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RESEARCH ARTICLE

CELLULASE PRODUCTION BY *BACILLUS SPP* AND *ASPERGILLUS NIGER* USING COIR WASTE AND SAW DUST AND PARTIAL PURIFICATION

Sajani Samuel[**], Muthukkaruppan, S.M., Gayathri Shanbhag, N[*] and Senthil Kumar, P.K

Department of Microbiology, Annamalai University, Annamalai Nagar, Tamilnadu - 608 002. INDIA.
*Department of Microbiology, Vels college of Science, Chennai, INDIA

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ABSTRACT

In this study four bacterial colonies (*Bacillus spp*, two *Pseudomonas spp* and *Proteus spp*.) and two fungal spp (*Aspergillus niger* and *Aspergillus fumigatus*) were isolated by spread plate technique and were checked for cellulase production (zone formation) with the help of congo red and NaCl/NaOH. The production of cellulase by bacteria (*Bacillus spp , Pseudomonas spp* and *Proteus spp*) and fungi (*A.niger* and *A. fumigatus*) were also confirmed by different assay (Gel punch assay, Dinitrosalicylic acid (DNS) and Carboxy Methyl Cellulose assay (CMC)). In DNS assay method high level of cellulase production was achieved by *Bacillus spp* (0.09 µl/ml and 0.08 µl/ml) for coir waste and saw dust as substrate and among fungi *A. niger* showed higher cellulase activity (0.1 µl/ml and 0.1 µl/ml) for coir waste and saw dust as substrate. In CMC assay high level of enzyme production was achieved by *Bacillus spp* (0.1 µl/ml and 0.09 µl/ml) for coir waste and saw dust and among fungi *A.niger* showed high level of cellulase activity (0.1µl/ml and 0.1µl/ml) .Regarding pH, Temperature, carbon source cellulase activity was high when pH was 6 for bacteria and 7 for fungi and for temp the optimum cellulase production was at 37ºC and 28ºC for bacteria and fungi. Among different carbon source cellulose showed higher yield in production for both bacteria and fungi. The crude enzyme after centrifugation of production media were used to determine the molecular weight of the enzyme produced which lied between 6 to 20 KDa for *Bacillus* for both substrate and that of *A. niger* was found to lie between 20 and 29 KDa for both the substrate by SDS-PAGE.

INTRODUCTION

Enzymes are found throughout nature in our bodies, in the environment and in living things .Enzyme speed up chemical and biochemical reaction and this process is called catalysis. Many enzymes have been successfully studied and applied to industrial and commercial uses. Among the plant and the animal enzymes , microbial enzymes find immense application, this is because microbes can be easily cultivated and their enzyme can be catalyzed by a wide variety of hydrolytic and synthetic reactions. A prominent carbonaceous constituent of higher plants and probably the most abundant organic compound is cellulose. Because a large part of the vegetation added to the soil is cellulose, the decomposition of carbohydrate has a special significance in the biological cycle of carbon. cellulose is a polysaccharide composed of glucose units a long linear chain linked by β-1,4 glycoside bonds (Mandels *et al.,* 1985).

Cellulases are group of hydrolytic enzymes capable of hydrolyzing cellulose to smaller sugar components like glucose units. Degradation of cellulose is brought about by fungi, bacteria and actinomycetes by secretion of extra cellular Enzyme cellulase. Celluloytic enzymes play an important role in nature biodegradation process where the plants cellulosic materials are efficiently degradable. Cellulases for instance have been utilized for extraction of valuable components from plant cell, improvement of nutritional values of animal feed and the preparation of plant protoplasts in genetic research (Sternburg and Dordal., 1979).The growing interest in microbial cellulases in recent years arises from research on utilization of waste materials as renewable bases. Microbes capable of producing cellulases are widely distributed in the various taxa. Cellulose is utilized by organism as carbon and energy sources. Thus cellulosic materials are classical renewable resources with world production calculated 10 tons per year (Pathak and Ghose, 1973). A wide variety of Gram positive and Gram negative species are reported to produce cellulose which

[**] ***Corresponding author:*** sajani.samuel123@rediffmail.com

includes *Clostridium thermocellum, Streptomyces spp, Ruminococcus spp, Pseudomonas spp, Cellulomonas spp, Bacillus spp, Serratia, Proteus, Staphylococcus spp, Bacillus subtilis*. The fungi such as *Penicillium fumiculosum, Aspergillus acculcatus, A.fumigatus, A.niger, Fusarium solani, T.viride, T.koningii, Rhizopus oryzae,Mucor pusillus (*Wood, 1982).

