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Isolation, Screening and Optimization of process parameters for enhanced production of cellulase by solid state fermentation

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Abstract

Cellulase producing 13 fungi were isolated from different soil samples, maintained as pure culture. Thus, P1 isolated was optimized with respect to Time course, pH, Temperature, Carbon source, Nitrogen source using solid state fermentation for increased cellulose production. The optimum cultural growth requirements were characterized at Time period of 144hrs and pH 6.0 and temperature 30ºC Lactose was found to be best a carbon source and peptone as best nitrogen source for enhanced cellulase production. Amongst various isolated fungi P1 produced the high concentration of cellulase within short fermentation period.

Keywords: Solid State Fermentation, Aspergillus, Cellulase, CMC.

Introduction

Cellulase belongs to EC 3.2.1.4 and catalyzes the endohydrolysis of 1,4-beta-D-glucosidic linkages in cellulose (Shaomin Y and Guang W., 2013). In the modern world, cellulase has many applications in industry because of the wide existence of cellulose, lichenin, and cereal beta-D-glucans (Parawira., 2012). Different types of organisms produce cellulases e.g. bacteria, fungi, protozoa, and some animal species (Watanabe and Tokuda., 2001). Cellulase are produce in various microorganisms such as Fungal species *Trichoderma, Humicola, Penicillium, Aspergillus, Thermomonospora fusca, H. insolens* and Bacterial species- *Pseudomonas, Cellulomonas*; and among *Actinomycetes-Streptomyces.*

Cellulase plays a important role in the extracellular matrix where the enzymatic reaction takes place (Coughlan., 1985). Cellulases involved in the whole process of hydrolysis in the conversion from biomass to biofuel which is divided into pretreatment, hydrolysis, fermentation, and distillation/evaporation (Dashtban., 2009) . Cellulase are classified into seven types, they are Endoglucanases or Endo-1,4-ß-D-Glucan Glucanohydrolases (EC 3.2.1.4), Exoglucanase or 1, 4-β-D-Glucan Cellobiohydrolases (EC 3.2.1.91) , Exoglucanases or 1, 4-β-D-Oligoglucan Cellobiohydrolases (Also Known as Cellodextrinases) (EC 3.2.1.74), β - Glucosidases or β-D-Glucoside Glucohydrolases (EC 3.2.1.21), Cellobiose Phosphorylase or Cellobiose: Orthophosphate Alfa–D-Glucosyl Transferase (EC 2.4.1.20), Cellodextrin Phosphorylase Or 1,4-B-D-Oligoglucan Orthophosphate Alfa –D-Glucosyl Transferase (EC 2.4.1.49), Cellobiose Epimerase (EC 5.1.3.11)

In order to produce cellulase Fermentation is carried out by using two processes , Solid State Fermentation (SSF) and Submerged Fermentation (SmF) ,SSF utilizes solid substrates, like bran, bagasse, paddy straw, other agricultural waste and paper pulp (Subramaniyam and Vimala ., 2012). The main advantage of using substrates is that nutrient-rich waste materials can be easily recycled in cheaper substrates. SSF is best suited for fermentation techniques involving fungi and bacteria that require less moisture content (Babu and Satyanarayana ., 1996).

In the present study various found culture were isolated from different soil sample and screened for cellulase production under solid state fermentation. The best screen isolate was selected as reference culture, which was further utilized for the optimization of various process parameter for enhanced production of cellulase under solid state fermentation. The selected fungal culture were further screened by solid state fermentation for cellulase production and the best selected fungal culture was further utilized for optimization process.

Materials and Methods

Reagents and media

Potato Dextrose Agar Medium (HiMedia, India), Carboxy Methyl Cellulase Sodium Salt (HPLC. Pvt. Ltd, Mumbai), Congo red (HPLC .Pvt. Ltd, Mumbai), $NaNo_3$ (Rankem, Mumbai), Yeast extract (HPLC. Pvt. Ltd, Mumbai), Peptone (HPLC. Pvt. Ltd, Mumbai), $CaCl_2$ (Loba Chemie, Mumbai), KH_2PO_4 (Thomal Baker, Mumbai), K_2HPO_4 (Rankem , Mumbai) used were of analytical grade.

