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RESEARCH ARTICLE

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Role of soil fungal isolates and yeast in cellulase and alcohol production through lignocellulosic substrate

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ABSTRACT

Present study was aimed at isolation of promising cellulase producing fungus, its identification and optimization of cultural conditions for production of cellulolytic enzymes. The cellulololytic activity of these cultures was studied by standard CM-cellulose and congo red plate assay method. The study related to process development involves optimization of different fermentation conditions (physical and nutritional) towards enhancement of cellulolytic enzymes production. Solid state fermentation cultural conditions (physical and nutritional factors) for cellulolytic enzymes production by the isolated promising Aspergillus niger were optimized. One factor at a time (OFAT) strategy was used for the optimization of medium components. Cellulase production with Aspergillus niger was highest at temperature 28˚C, pH-7.0, incubation time-72 hrs, salt-NaCl, nitrogen source-peptone and in presence of substrate-Apple peel and common grass

Key words: Cellulose, Cellulolytic, Optimization, Solid state fermentation

INTRODUCTION

The ever increasing demand for energy is one of the greatest challenges for human beings. Fossil fuels are dwindling rapidly and the current instability of oil supplies and the fluctuation of prices have further ignited widespread interest in alternative energy sources. Biofuels are among the promising transportation green energy sources for the future [1].The extensive range of organic materials used for biofuel production include starch from cereal plants, lignocellulosic materials and algae etc. First generation biofuels are manufactured using starch from food-based cereal plants like corn and wheat; sugar from sugar cane; oil from the seeds of rape; soya bean and jatropha; vegetable oils and animal fats. Second generation biofuels also known as cellulosic biofuels, are produced using lignocellulosic materials like forest and crop residues, straw, municipal and construction waste [2]. However, the public opinion on producing biofuels from edible sources is not favourable. Hence, there is more interest on research into the development of less expensive methodologies to produce biofuels from non- edible sources like lignocellulosic biomasses of plants and organic wastes [3]. Lignocellulose consists of lignin, hemicellulose and cellulose. Cellulase, a group of enzymes which catalyze the hydrolysis of cellulose and related oligosaccharide derivatives, is considered a potential tool for industrial saccharification [4],

Keeping the significance of cellulase enzymes in mind the current investigation was designed to isolate the cellulase producing fungi, optimization of environmental condition for higher enzyme production and also to estimate alcohol production by the isolates and their comparison with a well known producer species.

MATERIALS AND METHODS

Selection of sample sites : Bamboo forest soil was the sample site for present investigation. Three sites were chosen viz., TFRI area, SFRI area and GCF area in Jabalpur District of Madhya Pradesh.

Sample collection and processing: Soil samples were collected at different sample points from the undisturbed localities employing sterile soil augers, hand trowel and polythene bags. The soil was dug out using augers up to 20cm depth and was immediately scooped into sterile polythene bags using the hand trowel. The samples were collected from 5 spots in each site and then mixed together in order to obtain a representative sample, which were bought to the lab for further studies. Samples were serially diluted upto 10^{-5} dilution.

Isolation of fungi: Potato Dextrose Agar media and Rose Bengal Agar media were prepared. 1ml of serially diluted (10^{-5}) samples were inoculated into pre sterilized and pre solidified PDA and RBA agar plates by soil plate method and pour plate method and inoculated at 28°C for 2-3 days.

Identification of fungal isolates: The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28°C for 48 hours in order to obtain pure fungal culture. Colonial morphology and microscopic examinations of the fungal isolates were used to determine the reproductive and vegetative structures.

Preliminary estimation of cellulase production: Czapek Dox agar media supplemented with 1% CMC was prepared and fungal culture was inoculated by point inoculation method on the prepared plates and kept at 28°C for 72 hrs. After incubation the plates were listed for zone analysis for which the Petri plates were flooded with Congo red solution (1%). After 5 minutes the Congo red solution was discarded and the plates were washed with 1N NaCl solution and allowed to stand for 15-20 minutes. The clear zone was observed around the colony when the enzyme had utilized the cellulose.

