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Cellulase Activity Enhancement of Bacteria Isolated From Oil-Pump Soil Using Substrate and Medium Optimization

Amlan Chakraborty*, Alisha Mahajan

Amity Institute of Biotechnology, Amity University, Sector 125, Noida 201301, Uttar Pradesh
*Corresponding author: amlan.chakroborty@gmail.com

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Abstract Cellulase is composed of more than one distinctive enzymes which can degrade cellulose and is produced by a wide variety of fungal and bacterial species and the enzyme is transported across the cell membrane to the outside environment. The product of cellulose degradation is known to form glucose and important monomer highly used in food industry for producing various commercial products including alcohol. The problem lies in converting lipid rich cellulose source or cellulose from plants with a higher quantity of unsaturated fatty acids. This problem has created a need of finding bacteria capable of cellulose digestion without the inhibitory effects of lipids and poly-unsaturated fatty acids. To solve this problem, bacterial specie was isolated from a soil rich in oil, since it was collected from a petrol pump with little trace of vegetative cover. Also, due to lack of oxygen there was a chance of acclimatization of the bacteria and developing itself as an anaerobe. The cellulomonas medium was optimized by using different amino acids, Carbon sources, and Nitrogen sources. Extracted crude cellulase was subjected to change in pH, incubation temperature and metal ion supplementation and was inferred that Cysteine proved to be the best amino acid supplement followed by maltose being a good carbon supplement and ammonium chloride for nitrogen. At 60 ˚C and pH 7.15 the crude cellulase yielded higher glucose. Also a supplementation of Cobalt and Manganse enhanced the cellulase production enlightening the way that it may be used in traces for any metabolic pathway feeding the cellulase production. The colonies producing the crude cellulase had been tested on Whatman No.1 filter paper and have been found to grow colonied utilizing the filter paper as substrate. This infers the ability of the bacterium to produce cellulase and decay cellulose even at stressful conditions. The enhanced cellulase has negligible effect of poly-unsaturated fatty acids and capable of cellulose digestion from a diverse source.

Keywords: *cellulose, cellulase, oil-pump, filter paper substrate, cellulomonas sp.*

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1. Introduction

Cellulase is composed of more than one distinctive enzymes which can degrade cellulose and is produced by a wide variety of fungal and bacterial species and the enzyme is transported across the cell membrane to the outside environment [1]. In the first step, glycosidic linkage to cellobiose is broken [3] by beta-1, 4 glucanase [4], which is a glucose dimer having a beta-1, 4 bond [5] as unlikely to maltose, a counterpart with an alpha-1,4 bond. Subsequently, this beta-1, 4 glycosidic linkage is broken by betaglucosidase [2].

Bacterial species was isolated from a soil rich in oil, since it was collected from a petrol pump with little trace of vegetative cover. Also, due to lack of oxygen there was a chance of acclimatization of the bacteria and developing itself as an anaerobe. The cellulomonas medium was optimized by using different amino acids namely cysteine, lysine, tyrosine; Carbon sources like glucose, starch, maltose and Nitrogen sources like sodium nitrate, ammonium chloride, potassium sulphate, potassium nitrate, yeast extract and urea.

Furthermore, studies were conducted to observe the effects of different parameters on the stability and activity of Cellulase. These parameters include incubation time, temperature, pH and metal ions. The results were analyzed and used to optimize the working conditions of our experiments.

Usually in such experiments a separate carbon source is required for cellulase production as a substrate. For this, various substrates were used, during the course of our experiments, we observed that whatman filter paper I acted as the substrate instead of an additional carbon source with the enhancement of cellulase activity by varying the nutrient media. This observation attributes to the novelty of this research work.

2. Materials and Methods

2.1. Sample Collection

Collection of warm and moist oil pump soil was done which was kept overnight in 0.85% NaCl solution, further it was diluted and spread plating was done on cellulomonas sp. agar.

