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Deciphering Cow Dung for Cellulase Producing Bacteria

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ABSTRACT

Bacterial species isolated from cow dung were screened for cellulase production and the culture MJRV1102P was found to have maximum cellulase producing capacities. The culture was further characterized on the basis of staining and biochemical activities and was found to be gram positive Cocci capable of producing endospores, positive in catalase and mannitol fermentation Tests. The isolate produced significant amount of cellulases after 96 h of fermentation in CMC containing medium with a Total CMCase activity of 0.3825 U.

Key words: Cellulases; Cow dung; CMCase.

INTRODUCTION

Cellulose is mainly degraded by an enzyme known as cellulase. This is mainly produced by Bacteria, Fungi and Protozoan that catalyse cellulolysis (i.e. hydrolysis) of cellulose. Cellulolytic microbes have important role in environment for degradation of cellulose and convert it into useful products. Cellulases are mainly classified into 5 types on the basis of types of reaction catalyzed- (as cited from Wikipedia)-

- **Endo-cellulase:** It break internal bond to disrupt crystalline structure of cellulose and expose individual cellulose polysaccharide chains.
- **Exo-cellulase**: It cleaves 2-4 units from the ends of exposed chains produced by endocellulase, resulting in the tetrasaccharide or disaccharide such as cellobiose. There are 2 main types of exo-cellulases (cellobiohydrolases, abbreviate CBH)-one type working

processively from the reducing end, and one type working processively from the non-reducing end.
Processive /Progressive cellulase-This cellulase continue to interact with a single polysaccharide strand.
Non-Progressive/Non-Processive-This cellulase will interact once then disengage and engage another polysaccharide strand.

- **Cellobiose /beta-galactosidase:** It hydrolyses the exo-cellulase product into individual monosaccharide.
- **Oxidative cellulase:** It depolymerise cellulose by radial reactions, as for instance cellobiose dehydrogenase (acceptor).
- **Cellulose phosphorylases**: It depolymerise cellulose using phosphate instead of water.

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 [1]. Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals [2].

Production of Cellulase has been reported earlier in various microbial systems like fungi, bacteria or actinomycetes, most common producer being the fungi. High growth rate of bacterial species in comparisons to the fungal species can be explored in order to check the cost of production which is going high because of the larger generation time of the fungi. However, the application of bacteria in producing cellulase is not widely used. Bacterial cellulase usually lacks one of the three cellulase activities, that is FPase. However, cellulase produced by bacteria is often more effective catalyst. They may also be less inhibited by the presence of material that has already been hydrolyzed (feedback inhibition). The greatest potential importance is the ease with which bacteria can be genetically engineered. This is needed especially in order to enhance cellulase production. [3]

In the present study bacteria inhabiting cow dung have been deciphered for their cellulase producing properties.

Looking at the industrial applications and the previous research on cellulase production some of them being [3-10] on the production and purification of cellulase the current research work was carried out with the following objectives in mind:

- *Isolation and screening of cellulase producing bacteria from cow dung.*
- *Staining and Biochemical characterization of cellulase producing Bacteria.*
- *Production and extraction of crude Cellulase.*
- *Enzyme assay by DNS method and protein estimation by Lowry's method.*

MATERIALS AND METHODS

Collection of sample

The cow dung sample was collected in sterilized Petri plate using sterile spatula from the cow shed near Gomti Nagar Railway Station, Lucknow, India. Precaution was taken to minimize the contamination.

Isolation of cellulase producing bacteria from cow dung

Bacteria inhabiting cow dung were isolated using serial dilution agar plating (diluting upto 10^{-5} dilution) method on nutrient agar plates. The mixed cultures obtained were differentiated on the basis of colony morphology and were named as MJRV1101P-04P and further purified by quadrant streaking on nutrient agar plates. The purity of cultures was cross checked by Gram's staining procedure.

Screening of purified cultures for cellulase production

The purified cultures were screened for cellulase production by streaking them on CMC agar (minimal agar media supplemented with 1% CMC) plates centrally and incubating them for 72 hours at 37ºC, after the incubation period the plates were flooded with 0.1 % Congo red solution and destained with 1M NaCl for 15-20 minutes as explained by [4].

Staining and biochemical characterization of culture positive in screening:

The culture positive in cellulase screening was characterized for the staining (Gram's staining, endospore staining) and biochemical activities (Catalase, mannitol fermentation,Voges Proskeurs).

