Isolation and Biochemical Characterization of Xylanase Enzyme Producing Bacteria from Goat Rumen

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ABSTRACT

The rumen microbial communities of ruminants are thought to be the most promising biochemical source of inordinately diversified and multi-functional cellulolytic enzymes with unique functional adaptations to improve biotechnological processes. The exploitation of rumen microbial genetic variety has been limited due to a lack of effective screening culture techniques and a lack of understanding of the rumen microbial genetic diversity. This study is conducted to isolate and characterize rumen bacteria from goat rumen that have capability to produce xylanase enzyme. Serial dilutions technique is applied to isolate bacteria from goat rumen and repeated tubing of the selectively enriched microbial cultures by using the specific media for rumen bacteria. Following that, all of the isolates were underwent Methyl Red (MR) test & Voges-Proskauer (VP) test to identify organisms 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 where all isolates found promising which indicates that all five isolates are superior and capable to produce xylanase.

Keywords: Xylanase Enzyme, Goat Rumen.

1 Introduction

Rumen is one of the most well-studied anaerobic habitats, and it serves as a foundation for current knowledge of microbial ecology in anaerobic digestive systems [1]. The environment in the rumen encourages microorganisms to produce the enzymes needed to digest the nutrients [2]. The main goal of this study is to isolate and characterize xylanase producing bacteria. However, the isolation of xylanase producing bacterial species from goat rumen has never been described before because the rumen environment is entirely anaerobic, making aerobic bacteria impossible to live. The goals of this research were to isolate and identify the bacterium responsible Xylan degradation, and characterize the xylanase producing bacteria [3]. To do so at first we isolate bacteria from goat lumen via serial dilution and spread them on media for culture purpose. Primarily, we took ten isolates out of which we select five isolates randomly for further evaluation. Five isolates underwent through the following

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test namely Methyl Red (MR) test & Voges-Proskauer (VP) test, Triple Sugar Iron Agar (TSI) Test, Motility Indole and Urease activity test (MIU), Urease utilization test, Citrate utilization test, Oxidase test, Catalase test where majority of test result exhibit affirmative result which is an indication that all isolates are superior and capable to produce xylanase.

2 Materials & Methods

Isolation of bacteria was done from the goat rumen which were collected from different location of Dinajpur district. The sample were collected immediately after slaughtering of animals. About 2 goat rumen were collected and brought to the BMB Lab in HSTU for further processing.

2.1 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. 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 $(NH_4)_2SO_4$ 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 xylan 0.2% as a sole carbon source.

2.2 Biochemical Test

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

The ability of the isolates to ferment glucose and production of stable acid were determined by using Glucose Phosphate Peptone (GPP) medium. 2-3 drops of methyl red indicator were added to 48 hrs cultured tubes for MR test. In medium, the MR positive organism creates an acidic environment that will overcome the buffers (Potassium phosphate that resists pH). So, after adding methyl red, the broth stayed red. For VP test, after adding the 40% potassium hydroxide in the medium the VP positive organism form copper red colour [4, 5].

Like Methyl Red (MR), Voges-Proskauer (VP) broth are used to identify the metabolic pathway of an organism, this identification is performed by detecting the ability to produced stable acids end products. The positive VP result organisms further metabolize pyruvic acid to form acetyl-methyl carbinol (acetone). This end product, in the presence of atmospheric oxygen and 40% potassium hydroxide is converted to diacetyl and it under the catalytic action of alpha-naphthol and creatinine, is converted into a red complex [4, 5].

MR-VP broth was prepared with autoclaved distilled water and 5 ml broth was transferred to each test tubes. After that the selected isolates were inoculated into each tube and incubated at 37°C for 48 hours. After incubation, five drops of methyl red reagent were added for detecting MR positive isolates by searching for red colors in the test tubes. For VP test after inoculation and incubation at 37°C for 48 hours 0.6 ml of 5% alpha-naphthol was added to individual test tubes. Following after 0.2 ml of 40% KOH was added and the test tubes were shaken gently in atmospheric oxygen. After that the tubes were allowed to remain for 10-15 minutes.

