

Isolation, Screening and Purification of Cellulase from Cellulase Producing *Klebsiella variicola* RBEB3 (KF036184.1)

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Abstract: The cellulase producing bacteria were isolated from soil samples of paper industry waste, cloth industry waste, kitchen waste, garden, earthworm. Isolates were characterized on the basis of their morphological, biochemical and molecular characteristics. Total 32 isolates were obtained by primary screening technique from which 19 isolates showed cellulolytic activity, 06 isolates showed maximum cellulase activity and 13 showed a minimum cellulase activity. The Potential isolates were obtained from kitchen waste soil and garden soil and by characteristic study was found to be *Klebsiella variicola* RBEB3 and the sequence submitted to NCBI with accession number KF036184.1. Further purification of the cellulase enzyme was carried out by ammonium sulphate precipitation followed by gel filtration chromatography. The molecular weight of the enzyme was found to be 45 kDa by SDS PAGE method. On performing zymogram staining, the cellulolytic activity was confirmed, the enzyme activity was found to be 0.092 IU/ml and protein concentration was found to be 4.8 mg/ml. The use of microorganisms offers a promising approach for the large scale production of enzymes and as a food supplement or in pharmaceutical industry.

Keywords: Cellulase producing bacteria, purification, cellulase, *Klebsiella variicola*, Ammonium sulphate precipitation, Gel filtration chromatography.

1. Introduction

Cellulose is a renewable source of energy and a major structural component of plants [1]. It is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere [2]. It is the largest component of plant residues enter the terrestrial ecosystems and so represents a huge source of energy for microorganisms, the main agents responsible for soil organic matter decomposition [3]. Microorganisms like bacteria are responsible for the bioconversion of cellulose. It is a linear, unbranched polymer having glucose connected by β -1, 4 glycosidic linkages [4]. It is resistant to most forms of degradation and accumulates within the environment. Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic bacteria. Complete enzymatic hydrolysis of cellulase enzyme requires the synergistic action of three enzymes, namely endoglucanase, cellobiohydrolase or exoglucanase and β -glucosidase [5]. These enzymes convert cellulose into a utilizable energy source, glucose which provides a key role in utilization of biomass. Cellulases are the enzymes that help in the conversion of cellulose substrates into fermentable sugars. There is an increasing interest in cellulase production by bacteria as it has a high growth rate when compared to fungi and has a good potential in the production of cellulase. It is used in ruminant nutrition for

improving digestibility, fruit juices processing and in deinking of paper [6]. Cellulase producing bacteria has the potential to convert cellulose into glucose. The isolation and characterization of cellulase producing bacteria will be an important aspect of biofuel research, biodegradation and bioremediation. For this project purpose, cellulose degrading bacteria was isolated from soil samples of garden, earthworm, kitchen waste, paper industry waste and cloth industry waste. The Gram's iodine test was done to check the cellulolytic activity of the isolated bacterial strains. The morphological and biochemical analysis of the isolated microbes were conducted. It was then processed for cellulase activity and purification.

2. Literature Survey

Cellulase was produced by *Bacillus pumilus* EB3 using carboxymethyl cellulose (CMC) as substrate. Screening of bacteria, optimization of fermentation conditions and selection of substrates are important for the successful production of cellulase. Cellulase produced from *B.pumilus* EB3 was purified using ion exchange chromatography with an ion exchanger (HiTrap QXL) for characterization of the cellulase. Purification of cellulase from *B.pumilus* EB3 using ion exchange chromatography showed that 98.7 % of total CMCase was recovered. Characterization of the enzyme found that CMCase by *B. pumilus* EB3 has a molecular weight range from 30-65 kDa and was optimally active at pH

6.0 and temperature 60 °C [7].