Production of enzyme on laboratory scale by shake flask fermentation

The culture positive in cellulase screening was grown under optimal condition for cellulase production in 100 ml production medium (Peptone 5.0 gm/lt; yeast extract 5.0 gm/lt; KH_2PO_4 1.0 gm/lt; NaCl 5.0 gm/lt; $MgSO_4$ 0.2 gm/lt; pH- 7) containing 1% CMC and incubated for 96 hrs in shaker incubator (120 rpm) at 37°C. The fermented broth was filtered at 4°C and the filtrate was centrifuged at 5000 rpm for 5 minutes at 4°C to extract the crude extracellular cellulases. It was found to be 85 ml.

Estimation of protein in crude extract by lowry's method

Concentration of protein in crude extracellular extract was determined by Lowry's method [11]. Different concentrations of BSA (Bovine Serum Albumin) as standard protein in a range of 0.02-0.2mg/ml, were reacted with Lowry's reagent C and D and the absorbance at 660nm were read. A standard graph was plotted between concentration of protein in X axis and absorbance at 660nm in Y axis. 100 µl of extract was reacted with Lowry's reagent C and D and absorbance at 660nm was read. This absorbance was compared with the standard graph in order to get the concentration of protein in crude extracellular extract.

Enzyme assay by DNS method

CMCase activity was assayed using a standard method [12]. Different concentrations of glucose in a range of 0.05 to 0.5 mg/ml were reacted with Dinitro salicylic acid and absorbance readings

at 540 nm were recorded. A standard graph was plotted between concentration of glucose in X axis and absorbance at 540 nm in Y axis. The activity was estimated using 1% solution of carboxymethyl cellulose (CMC) in 0.1M Tris buffer (pH 7.0) as substrate. The reaction mixture contained 0.5 ml substrate and 0.5 ml of enzyme solution. The reaction was carried out at 37^{o}C for 15 min. The amount of reducing sugar released in the hydrolysis was measured. One unit of CMCase activity was expressed as micromoles of glucose librated per ml enzyme used per minute.

RESULTS

Isolation of cellulase producing bacteria from cow dung

Cellulase producing bacterial strain were isolated by the help of serial dilution agar plating method and the mixed colonies obtained were differentiated on the basis of morphological characteristics, the differentiated cultures were named as MJRV1101P, MJRV1102, MJRV1103P, MJRV1104P. All the four colonies were purified by quadrant streaking.

Screening of purified cultures for cellulase production

After flooding the screening plates with 0.1% Congo red dye and washing with 1N NaCl solution a clear zone of hydrolysis of CMC was obtained, culture MJRV1102P showed largest zone of hydrolysis around its colony. Screening results were ranked based on the hydrolysis zone as intense+++, moderate++, slight +, and no hydrolysis–. The same can be seen in Table 1 and figure 1 below.

Table 1: Ranking of different isolates on basis of zone of hydrolysis

S. No.	Culture	Ranking (zone of hydrolysis)
1	MJRV1101P	–
2	MJRV1102P	+++
3	MJRV1103P	++
4	MJRV1104P	+

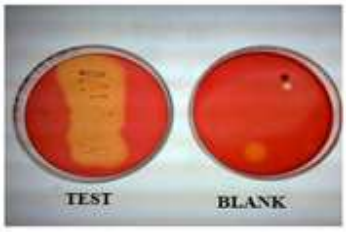

Figure 1: Zone of hydrolysis of CMC in MJRV1102P.

Staining and biochemical characterization of MJRV1102P

The Table 2 below shows the results of staining and biochemical activities of the cellulase positive culture MJRV1102P.

Table 2: Staining and Biochemical properties of MJRV1102P.

S. No.	STAINING/ TEST	RESULT
1	Gram's Staining	+ve Cocci
2	Endospore Staining	+ve
3	Catalase Test	+ve
4	Mannitol Fermentation	+ve

Protein estimation in crude enzyme by Lowry's method

Standard Graph:

Table 3 and figure 2 below show the standard graph for the estimation of protein in crude extract.