Mass production: Common grass (*Cynodon dactylon*) was used as the main substrate for SSF. 10 gms of finely chopped grass was taken in flask and moistened with 20 ml of mineral salt solution (peptone-0.1%; malt extract-0.1%, yeast extract-0.2%, calcium carbonate-0.2%, ammonium phosphate-0.2%, and ferrous sulphate.7H2O-0.001%) [5], sterilized, cooled, inoculated with fungal culture and incubated at 28°C for 120 hrs. Also PD broth was inoculated with fungal spores and after 7 days of incubation the fungal biomass was filtered on a pre weighed filter paper and weighed after complete drying.

Trichoderma reesei is also a good source of cellulase enzyme. Hence, comparative study was carried out between *T. reesei* (procured from MTCC) and *A. niger* with respect to cellulase production by using DNS method. Solid State Fermentation was done by inoculating *T. reesei* in 10 gms grass moistened with mineral salt solution and sterilized, cooled and incubated at 28°C for 120 hrs.

Extraction of crude enzyme: A solution of tween 80 (1%) was added to 100 ml of distilled water. 10ml of this solution was added to 2 gms of fermented substrate and homogenized on rotary shaker at 180 rpm for 1hr. The pellet was removed by centrifuging the homogenate at 8000 rpm at 4^0C for 15 min and the resultant clear supernatant was used as Cell Free Extract (CFE) for further studies.

Quantitative assay for cellulose: DNS Method:
Cellulase activity was assayed using Dinitrosalicylic acid (DNS) method [6]. 2 ml enzyme solution was taken and 1ml DNS reagent was added to it, then the contents were incubated at boiling water bath for 15 minutes. 1ml of sodium potassium tartrate (Rochelle sodium tartrate) was added and incubated for 10 minutes at room temperature. Then the contents were cooled and absorbance of the red colour developed was taken at 500 nm.

Optimization: Optimization of culture conditions was carried out for different parameters *viz* carbon source, pH, temperature, nitrogen source, incubation period and salt concentration for cellulase production.

Carbon source / Substrate: Although lignocellulosic material was the main substrate used during this investigation but other substrates were also tested for their performance for the purpose. For this Grass, Broken rice, Apple peel, Banana peel, Orange peel were used and 20 ml mineral salt solution was added and sterilized. Inoculation was done with the selected fungus and incubated at 28^0C for 120 hrs.

pH: 10 gms of substrate was taken and 20 ml mineral salt solution was added to it and sterilized and cooled. pH was adjusted to 5, 7 and 10 using 0 .1 N HCl and 0.1 N NaOH. Fungal inoculum was added and incubated afterwards.

Temperature: 10 gms of substrate was taken and 20ml mineral salt solution was added to it and sterilized and cooled. Fungal inoculation was done and incubated it at different temperature 28^0C, 40^0C, 60^0C for 10 hrs.

Nitrogen source: 10 gms of substrate was taken and 20ml mineral salt solution was added to it and supplemented with different nitrogen source viz peptone, beef extract, ammonium sulphate and sterilized it and cooled. Inoculation was done of a fungus at different nitrogen source and incubated at 28^0C for 120 hrs.

Salt concentration: 10 gms of substrate was taken and 20 ml mineral salt solution was added to it and supplemented with different salts viz NaCl, $CaCl_2$ & $FeCl_3$. After sterilization the fungus was inoculated and incubated at 28^0C for 120 hrs.

Incubation period: 10 gms of substrate was taken and 20ml mineral salt solution was added to it and sterilized it and cooled. Fungal inoculum was added and incubation was carried out at different time intervals viz 24 hrs, 48 hrs, 72 hrs.

Immobilization: Immobilization was done by two ways: whole cell immobilization and Cell free immobilization.

Whole cell immobilization: Fungus was inoculated in PDA broth and incubated at 28^0C for 72hrs. After 72hrs fungal mat was separated from broth by filtration and fungal mat was used for further process. Fungal mat was crushed and dissolved in 2% sodium alginate solution (1:1). This mixture was then dropped into the chilled solution of 0.2M $CaCl_2$ with continuous shaking at 4^0C. Finally beads of calcium alginate were formed. Beads were washed 3-4 times with deionised water and finally with 50mM Tris HCl buffer (7.5 pH), weighed and the activity was further checked.