A microorganism hydrolyzing carboxymethyl cellulose was isolated from a paddy field, identified as *Bacillus* sp. Production of cellulase by this bacterium was found to be optimal at pH 6.5, 37 °C. This cellulase was purified to homogeneity by the combination of ammonium sulphate precipitation, DEAE cellulose, and sephadex G-75 gel filtration chromatography. The enzyme was a monomeric cellulase with a relative molecular mass of 58 kDa, as determined by SDS-PAGE. The enzyme exhibited its optimal activity at 50 °C and pH 6.0. The enzyme was stable in the pH range of 5.0 to 7.0 and its stability was maintained for 30 min at 50 °C and its activity was inhibited by Hg²⁺, Cu²⁺ [8].

An alkaline cellulase was purified from a newly isolated alkalophilic *Bacillus* sp. HSH-810. It was optimally active at pH 10 and 50 °C and was stable from pH 6-10 with more than 60 % activity remaining after heating at 60 °C. The molecular mass of cellulase was found to be 80 kDa [9].

3. Methodology/ Approach

3.1 Isolation and screening for cellulase producing bacteria

Soil samples of garden, earthworm, paper industry waste, cloth industry waste, kitchen waste were collected from Bangalore district in sterile containers using a sterilized spatula [10]. 1 g of collected soil sample was suspended in 9ml of saline solution and was shaken thoroughly. Dilutions were made from it upto 10⁻⁶. 100 µl of solution from each dilution was taken and spread on CMC agar media plates. Plates were then incubated overnight at 37°C (M. L. Maki et al., 2011).

The plates were flooded with 1 % Grams iodine solution to see the cellulolytic activity of the isolated strain which gave dark blue plates. The formation of a clear zone of hydrolysis was observed around the colonies indicated cellulose degradation [11].

3.2 Enrichment of pure culture

The colonies showing a significant clear zone were plated onto the minimal agar medium and were analyzed for colony characteristics and subcultured onto the minimal agar medium containing 1% Carboxy Methyl Cellulose (CMC), incubated at 37°C for 24 hours and then stored at 4°C [10].

3.3 Identification for cellulase producing bacteria

The isolated bacterial strains were identified by means of morphological, biochemical and molecular characterization.

3.3.1 Morphological Characterization

The plates were examined by Gram staining for identification [12]. It is a differential staining technique by which we can distinguish the gram positive and gram negative bacteria. Bacteria are divided into two groups, based on whether they retain or lose crystal violet after treatment with iodine and alcohol and counter staining with

safranin.

3.3.2 Biochemical Characterization

The bacterial isolates were identified by performing biochemical tests like Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Sucrose fermentation test, Starch hydrolysis test, Urease test by standard methods [13]. The various media was prepared in sterile distilled water and pH was adjusted accordingly.

3.3.2 Molecular Characterization

The molecular characterization of cellulose degrading bacteria was done by 16S rRNA technique [14].

3.4 Secondary screening and production of cellulase enzyme

For preparation of standard inoculum, the isolate that showed a maximum zone of hydrolysis was cultured in 100 ml LB Broth medium and incubated at 37 °C for 24 hours.

3.4.1 Preparation of crude enzyme

After incubation, the cultures were centrifuged at 8000 rpm for 10 minutes and supernatant was used as a source of crude enzyme. The crude enzyme solution was used for the determination of enzyme activity.

3.5 Purification of Cellulase

3.5.1 Ammonium sulphate precipitation

100 ml of the crude enzyme prepared was brought to 75% saturation with solid ammonium sulphate. The mixture was left overnight at 4°C in a magnetic stirrer. The mixture was centrifuged and the pellet was dissolved in 1 ml of phosphate buffer saline for further purification.

3.5.2 Gel Filtration Chromatography

100µl of the enzyme was applied to 6 % CL Agarose matrix column which was previously equilibrated with 50 ml of 1 X Phosphate Buffer Solution. The column was run with 1 X PBS as mobile phase. The collected elute fractions (2 ml) was monitored for enzyme activity as well as for protein concentration at 280 nm. The fraction showing high activity was pooled and used for SDS-PAGE analysis.