The actinomycetes such as *Streptomyces spp, Thermoactinomycetes spp, Thermonospora curvata* etc. (Emert *et.al*., 1974). More recently it is found that ability to produce cellulases is widespread among fungi that too ability to in last decade. Cellulase is used in fermentation of biomass into bio fuels and also in food processing (coffee).It is used in treatment of phytobenzoars and in animal health care, textile industry as a fading agent. It is used in SCP production as a supplement source of protein in human and animal diet. Research is going on in utilization of waste water materials as renewable resources. (Emert *et* al., 1974).

The raw materials commonly used are saw dust, coir waste, soyabean, bagasse and wheat straw, vegetablepotato tubers, corn waste, bannana waste etc. This study is focused on the production and partial purification of cellulose from bacteria and fungi (Cruick Shank, 1976).

MATERIALS AND METHOD

Collection of soil sample

Soil sample was collected from coir and saw dust industries at Perundurai village in Erode District. Upper layer of the soil was removed and the next layer of the soil was collected using sterile spatula in polythene bags and stored for further analysis.

Screening and isolation of the organism

One gram of the soil sample was weighed and mixed in 10 ml of water which was serially diluted from 10^{-1} to 10^{-7} dilution for both the substrate for bacteria and for fungi the dilution used was from 10^{-1} to 10^{-5} for both the substrate. Minimal salt mineral media (Sodium nitrate - 0.2 g, potassium dihydrogen phosphate - 0.1g, magnesium sulphate- 0.2 g, ferrous sulphate - 0.005 g, potassium chloride - 0.2 g, yeast extract - 0.5 g, tryptone -1g, cellulose -1 g, distilled water-100ml agar - 0.25g, pH-7) for bacteria and Carboxy methyl cellulose agar for fungi(potassium hydrogen phosphate - 0.19 g, di potassium hydrogen phosphate - 0.29 g, sodium nitrate - 0.25 g, magnesium sulphate - 0.12g, yeast extract - 0.12g, casein hydrolyzate - 0.12g, cellulose - 0.25g, agar – 0.25g, pH-5.5) plates were prepared and spread plate technique was done for isolating the colonies. The plates were incubated at 37ºC for 24 hr for bacteria and 27ºC for one week for fungi. After the incubation period the plates were flooded with congo red along with NaCl which was used to detect the organism producing cellulase (for bacteria and fungi).The colonies that produced zones were selected for both bacteria and fungi were purified and subcultured for other purpose. The bacterial and fungal isolates were identified using normal laborarty technique. The bacterial culture isolated was *Bacillus spp*,two *Pseudomonas spp* and *Proteus spp* and the fungal colonies isolated was *Aspergillus niger* and *Aspergillus fumigatus.*

Pretreatment of the substrate

Saw dust and coir waste were used as substrate. The raw substrate were sundried and crushed to powder. The substrate were soaked individually in 1%NaOH (1:10, substrate:solution) for two hours at room temperature. The substrate was washed free of chemicals and autoclaved at 121ºC for one hour. Treated substrates were then washed with distilled water which was used for inoculum preparation.

Inoculum preparation

The isolated bacterial colonies were inoculated individually into sterile MSM broth with saw dust and coir waste separately and incubated at 37 ºC in shaker for 24 to 48 hrs and used for further processes. Similarly the fungal colonies were inoculated into carboxy methyl cellulose agar broth and kept in shaker at 30º C for one week.

Optimisation

Opitimisation of cellulase producing bacteria and fungi was carried out using various parameters like pH, Temperature,carbon source etc. About 100ml of bacterial production media and fungal production media with varying pH 5,6,7,8 and 9 (both the substrate) and the temperature employed was 25ºC, 30ºC, 37ºC and 45ºC for both bacteria and fungi, the carbon source employed was glucose, sucrose, lactose, and cellulose was prepared. The media was inoculated with respective organism and incubated. The results were noted by taking the O.D value using calorimeter (Gascoigne and Gascoigne, 1960).

Production Media

100ml of production media was prepared Minimal Salt Media and Carboxy Methyl Cellulose Agar for two different substrates(for bacteria and fungi) sterilized, after cooling about 10ml of respective culture was inoculated and incubated at the pH and Temperature which was found to be optimum for the production of cellulase in bacteria and fungi. After incubation the production media was centrifuged at 4000 rpm for 10 min and the supernatant collected was filtered through millipore syringe filter were the filterate served as crude enzyme for further assay.