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2.2.2 Oxidase Test

Oxidase test was used to check the isolates can utilize cytochrome c oxidase which is basically an enzyme of the electron transport chain. On a clean filter paper a drop of oxidase reagent (1% tetramethyl-p-phenylenediamine-dihydrochloride) was added. After that a small amount of bacterial pure culture of the selected isolates were streaked onto the oxidase droplet by an inoculating loop. Development of dark blue color is a sign that isolates are positive for Oxidase test [6].

2.2.3 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 [6].

2.2.4 Motility Indole Urease (MIU) Test

MIU test can combine three individual tests into a single medium. The MIU test used to detect if the selected isolate is mobile or not, it can utilize urea and also check if it can produce indole [6]. A color change from yellow to orange to pink-red showed urease positive, for motility a cloudy mass of bacteria was shown and for pink-mauve color on the bottom of the tube formed for indole production. The media was prepared using MIU agar medium and autoclaved at 15 psi. pressure (121°C) for 15 minutes. After that, 5 ml sterile 40% urea solution was added per 95 ml basal medium. Then 5 ml solution was transferred to each sterilized test tube and the media was left to cool down completely 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.

2.2.5 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 butt formation of yellow color showed the capability of each isolates to ferment each sugars. Where Production of black color means the bacterial isolate can utilize iron and have produced H_2S.

2.2.6 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 distinguished 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 [7].

2.2.7 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 5.0 ml 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 [5].

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

By flooding the culture plates with freshly generated Congo red solution, purified strains' xylanase utilization was identified (1.0 percent). This solution colors the xylene-containing medium, resulting in a translucent halo zone where the xylene is destroyed, indicating cellulytic activity (Figure 1).

Figure 1.Cellulytic activity of five isolates where A,B,C,D & E indicates Xylan (1,3,5,7 & 8) isolates gradually**.**

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

After each test was conducted, we observed that all five isolates remain yellow including control that indicates negative result for Methyl Red (MR) test where all five isolates showed a red color that indicates a positive result for Voges-Proskauer (VP) test (Figure 2)**.**

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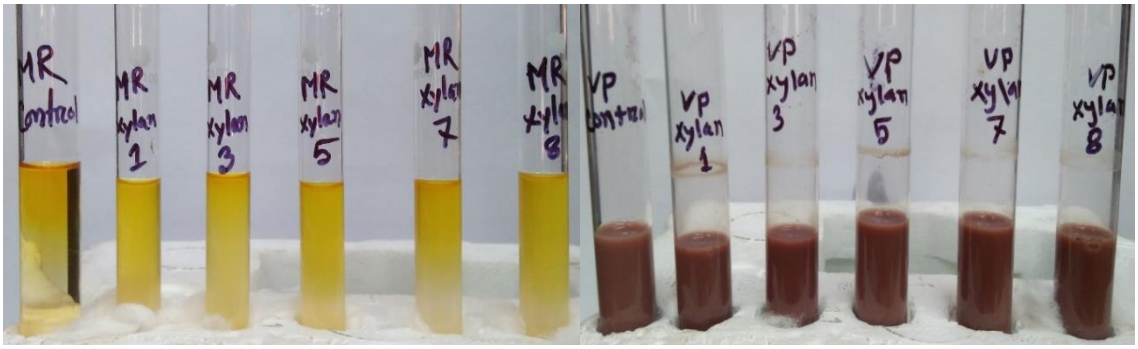

A **B**

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

3.3 Oxidase test result

All isolates xylanase (1, 3, 5 & 8) except xylanase 7 showed dark blue that indicates these are positive for Oxidase test and the isolates are aerobic and can use oxygen where number 7 is partially positive with slight blue color (Figure 3).

