Isolation and Biochemical Characterization of Cellulase Producing Goat Rumen Bacteria

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doi: https://doi.org/10.21467/proceedings.123.12

ABSTRACT

Cellulose is the most prevalent polymer on the planet and has long been utilized for a variety of industrial applications. The study's goal was to screen and isolate cellulase-producing bacteria from the rumen of a goat collected from different location of Dinajpur district. To do so, rumen content samples from two distinct goats were collected. In this investigation, rumen cellulase-producing bacteria were isolated and characterized after serial dilution of five isolates up to six fold and inoculation into Nutrient agar. Following that, all of the isolates were underwent Methyl Red (MR) test & Voges-Proskauer (VP) test to identify organism's metabolic pathway, Triple Sugar Iron Agar (TSI) Test to determine bacterial ability to utilize sugar, Motility Indole and Urease activity test (MIU) to determine motility, Urease utilization and can produce Indole or not, Citrate utilization test to utilize citrate as carbon and energy source, Oxidase test, Catalase test to check the presence of catalytic enzyme. The result revealed the colonial characterization of bacteria and also where proven all five isolates are promising enough and superior in quality to produce cellulose.

Keywords: Cellulose, Goat Rumen

1 Introduction

A ruminant is any mammal that digests its food in two stages: first by consuming the raw material and regurgitating a semi-digested form called cud, and then by ingesting the semi-digested form known as mash [1]. Although, Ruminant animals do not have enzymes to break down fibrous foods, but they do have bacteria in their rumen that degrade can such food stuff [2]. The rumen is a unique organ found in ruminant animals like goat that aids in the digestion of cellulose and other plant polysaccharides through the action of certain microbial populations through the production of enzymes. Among several cellulose is one of the enzyme that is the most prevalent polymer on the planet and has long been utilized for a variety of industrial applications [3]. Therefore, isolation and characterization of potent rumen cellulolytic microorganisms can be a novel finding. During growth on cellulosic materials, microbes such as fungi, bacteria, and actinomycetes can produce cellulases. Cellulases produced by bacteria have some advantages over other microbes like hydrolyzed material may also make them less inhibited, more efficient catalysts and ease of developing genetically engineered with bacteria holds the most potential importance [4, 5]. Thus, we conduct this study to isolate cellulase-producing bacteria from mammals like goat and determining their cellulolytic activity via culturing

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Proceedings DOI: 10.21467/proceedings.123; Series: AIJR Proceedings; ISSN: 2582-3922; ISBN: 978-81-954993-5-9

them in selective enrichment media and optimizing their physiological criteria via performing several biochemical test. The goal of this research is to extract and describe microorganisms from the rumen, as well as to investigate their ability to hydrolyze cellulose.

2 Materials & Methods

2.1 Sample collection and processing

In a clean sterile polythene bag, samples were taken from the rumen of goat in the morning which were collected from different location of Dinajpur district. The samples were collected immediately after slaughtering of animals. About 2 goat rumen were collected and brought to the BMB Lab in HSTU for screening cellulolytic activity and further processing.

2.2 Procedure for Isolation

The rumen samples were cut by sharp blade after cleaning the upper portions of the samples with autoclaved distill water. The isolation was then carried out using conventional techniques of serial dilutions (10^{-1} ~10^{-6}) for each sample in autoclaved distilled water and spreaded on agar plates containing 0.5% yeast extract, 0.5% NaCl, 1% trypton, 1.5% agar having pH 7.0.

2.3 Maintenance of colonies

After 24h of incubation at 37°C single bacterial colonies were picked from each plate and streaked on agar plates for further purification at the same conditions for 24h. The purified colonies were then subjected on the specific substrate agar plate containing $(NH4)_2SO_4$ 0.1%, KH_2PO_4 0.05%; K_2HPO_4 0.05%; $CaCl_2$ 0.01%; NaCl 0.01%; $MgSO_4.7H_2O$, 0.02%; yeast extract 0.1%, and CMC (carboxy methyl cellulose) 0.2% as a sole carbon source.