Isolation and screening of cellulase producing fungi

Fungal culture were isolated from soil samples collected from the garden of KBS Nataraj college, Dairy farms, Plastic industry, Dump Site in outer region of Vapi,Gujarat-396195, India. The soil samples were suspended in sterile water and appropriate dilutions were plated by spread plate technique in the basal medium agar plate (g/100ml) $NaNo_3$ 0.05g; yeast extract 0.2 g; $CaCl_2$ 0.01g; K_2HPO_4 0.2g; KH_2PO_4 0.1g; Peptone 0.1g; Agar 3g; pH 6 containing CMC as cellulase substrate. The plates were incubated at 30°C for 3-7 days. After incubation period the basal agar plates were observed for colonies showing zone of clearance against red background after addition of Congo red and NaCl. The fungal isolates showing large zone of clearance were considered as cellulase producing fungi. The cellulase producing fungi were stored and maintained in PDA agar (pH 5.6) by subculturing periodically.

Solid State Fermentation

Preparation of inoculums

The preserved culture on the PDA slant was first inoculated on fresh PDA slants and incubated at 30 °C for 3-7 days. The activated spores were removed and suspended in Tween-80 (0.01%, v/v) and 2 mL of spore suspension (2×10^6 spores per mL) was used for inoculation in SSF flasks.

Enzyme production

Enzyme production was carried out under solid state fermentation (SSF). For SSF, 5 grams of rice straw was weighed into 250 ml Erlenmeyer flasks and were autoclaved at 121°C for 30 minutes at 15 psi pressure. After cooling, the rice straw was moistened with 20 ml of sterile basal medium containing : 0.2% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$, 0.001% $FeSO_4$, 0.1% K_2HPO_4, 0.2% peptone. The fermentation flasks were inoculated with 2 ml of spore suspension (2×10^6 spores per mL). The contents were mixed thoroughly and incubated for 7 days at 30°C under static condition.

Extraction of enzymes

Enzyme was extracted using 20 mL of buffer. The contents of the flask were mixed with buffer and crushed with the help of a glass rod and were allowed to shaken for 30 min after appropriate incubation period .The whole contents were then filtered through a four layered cheese cloth. The filtrate obtained was centrifuged at 5000 rpm for 20 min at 4 °C. The clear brown coloured supernatant was used as crude enzyme and stored at -20 °C until used.

Enzyme assays

Cellulase activity was determined as described by (Mandels M., 1975). The assay mixture of a total volume of 2 mL, contained 1 mL of 1 mM of carboxyl methyl cellulose (CMC) in 0.1M sodium acetate buffer (pH 4-5) and 1 mL crude enzyme. The mixture was incubated at 50 °C for 30 min. The reaction was stopped by the addition of DNS reagent. The treated samples were boiled for 10 min, cooled in water for

color stabilization, and the optical density was measured at 550 nm. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

Optimization of Cellulase Production

The present work involves optimization of different parameters governing cellulase production. The effects of various nitrogen sources, carbon source, incubation temperature, etc on cellulase production were examined by one factor at a time method.

Effect of Time Period

Effect of time period was performed by inoculated 2 mL of spore suspension (2×106 spores per mL) of selected fungal strain on SSF flask containing basal nutrient medium. The SSF flask were incubated for different time period (24h, 48h, 72h, 96h, 120h, 144h) at 30°C for 6 days. The crude enzyme was extracted periodically, centrifuged and the cell-free supernatant was used as the source of crude cellulase enzyme.

Effect of pH

Effect of initial pH of the medium on cellulase production was studied by adjusting the pH of the production medium used for moistening of solid substrate in the range of 4 to 9 using sterile 1 N NaOH and 1N HCL after sterilization . Then each flask was inoculated with 2 mL of spore suspension (2×10^6 spores per mL) and incubated at 30°C for 6 days under static conditions. The crude enzyme was extracted after 6 days, centrifuged and the cell-free supernatant was used as the source of crude cellulase enzyme.

