IJBPAS, May, 2025, 14(5): 2413-2425

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ISOLATION, SCREENING AND CHARACTERIZATION OF CELLULASE PRODUCING BACTERIAL STRAIN FROM FECAL SAMPLE OF NILGAI (*Boselaphus Tragocamelus)*

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Received 15th Nov. 2023; Revised 19th Dec. 2023; Accepted 8th June 2024; Available online 1st May 2025

https://doi.org/10.31032/IJBPAS/2025/14.5.8867

ABSTRACT

Increasing concerns about fossil fuel availability, greenhouse gas emissions, and pollution caused by the partial combustion of fossil fuels have resulted in a greater emphasis on the use of cellulases to perform enzymatic hydrolysis of lignocellulosic biomass. The primary barrier is the complex structure and composition of lignocellulosic biomass, which are mostly composed of cellulose, hemicellulose, and lignin. Microbes have enzymes capable of breaking down lignocellulose and producing fermentable sugars.

The aim of this study was to isolate and screened out potential cellulolytic bacteria from fecal sample of Nilgai (*Boselaphus tragocamelus).* Based on experiment, total of five bacteria were isolated and out of five only NE bacterial isolate produced clear zone into carboxymethyl cellulose (CMC) agar plate and were identified as cellulase producing bacteria. According to the morphological and biochemical tests, NE bacterial isolate was identified as *Pseudomonas* sp. NE showed maximum cellulase production (0.061 U/ml) at pH 7.0 after 60 h of incubation at 40°C in a medium containing 1.0% CMC. This bacterial isolate's mesophilic characteristic and ability to produce CMCase supports its potential viability for the present industrial processes that convert lignocellulosic biomass into biofuel.

Keywords Cellulase, lignocellulosic biomass, bacterial isolates, characterization, herbivores

INTRODUCTION

The worldwide ecological economy depends heavily on lignocellulosic biomass, abundant renewable resource obtained from a variety of plant resources **[1]**. Since there is a growing emphasis on lowering fossil fuel consumption and addressing climate change, lignocellulosic biomass has gained a lot of interest as a sustainable alternative to fossil fuels for energy **[2]**. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin, with each adds special qualities and functions to the structure of the lignocellulosic biomass **[3]**. Cellulose is the primary polysaccharide found in the plants and it's an essential part of plants structure and serves as a renewable energy source in biospheres **[4-6]**. According to Lynd et al., cellulose is composed of linear chains of β-D-glucose units joined by β-1,4 glycosidic linkages. The effective exploitation of cellulose from lignocellulosic biomass remains a considerable problem due to its resistant nature **[2]**.

Cellulases, which are generated by bacteria, fungi, and algae have a varied set of enzymes with distinct roles **[7]**. Numerous cellulase producing microbes were discovered over the years including bacteria belonging to the families of *Aeromonas*, *Bacillus*, *Micromonospora*, *Acidothermus*, *Paenibacillus*, *Streptomyces*, and *Pseudomonas* **[8-10]**, cellulolytic fungi belonging to the genus of like *Aspergillus*, *Humicola*, *Trichoderma*, *Talaromyces* **[11-13]** and microalgae like *Chlamydomonas reinhardtii* and *Volvox carteri* **[14]**. (Guerriero et al. 2018). Cellulase enzyme is composed of mainly three group of enzymes. Endoglucanases (E.C. 3.2.1.4), Cellobiohudrolase (EC 3.2.1.91) and β-glucosidase (E.C.3.2.1.21) with each enzyme contributes to a distinct phase of cellulose hydrolysis, which works in harmony to break down cellulose into glucose units and soluble sugars **[3,15]**. Bacterial cellulases are widely employed because to their rapid growth, durability at pH as well as temperature extremes, low feedback inhibition, and ability to thrive in a variety of environmental habitats **[16]**. Numerous sources such as compost piles, decaying agricultural waste, ruminant feces of cow, buffalo, horses or wild herbivores like deer, nilgai etc. have been used to identify the cellulase-producing **[17, 18]**. The processing and extraction of fruit and vegetable juices in the food business, as well as the brewing, agricultural, biofuel, laundry, pulp and paper, and biopolishing and biostoning in the textile industries, are some

possible commercial applications of the cellulases **[19]**.