2.2. Isolation of Cellulomonas sp. Bacteria

Cellulomonas sp. media was prepared with composition (all percentage in w/v): Peptone 1%, Carboxymethyl cellulose 1% (substrate), K2HPO4 0.2%, MgSO4 0.03%, NH2(SO4)3 0.25%, Gelatine 0.2%, Agar 1%, pH 7.0±0.2. The autoclaved media was poured in sterile petriplates. Serial dilutions up to 10-7 for the soil suspension were made and spreading was done. For comparative study, standardized Cellulomonas sp. media lacking the cellulose source (i.e. substrate) was prepared. The autoclaved media was poured in sterile petri plates containing Whatman No. 1 Filter paper (as the source of cellulose / substrate for cellulase) as sandwich in between molten medium. Serial dilutions up to 10-3 were made for the soil suspension and spreading was done. Also complete cellulomonas sp. Media with 1% carboxymenthyl cellulose was prepared and colony morphology was studied hence and compared to that of the filter paper.

2.3. Screening and Identification of Cellulomonas sp. Bacteria

Colonies were investigated for their morphological characteristics by studying under the compound microscope. Furthermore, the colonies were subjected to gram staining and were checked for spore formation, shape and arrangement of the colonies which grew on the agar plates. The agar plates were flooded with Congo-Red and were de-stained with 9% (w/v) Sodium Chloride. Eventually, bacterial colonies were observed along with zone of clearance; which denoted digested cellulose. White colored colonies or zones of bacteria were visible on the plates.

2.3.1. Biochemical Tests Carried out on Cellulomonas sp.

Catalase test, Indole test, Citrate test, Voges- Proskauer test, and Nitrite and Nitrate Reduction test were performed on the isolated bacteria and the result was compared with standard test results.

2.4. Inoculum Development

Colonies of cellulolytic bacteria were picked up from each of the prepared plates and were inoculated in the test tubes that contained cellulomonas sp. media. Growth was accounted for Cellulomonas sp. after incubation at 37°C for 48 hours.

2.5. Optimization of Nutritional Condition for Cellulase Production

For the optimization, variation in amino acid, carbon source and nitrogen source was carried out. For amino acid variation, cellulomonas specific media was supplemented with cysteine, lysine and tyrosine. These amino acids were chosen since it has been found that they play a key role in producing cellulase. For carbon source variation, cellulomonas specific media was supplemented with glucose, starch and maltose. For nitrogen source variation, Cellulomonas specific media was supplemented with sodium nitrate, ammonium chloride, ammonium sulphate, potassium nitrate, yeast extract and urea. After the variation of nutritional sources, all the test tubes were inoculated with cellulolytic bacteria and growth was observed. The tubes were centrifuged, and supernatant was collected. Cellulase specific activity was calculated for each of the variations in each category using the Lowry method for finding the total protein content and Di-nitro Salicylic acid assay (DNS Assay) for the production of the orange chromatophore at 540 nm.

For the DNS assay, the supernatant of the individual variations were taken and was incubated with Carboxymethyl cellulose as substrate under specific incubation time and temperature. Due to the enzyme's activity on cellulose glucose was produced which was quantified by the DNS assay using a glucose standard curve.

4.6. Characterization of Crude Cellulase of Newly Isolated Cellulolytic Strain

4.6.1. Effect of Incubation Temperature on Activity and Stability of Crude Cellulase

The inoculated Cellulomonas sp. media was kept at 30°C, 42°C, 60°C, 82°C and 100°C. The supernatant collected after centrifugation was supplemented to 500µl of phosphate buffer having 1% (w/v) Carboxymethyl Cellulose (as substrate) and the specific activity was recorded using the DNS assay procedure (discussed earlier).

4.6.2. Effect of pH on Activity and Stability of Crude Cellulase

The supernatant which was collected after centrifugation of inoculated standardized Cellulomonas sp. media was added to 500µl of phosphate buffer having 1% (w/v) Carboxymethyl Cellulose (as substrate) and the pH was maintained at 2, 5.51, 7.15, 10.35, 12.58. The specific activity was recorded using the DNS assay procedure (discussed earlier).