Cell free immobilization:: Enzyme solution was taken after centrifugation and crude enzyme was precipitated by adding 80% ammonium sulphate solution at 4^0C. Precipitate was dissolved in Tris HCl buffer (50mM, 7.5 pH). This partially purified enzyme solution was mixed with 2% sodium alginate solution (1:1). This mixture was then dropped into the chilled solution of 0.2M $CaCl_2$ with continuous shaking at 4^0C. Finally beads of calcium alginate were formed. Beads were washed 3-4 times with deionised water and finally with 50mM Tris HCl buffer (7.5 pH). Beads were dried and weighed and enzyme activity was again checked by using beads.

Ethanol production: An attempt was made to study the capability of fungi to produce alcohol using lignocellulosic material as substrate. Also a comparison was carried out with a well known yeast strain *Kluyveromyces* procured from Microbial Type Culture Collection, Chandigarh. For this purpose, 3 different conical flasks with 10 gms of finely chopped grass were taken and 20ml of mineral salt solution was added to it. Inoculation was done with *A. niger (Flask 1), Kluyveromyces(Flask 2)* and combination of both the microbes(flask 3) in 3 different flasks respectively and incubated at 28°C for 168 hrs and observed for alcohol production.

Estimation of ethanol production

Qualitative estimation: The fermented substrate was centrifuged at 10000 rpm for 15 minutes. The supernatant was then used for further study. The qualitative analysis was carried out using ethanolic acid. 2ml of ethanolic acid was added to 1ml of the test sample (supernatant) and heated in the water bath for 5 min until characteristics sweet smell of esters was perceived [7].

Quantitative estimation: The quantitative estimation of ethanol was done by biochemical method using potassium dichromate mehtod. 30 microlitres of test sample was taken and the volume was made up to 500 microliters by adding distilled water in test tube.1 ml of potassium dichromate reagent followed by 2 ml of sodium hydroxide solution were added in all the test tubes including sample test tube. Incubation was carried out at 50^{o}C for 30 minutes. The absorbance was measured at 600 nm by using a spectrophotometer [8] and concentration was measured using standard graph of ethanol.

RESULTS AND DISCUSSION

Isolation and identification: Soil samples from different bamboo forest were inoculated on PDA and RBA plates and after incubation fungal isolates were obtained on all the plates. There were total 30 isolates obtained which were further tested for their cellulose producing potential. Primary screening of each fungal culture was performed by point inoculation on Czapek Dox agar medium supplemented with 1% CMC for cellulose production followed by incubation which resulted into yellow or clear zone of hydrolysis on application of freshly prepared 1% congo red solution followed by 1N NaCl solution.

As shown in figure 1, out of 30 isolates tested, 4 isolates were found as positive for cellulase production. The fungal cultures were identified as *Aspergillus niger, Aspergillus flavus, Fusarium sp.* and *Alternaria sp.* Out of 4 fungal

isolates *Aspergillus niger* was selected for further studies as it had produced largest zone of hydrolysis with diameter 16 mm.

Table 1: Preliminary estimation of cellulose production

S. No.	Fungus	Media	Zone test	Zone diameter
1.	*Aspergillus flavus*	Czapek Dox Agar media	Positive	14.5 mm
2.	*Aspergillus niger*	Czapek Dox Agar media	Positive	16mm
3.	*Alternaria spp.*	Czapek Dox Agar media	Positive	9.2mm
4.	*Fusarium spp.*	Czapek Dox Agar media	Positive	7mm

Optimization: Optimization of different atmospheric conditions like pH, temperature, incubation period, salts, different substrates and nitrogen sources, etc resulted into presence or absence of zone with varying sizes and differences in the concentraion of enzyme production.