Isolation and characterization of high-yielding strains of cellulase-producing bacteria from less expensive sources remains a challenge for industrial applications. According to Varga and Kovler, 20 – 70 % of cellulose in animal feed may not be digested by the animal **[20]**. The crude fiber degradation in the gut is not ideal, and the amount of fiber in feces is still significant **[21]**. This study aimed to identify and characterize cellulase producing strain from fecal sample of the Nilgai (*Boselaphus tragocamelus*) from sayaji Baug Zoo, Vadodara, Gujarat, India and determine optimal conditions for optimum production of cellulase enzyme for commercial usage.

MATERIAL AND METHODS

Collection of sample

The Fecal sample of Nilgai (*Boselaphus tragocamelus*) was collected from its natural habitat, Sayajibaug Zoo, Gujarat, India. For sample collection, a pre-sterilized spatula, gloves, and plastic zip bags were used, and before bacterial isolation samples were maintained in the cold box for approximately 12 hours at 4 °C.

Isolation of cellulolytic bacteria

Cellulolytic bacterial isolates from fecal samples were identified using serial dilutions and the pour plate method. A fecal sample (0.5g) suspended in 50 ml of 0.85% w/v saline solution in a 250 ml conical flask is used to isolate cellulolytic bacteria. It was then stirred at a temperature of 37°C and 180 rpm for an hour. Saline solution was used to create successive dilutions up to 10^{-8}. Carboxymethylcellulose (CMC) Agar plates contains following components (g/L): Agar powder (15), carboxymethylcellulose (CMC) (10), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.2), NH_4NO_3 (1.0), $FeCl_3.6H_2O$ (0.05), and Bushnell Hass Broth (3.27) and an aliquot of 0.1 ml of each dilution was added to plates. The plates were incubated for 72 hours at 37°C. Discrete colonies were selected and through repetitive streaking, bacterial colonies were purified. Purifies colonies were kept at 4°C for further identification **[22, 23]**.

Screening of Cellulolytic Bacteria

Discrete colonies with distinct morphologies were selected from various dilution plates and inoculated into LB broth for overnight incubation at 37°C. From freshly grown bacterial culture, 3µl were dropped on CMC-BH agar plates and further incubated at 37°C for 72 hr. After incubation period 1% Congo red solution was flooded onto the plates for around 20 min. The stain was discarded and plates were then counter strain with 1 M NaCl for another 15 min **[24]**. The isolates that had

a clear hydrolytic zone around the colonies were selected and employed to produce enzymes in a liquid medium. As for quantitative analysis, isolates with a significant zone of hydrolysis were grown in LB broth and incubated for overnight at 37°C in shaking incubator at 180 rpm. 1 ml of grown culture was inoculated into 100 ml enzyme production media and incubated for 72 hr at 37°C and 180 rpm. After incubation cultures were centrifuged and resulting crude enzyme used for estimation of enzymatic activity by 3,5-dinitrosalicylic acid (DNS) method **[25]**.

Identification of Cellulase producing bacteria

Morphological Characterization

Colony morphologies including their shape, size and pigmentations, was used to identify the isolates. Isolates were stained using gram stain reagents and then observed under oil immersion lens (100X) magnification under a light scope microscope to determine their Gram's nature **[26]**.

Biochemical Characterization

Bacterial isolates were characterized biochemically by performing different tests like MR-VP test, citrate utilization, catalase test, indole production, starch hydrolysis and triple sugar iron Agar (TSI) test and compared as described in Bergey's Manual of Systematic Bacteriology **[27]**.

Enzyme Assay

Cellulase production medium

The isolates with best zone of hydrolysis were grown overnight in LB broth medium. To obtain crude enzyme, 500 µl of overnight grown bacterial culture were transferred to 50 ml of enzyme production media containing (g/L) carboxymethyl cellulose (CMC) (10), yeast extract (4), and Bushnell Hass broth (3.27) and incubated for 3 days at 37°C and 180 rpm in orbital shaker. Production media was centrifuged at 10,000 g for 10 min and resultant crude enzyme was used for various enzyme assay **[20, 28]**.