Effect of Temperature

Effect of temperature on cellulase production was studied by incubating the production medium used for moistening of solid substrate with 2 mL of spore suspension (2×10^6 spores per mL) at temperature range 15°C- 60°C for 6 days under static condition. The crude enzyme was extracted after 6 days, centrifuged and the cell-free supernatant was used as the source of crude cellulase enzyme.

Effect of Carbon source

Effect of carbon source on cellulase production was studied by taking different carbon sources i.e fructose, maltose, lactose, sucrose, glucose, mannitol in the medium used as moistening agent for solid substrate. The SSF flasks were inoculated with 2 mL of spore suspension (2×10^6 spores per mL) and incubated at 30°C for 6 days with static condition. The crude enzyme was extracted after 6 days, centrifuged and the cell-free supernatant was used as the source of crude cellulase enzyme.

Effect of Nitrogen source

Effect of different nitrogen sources i.e yeast extract, peptone, beef extract, and their combinations, ammonium sulphate, urea was determined by replacing peptone in the production medium used for moistening of solid substrate. A control is represented with peptone use as nitrogen source was also performed. Each flask containing the medium was inoculated with 2 mL of spore suspension (2×106 spores per mL) and incubated at 30°C for 6 days under static conditions. The crude enzyme was extracted after 6 days, centrifuged and the cell-free supernatant was used as the source of crude cellulase enzyme.

Results and Discussion

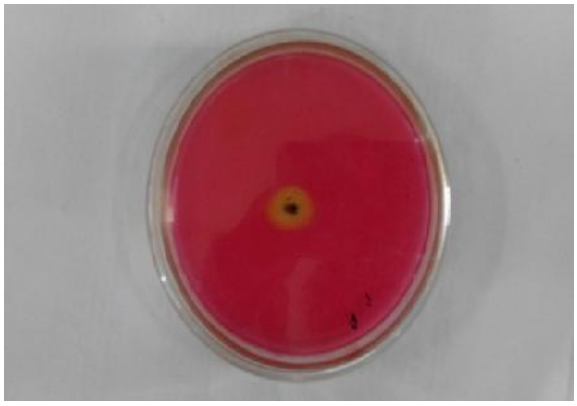
Isolation and Screening of cellulolytic fungi

Screening of fungal isolates for cellulase production was done by CMC hydrolysis on CMC agar plate. The isolated fungal culture were spotted at the centre of CMC agar plate and incubated for 3 - 6 days. Upon incubation the growth of fungal culture were observed and the plates were flooded with Congo red dye . The fungal culture shows zone of CMC hydrolysis were considered as cellulase producing fungus and selected for further study.

A total of 13 fungal culture were selected for the production of cellulase under solid state fermentation using rice straw as solid substrate. The results observed shows that isolate P1 shows maximum cellulase activity of 1.6 U/gram of dry substrate at 144 hrs of incubation under static condition (Table 1). Isolate P10 shows 1.3 U/gram of dry substrate cellulase activity after 168 hr of incubation. However, isolate P6, P9 and P13 shows negligible amount of cellulase activity even after 168 hrs of incubation under solid state fermentation. Thus, isolate P1 as selected for further study.

Table 1- Screening for cellulase producing strain

Sample	Isolate No.	Zone of CMC hydrolysis on CMC agar plate	Cellulase activity (U/gm of dry substrate)
Garden soil, KBS NATRAJ college, vapi	P1	1.2cm	1.6
	P2	0.9 cm	0.86
Aarti garden soil, vapi	P3	0.5 cm	0.45
	P4	1.1 cm	0.89
	P5	0.7 cm	0.82
Dairy farm soil, GIDC, vapi	P6	-	-
	P7	0.6 cm	0.71
	P8	0.4 cm	0.45
Plastic Industry soil, GIDC, vapi	P9	-	-
	P10	0.7 cm	0.78
Dump sites soil, GIDC, vapi	P11	0.9 cm	0.82
	P12	0.3 cm	0.32
	P13	-	-



(A)

Fig. 1 Screening on cellulase production: (A) Zone of clearance of cellulase on CMC agar plate

Optimization of Cellulase Production

Time Course study of cellulase production

The effect of Time period on cellulase production by fungal culture by inoculating the SSF flasks at various time period and harvested to assay enzyme activity. The cellulase activities of fungal increased steadily with increasing (Fig 2) Time period and attained maximum (1.8 U/gm of dry substrate) on the 144 hrs of incubation. However, further incubation results in decreased cellulase production.