Effect of pH: pH highly affect the production of cellulase enzyme. Out of 3 pH ranges tested i.e, 4, 7 and 11 the diameter of zone was found to be no zone, 11mm and 7mm respectively. The highest diameter 11mm and highest glucose concentration 86.69mg/ml at pH 7 indicates that pH 7 is the optimum pH for growth and highest cellulase production. Similar study was conducted by [9] and their results were found very similar to these results.

Effect of temperature: Temperature is a critical factor for maximum enzyme activity and is necessary to be optimized for industrial enzyme production. Out of 3 temperature ranges tested i.e. 28^0C, 40^0C and 60^0C, the highest zone of 16mm diameter was found at 28°C and glucose concentration to be 113.07 mg/ml. In the study conducted by Devi MC *et al.*, [10] maximum enzyme production was achieved at temperature of 30°C by *Aspergillus niger* in paper cellulose.

Effect of incubation period: Zone of hydrolysis was best seen after 48 hrs of incubation while overgrowth was obtained upon extending the incubation period upto 72 hrs. on the contrary, upon performing SSF, the glucose concentration was found to be 89.32 mg/ml at 72hrs which was highest amongst other temperature ranges tested. Similar result was expressed early in the study conducted by Mrudula *et al.*, (2011) with maximum production of cellulase after 72 hrs of incubation in SSF using *A. niger*.

Table 2: Quantitative estimation of cellulase production by DNS method

S.No.	Environmental Condition		Optical Density (500 nm)	Concentration of glucose (mg/ml)
1	**pH**	4	1.489	47.49
		7	2.679	86.69
		11	2.656	85.93
2	**Temperature (°C)**	28°C	3.480	113.07
		40°C	3.354	108.92
		60°C	0.243	6.46
3	**Incubation Time (Hours)**	24 Hrs	0.432	12.68
		48 Hrs	1.576	50.36
		72 Hrs	2.759	89.32
4	**Salt**	NaCl	2.239	72.20
		$CaCl_2$	1.016	31.92
		$FeCl_3$	1.382	43.97
5	**Carbon Source**	Broken rice	2.815	91.17
		Apple peel	3.48	113.07
		Banana peel	2.652	85.80
		Orange Peel	2.817	91.23
		Common Grass	2.821	91.37

Effect of salts: The production of cellulase seems to vary with use of different salts in the media. NaCl salt was found to be optimum for maximum cellulase production as compared to $CaCl_2$ and $FeCl_3$ salts and the zone diameter using NaCl was 13 mm and glucose concentration was found to be 72.20 mg/ml.

Effect of different Carbon sources: In the present study different substrates were used as carbon source durning SSF. Agro wastes and lignocellulosic material such as Apple peel, Banana peel, Orange peel, Broken rice and common Grass were tested for their potential. High glucose concentration of 113.07mg/ml was obtained in the appled peel. Grass also resulted into comparable glucose concentration 91.37mg/ml. Being costly, use of apple peels as a substrate for cellulase production is not economical, therefore common grass being easily available and less costlier can be used as a good alternative for cellulase production at industrial levels.

Mass production by Solid State Fermentation: The isolate *Aspergillus niger* was mass produced using Solid State Fermentation (SSF) method and the results obtained in this study are comparable with the results obtained by Krishna [11] who used banana stalk and coconut coir respectively for production of cellulases. *A. niger* mat was obtained from PD broth and was weighed, it was found to be 0.4202gm .

Lignocellulosic material was used as substrate which resulted in good production of fungi with high biomass. Cellulase production is hindered because of the high costs of substrate (pure cellulose) and of some chemicals, such as proteose peptone, and also because of low yields of cellulases per unit of cellulose. To overcome these problems, cheap sources of cellulose like lignocelluloses and agricultural wastes are used in SSF. Out of such sources, grasses are the cheapest and easily available substrate for cellulase production and they also proved fruitful in current investigation.

Comparative study of *A. niger* with *Trichoderma reesei*: *Trichoderma reesei* being one of the most important fungal species for cellulase production gave a good response in the present study. It resulted in the biggest zone of hydrolysis with diameter 18.2 mm and highest glucose concentration 113.73 mg/ml (Table 3) The results obtained on using A.niger are also very much similar to *T. reesei* and hence this study shows that *Aspergillus niger* can also be used as a good source for cellulase production.