Assay of Endo-β-1,4-glucanase activity

Enzyme activity of bacterial isolates was measured by 3,5-dinitrosalicylic acid (DNS) method. Reaction mixture comprised 0.6 ml of crude enzyme and 2.4 ml of substrate (1% carboxymethyl cellulose (CMC) in 50mM sodium phosphate buffer pH – 7.0) was incubated at 37°C for 20 minutes in a water bath. The addition of 3 ml of DNS reagent stopped the reaction. The mixture was then boiled for 10 minutes. At 540 nm, the absorbance of the samples was evaluated in comparison to a blank that contained all the reagents excluding crude enzyme **[29]**. One unit activity was described as the amount of

enzyme needed to hydrolyze CMC and release 1 µmol of reducing sugars per minute per milliliter under specific assay condition.

Assay of β-glucosidase

β -glucosidase activity of isolates was assayed using p-nitrophenyl- β-D-glucopyranoside (pNPG) method. A reaction mixture of 1.5 ml containing 1 ml of substrate (5mM pNPG in 50mM sodium phosphate buffer, pH – 7.0.) and 0.5 ml of enzyme was incubated at 37℃ for 1h. After incubation reaction was terminated by addition of 1.5 ml of 1 M Na_2CO_3. The developed colour was read using spectrophotometer at 405 nm using p-nitrophenol (pNP) as standard **[30]**.

Optimization of culture condition for enzyme production

To examine the effects of pH, temperature, and incubation time on cellulase production, selected bacterial isolates were tested under various conditions. Enzyme reaction mixture was incubated with range of different temperature (35°C-50°C) and different pH range (pH 5-8) the enzyme activity determined using 3,5-dinitrosalicylic acid (DNS) method **[31]**. Also, enzyme production media incubated for 72 h at 37°C and every 12 h culture was taken and centrifuged and collected supernatant was used for estimation of enzymatic activity **[28]**. Each experiment was conducted in triplicates.

Determination of Substrate specificity

Various substrates like carboxymethyl cellulose (CMC), p-nitrophenyl- β-D-glucopyranoside (pNPG), beechwood xylan and amylum were used to determine the substrate specificity of the strain NE **[32]**.

RESULTS AND DISSUASION

Isolation and screening of potential cellulase producing bacteria

In this study, *Boselaphus tragocamelus* fecal sample was examined for the presence of cellulase producing bacteria. Five bacterial strains were isolated from the fecal sample on CMC-BH agar plates. Plates were stained with 1 % congo-red solution for 20 min followed by 1M NaCl counterstain for 15 min. Among five bacterial isolates only one isolate (NE) showed clear hydrolysis zone around colony which reflect their extent of cellulolytic activity **(Figure 1)**. Zones of hydrolysis can be difficult to identify using this procedure, and it can be time-consuming. Plate-screening techniques with dyes are not quantitative due to a tenuous relationship between enzyme activity and halo size **[33, 34]**. Initial enzyme activity of bacterial isolates before optimization are shown in **Table 1**. CMCase activity of bacterial isolate NE was greater than the CMCase activity generated from certain known natural isolates. for example,

Bacillus sp. (0.0197 U/ml), *Geobacillus* sp. DUSELR7 (0.058 U/mL) **[30, 31]**.

Characterization of cellulose producing bacteria

Following gram staining, a microscopic analysis showed that the bacterial isolate NE was rod-shaped and gram positive in nature **(Figure 2)**. The isolate gave positive test for methyl-red, voges-proskaur and catalase test. TSI agar test results for bacterial isolate NE suggests that it is capable of fermenting sugars to produce gas, leading to the observed gas production with slant and butt colour change. However, it does not produce hydrogen sulphide, as evidenced by the absence of blackening in the agar medium. Whereas a negative result in the test for the formation of indole signifies the lack of tryptophanase, an enzyme necessary for the conversion of tryptophan into indole, while a negative result in the test for starch hydrolysis shows the absence of amylase, an enzyme that breaks down starch into simpler sugars.

Morphological and biochemical characteristics of the isolate NE have been summarized in **Table 2**. Both characteristics led to the identification of the isolated strain as *Pseudomonas* sp. Biochemical and morphological information provides valuable insights into the metabolic capabilities of bacterial isolate NE, helping to characterize its phenotype and potentially aiding in its identification and classification.