4.6.3. Effect of Metal Ions on Activity and Stability of Crude Cellulase

The inoculated standardized Cellulomonas sp. media was supplemented with metal ions Cobalt, Mercury, Calcium, Manganese, Iron, Magnesium and even the chelating agent EDTA. The supernatant which was collected was added to 500µl of phosphate buffer having 1% (w/v) Carboxymethyl Cellulose (as substrate) and the specific activity was recorded using the DNS assay procedure (discussed earlier).

3. Results and Discussion

3.1. Identification of Cellulomonas sp. Bacteria

Cellulolytic bacteria were successfully isolated from soil. The morphology of potential cellulolytic strains from all seven petri plates was analyzed. Colonies of Cellulomonas sp. on CMC agar were pale yellow color, shining with prominent margins, average diameter being 1 mm forming a convex and sometimes circular structure at 30°C. When scraped with a loop, colonies were slimy or

viscous and tend to clump. For comparative study cellulolytic colonies from a petri plate containing filter paper was also analyzed for better interpretation of results. Fresh culture of this isolate consists of Gram positive, slender, and rod shaped cells but the older cultures contain cells which were identified to be coccoid. Upon examination under microscope of this isolate, it was revealed that it was gram negative upon gram staining along with no spore formation taking place. Cellulolytic bacterial colonies (distinct) with clear white zones in the plates stained with Congo-red and de-stained with Sodium Chloride is noticeable marked with arrows and Carboxymethyl cellulose as substrate (Figure 1) and with Whatman No.1 Filter paper as substrate (Figure 2).

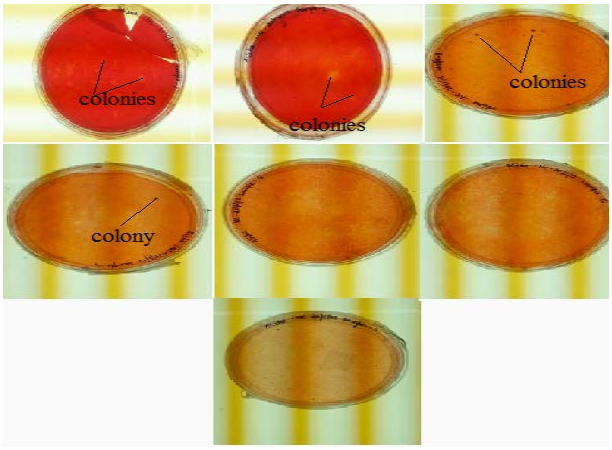

Figure 1. (Color online) Cellumonas sp. growing on CMC (as subsrate) stained and de-stained with congo-red

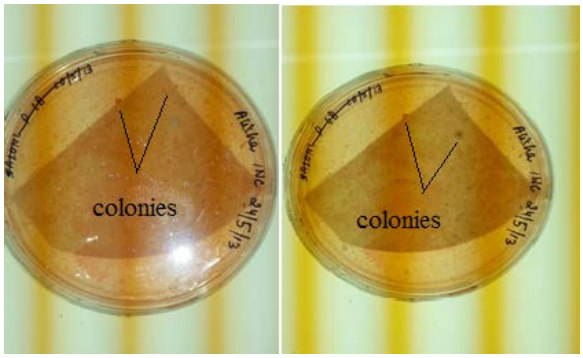

Figure 2. (Color online) Cellumonas sp. growing on Whatman No. 1 Filter paper (as subsrate) stained and de-stained with congo-red

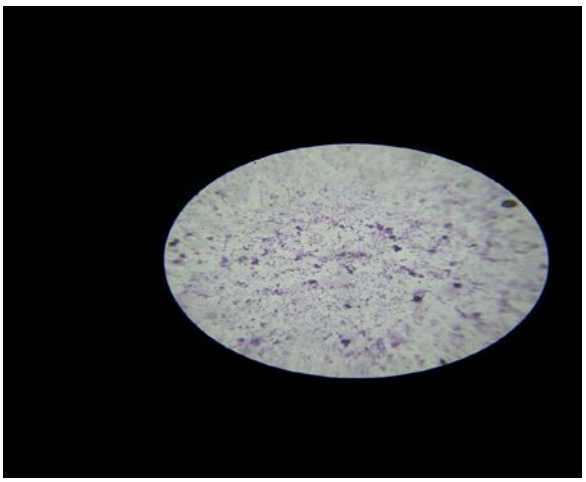

Figure 3. (Color online) Cellulomonas sp. was observed under 40X microscope showing Gram positive

The result of the biochemical tests conducted has been summarised in Table 1 below. Cellulomonas bacteria was found to be Gram-positive as was inferred by Gram staining which showed violet colour (Figure 3) with no spore formation. The bacterium is rod shaped as was visualised under microscope.