Enzyme Assay

Three different assays were followed to check the cellulase activity (Eriksson and Petterson., 1972). The assays are Gel punch assay, Dinitrosalicyclic method and Carboxy methyl cellulase assay.

Partial purification of cellulose

To 10ml of crude enzyme 50ml of ethyl alcohol was added and allowed for precipitation for an hour and then centrifuged at 5000rpm for 10min.The precipitate was collected from each source was dissolved individually in 3ml of sodium acetate buffer at pH7 for bacteria and pH5.5 for fungi.

Dialysis

Dialysis membrane about 5cm was cut and put into warm water for 15min and washed thoroughly with de-ionized water. Then the precipitate enzyme dissolved individually in sodium acetate buffer was poured into dialysis membrane and tied at both ends using thread and dialysed against the sodium acetate buffer at 4C overnight.

The dialysed sample were used for studing its molecular weight using SDS-PAGE.

RESULTS AND DISCUSSION

Spread plate technique

A total of about twenty different types of bacterial colonies were observed on Minimal Salt Media from both substrate (coir waste and saw dust), among which four types of colonies showed cellulase activity. The bacteria isolated was confirmed as *Bacillus spp*,two *Pseudomonas spp* and *Proteus spp* on gram staining and various other biochemical test, the colonies were subcultured on the same media and were labeled as(B,P1,P2,P3). The two fungal colonies were subjected to Lactophenol Cotton Blue staining (LPCB) and were found to be *Aspergillus niger* and *Aspergillus fumigatus*, the fungal colonies were also sub cultured on Carboxy Methyl Cellulose (CMC) agar slant and were labeled as (A1 and A2).

Table 1. STANDARD ENZYME

Percentage	*OD Value(µl/ml)*
5%	0.02
10%	0.03
15%	0.05
20%	0.06
25%	0.08
30%	0.09
40%	0.1
50%	0.12

Table 2. SUPERNATANT Zone Size

Culture	*Coir waste*	Saw dust
B	1.2	1
P1	0.8	0.8
P2	0.7	0.6
P3	0.5	0.4
A1	1.3	1.2
A2	1.1	1
Enzyme Standard	1	1

Table 3. CMC Assay OD Value

Culture	*Coir Waste (µl/ml)*	*Saw Dust (µl/ml)*
B	0.1	0.09
P1	0.07	0.07
P2	0.06	0.06
P3	0.03	0.05
A1	0.12	0.1
A2	0.09	0.08
Enzyme Standard	0.2	0.22

Table 4: DNS Assay OD value

Culture	*Coir waste(µl/ml)*	Saw Dust(µl/ml)
B	0.09	0.08
P1	0.07	0.06
P2	0.06	0.05
P3	0.03	0.01
A1	0.1	0.1
A2	0.09	0.08
Enzyme Standard	0.2	0.2

Table 5. pH Coir Waste (OD Value µ/ml)

pH	B	P1	P2	P3	A1	A2
5	0.06	0.05	0.05	0.04	0.1	0.09
6	0.08	0.06	0.05	0.05	0.08	0.07
7	0.1	0.09	0.07	0.06	0.06	0.05
8	0.07	0.05	0.05	0.04	0.05	0.04
9	0.06	0.04	0.05	0.03	0.04	0.03

Table 6: pH Saw Dust (OD Value µ/ml)

PH	B	P1	P2	P3	A1	A2
5	0.07	0.05	0.04	0.04	0.12	0.09
6	0.08	0.07	0.05	0.06	0.08	0.06
7	0.1	0.08	0.07	0.07	0.05	0.04
8	0.06	0.05	0.04	0.05	0.05	0.03
9	0.05	0.04	0.03	0.03	0.03	0.03

Table 7: Temperature Coir Waste (OD Value µ/ml)

Temperature	B	P1	P2	P3	A1	A2
25ºC	0.08	0.07	0.06	0.05	0.07	0.08
30ºC	0.09	0.08	0.07	0.05	0.12	0.09
37ºC	0.1	0.08	0.08	0.06	0.08	0.07
45ºC	0.07	0.07	0.05	0.04	0.06	0.06

Table 8: Temperature Saw Dust (OD Value µ/ml)

Temperature	B	P1	P2	P3	A1	A2
25ºC	0.05	0.06	0.05	0.04	0.06	0.05
30ºC	0.07	0.07	0.06	0.05	0.09	0.09
37ºC	0.09	0.08	0.07	0.07	0.05	0.05
45ºC	0.07	0.06	0.05	0.03	0.04	0.03

