1.506	6.464	94.128

strains obtained after secondary screening. The data in Table 1 and 2 indicates that strain YPK2011 was having highest activity.

Optimization of *cellulases* production

Time dependent activity profile of *cellulases* under SSF conditions is depicted in Fig. 1. Maximum activity was obtained at 5th day after incubation. This result was used for optimization of other parameters and every time the sample was assayed for activity on 5th day after incubation.

The effect of temperature on the production of *cellulases* under SSF is shown in Fig. 2. The activity of *FPase* changes slightly with temperature but there is marked increase in the activities of *CMCase* and *α glucosidase* at an incubation temperature of 30°C. It was also observed that there was no production of cellulases at 45 C though there was growth of the fungi at this temperature, which was also restarted.

Fig. 3 shows the activities of *cellulases* with different initial pH of the inoculum. Maximum

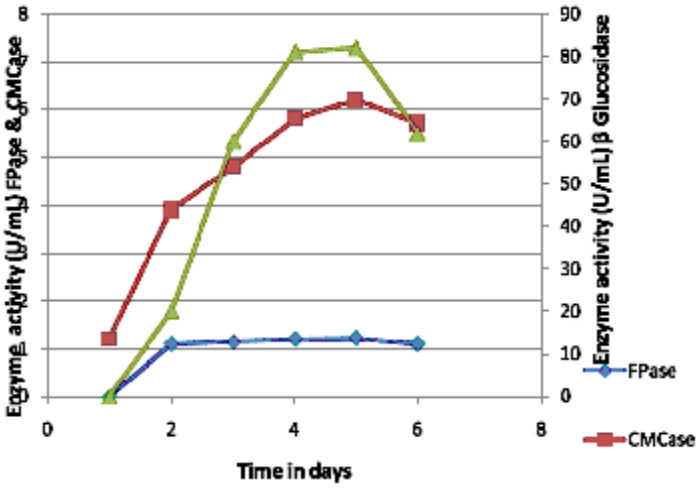

Fig. 1. Activity profile of FPase, CMCase and β Glucosidase with time

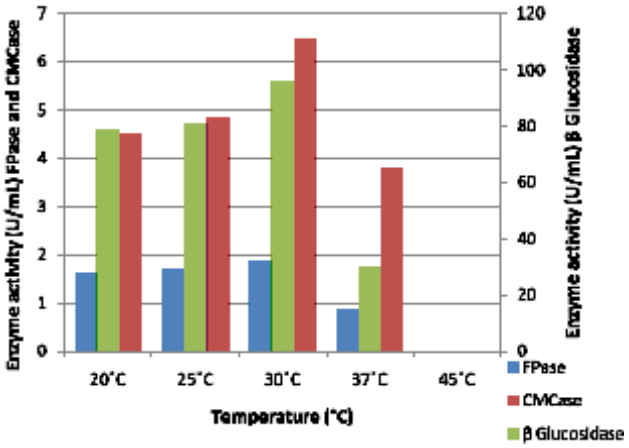

Fig. 2. Activities of FPase, CMCase and β Glucosidase at different incubation temperatures 5th day of incubation

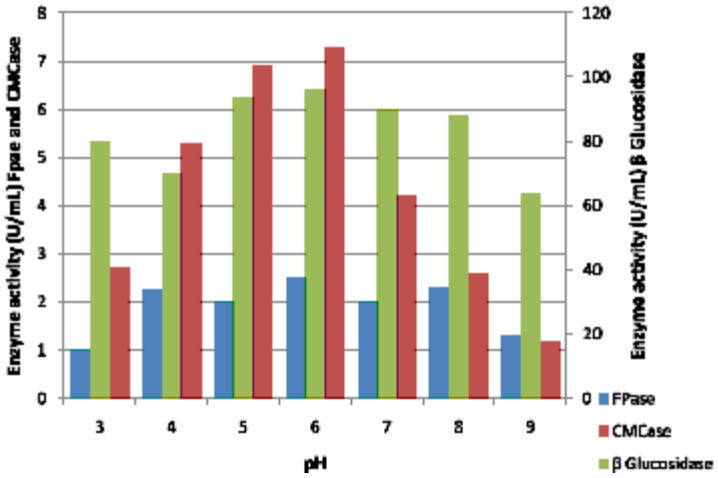

Fig. 3. Activities of FPase, CMCase and β Glucosidase at different initial pH on 5th day of incubation

