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ISOLATION, SCREENING AND PARTIAL PURIFICATION OF CELLULASE FROM CELLULASE PRODUCING BACTERIA

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ABSTRACT:

The cellulase producing bacteria was isolated from cow dung and characterized by morphological and biochemical analysis. Further partial purification of cellulase was carried out by dialysis and ammonium phosphate precipitation and also to determine molecular weight by SDS-PAGE. Bacterial isolated were grown on Carboxy Methyl Cellulose (CMC) agar at various optimum conditions such as parameters like pH, temperature, incubation period, carbon and nitrogen sources prior to examined and identified primarily as genus, *Bacillus* species for cellulase production by morphological and biochemical analysis. Optimized conditions of CMC and Coir waste as substrates were Glucose, Yeast extract of pH-6 at 40ºC for 48 hour but saw dust as substrate has optimized pH-7 with Xylose, Yeast extract at 50ºC for 48 hours. Among three substrates used, Carboxy Methyl Cellulose was found to be the best substrate for cellulase production, when compared to coir waste and saw dust as substrates. This is due to the less complexity and hence easy assimilation of Carboxy Methyl Cellulose by the isolated microbe. Ammonium sulphate precipitation followed by dialysis was performed to partially purify the cellulase enzyme. The molecular weight was found to be 32.5 kilodaltons by SDS-PAGE method. On performing zymogram staining, the cellulolytic property was confirmed. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production and as a possible food supplement or in pharmaceutical industry.

Key words: Cellulase producing bacteria, Cellulase, Coir wastes, Saw dust, Cow dung, Partial purification

[I] INTRODUCTION

Cellulose is the most abundant biomass on the earth. Cellulases are inducible enzymes which are synthesized by large number of microorganisms either cell-bound or extracellular during their growth on cellulosic materials [1]. Cellulose, acrystalline polymer of D-glucose residues connected by β-1, 4 glucosidic linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature [2]. Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources.

Complete enzymatic hydrolysis of enzyme requires synergistic action of 3 types of enzymes, namely cellobiohydrolase, endoglucanase or carboxy methyl cellulose (CMCase) and α-glucosidases [3]. Cellulose is commonly degraded by an enzyme called cellulase. Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyse the cellulolysis (or hydrolysis) of cellulose [4]. This enzyme is produced by several microorganisms, commonly by bacteria and fungi. Cost of cellulase in enzymatic hydrolysis is regarded as a major factor [5].

Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness [6].Application of enzymes in textile, food, detergent, leather and paper industries demands identification of highly stable enzymes active at

extreme pH and temperature [7]. Cellulase is used in the fermentation of biomass into biofuels [8], fibre modification and they are even used for pharmaceutical applications.

Bacteria has high growth rate as compared to fungi has good potential to be used in cellulase production. Celluloytic property of some bacterial genera such as *Cellulomonas species*, *Pseudomonas species*, *Bacillus species and Micrococcus species* were reported [9]. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH, temperature, presence of inducers, medium additives, aeration, and growth time [10].

The aim of this study was to isolate and screening of cellulase producing bacteria from cow dung, optimization of conditions, production of cellulase, partial purification determination of Molecular weight.

[II] MATERIAL AND METHODS

2.1 Isolation and screening for cellulase producing bacteria

Cow dung samples were collected from Coimbatore district from two inches deep using a sterilized spatula in sterile containers [4]. The collected soil samples were suspended and 1g of sample was serially diluted in 10ml of sterile distilled water and mixed properly upto 10^{-7}. 100 µl of each dilution and water samples (without dilution) were spread on agar plates and then incubated at 37ºC for 24h.

2.1.1 Enrichment of pure culture

The colonies were diluted and plated on minimal agar medium were analysed for colony morphology and subcultured in minimal agar slants supplemented with 1% Carboxy Methyl Cellulose (CMC), mixed and incubated at 37˚C for 24h and then stored at a 4˚C [4].