2.4 Identification and characterization of isolated colonies

The isolates were then undergo with various biochemical tests such as: Citrate utilization, indole, catalase, urease, methyl red, vogues proskauer, motility, sugar fermentation, starch hydrolysis, hydrogen sulphide generation (H_2S) from triple sugar iron (TSI) agar, and sugar fermentation. The tests were carried out according to Cheesbrough's instructions [6].

2.5 Methyl Red Voges-Proskauer Test (MR-VP) Test

Glucose Phosphate Peptone (GPP) medium was used to test the isolates' capacity to ferment glucose and produce stable acid. For the MR test, 2-3 drops of methyl red indicator were added to 48-hour cultured tubes. The MR-positive organism in medium creates an acidic environment that overcomes the buffers (Potassium phosphate that resists pH). As a result, the soup remained crimson after adding methyl red. After adding 40 percent potassium hydroxide to the medium for the VP test, the VP positive organism turns copper red. Voges-Proskauer (VP) broth, like Methyl Red (MR), is used to identify an organism's metabolic pathway. This identification is done by detecting the ability to create stable acids end products. Organisms with a positive VP result metabolize pyruvic acid to produce acetyl-methyl carbinol (acetoin). This final product is transformed to diacetyl in the presence of ambient oxygen and 40% potassium hydroxide, and then to a red complex under the catalytic action of alpha-naphthol and creatine [7, 8].

With autoclaved distilled water, MR-VP broth was made, and 5 ml broth was put to each test tube. The chosen isolates were then injected into each tube and incubated for 48 hours at 37°C. After

Proceedings DOI: 10.21467/proceedings.123
ISBN: 978-81-954993-5-9

Series: AIJR Proceedings
ISSN: 2582-3922

incubation, five drops of methyl red reagent were added to the test tubes to detect MR positive isolates by looking for red hues. After inoculation and 48 hours of incubation at 37°C, 0.6 ml of 5% alpha-naphthol was applied to individual test tubes for the VP test. After that, 0.2 mL of 40% KOH was added and the test tubes were gently shaken in air oxygen. The tubes were then left to sit for another 10-15 minutes.

2.6 Oxidase test

The oxidase test was done to see if the isolates could utilize cytochrome c oxidase, which is an electron transport chain enzyme. A drop of oxidase reagent (% tetramethyl-p-phenylene-diamine-dihydrochloride) was placed on a clean filter paper. After that, an inoculating loop streaked a little quantity of bacterial pure culture of the selected isolates onto the oxidase droplet. Isolates that develop a dark blue color are positive for the Oxidase test.

2.7 Catalase test

The catalase test was used to check the presence of the catalase enzyme. A small amount of 3% H_2O_2 was added into the bacterial colony on a clean glass slide. Production of bubbles indicates positive outcome.

2.8 Motility Indole Urease (MIU) Test

Three separate tests can be combined into one medium with the MIU test. The MIU test is used to determine whether or not the chosen isolate is mobile, as well as if it can use urea and generate indole [6]. Urease positivity was shown by a color shift from yellow to orange to pink-red; motility was indicated by a hazy mass of bacteria; and indole production was indicated by a pink-mauve hue on the tube's bottom. MIU agar medium was used to make the media, which was then autoclaved for 15 minutes at 15 psi (121°C). Then, per 95 ml of basal medium, 5 mL of sterile 40% urea solution was added. After that, 5 mL of solution was added to each sterilized test tube, and the medium was allowed to cool fully until it had a semi-solid consistency by gently placing the tubes in a horizontal manner. The selected isolates were inoculated and incubated at 37°C for 24 hours.