Optimizing parameters for the production of cellulase

The findings showed that at 40°C temperature and pH 7.0, bacterial isolate NE displayed its maximal enzyme activity **(Figure 3a and Figure 3b)**. Both higher and lower temperatures as well as pH values outside of the neutral range resulted in a reduction in enzyme activity. Bacterial isolate NE showed maximum production between 36 to 60 hrs and the highest activity was found at 60 hrs **(Figure 3c)**. Furthermore, enzyme activity rose with longer incubation times up to 60 hours, after which it decreased. The reported maximal enzyme activity of bacterial isolate NE at 40°C and pH 7.0 is consistent with the physiological conditions suitable for many bacterial enzymes. Also 40°C is in the mesophilic temperature range, which is ideal for the development and activity of many bacteria. Furthermore, pH 7.0 is a neutral pH that has been associated to the most optimal enzyme activity for a variety of bacterial enzymes.

Determination of Substrate specificity

The bacterial isolate NE has the highest affinity for the carboxymethyl cellulose (CMC) substrate as shown in **(Figure 3d)**. This indicates that it is very effective in

breaking down or utilizing CMC as a substrate. NE exhibits some degree of specificity towards the substrate p-nitrophenyl-β-D-glucopyranoside (pNPG), albeit not as much as it does for CMC. NE has minimal specificity for the substrates beechwood xylan and amylum, indicating that it is not as active or effective with these specific substrates. This information might be useful for better understanding the enzymatic capabilities and prospective uses of this bacterial isolate in biotechnological or industrial processes involving substrate breakdown or modification.

Table 1: Initial CMCase activity (U/mL) of bacterial isolates after 72 h incubation at 37°C and 180 rpm

Isolate no.	CMCase Activity (U/ml)
NA	0.0098
NB	0.0294
NC	0.0348
ND	0.0139
NE	0.0582

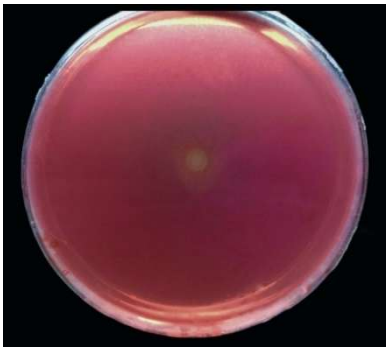

Figure 1: Primary screening of cellulase producing bacterial isolate NE on CMC-BH agar plate

Table 2: Morphological and biochemical characteristics of the bacterial isolate NE

No.	Biochemical test	Observation
1	Gram's reaction	-
2	Cell shape	Small
3	Cell arrangement	Rod
4	Methyl-red	+
5	Voges-Proskaur	+
6	Indole production	-
5	Catalase test	+
6	Starch hydrolysis	-
T	Slant Colour	+
S	Butt Colour	+
I	Gas Production	+
	H_2S production	-