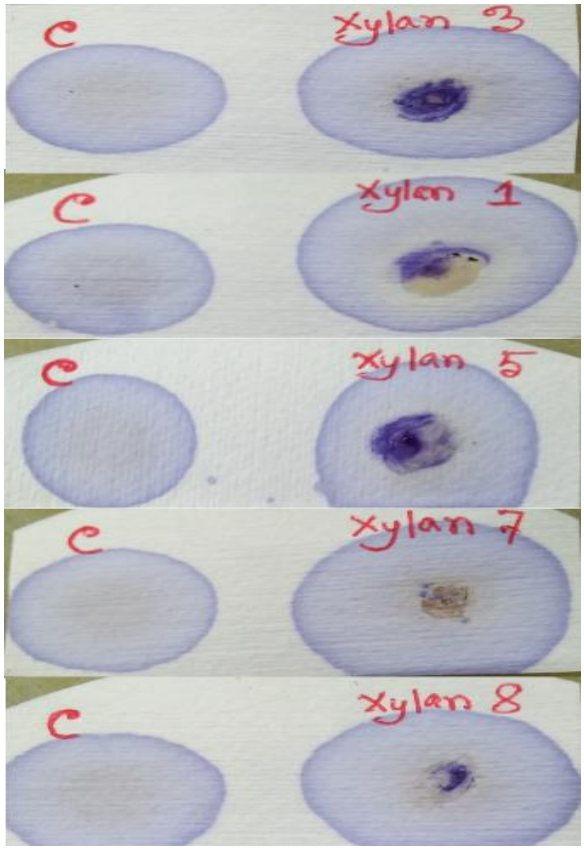

Figure **3**. Oxidase test result where four out of five isolate is positive & one isolate is partially positive**.**

3.4 Catalase test

The rapid production of bubbles for four isolates namely xylanase (1,3,7 & 8) indicates that the organism can utilize catalase where the xylanase 5 is partially positive for Catalase test as it produced minimal bubbles compared to other isolates (Figure 4).

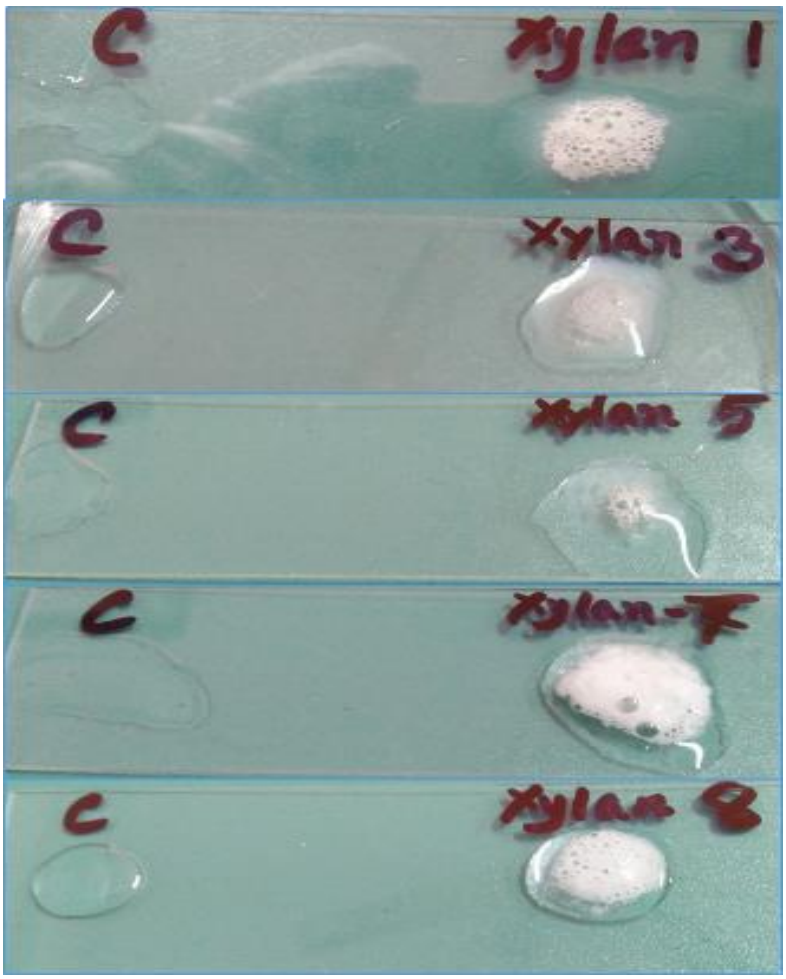

Figure 4. Formation of bubbles in Catalase test.