2.9 Triple Sugar Iron (TSI) test

The Triple Sugar Iron (TSI) media contain three sugars (Lactose, Sucrose, and Glucose) and also iron which was done to confirm the ability of the bacteria to utilize these sugars. No color change and remain red in color indicate these isolates cannot ferment any of the sugar, where yellow color indicates these isolates can only ferment Glucose. In acidic slant or acidic but formation of yellow color showed the capability of each isolate to ferment each sugar. Where Production of black color means the bacterial isolate can utilize iron and have produced H_2S.

2.10 Citrate Utilization Test (CIU)

The citrate test was used to identify the ability of the bacterial isolate to utilize citrate as a carbon and energy source. CIU basically identify gram negative bacteria based on their metabolic product. The gram negative isolates contain citrate-permease which uses citrate present in the medium as a carbon source of energy. This test is also distinguish between members of the *Enterobacteriaceae* family. A CIU positive test depends on the production of the alkaline products of citrate metabolism which will subsequently increase the pH of the medium identified by the color change of a pH indicator. Citrate

enters into the cell and is cleaved by citrate lyase releasing oxaloacetate and acetate, this oxaloacetate is metabolized to pyruvate and CO_2. After that CO_2 reacts with water and sodium carbonate to release an alkaline by-product. The ammonium salts that are present in the media are also used by the microorganisms which ultimately produces another alkaline product ammonia and turning the media from deep green to intense Prussian blue [9].

2.11 Carbohydrate utilization test (Dextrose, Maltose)

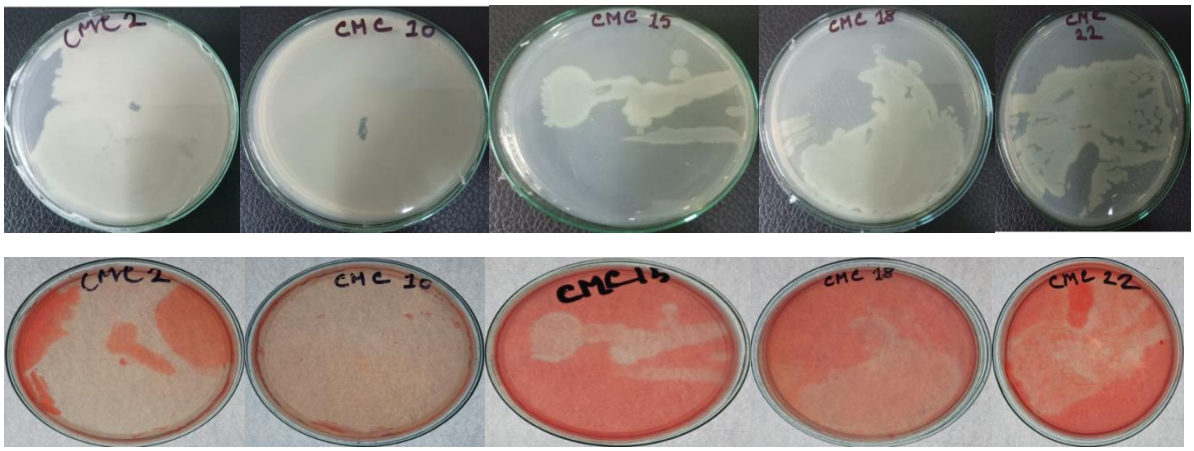
Carbohydrate utilization test was done using phenol red carbohydrate broth to observe if the selected isolates can utilize these sugars. Dextrose, maltose containing media were prepared according to protocol provided by the manufacturers and 5ml of the media were transferred into each test tubes at pH of 7.4 ± 0.2. After that, the selected isolates were inoculated and incubated at 37°C for 24 hours. After incubation, the tubes that turned yellow as a result of acid production by the bacteria were taken as a positive and the ones with no color change were indicated as negative [8].

3 Result

The isolation was done using standard techniques such as serial dilutions and repeated tubing of selectively enhanced microbial cultures using rumen bacteria-specific medium. During the isolation process, strict anaerobic conditions were maintained.