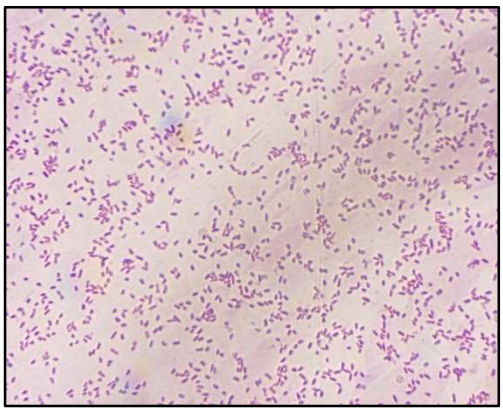

Figure 2: Microscopic observation of bacterial isolate NE after Gram staining

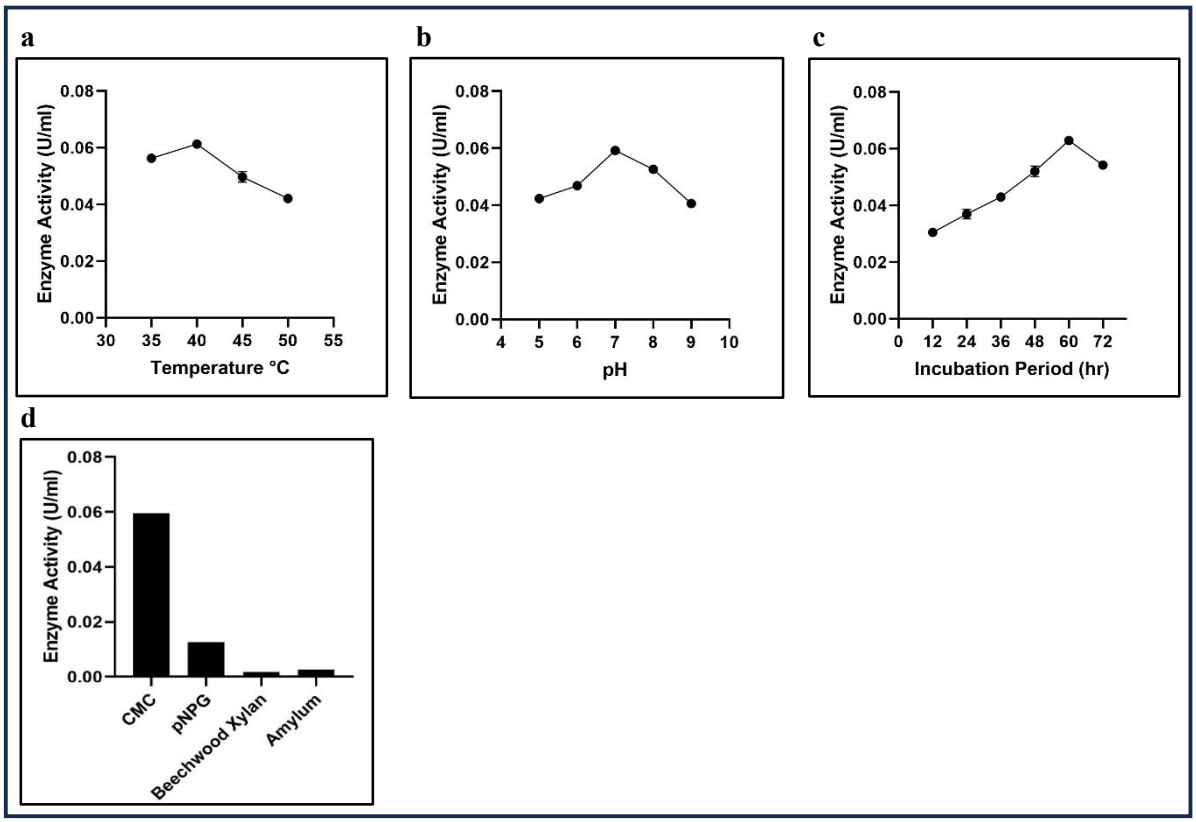

Figure 3: Optimization of different parameters of cellulolytic bacterial strain NE. a effect of temperature, b effect of pH, c effect of incubation period, d substrate specificity

CONCLUSION

Cellulases have been investigated for decades and are added to the pre-treated materials to hydrolyse the cellulose part into simple glucose. However, the freshly found cellulolytic bacteria were still required. Aim of this study was to harness the use of certain cellulase producing bacteria from fecal sample of *Boselaphus tragocamelus* from Vadodara, Gujarat, India. After optimizing the

parameters, results indicates that cellulase producing strain NE (*Pseudomonas* sp.) showed maximum cellulase activity at pH 7.0 and 40 °C temperature on 60 h incubation period. Because of its mesophilic characteristics, the isolated strain NE have the potential to produce cellulase enzymes for prospective use as environmentally friendly product in many industries including food, fermentation, agriculture, pulp and paper, and textile industry and a useful biotechnological tool for managing cellulosic waste. Yet, additional research like molecular characterization of this bacterial strain is required to improve its cellulolytic characteristics for industrial applications.

ACKNOWLEDGEMENT

We would like to thank Dr. Pratyush Patankar (Curator) and Mr. Manav Mehta (Education Officer) at Sri Sayaji Baug Zoo, Vadodara Municipal Corporation, Gujarat for cooperating with us.

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