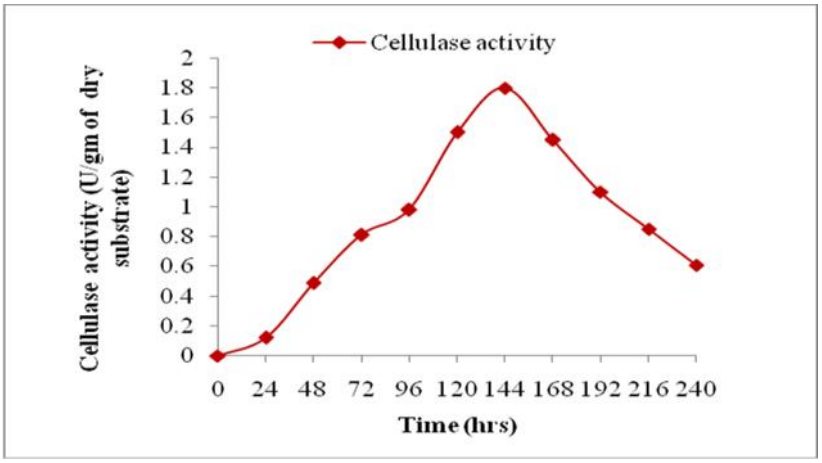


Fig -2 Time course study of cellulase production

Effect of pH on cellulase production

The effect of pH on the activity of the cellulase enzyme was done by adjusting the moistening agent pH in the range of 3.0 to 11.The maximum cellulase activities (2.2 U/gm of dry susbtrate) at pH 6. A further increase in pH reduced the cellulase activity was obtained. The reason for decreasing production at higher pH was probably due to proteolytic inactivation of the cellulase. Hence, it suggested that slightly acidic pH values favoured cellulase production, which is in agreement with earlier results of other researchers (Eriksson,1976).

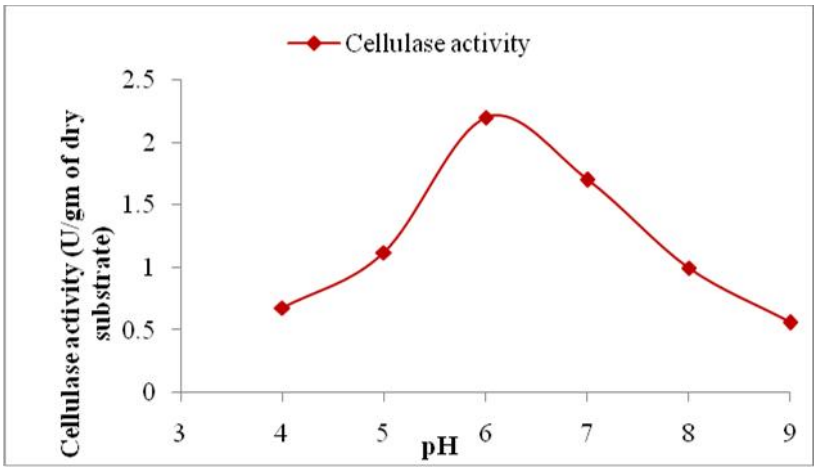


Fig -3 Effect of pH on cellulase production

Effect of incubation temperature on cellulase production

Effect of Temperature significantly influenced the growth, development and, in general, metabolic activities of an organism. Hence, it was essential to optimize temperature for maximum cellulase production in fungal strains under SSF. Thus, SSF flasks were incubate at different incubation temperature for 144 hrs of incubation and analyzed for cellulase (1.96 U/gm of dry substrate) was obtained at 30°C incubation temperature (Fig-4). Similarly (Dharm dutt and Alok kumara., 2012) has shown that *A. flavus* AT-2 produced maximum cellulase activity (9.50 IU/mL) at 30 °C, whereas *A. niger* AT-3 produced maximum cellulase activity (10.75 IU/mL) at 35 °C.