Table 3: Lowry's Standard Graph

S. No.	BSA (0.2mg/ml) (in ml)	D/W (in ml)	Conc. of BSA (mg/ml)	Reagent C (in ml)		Reagent D (in ml)		O.D (660nm)
1	0.0	1.0	0.0	5	**Incubate at RT for 10 minutes**	0.5	**Incubate in dark for 30 minutes**	0.0
2	0.1	0.9	0.02	5		0.5		0.03
3	0.2	0.8	0.04	5		0.5		0.08
4	0.3	0.7	0.06	5		0.5		0.13
5	0.4	0.6	0.08	5		0.5		0.18
6	0.5	0.5	0.1	5		0.5		0.23
7	0.6	0.4	0.12	5		0.5		0.28
8	0.7	0.3	0.14	5		0.5		0.33
9	0.8	0.2	0.16	5		0.5		0.38
10	0.9	0.1	0.18	5		0.5		0.43
11	1.0	0.0	0.20	5		0.5		0.48

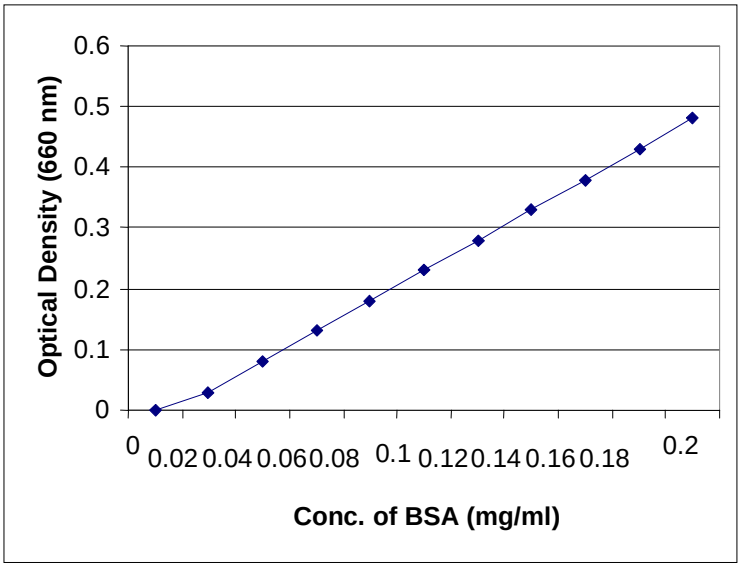

Figure 2: Standard Graph for Protein Estimation by Lowry's method.

Protein Estimation in Crude Enzyme:

Concentration of protein in the extracellular crude extract (85 ml) was determined by comparing the absorbance readings of the test sample with the standard graph and it was found out to be 0.268 mg/ml. Table 4 and Figure 3 below show the results of protein estimation.

Table 4: Protein Estimation in Crude Extract by Lowry's method

Test Tube	Crude Enzyme (In ml)	D/W (In ml)	Reagent C (in ml)	Incubate at RT for 10 minutes	Reagent D (In ml)	Incubate in dark for 30 minutes.	O.D (660nm)	Conc. of Protein (mg/ml)	Total protein (mg)
Blank	0.0	1.0	5		0.5		0	0.0	0.0
Crude Enzyme	0.5	0.5	5		0.5		0.56	0.268	86.36

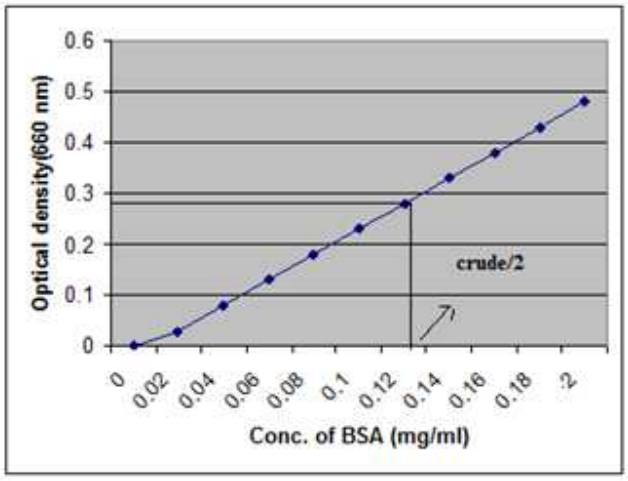

Figure 3: Estimation of Protein in Crude Cellulase by Lowry's method.

DNS assay of crude cellulase enzyme

Standard Graph:

Table 5 and Figure 4 below show the standard graph for DNS assay.