Table 1. Summary of Biochemcal test conducted on the isolated Cellulomonas sp. of bacteria

Biochemical tests	Inference	Observation
Catalase test	Positive	Immediate effervescence or bubbles were observed upon addition of 3% H_2O_2 and also it can be inferred that the bacterium is an aerobe and possess the enzyme catalase.
Indole test	Negative	The culture was a bit cloudy with persistent yellow colour and no pink red colour was formed which would have been formed for a positive Indole test
Citrate test	Negative	Growth was not visible on the slant surface and the medium was an intense Green.
Nitrate and Nitrite Reduction test	Positive	The addition of zinc dust to the uninoculated broth in forces the reduction of the NO_3 to NO_2. Reagents A and B are already present, therefore the reagents react with NO_2 resulting in a red color change.
Voges-Proskauer test	Negative	The Methyl Red test or the Voges- Proskauer test did not give any positive result. No red colour was observed instead a yellow colour was observed

Specific enzyme activity of the enzyme was calculated using the following formula:

$$\text{Units}(\text{U})/\text{ml}/\text{min of enzyme}\ (\mu g \text{ of glucose } released) = \frac{\times(Toal\ assay\ volume)\times Dilution\ Factor}{(Vol\ of\ enzyme\ used)\times(Volume\ in\ cuvette)} \times Duration\ of\ Incubation\ in\ \text{minute}$$

Cellulase Specific activity was calculated using the formula:

$$\text{Specific activity of cellulase} = \frac{\text{Units of Enzyme used}}{\text{Total protein present}}$$

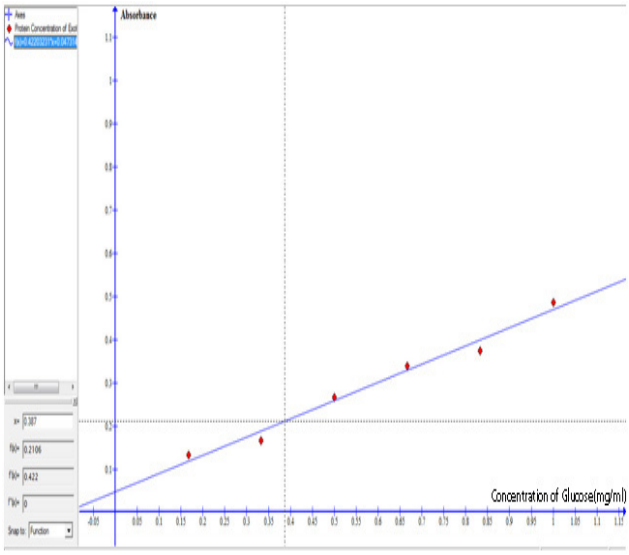

Figure 4. (Color online) Graph showing Concentration of Cellulase (x = 0.387) on the Glucose standard curve

It was found that 19 µg of enzyme was present, which was inferred from the absorbance plot on y-axis of the glucose standard curve (Figure 4).

Therefore cellulase specific activity was calculated to be 0.516 ± 0.2 U/ µg.

3.2. Amino Acid Variation

A negative control was considered for the media in which no amino acid was added as a supplement. Figure 5 shows the variation in color intensity upon 1:10 dilution with Phosphate buffer of the various amino acid upon DNS reagent addition. It has been found that Cysteine showed a higher specific activity of 0.516 ± 0.2 U/µg (Table 2) which was higher than the control set.