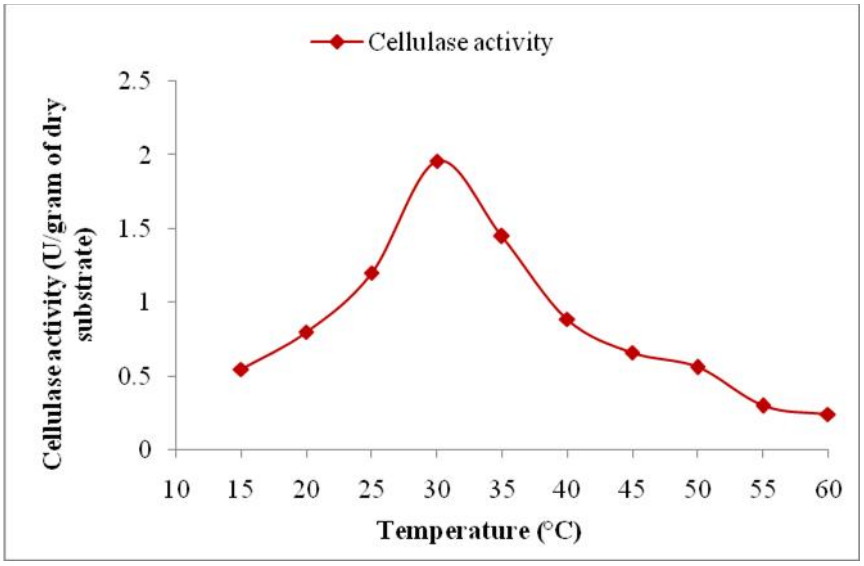


Fig -4 Effect of incubation temperature on cellulase production

Effect of Carbon Source on cellulase Production

The effect of carbon source on growth and enzyme production by the isolate was determined by growing the test fungal isolated in SSF medium in which any of the following carbon sources: glucose, sucrose, lactose, fructose, mannitol and maltose were used as a sole carbon source. The maximum cellulase activity was shown when Lactose was used as carbon source (2.3 U/gm of dry substrate) (Fig-5). In contrast to (Chundakkadu K., 1998) the various carbon sources tested, glucose was found to be the best carbon source for the substrates CMC and coir waste, because glucose is the good inducer of the cellulase production.