Table 9:Carbon Source Coir Waste (OD Value µ/ml)

Carbon Source	B	P1	P2	P3	A1	A2
Lactose	0.04	0.03	0.03	0.02	0.05	0.05
Glucose	0.06	0.07	0.05	0.04	0.07	0,07
Cellulose	0.08	0.08	0.07	0.05	0.08	0.07
Sucrose	0.05	0.05	0.03	0.01	0.06	0.06

Table 10: Carbon Source Saw Dust (OD Value µ/ml)

Carbon Source	B	P1	P2	P3	A1	A2
Lactose	0.05	0.04	0.04	0.03	0.05	0.06
Glucose	0.07	0.06	0.07	0.04	0.08	0.07
Cellulose	0.09	0.07	0.08	0.05	0.1	0.08
Sucrose	0.06	0.05	0.06	0.02	0.05	0.06

PURIFICATION

Turbidity occurred in the production media it was concentrated by centrifuging at 3500 rpm and then concentrated with ethyl alcohol and then dialyzed extensively in sodium acetate buffer the setup was placed at 4^0C incubation overnight. Dialysed enzyme thus obtained was checked for cellulase activity and was subjected to run on the SDS-PAGE and the molecular weight was found to lie between 6 to 20 KDa for *Bacillus spp* and for *A.niger* the molecular weight was found to lie between 20 to 29 KDa for both the substrate. The enzyme thus obtained was noted for its enzyme activity by using different assay (gel punch assay, Dinitrosalicylic acid and Carboxy Methyl Cellulose assay) . (Table 2, Table 3 and Table 4). The crude enzyme cellulase from supernatant was also used for optimization study(pH, Temperature, carbon source). In DNS method high level of cellulase production was achieved by *Bacillus spp* (0.09µl/ml and 0.08µl/ml) with coir waste and saw dust (Table 4). In case of fungi *A.niger* showed high level of cellulase activity(0.1µl/ml and 0.1µl/ml) with coir waste and saw dust (Table 4). Similarly in Carboxy Methyl Cellulose assay high level of cellulase production was achieved by *Bacillus spp*(0.1µl/ml and 0.9µl/ml) with coir waste and saw dust as substrate (Table 3). In case of fungi *A.niger* showed high level of cellulase activity(0.12µl/ml and 0.1µl/ml) with coir waste and saw dust (Table 3). Regarding pH, at pH7 maximum cellulase production(0.1µl/ml and 0.1µl/ml) was recorded by *Bacillus spp* using coir waste and saw dust as substrate (Table 6). Among fungi *A.niger* showed higher cellulase production(0.1µl/ml and 0.12µl/ml) at pH5 when coir waste and saw dust were used as substrate (Table 6). Regarding temperature at 37ºC maximum cellulase production (0.1µl/ml and 0.09µl/ml)was recorded by *Bacillus* and in fungi *A.niger* (0.12µl/ml and 0.09µl/ml) showed good cellulose activity at 30^0C when coir waste

and saw dust were used as substrate (Table 7 and Table 8).

Among different carbon source, cellulase activity was high in cellulose degrading (0.08µl/ml and 0.09µl/ml) by *Bacillus spp* and (0.08µl/ml and 0.1µl/ml) *A.niger* when coir waste and saw dust were used as substrate (table 8 and Table 9). The enzyme thus obtained was partially using sodium acetate buffer (pH 7and pH 5.5) and then the dialysed sample was run on SDS-PAGE and thus molecular weight was found to lie between 6 and 20kda for both substrate for *Bacillus spp* and *A.niger* it was found to lie between 20 and 29KDa for both substrate.

CONCLUSION

This work demonstrated that some bacteria and fungi are good producers of cellulose. This result indicated that suitability of using cheap and abundantly available coir waste and saw dust as solid substrate for large scale production of cellulase in Solid State Fermentation system thereby minimizing the high cost when other substrate and chemicals are used for cellulose enzyme. From this study it is evident that coir waste is a best carbon source for the production of cellulase than with the saw dust as it gives the higher yield of cellulase enzyme. Cellulase enzyme plays an important role in natures biodegradation process.To reduce the cost of production, the lignocellulosic substrates are used instead of systhetic cellulase due to reasonable cost, high enzyme production capacity etc, It is an important issue to deal with the residue both the comprehensive utilization of cellulosic resources and for prevention of environmental pollution.

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