3.1 Identification of cellulytic activity

Cellulase-producing microorganisms are being screened using the process of enhancing nutritional agar with 1 percent carboxymethyl cellulose [4]. Carboxymethyl cellulose (CMC) agar plates were created. The isolate was stabbed into the solidified agar and incubated for 48 hours to express cellulose depolymerization into the surrounding medium via cellulase producing bacteria. The plates were soaked with an aqueous solution of 1% Congo red for 15 minutes to visualize the hydrolysis zone of inhibition. 1 m sodium chloride was used to wash these. The diameter of the cleared zone around the colonies on carboxymethyl cellulose agar was measured to evaluate the organism's cellulase activity (Figure 1).



A **B** **C** **D** **E**

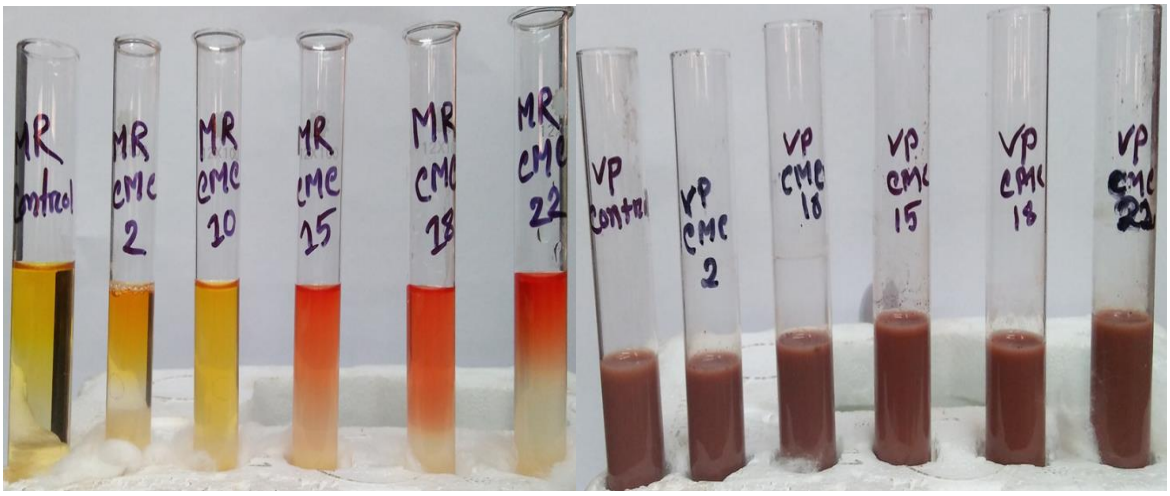
Figure 1. Cellulytic activity of five isolates where A, B, C, D & E indicates CMC (2, 10, 15, 18 & 22) isolates gradually.

Proceedings DOI: 10.21467/proceedings.123
ISBN: 978-81-954993-5-9

Series: AIJR Proceedings
ISSN: 2582-3922

3.2 Methyl Red (MR) & Voges-Proskauer (VP) test result

After both tests were conducted, we observed that three isolates (15, 18, 22) out of five shows positive output as these turned into red where remaining two remain yellow that indicates negative result for Methyl Red (MR) test where all five isolates showed negative result for Voges-Proskauer (VP) test (Figure 2).

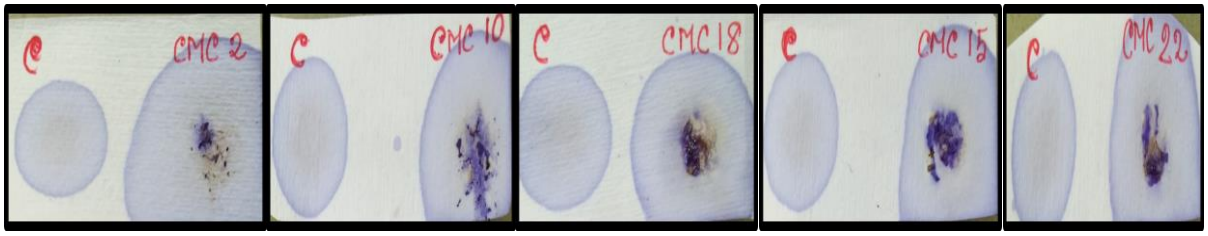


A **B**

Figure 2. A & B indicate Methyl Red (MR) & Voges-Proskauer (VP) test result analysis gradually.