3.5 Motility Indole Urease (MIU) Test result

All the isolates except xylanase 8 were positive for Urea utilization, have motility capability with indole production ability as these gives pink red color with cloudy mass with pink-mauve color on the bottom of the tube. But xylanase 8 was negative for the whole MIU test (Figure 5).

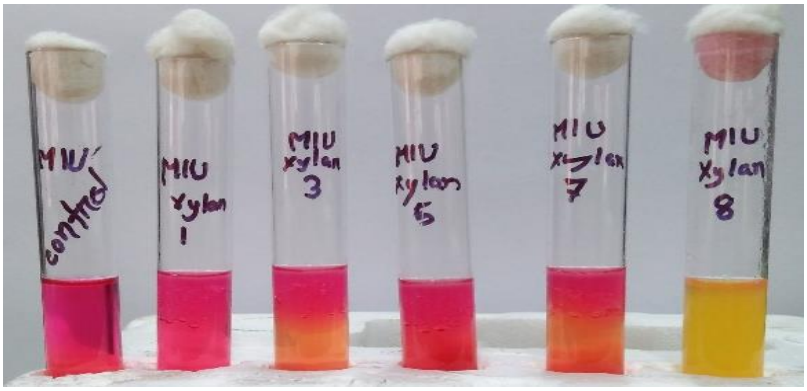

Figure 5. Motility Indole Urease (MIU) Test result that contain both positive & negative results.

3.6 Triple Sugar Iron (TSI) test result

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

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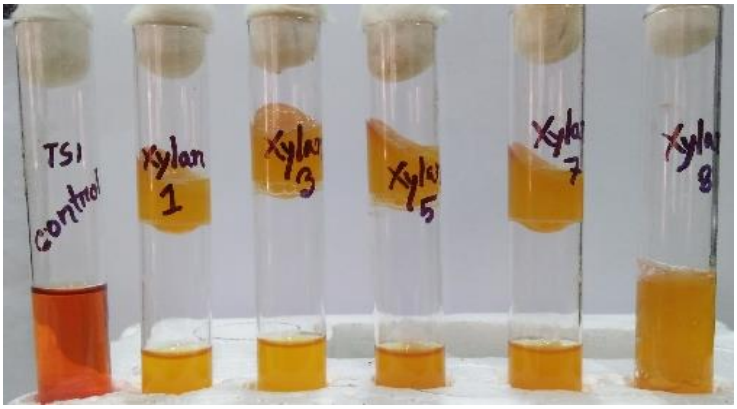


Figure 6. Triple Sugar Iron (TSI) test result analysis.

3.7 Citrate Utilization Test (CIU) result

All five isolates converted into intense Prussian blue from deep green that signal the positive CIU test (Figure 7).