3.6 Determination of molecular weight

3.6.1 SDS- Polyacrylamide Gel Electrophoresis

SDS PAGE was performed to determine the molecular weight of the purified enzyme sample. The standard protein marker was loaded next to the purified sample, followed by the crude. The eluted fraction collected from gel chromatography was used for analysis by SDS-PAGE [15]. Resolving gel consisted of 12% polyacrylamide in Tris-HCl (1.5 M, pH 8.8), while stacking gel consisted of 5% polyacrylamide in Tris-HCl (1 M, pH 6.8).

3.6.2 Native PAGE and Zymogram staining for checking cellulase bands

SDS PAGE (12 %) was prepared with substrate Carboxy Methyl Cellulose (1 %). The samples were loaded with zymogram sample loading buffer. The gel was removed and washed with water. Renaturation and Denaturation buffer was added to the gel. The gel was incubated at 37°C for

overnight. The gel was stained with Congo red solution and treated with sodium chloride for 15 minutes. A clear zone was observed as the cellulase bands [10].

3.7 Enzyme assay

3.7.1 DNS method

Carboxy methyl cellulase (CMCase) activity was measured by the DNS method through the amount of reducing sugars released during hydrolysis. 1% solution of carboxy methyl cellulose (CMC) prepared in 0.05 M citrate buffer (pH 4.8) was taken as substrate. 1 ml citrate buffer, 0.5 ml of substrate solution and 1ml of crude enzyme solution was added. The reaction mixture was incubated at 45°C for 30 min. The amount of reducing sugars released during hydrolysis was measured by DNS method. The reaction was stopped by the addition of DNS reagent. The treated samples were boiled for 10 min and cooled in water to stabilize the colour. The optical density was measured at 550 nm. The cellulase activity was measured using a calibration curve for glucose [16].

3.7.2 Filter paperase activity

Whatmann no. 1 filter paper strip (1 x 3 cm) soaked in 1.8 ml 0.05M sodium citrate buffer (pH 4.8) was taken as the substrate. The reaction mixture was incubated at 40°C for 60 minutes. The reaction was stopped by the addition of 3 ml of di nitro salicylic acid (DNS) reagent. The reagent tubes were then placed in water bath at 100°C for 15 minutes. 1 ml of Rochelle salt solution was added to the reagent tubes. The absorbance was measured at 575 nm [17].

3.7.3 Protein estimation

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

4. Results and Discussion

4.1 Isolation and screening of cellulose degrading bacteria

Nineteen isolates were obtained from soil samples (as shown in table 1) and maintained in pure culture in CMC agar. The bacterial colonies were spotted on fresh CMC agar media. The cellulases producing bacterial strain were identified by the zone of clearance. This was revealed by adding Gram's iodine solution. The isolates were studied for colony morphology.

Table 1: Details of the isolates isolated from different soil samples

S. No.	Source of soil sample	Representative isolates
1	Cloth waste	C ₅ , C ₉
2	Kitchen waste	K ₂₅ , K ₃₁ , K ₃₈
3	Garden soil	G ₁ , G ₁₄ , G ₁₇ , G ₁₈ , G ₁₉ , G ₂₂ , G ₂₉ , G ₃₀
4	Paper waste	P ₁ , P ₄ , P ₅ , P ₈
5	Earthworm	E ₅ , E ₁₈

4.2 Identification of cellulose degrading bacteria

4.2.1 Morphological identification

The bacterial isolates were identified by gram staining technique, out of 19 isolates 6 isolates showed maximum zone of clearance and was found to be gram negative rod shaped bacteria and the other 13 isolates showed a minimum zone of clearance and was found to be Gram positive rods and Gram positive cocci. These bacterial isolates have firmly bound cellulases. Furthermore, the organism which showed maximum zone of clearance ie. gram negative rod was biochemically identified.