3.3 Oxidase test result

All isolates namely CMC (2, 10, 18, 15 & 22) showed dark blue which indicates these are positive for Oxidase test and the isolates are aerobic and can use oxygen (Figure 3).



A **B** **C** **D** **E**

Figure 3. Oxidase test result, where examined five isolates are positive.

3.4 Catalase test

The rapid production of bubbles for four isolates namely CMC (2, 10, 15 &18) indicates that the organism can utilize catalase where the CMC 22 is partially positive for Catalase test as it produced minimal bubbles compared to other isolates (Figure 4).

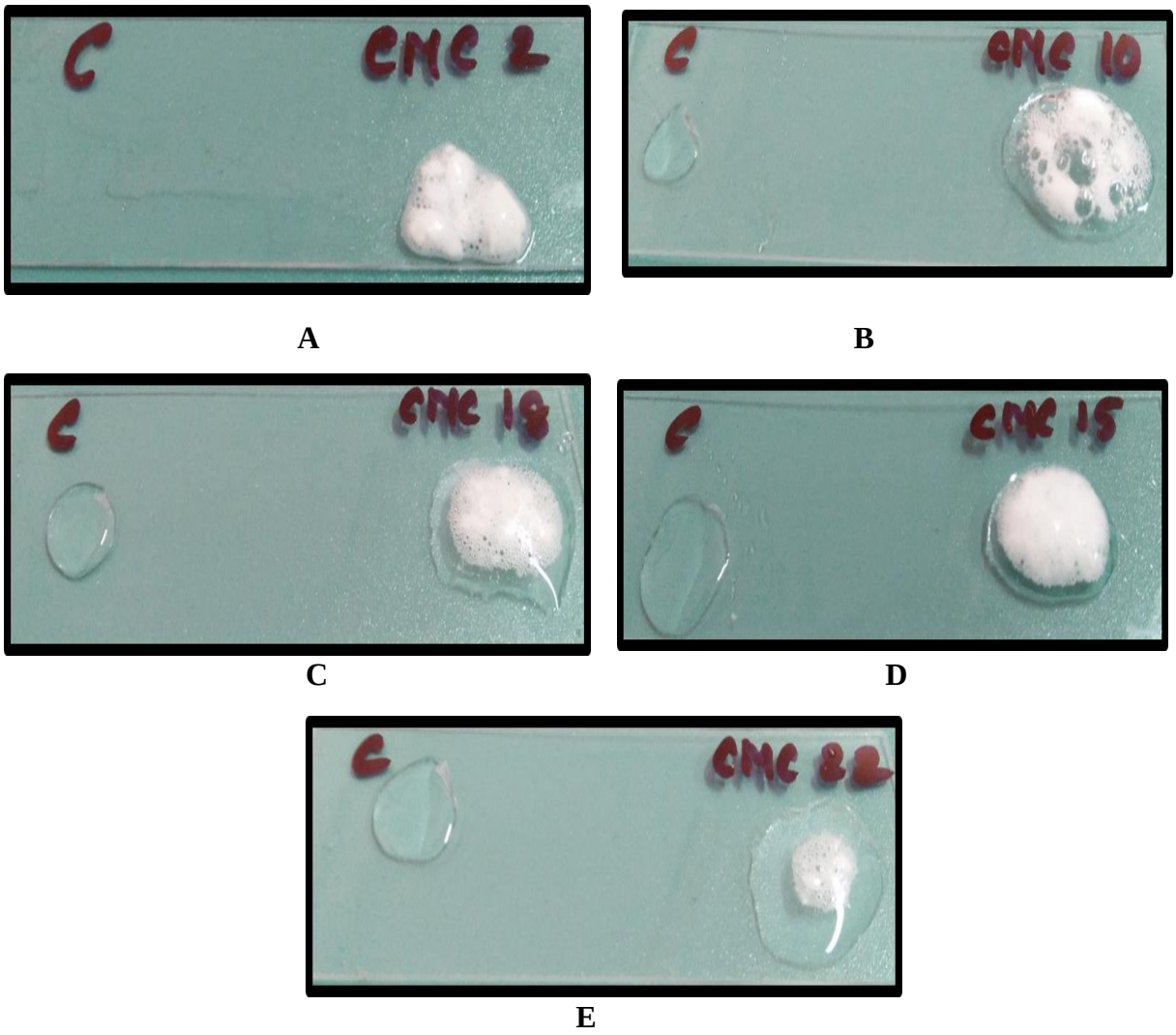


Figure 4. Formation of bubbles in Catalase test.