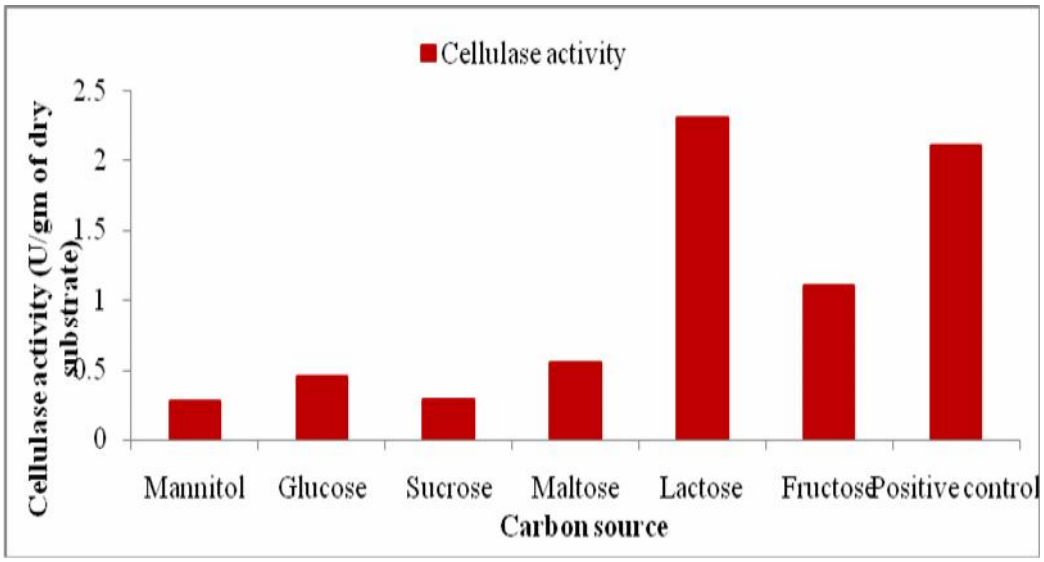


Fig -5 Effect of carbon source on cellulase production

Effect of Nitrogen Sources on cellulase Production

In this study the effect of supplementary organic nitrogen sources (Peptone, Urea) and inorganic nitrogen sources ($(NH_4)_2SO_4$, NH_4Cl) on the production of cellulase was done under Solid State Fermentation. The maximum cellulase activity was observed Peptone when (2.75 U/gm of dry substrate) was used as nitrogen source . However, urea and Ammonia also shows comparable cellulase activity when used as nitrogen source (Fig-6). In contrast to (Dharm dutt and Alok kumara., 2012) the maximum cellulase activity (*A. flavus* AT-2, 13.40 IU/mL and *A. niger* AT-3, 17.10 5 IU/mL) were observed with yeast extract.

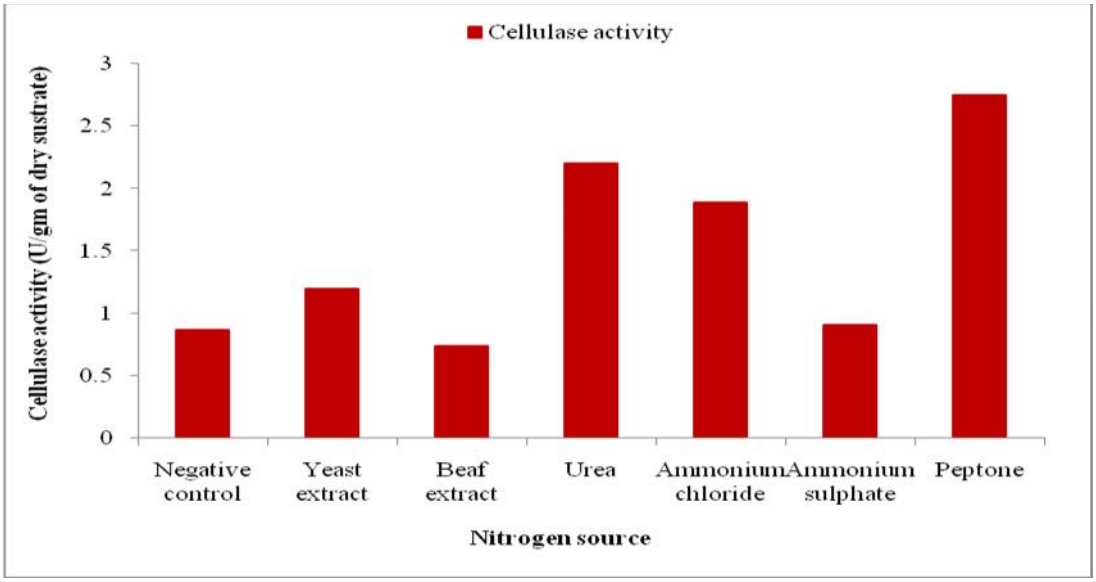


Fig -6 Effect of nitrogen source on cellulase production

Summary and Conclusion

The present investigation concludes that fungal isolate P1 shows higher fermentation yielding point for cellulase. The optimum temperature and pH was found to be 30°C and pH 6 for cellulase production. The best carbon and nitrogen source was found to be lactose and peptone for cellulase production by fungal isolate P1 when rice straw was used as solid substrate. Thus, in present study P1 fungal isolate found to be promising culture for cellulase production under solid state fermentation.

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