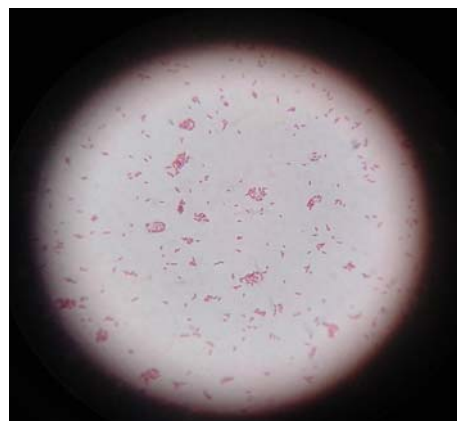


Figure 1: Microscopic view of gram negative rods

4.2.2 Biochemical identification

The biochemical characterization of the isolated strains which showed maximum zone of clearance were done and the results are tabulated in **Table 2**

Table 2: Physiological and biochemical characteristic of isolated strains

Characteristic	G ₃₀	K ₃₁
Gram stain	-	-
Morphology	Rod	Rod
Indole test	-	-
Voges-Proskauer	+	+
Methyl Red test	-	-
Citrate Utilization	+	+
Catalase test	+	+
Oxidase test	-	-
Starch Hydrolysis	+	+
Sucrose Fermentation	-	-
Urease test	-	-

4.2.3 Molecular identification:

The culture that was labeled as K₃₁ and G₃₀ was identified as *Klebsiella variicola* based on sequence homology and phylogenetic analysis. The 16S rRNA sequence of *Klebsiella variicola* was deposited at NCBI with accession number KF036184.1.

4.3 Purification of cellulase

Purification of cellulase enzyme was carried out by ammonium sulphate precipitation method and gel filtration chromatography.

4.4 Molecular weight determination

The crude and the ammonium sulphate precipitated sample were loaded on SDS-PAGE and a protein profile was obtained. The molecular weight of the enzyme was determined and found out to be 45 kDa by 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.

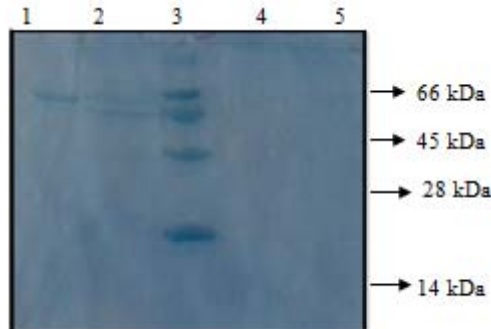


Figure 2: SDS PAGE of purified cellulase enzyme

Lane 1- Sample K₃₁, Lane 2- Sample G₃₀, Lane 3- Four band marker.

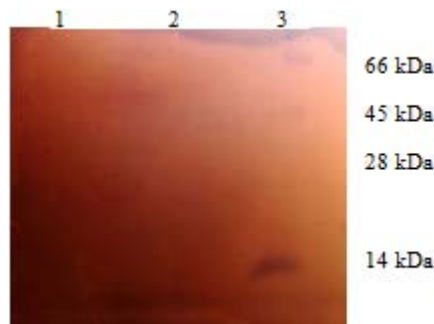


Figure 3: Zymogram staining

Lane 1- Sample G₃₀, Lane 2- Sample K₃₁, Lane 3- Four band marker

4.6 Protein estimation by Lowry's Method

The protein concentration in crude sample was determined with bovine serum albumin (BSA) as a standard and was found to be 4.8 mg/ml.

Table 3: Protein Estimation by Lowry's Method

S. No	Amount of BSA (μl)	Concentration of BSA (mg/ml)	Volume of Distilled water (μl)	Complex Reagent	Incubate at room temperature for 30 minutes	F.C. Reagent	incubate at room temperature for 10 minutes	O.D at 660 nm
1	0	0	200	↓ 3 ml ↑		↑ 0.3 ml ↓		0
2	20	20	180					0.1
3	40	40	160					0.2
4	60	60	140					0.3
5	80	80	120					0.3
6	100	100	100					0.4
7	120	120	80					0.3
8	50		150					1.0