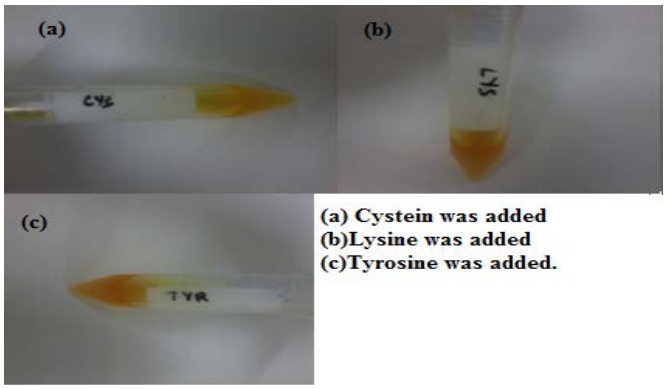

Figure 5. (Color online) Variying color intensity after 1:10 phosphate buffer dilution of the amino acid variation with DNS added.

3.3. Carbon Source Variation

A negative control was considered for the media in which the carbon source was Carboxymethyl cellulose. Figure 6 shows the variation in color intensity upon 1:10 dilution with Phosphate buffer of the various carbon source upon DNS reagent addition. It has been found that Maltose showed a higher specific activity of 0.581 ± 0.2 U/ µg (Table 2) which was higher than the control set. This was followed by starch and hence proved the ability of the cellulase enzyme to cleave the glyocosidic bond.

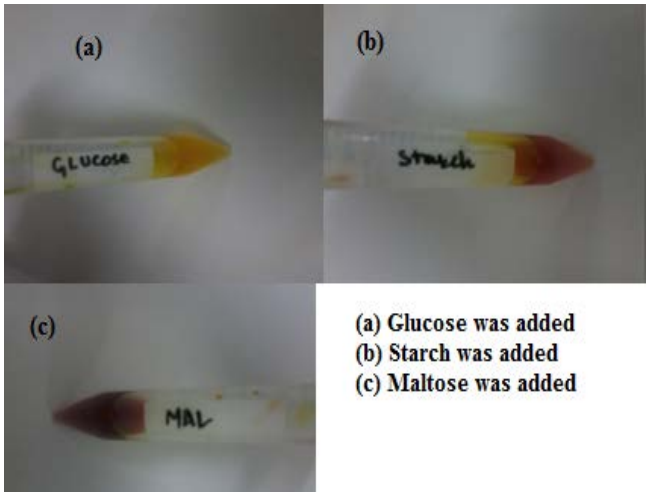

Figure 6. (Color online) Variation in carbon source leading to color enhancement of DNS assay

3.4. Nitrogen Source Variation

A negative control was considered for the media in which no nitrogen source was added as a supplement. Figure 7 shows the variation in color intensity upon 1:10 dilution with Phosphate buffer of the various nitrogen source upon DNS reagent addition. It has been found that Ammonium Chloride showed a higher specific activity of 0.482±0.2 U/ µg (Table 2) which was higher than the control set. Nitrates of sodium and potassium has been fruitful too and showed nearby activity in considerable higher ranges than that of the control set.

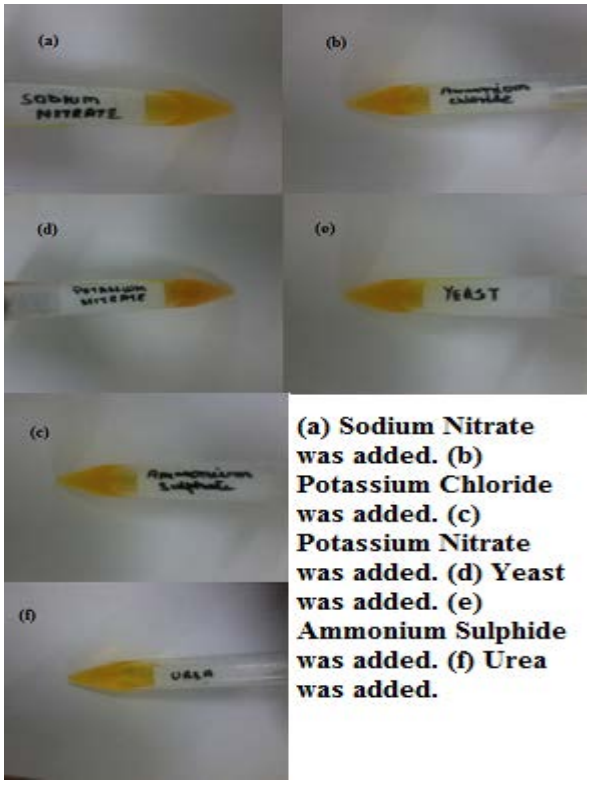

Figure 6. (Color online) Nitrogen source variation showed by difference in color intensity upon DNS reagent addition