3.5 Motility Indole Urease (MIU) Test result

The isolates namely CMC (2 & 10) were positive for Urea utilization test. All the isolates except CMC 2 showed positive for Motility test and also have indole production ability. (Figure 5).

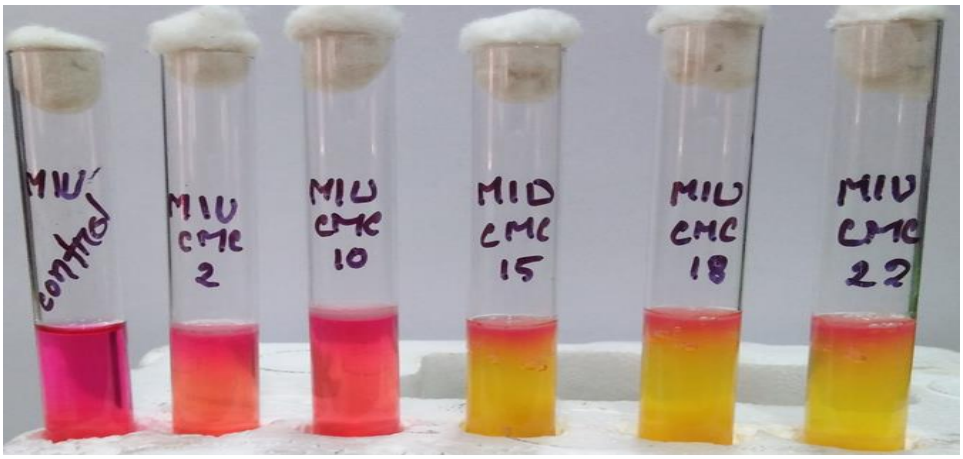


Figure 5. Motility Indole Urease (MIU) Test result that contain both positive & negative results.

Proceedings DOI: 10.21467/proceedings.123
ISBN: 978-81-954993-5-9

Series: AIJR Proceedings
ISSN: 2582-3922

3.6 Triple Sugar Iron (TSI) test result

Formation of yellow color with bubbles in all isolates except CMC 10 is a sign that all the bacterial isolates are capable to ferment all three sugars (Figure 6).