PROTEIN ESTIMATION

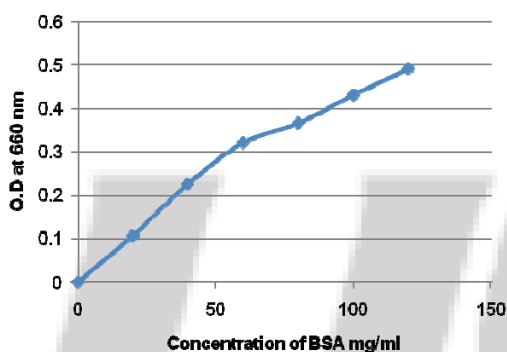


Figure 4: Protein estimation curve

4.7 Enzyme activity assay

The crude enzyme solution was utilized for the determination of enzyme activity. Cellulase activity was measured by the DNS method from Carboxy methyl cellulose (CMC). The enzyme activity of the crude sample was found to be 0.092 IU/ml.

Table 5: Determination of enzyme activity by DNS method

S.NO	Glucose Standards (mg/0.5 ml)	O.D at 540 nm
1	1	1.66
2	0.8	1.707
3	0.6	1.239
4	0.4	0.899
5	0.2	0.778

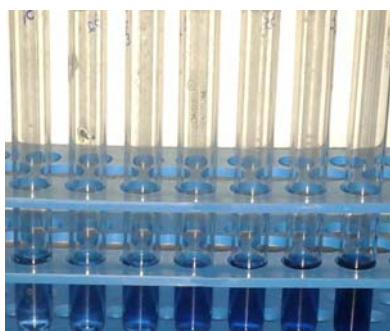


Figure 5: Colour formation by Lowry's Method

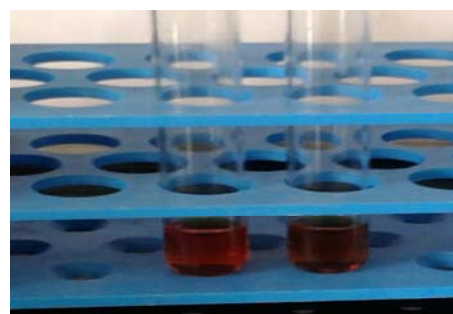


Figure 6: Colour formation by DNS Method

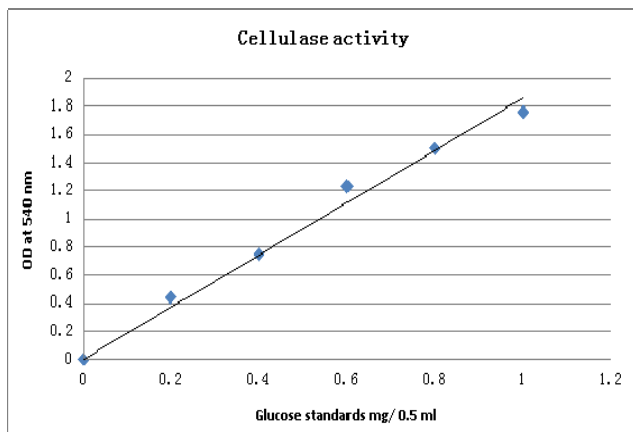


Figure 7: Linear glucose standard curve for the determination of cellulase activity

Enzyme activity = Amount of glucose released in 0.5 ml sample (mg) * dilution rate / 0.18 mg . min⁻¹* 60 min
 = 0.25 mg * 4/ 0.18* 60
 = 0.092 IU/ ml