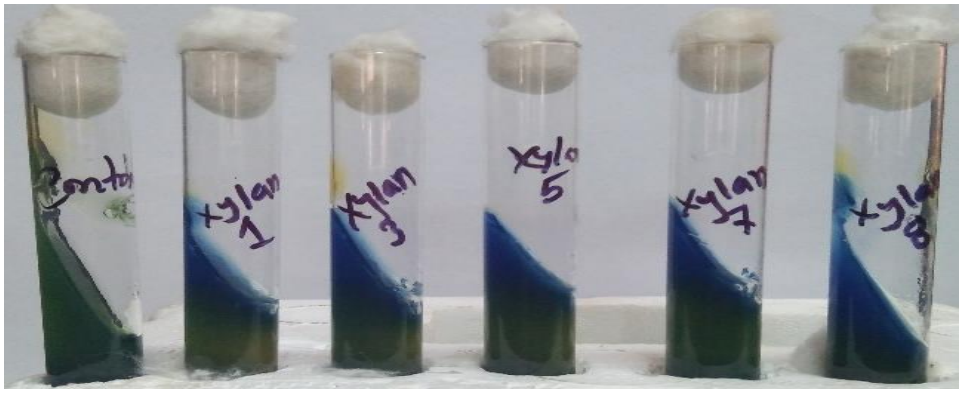
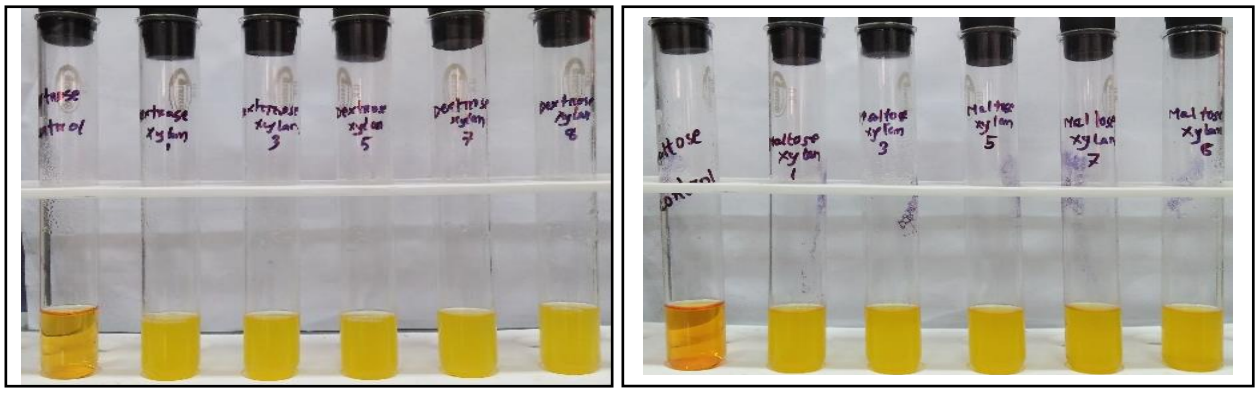


Figure 7. Citrate Utilization Test (CIU) result analysis.

3.8 Carbohydrate utilization test (Dextrose, Maltose)

Formation of yellow colour has proven that all the isolates can utilize carbohydrate (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 xylanase. To conduct this study, a total of 10 samples were collected from goat rumen, underwent serial dilution and finally 5 isolates are randomly selected for further evaluation by plating them into specific nutrient medium. The culture plates were flooded with Congo red solution, purified strains xylanase utilization was identified (1.0 percent). This solution colors the xylenen-containing medium, resulting in a translucent halo zone where the xylene is destroyed, indicating the all 5 isolates exhibited xylanase 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 organisms metabolic pathway where all isolates showed negative for MR test but possitive for VP test. Triple Sugar Iron Agar (TSI) Test was also applied to examine bacterial sugar utilization ability ennded with possitive result, Motility Indole and Urease activity test (MIU) to determine motility, Urease utilization and these can produce Indole or not where all isolates except one named xylanase 8 exhibit possitive result. Besides that, Citrate utilization test result were affermative for all isolates and so for the Carbohydrate utilization test. Oxidase test, Catalase test were also performed to check the presence of catalytic enzyme activity ended with promising output. In A nutshell , the obtained isolates are able to produce Xylene enzyme where additional biochemical test evaluate therir additional characterstics as well.

5 Conclusion

Based on the findings of the result, it can be concluded that the isolated species of microbes are superior and may have potential to produce Xylanase enzyme in higher quantity. However , it warrants further experiments to ensure which kind of bacterial species are there in the isolates.

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