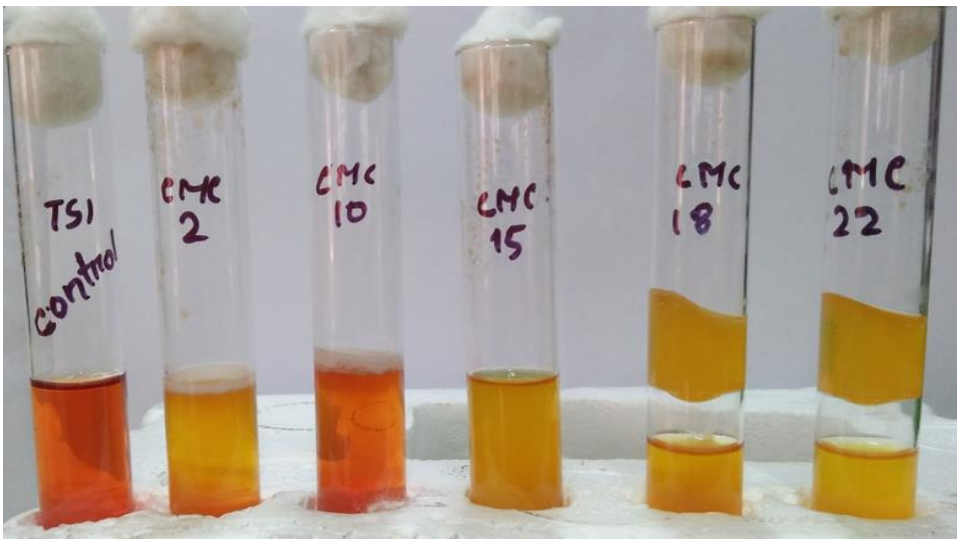


Figure 6. Triple Sugar Iron (TSI) test result analysis.

3.7 Citrate Utilization Test (CIU) result

Two isolates (CMC 2 & CMC 10) are positive for CIU test as converted into intense Prussian blue from deep green that signal the positive CIU test where rest of the isolates are partially positive as these partially change in previously mentioned color (Figure 7).

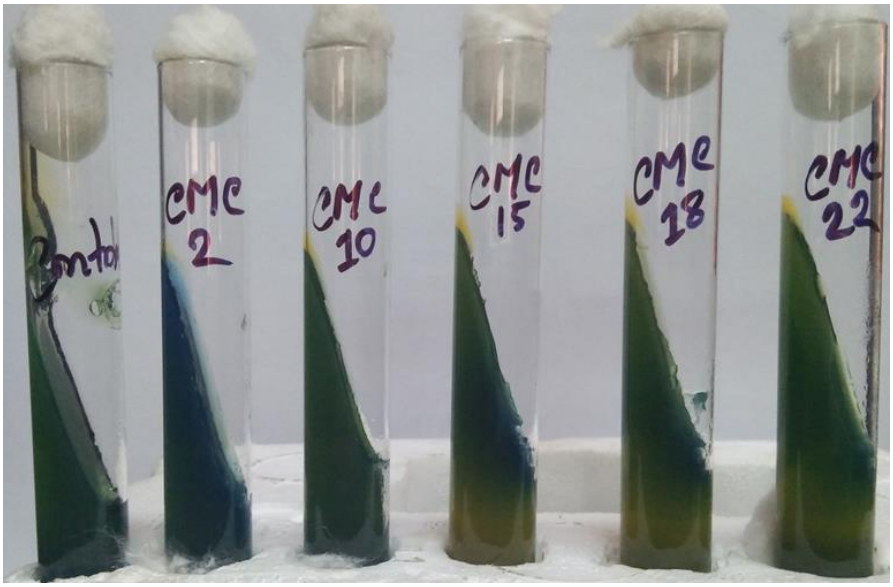
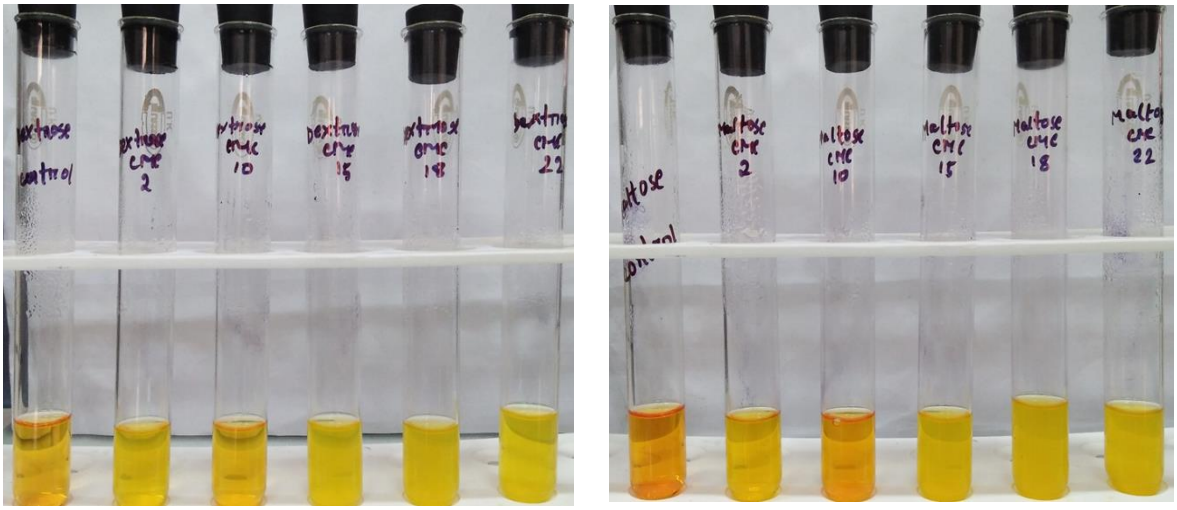