Table 3: Comparative study of cellulose activity by *A. niger* and *T. reesei*

S. No.	Parameter	Glucose Concentration *(Trichoderma reesei)*	Glucose Concentration (*A. niger*)
1.	**Temperature (28°C)**	113.73mg/ml	113.07mg/ml
2.	**pH (7)**	86.72mg/ml	86.69mg/ml
3.	**Incubation period (72hrs)**	90.67mg/ml	89.32mg/ml
4.	**Carbon source (Grass)**	92.98mg/ml	91.37mg/ml
5.	**Nitrogen source (peptone)**	67.95mg/ml	68.94mg/ml

Partial purification and immobilization : Enzyme purification is one of the crucial step during industrial enzyme production process. In present study the cellulase enzyme was partially purified by ammonium sulphate precipitation method. The best precipitation was obtained with 80% is saturation which was properly dissolved in Tris – HCl and gave best enzyme activity after immobilization.

The partially purified cellulase enzymes were immobilized in calcium alginate beads. The beads were weighed to be 2.461 gm (Fig 3). The beads were further tested to check their functionallity. The optical densities for cell free immobilized beads was observe to by 0.423 and 0.220 for sucrose and starch respectively while ODs for that of whole cell immobilized beads were 0.763 and 0.511 respectively.

Qualitative and quantitative estimation of alcohol: The supernatant obtained upon centrifuging the fermented substrate was assumed to contain monosaccharides (glucose). This glucose was converted into alcohol by using *A. niger, kluyveromyces* and mixture of the two above mentioned microbes. All the 3 test tubes resulted into ester like smell indicating the alcohol production. Also the quantitative estimation of alcohol production was successfully carried out. The ODs obtained by incubating 3 types of cultures were 0.053, 0.097 and 0.168 respectively. The concentration of alcohol produced after SSF was measured to be 1.76 µl, 4.8 µl and 15.09 µl by using ethanol standard graph (Table 4).

Table 4: Quantitative estimation of alcohol production

Organism	*A niger*	*kluyveromyces*	*A. niger + Kluyveromyces*
Optical density (600 nm)	**0.053**	**0.097**	**0.168**
Concentration of alcohol (µl)	**1.76µl**	**4.8µl**	**15.09 µl**

Fig 1: CMC test for qualitative analysis of cellulase production

Fig 2: Microscopic image of *A.niger*

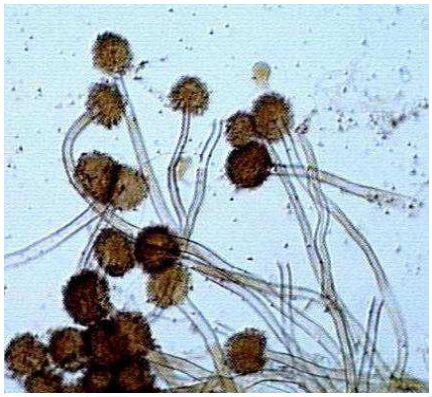

Fig 3: Immobilized Enzyme in gel beads

CONCLUSION

The study indicated that the fungi as enzyme sources have many advantages that, the enzymes produced are normally extracellular, making easier for downstream process. The development of economically feasible technologies for cellulase production and for the enzymatic hydrolysis of cellulosic materials will enable to utilize the large quantities of biomass such as the residues of food industries, agriculture and also lignocellulosic waste materials and *A. niger* is one such beneficial and commercially applicable fungi. Thus the present investigation suggests that lignocellulosic material can be highly used for biofuel production and *A. niger* in combination with *Kluveromyces spp*. is one of the promising fungal strain for this purpose.

This indicates that the isolated fungal culture has good potential to produce alcohol. Although the implementation of well known yeast strain *Kluyveromyces* along with *A. niger* had increased the alcohol production to a great pace. Hence, it can be concluded that the *A. niger* is an economical, beneficial and commercially applicable fungus and the lignocellulosic materials can be a good, cheaper and better source of biofuel production.

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