2.2 Identification for cellulase producing bacteria

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations.

2.2.1 Morphological examinationby staining techniques

The plates were stained by Gram staining and Endospore staining for identification of bacillus species [11].

2.2.2 Biochemical Characterizations

The parameters investigated included Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Gelatin test, Casein hydrolysis test, Congo red test, Motility test, Amylase test, Nitrate reduction test, Carbohydrate fermentation test by standards methods [12]. The various media was prepared in sterile distilled water and pH was adjusted accordingly.

2.3Pre-treatment of substrates

The biowaste substrates were sun dried individually to reduce the moisture content and to make powder form. Then the substrates were soaked individually in 1% Sodium hydroxide solution (NaOH) in the ratio 1:10 (substrate: solution) for 2h at room temperature and autoclaved at 121°C for 1 hour [13]. The treated substrates were then filtered and washed with distilled water until the wash water becomes neutral. The Cellulase fermentation medium was seeded with 10% inoculum (CMC broth) incubated at 37ºC.

2.4 Submerged Fermentation process

10ml of broth culture was inoculated with 90ml of cellulase fermentation medium. Then it was kept in a shaker at 37ºC for 24h. Simultaneously, separate media were prepared for CMC, coir waste and saw dust as substrates [14].

2.5 Optimization of Culture Conditions on cellulase activity

Effect of pH, Temperature, Incubation period, Carbon and Nitrogen source on cellulase production

In order to determine all effects on cellulase production, the selected bacterial isolate was grown in CMC broth and incubated at various parameters. The influence of all factors on enzyme activity was determined by measuring cellulase activity at varying pH values from 4 to

8and temperature varying from 35 to 55ºC and incubation period varying from 24 to 120 h at 37°C. Both carbon and nitrogen sources have wide variety of impacts on the various organisms and enzyme production. Carbon and nitrogen sources have been replaced with various substances. Carbon source such as glucose, xylose, lactose, maltose, sucrose and Nitrogen source such as sodium nitrate, urea, beef extract, yeast extract, ammonium phosphate. All factors influence on enzyme activity was determined by measuring cellulase activity [15].

2.6 Enzyme activity assay

2.6.1 Preparation of crude enzyme

After incubation, the cultures were centrifuged and supernatants were used as source of crude enzyme [16]. The crude enzyme solution was utilized for determination of enzyme activity.

2.6.2 DNS method

Cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller, G.L., 1959) [17], through the determination of the amount of reducing sugars liberated from carboxy methyl cellulose (CMC) solubilized in 50 mM Tris–HCl buffer, pH 7.0. This mixture was incubated for 20 min at70 °C. For crystalline cellulose substrates, incubation times were extended to 2h h and the reaction was stopped by the addition of DNS solution. 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.

2.6.3 Protein determination

Protein concentrations in crude sample were determined by using a Lowry method [18] with bovine serum albumin (BSA) as a standard.

2.7 Partial purification of Cellulase

2.7.1 Ammonium sulphate precipitation

About 20ml of the crude enzyme prepared was brought to 80% saturation with solid ammonium sulphate [19]. The mixture was left overnight at 4ºC in a magnetic stirrer. Centrifuge the mixture and the pellet was dissolved in 10ml of 50mM sodium acetate buffer (pH-5.5) for further purification.

2.7.2 Dialysis

The pre-treated dialysis bag was used for the dialysis of the enzyme collected after the ammonium sulphate precipitation [19]. 8ml of the partially purified enzyme was dialyzed against 30mM sodium acetate buffer (pH-5.5) at 4ºC with three changes of buffer. The partially purified sample was assayed for enzyme activity and protein content.

2.8 Determination of molecular weight

2.8.1 SDS- Polyacrylamide Gel Electrophoresis

SDS PAGE was carried out in order to determine the molecular weight of purified enzyme sample [20]. It was carried out in such a way that the standard protein marker was loaded next to the purified sample, followed by the dialyzed and the crude. The silver staining was used to separate proteins by SDS- PAGE analysis.