3.5. Physical Parameters Variation

Figure 7. (Color online) Metal ion variation supplementing cellulase production, showing variation in color of DNS assay

The variation in incubation temperature for the activity of the enzyme, was found maximum at 60°C infering the optimum incubation temperature (Table 2) producing a higher specific activity of the enzyme cellulase. A pH of 7.15 was the optimum pH (Table 2) producing a higher specific activity of the enzyme cellulase as well as for its stability. Further, a supplement of Cobalt showed a higher

specific activity followed by Manganese in close quarters (Table 2), of the enzyme cellulase as well as for its stability. Figure 8 shows the variation in color on metal ion supplementation. The color intensity variation of the DNS assay proves that there is a positive effect on cellulase activity enhancement.

Table 2. Specific activity of the variations done for the enhancement of cellulase activity

Amino acid	**Amino acid variant**	**Specific activity (U/µg)**
	Control	0.413±0.2
	Cysteine	0.516±0.2
	Lysine	0.473±0.2
	Tyrosine	0.378±0.2
Carbon source	**C-source variant**	**Specific activity (U/µg)**
	Control	0.217±0.2
	Maltose	0.581±0.2
	Starch	0.342±0.2
	Glucose	0.306±0.2
Nitrogen source	**N-source variant**	**Specific activity (U/µg)**
	Control	0.207±0.2
	Sodium Nitrate	0.443±0.2
	Ammonium Chloride	0.482±0.2
	Ammonium Sulfate	0.421±0.2
	Potassium Nitrate	0.411±0.2
	Urea	0.309±0.2
Incubation Temperature	**Temperature (°C)**	**Specific activity (U/µg)**
	30	0.078±0.2
	42	0.233±0.2
	60	0.245±0.2
	82	0.198±0.2
	100	0.004±0.2
pH	**pH**	**Specific activity (U/µg)**
	2.00	0.058±0.2
	5.51	0.198±0.2
	7.15	0.544±0.2
	10.35	0.103±0.2
	12.58	0.009±0.2
Metal ions and chellating agent	**Metal Variant**	**Specific activity (U/µg)**
	Control	0.319±0.2
	Cobalt	0.546±0.2
	Mercury	0.088±0.2
	Calcium	0.324±0.2
	Manganese	0.471±0.2
	Iron	0.011±0.2
	Magnesium	0.276±0.2
	EDTA (chellating agent)	0.241±0.2

4. Conclusion

In the prior art it is known that in an oil pump soil generally contains bacteria which can degrade oil to form simple carbon compounds and thereby shows biosurfactant property. Here in this work we have got results that similar bacteria is capable of degrading cellulose, a compound that is widely available in nature and whose monomers are essential for producing alcohol of commercial grade [6,7]. This work optimizes the isolated bacteria capable of cellulose degradation and also we have screened and got results of degradation of filter paper used as a carbon source. It has been found by our work that Cysteine supplementation proved to be effective in producing the enzyme cellulase in higher amounts. Further, a carbon source supplementation of maltose and nitrogen source of Ammonium chloride is fruitful in enhancing the cellulase production. Also, an incubation temperature of 60°C, for the action of enzyme on cellulose and pH of 7.15 proved to be effective than the rest of the conditions. Both Cobalt and Manganese served to be an effective supplement to enhanced cellulase production and may have a role in the bacteria's biochemical pathways. The novelty lies in the source of the organism as a high lipid containing cellulose substrate would be tedious and impossible for cellulase to act on, but our isolated bacterium would be highly effective in use of the same, in degrading cellulose.

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