Figure 7. Citrate Utilization Test (CIU) result analysis.

3.8 Carbohydrate utilization test (Dextrose, Maltose)

Formation of yellow color has proven that all the isolates can utilize carbohydrate except CMC 10 that is capable to utilize Dextrose but not Maltose (Figure 8).



A **B**

Figure 8: A and B represents Carbohydrate utilization test for Dextrose & Maltose gradually.

4 Discussion

Rumen is one of the most well-studied anaerobic habitats for bacteria which are metabolically active that leads to the production of various enzymes and bioactive compounds in comparison to other environmental condition. Such bacterial source can be good option to screen out bacteria that produce enzyme of our target of interest. In this study we aimed goat rumen to isolate and characterize bacteria that produce enzyme named cellulose. To conduct this study, a total of 5 samples were collected from goat rumen that underwent serial dilution and further evaluated by plating them into specific nutrient medium. The culture plates were flooded with Congo red solution, purified strains cellulase utilization was identified (1.0 percent). This solution colors the cellulose containing medium, resulting in a translucent halo zone where the cellulose is destroyed, indicating the all 5 isolates exhibited cellulase production with high titer value. Following that we also conduct a series of biochemical test like Methyl Red (MR) test & Voges-Proskauer (VP) test to identify organism's metabolic pathway where two isolates showed negative for (MR) test but all isolates are negative for (VP) test. Triple Sugar Iron Agar (TSI) Test was also applied to examine bacterial sugar utilization ability ended with positive result except one, Motility Indole and Urease activity test (MIU) to determine motility, Urease utilization and these can produce Indole or not where isolates exhibit mixed results. Besides that, Citrate utilization test result were affirmative for two isolates and partially positive for three isolates. Carbohydrate utilization test shows all positive for Maltose except one and all positive for Dextrose. Oxidase test, Catalase test were also performed to check the presence of catalytic enzyme activity ended with promising output. In A nutshell, the study revealed the presence of the cellulose producing organisms in goat rumen. Moreover, Evidences based on zones of clearing in cellulose agar led to the conclusion that isolated bacteria are superior and have the potency to produce cellulose.

Proceedings DOI: 10.21467/proceedings.123
ISBN: 978-81-954993-5-9

Series: AIJR Proceedings
ISSN: 2582-3922

5 Conclusion

According to the findings, goat rumen samples include a considerable number of Bacteria which are superior and have the potential to be utilized in cellulase synthesis. As a result, it can be inferred that goat rumen can be beneficial source to isolate Cellulase-producing bacteria. However, it warrants further experiments to ensure which kind of bacterial species are there in the isolates.

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