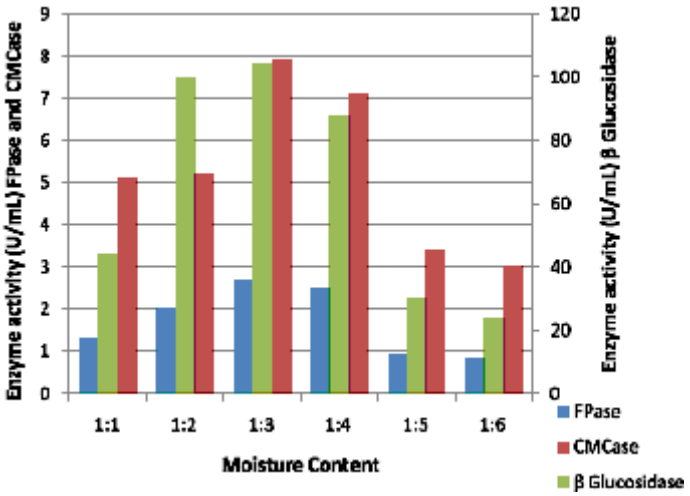

Fig. 4. Activities of FPase, CMCase and β Glucosidase at different moisture content on 5th day of incubation

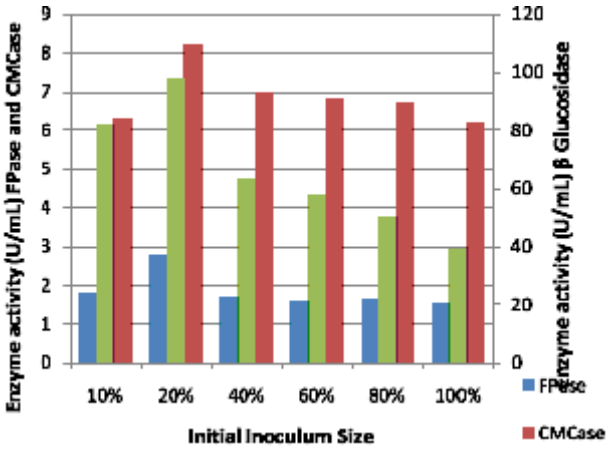

Fig. 5. Activities of FPase, CMCase and β Glucosidase at different intial inoculum size on 5th day of incubation

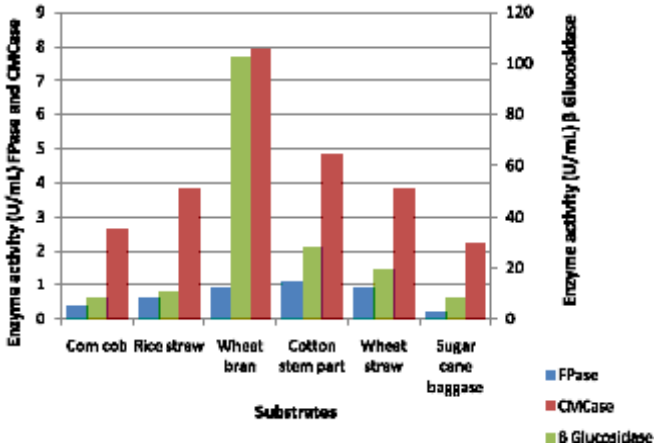

Fig. 6. Activities of FPase, CMCase and β Glucosidase with different substrates on 5th day of incubation.