Table 5: Standard graph for DNS assay

S. No	Glucose (0.5mg/ml) [in ml]	Distilled water (in ml)	Conc. of Glucose (in mg/ml)	DNS (in ml)	Boil for 15 minutes	Optical Density (At 540nm)
1	0.0	1.0	0.0	1		0.00
2	0.1	0.9	0.05	1		0.04
3	0.2	0.8	0.10	1		0.10
4	0.3	0.7	0.15	1		0.16
5	0.4	0.6	0.20	1		0.21
6	0.5	0.5	0.25	1		0.26
7	0.6	0.4	0.30	1		0.31
8	0.7	0.3	0.35	1		0.36
9	0.8	0.2	0.40	1		0.41
10	0.9	0.1	0.45	1		0.46
11	1.0	0.0	0.50	1		0.51

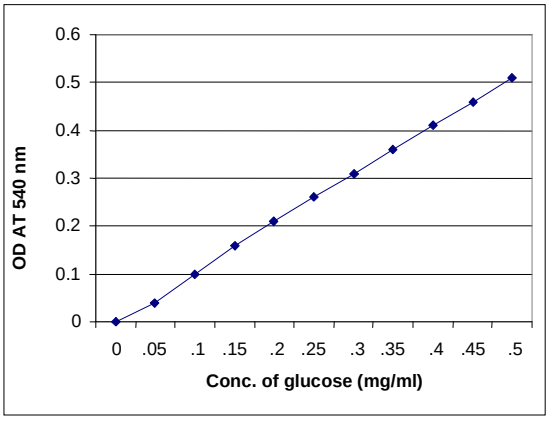

Figure 4: Standard Graph for DNS Assay.

DNS Assay of Crude Cellulase Enzyme:

After DNS assay O.D of reaction tube was 0.35 and thus the liberated concentration of glucose was 0.375 mg/ml which was calculated according to standard graph. Activity was calculated to be 0.0045 U/ml/min. Total activity of enzyme was 0.3825U, specific activity was 0.004U/mg. Results of the same are depicted in Table 6 and figure 5 below.

Table 6: Estimation of Cellulase Activity by DNS Assay

S.No.	1% CMC (In ml)	Vol. (In ml)	Incubate at 37°C for 15 minutes	Vol. of DNS (In ml)	Boil For 15 minutes	Optical Density (540nm)	Conc. of glucose (In mg/ml)	Activity (U/ml/min)
1	0.5	0.5 (D/W)		1		0.0	0	-
2	0.5	0.5 (Crude Enzyme)		1		0.35	0.375	0.0816

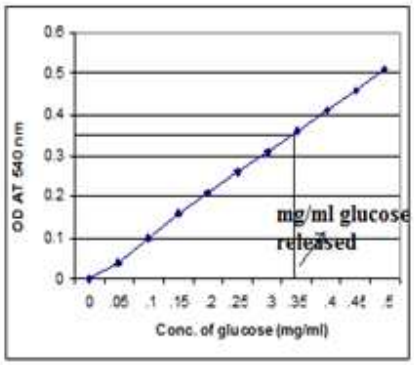

Figure 5: Graph for DNS Assay of Crude Enzyme.

DISCUSSION

Cow dung which can be a very good source for cellulase producing microbes was evaluated for the presence of cellulase producing bacteria in the present study. Cellulosic material if stands as such in the environment can be very problematic that's why in this study we are searching for the cellulase producers, So that we can convert the wastage celluloses into useful products.

Bacterial samples isolated and purified from cow dung [4] were screened for their cellulase producing capacity on to the Minimal Agar Medium supplemented with cellulose (CMC) further the hydrolysis was checked by Congo red dye test as done earlier by [4].

The culture showing maximum hydrolysis in the Congo red screening was characterized for various staining and biochemical activities.

Production of cellulase by the help of isolated bacteria was done through shake flask fermentation process by using the same production media as has been used earlier by [3].

After incubating for four days at 37°C cell free extract was obtained from fermented production media by filtering the production media through filter paper and spinning the filterate as has been done earlier by [13].

Cellulase production was assayed by using DNS method of [12]. The crude enzyme activity (0.0045 U/ml/min.) was observed after 4 days of incubation at pH 7 and 37°C in the present study, activity of 0.079U/ml/min has been reported earlier by [3].

CONCLUSION

Finally based on the present research it can be concluded that Cow dung can be a very good source for the isolation of Cellulase producing bacteria. Cellulases purified here can be used for all its applications.

Future work of the present work includes purification of enzyme in order to gain higher specific activity of cellulase by the help of sophisticated purification procedures including Salt/Solvent precipitation, Dialysis, Ion Exchange chromatography, Affinity chromatography, and HPLC, also the purified enzyme can be characterized for the effect of temperature, pH, activators and inhibitors. Molecular weight of purified enzyme can be determined by SDS-PAGE.

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