5. Discussion

Degradation of cellulosic materials is a complex process requiring microbial enzymes. Habitats that contain these substrates are the best sources to find these microorganisms [15]. The sites for sample collection were selected as those rich in cellulosic biomass such as soil samples of paper industry waste, cloth industry waste, kitchen waste, garden and earthworm. These were the maximum possibilities to get cellulase producing bacterial strain. The wide availability, ease of processing and cost effectiveness plays an important role for its selection. The cellulolytic activity shown by the isolated bacterial strains was found to depend on the source of occurrence in natural environments which enables them to be responsible for cellulose degradation that occurs in various amounts of biowaste [5]. Screening of the isolates was carried out for their cellulase activity by using media containing 1 % CMC as a source of carbon. After incubation, the plates were flooded with 1 % Gram's iodine solution. It resulted in dark blue plates and the zone of hydrolysis was appeared to be uncoloured which showed the cellulase activity. The selected bacterial isolates were characterized for their morphological and biochemical characteristics. The isolated bacterial strain was identified to be gram negative rods. Secondary screening was done on the basis of their ability to show maximum enzyme activity. The extracellular cellulase produced by the isolate was purified by ammonium sulphate precipitation and gel filtration chromatography [11]. The molecular weight of the enzyme was determined by SDS PAGE. Zymogram staining was done and a clear zone was observed confirming the presence of cellulose degrading enzyme. The protein concentration of the crude enzyme was determined carried out by Lowry's method [18]. Genomic DNA was isolated from the bacteria. PCR amplification of this DNA was done with universal primer specific to 16S rRNA gene. the size of amplified PCR product was around 1.5kb. For analyzing the sequence, direct sequencing of the PCR product was carried out. The sequence of the 16S rRNA gene was analyzed by bioinformatics tools like BLAST. The results showed 99 % resemblance with *Klebsiella variicola*. Cellulase is an inducible enzyme and it is affected by the

nature of substrates used for production. CMC as a substrate gave the highest yield of enzyme. It is due to the less complexity and easy assimilation of it by the isolated microbe [17]. Glucose was found to be the best carbon source for the substrate CMC as glucose is a good inducer of the cellulase production.

6. Conclusion

The cellulase producing bacterial species was isolated from soil samples of garden, earthworm, kitchen waste, paper industry waste, cloth industry waste and characterized by various staining procedures, biochemical analysis. Purification of cellulase enzyme was done by ammonium sulphate precipitation. The enzyme solution from ammonium sulfate precipitation was subjected to gel filtration chromatography on CL-agarose column. The proteins of crude enzyme extract were separated in different eluted fractions. All the fractions were assayed for cellulase activity by DNS method and it was found that the cellulase activity was 0.092 IU/ ml. The molecular weight of the enzyme was determined by SDS PAGE and was found to be 45 kDa. Zymogram staining was done and the cellulase bands were seen as a clear zone which confirmed the presence of cellulose degrading bacteria. The concentration of the cellulase protein estimated by Lowry's method was found to be 4.8 mg/ ml. Genomic DNA was isolated from the bacteria. PCR amplification of this DNA was carried out using universal primer specific to 16S rRNA gene. The size of the amplified PCR product was 1.5 kb. The sequence of the 16S rRNA gene was analyzed by bioinformatics tools like BLAST. The results showed 99 % resemblance with *Klebsiella variicola*. The cellulase producing bacteria has the ability to convert cellulose into reducing sugars which could be readily used in animal foods, food supplement and in pharmaceutical industry. Improvement in the performance of cellulase can be imparted by mutagenesis and protein engineering techniques for the better industrial applications.

7. Future Scope

The isolated strains that are screened can be used for large scale fermentation for the conversion of cellulose into glucose and production of cellulase enzyme.

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Shreya Ranjit, final year M. Tech student at Department of Biotechnology, Acharya Institute of Technology, Bangalore. For the partial fulfillment of M.tech the project was carried out under the guidance of Dr. S.M.Gopinath, Prof and HOD, Prof Ismail Shareef.M and under the mentoring of Prof.Ashalatha Asst Prof, Biotechnology department, Acharya Institute of Technology at Aristogene Biosciences Private Limited, Bangalore. The isolation, screening, amplification of the strain was carried out independently and the sequence of the strain *Klebsiella variicola* RBEB3 is submitted to NCBI having accession number KF036184.1.