2.8.2 Native PAGE and Zymogram staining for checking cellulase bands

PAGE also called native gel electrophoresis was performed according to the standard procedure, using a discontinuous buffer system without SDS [4]. The gel was removed and placed on a CMC-agar gel (CMC 1% and agar 1%). Both the gels were incubated at 37ºC for overnight. The CMC-agar gel was treated with sodium chloride for 15 minutes and then stained with congored solution. The bands of cellulase were seen as a clearance zone under black background.

[III] RESULTS

3.1 Isolation and screening for cellulase producing bacteria

Five isolates were obtained from cow dung sample and maintained in pure culture in CMC agar slants. The isolates were studied for colony morphology.

3.2 Identification for cellulase producing bacteria

The isolate appeared white colonies on CMC agar. A microscopic examination of the isolate revealed that it was a Gram positive bacterium with a centrally oval shape spore and produced

enzyme cellulase. Furthermore, the biochemical analysis of the isolates was performed and identified to be *Bacilli species.*

Thepositive results were tabulated **[Table-1]**.

S.no	Biochemical test	Observation	Result
1	Catalase	Effervescence	+
2	Oxidase	Deep blue colour	+
3	Indole	Cherry red ring	+
4	Voges-proskauer	Red colour	+
5	Methyl red	Red colour	+
6	Gelatin hydrolysis	Liquefaction	+
7	Citrate	Green to blue	+
8	Casein	Opaque	-
9	Starch hydrolysis	Clear zone	+
10	Nitrate reduction	Red colour	+
11	Motility	Motile	+
12	Sugar fermentation of		
	Glucose	Red to yellow	+
	Sucrose	Red to yellow	+
	Lactose	Red to yellow	+
	Maltose	Red to yellow	+
	Mannitol	Red to yellow	+
	Xylose	Red to yellow	+

- Negative and + Positive reaction.

Table: 1. Biochemical characteristics of the bacterial isolate

3.3 Optimization of Culture Conditions

The optimum conditions of cellulase producing bacteria were observed in three substrates and tabulated **[Table-2]**. Enzyme activity of CMC and coir waste were used as substrate was observed to increase in pH range of 5 to 8 but the maximum enzyme activity was recorded at a pH-6 with yeast extract as nitrogen source and glucose as carbon source at 40ºCfor 48h.On further incubation the values were found to decrease. When sawdust was used as substrate, the maximum enzyme activity was observed with yeast extract as nitrogen source and xylose as carbon source inpH-7 at 50ºC for 48h.

3.4 Enzyme activity assay

The crude enzyme solution was utilized for determination of enzyme activity. Cellulase activity was measured by the DNS method [17], from carboxy methyl cellulose (CMC), sawdust and coir waste. The specific enzyme activity of the crude sample was found to be 4.32U/mg.The protein concentrations in crude sample were determined with bovine serum albumin (BSA) as a standard.

[Table-2]

Optimization of Culture Conditions

Different parameters	Different values	Coir waste	Saw dust	CMC
Incubation period	24h	1	1.16	1.69
	48h	**1.72**	**1.82**	**1.89**
	72h	1.43	1.19	1.19
	96h	0.38	0.31	0.71
	120h	0.34	0.04	0.59
pH	4	4.69	1.75	4.48
	5	5.68	3.25	5.79
	6	**5.78**	3.40	**5.90**
	7	4.70	**3.43**	4.57
	8	4.37	1.86	4.50
Temperature	35°C	2.81	1.47	5.98
	40°C	**4.16**	2.31	**6.05**
	45°C	2.92	3.16	5.06
	50°C	2.47	**3.43**	4.82
	55°C	2.14	3.02	4.80
Carbon source	Sucrose	5.29	5.40	5.2
	Lactose	4.40	5.03	5.4
	Glucose	**6.01**	7.03	**10.96**
	Xylose	5.57	**8.68**	9.23
	Raffinose	4.62	3.11	4.30
Nitrogen source	Urea	2.17	1.56	3.51
	Yeast extract	**4.62**	**8.58**	**12.29**
	Beef extract	4.09	4.01	6.13
	Ammonium phosphate	3.90	3.56	6.00
	Sodium nitrate	1.17	1.92	5.18