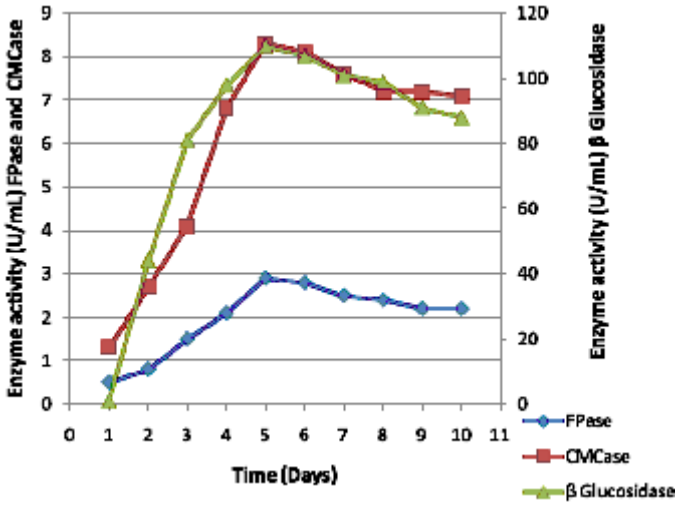

Fig. 7. Activity profile of FPase, CMCase and β Glucosidase with time at optimized parameters

activity was obtained with initial pH of 6. However there was no significant change between activities at pH 5 and 6.

Effect of initial moisture content on the activity of *cellulases* on the 5th day of incubation is shown in Fig. 4. It is observed that best activity was obtained with initial moisture content was 1:3 and lower moisture content adversely affects the activity of the *cellulases* produced.

Activities measurement with varying inoculums size (Fig. 5) shows the optimum inoculums size for cellulases production to be 20% W/W. Activities of *cellulases* do not change significantly by varying inoculum size from 40% to 100% (W/W).

As evident from Fig. 6, among the substrates studies for SSF, wheat bran gives the best productivity for all the three *cellulases*. Activities of *CMCase* and *β Glucosidase* were found to be significantly higher when wheat bran was used as substrate for SSF, while it varied slightly for *FPase.*

3 To further validate the conditions optimized in this study, SSF was carried out with optimized parameters. The time dependent profile is shown as given in Fig. 7. The activities of *FPase, CMCase* and *β Glucosidase* are observed to be higher when produced under the optimized culture conditions. There was a trend of increase in activity at every step of the optimization process which continuously validates the optimization of earlier process.

DISCUSSIONS

Considering the commercial importance of cellulases, we have attempted to optimize the physical conditions for production of cellulase under SSF conditions by locally isolated fungus. Physical conditions significantly affect the production of metabolites[13]. Cellulases are produced extracellularly to breakdown and utilize the complex sources of carbon which are present in ecological surrounding. The optimum temperature and pH for cellulase production was found to be 30 C and 6.0 respectively. This data is correlated well with the conditions prevailing in the environment from which the fungus was isolated. However, data from available literature shows that cellulases are produced under a wide range of temperature and pH. Mekala *et al*., (2008) showed that cellulases production was maximum in flasks incubated at 33C and decreased with high temperature[14]. Loss of activity at higher temperature may be attributed to denaturation of enzyme inhibition of microbial growth at higher temeperaute. Enzyme production is greatly influenced by initial pH of the culture medium. Fungal metabolism progressed from imminent to actual exhaustation of carbon source. This may lead to the situation in which at least part of the biomass started to sporulate, after which a return to the productive phase no longer occurred. After pH value of 6.0, the production of cellulases decreased which might be due to the fact that cellulases are acidic proteins and are greatly affected by the neutral pH values[15, 16].

Matrix morphology and mass transfer phemomena in SSF are attributed for the observed effect of inoculums size on production of cellulases. At lower size the full capacity of the system was not utilized and an increase in inoculums concentration thus increased the production. While at higher inoculums sizes the transfer of nutrients and oxygen is not so proper which results in almost constant activity with no further decrease in activity with increasing inoculums size.

CONCLUSION

A locally isolated fungus was successfully cultured and optimized for production of cellulases under SSF in shake flask system. The maximum activity were recorded as 2.9 U/mL, 8.3 U/mL and 110 U/mL for FPase, CMCase and β-glucosidase respectively at 30 C and pH 6 with wheat bran as substrate with 20% (w/w) inoculums concentration on 5th day of incubation.

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