Table: 2. Optimization of Culture Conditions

3.5 Partial purification of Cellulase

Partial purification of cellulase enzyme was carried out by dialysis followed by ammonium sulphate precipitation method. The specific

activity of the crude sample was found to be 6.57U/mg of dialysed sample and ammonium sulphate precipitated sample was found to be 4.21U/mg and 6.73U/mg respectively.

3.6 Molecular weight determination

The crude dialysed and the ammonium sulphate precipitated sample was loaded on SDS-PAGE and a protein profile was obtained. The molecular weight of the enzyme was determined and found out to be 32.5KDaby comparing with the molecular weight marker. The ammonium sulphate precipitated sample was run on native PAGE. The native gel placed over the CMC-agar gel was then subjected to zymogram staining. Clearance zone was observed confirming the presence of cellulose degrading enzyme.

[IV] DISCUSSION

Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes. Habitats that contain these substrates are the best sources in which to find these microorganisms [20]. Screening for the isolates with cellulolytic activity revealed that the spore formers were more prolific producers of the enzyme.

Cow dung was selected as a source for obtaining desirable cellulose producing organisms, because it is a rich source of diverse group of cellulolytic microorganisms owing to diet of the ruminants which consists of high amounts of cellulosic matter [10]. Further, its wide availability, ease of processing and cost effectiveness also plays an important role for its selection [20]. The isolated bacterial colonies were characterized for their morphological and biochemical characteristics as described [21] by Cappuccino and Sherman. Further, selected colonies were screened for their cellulase activity by Congo red test.

Media optimization is an important aspect to be considered in the development of fermentation technology. The isolated *Bacillus species* was inoculated in fermentation medium and production of cellulase was assayed [14]. Enzyme production was tested with different pH, different temperature, different carbon sources and nitrogen sources [22]. Based on the results, the fermentation media has been designed and the production of cellulase was carried out. The culture used for inoculation in the fermentation medium must be in healthy, active state and of optimum size, possibly in the log phase, thus it will be in its high rate for substrate conversion.

Cellulase is an inducible enzyme and it is affected by the nature of the substrates used for production. When CMC was used as substrate, the enzyme activity was observed to increase at 48h incubation on further incubation the values were found to decrease [20]. CMC as the substrate gave the highest yield of enzyme. It is assumed that this is due to the less complexity and hence easy assimilation of it by the isolated microbe [23]. Likewise the yield of enzyme using sawdust as the substrate was found to be very low and this is obviously is due to its complexity and hence less susceptible to degradation [22].

Among 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 [14]. Data are in accordance with the results of [24] who reported that organic nitrogen sources were found to be more suitable for optimizing cellulase production by *Bacillus species* than inorganic sources.The extracellular cellulase produced by the *Bacillus species* isolate was partially purified by ammonium sulphate precipitation and dialysis [9].

[V] CONCLUSION

The present work was carried out to optimize the nutritional and environmental parameters for improving cellulase production by the cellulolytic bacteria. The cellulase producing *Bacillus species* was isolated from cow dung and characterized by various staining procedures, biochemical analysis and partial purification. From my present study, the result showed that cellulase producing bacteria can grow at optimized condition. Thereby, partial purification of cellulase enzyme was done and determined molecular weight of the enzyme.The *Bacillus* species showed a potential to convert cellulose into reducing sugars which could be

readily used in many applications such as animal foods and a feed stock for production of valuable organic compounds [25]. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production [26] and as a possible food supplement or in pharmaceutical industry.

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