Production of Chitosanolytic Bacteria from Soil Sample

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Abstract

Bacillus megaterium produces penicillin amides used to make synthetic penicillin and several enzymes. This study focuses on showing the presence of *Bacillus megaterium* in the market area. In this research, the soil samples (A1, A2, A3, A4, A5, A6, A7, A8, A9, and A10) were collected from the Htauk-kyant market area, Yangon Region, and a preliminary screening of the bacteria present in the sample was carried out. The bacteria were isolated from soil samples by the serial dilution plate method and then cultured in nutrient agar medium. Ten bacterial strains (A1 to A10) were isolated and characterized by microscopic examination and gram staining methods. Among these bacterial strains, eight gram-positive bacterial strains: A1, A2, A3, A4, A6, A8, A9, and A10 were identified by phenotypic analysis. The confirmation of chitosanase producing bacteria was performed by qualitative methods such as a chitosan detection agar assay and the DNS (3, 5-dinitrosalicylic acid) assay. In these tests, A2 was found to show chitosanolytic activity.

Keywords: *Bacillus megaterium*, serial dilution plate method, gram staining test, phenotypic, chitosanolytic activity

Introduction

Bacillus megaterium is a rod-like and motile, gram-positive bacteria, mainly an aerobic spore-forming bacterium, found in widely diverse habitats. With a cell length (up to 4 μm) and a diameter (1.5 μm). It is the biggest known bacteria. The cell occurs in pairs and chains, where the cells are joined together by polysaccharides on the cell. It grows at temperatures ranging from 3 °C to 45 °C, with the optimum being around 30 °C. It is a potential agent for the biocontrol of plant diseases. Using *B. megaterium* scientists have developed numerous proteins that are commonly used in medical and agricultural fields. It produces enzymes for several amino acid dehydrogenases. It has some phenotypic and phylogenetic similarities with the pathogens *B. anthracis* and *B. cereus*, although it is relatively harmless. It is ubiquitous in the environment around us. It can be found in various foods (including honey) and on a variety of surfaces, including clinical specimens, paper, stone, etc. It has the ability to produce chitosanase, an enzyme which hydrolyzes chitosan to chitooligosaccharide (Pelletier and Sygusch, 1990).

Materials and Methods

Sampling

The soil sample was collected from the Htauk-kyant Township market area, Yangon region. The dust and dead matter on the upper layer of the solid were removed and it was dug down to 6 inches deep. Then the soil sample was put into the sterilized plastic zip bag and kept in the refrigerator.

Isolation and Identification of *Bacillus megaterium*

1 g of soil sample was suspended in 100 mL of distilled water and thoroughly mixed well for 15 minutes with the vortex and label as 10^{-2}. Then the sterile test tubes were labelled as 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Each suspension was serially diluted from 10^{-2} to 10^{-6}. In addition, 100 mL of medium was boiled on a hot plate and sterilized by autoclaving for 15 minutes at 121 °C. The sterilized media was cooled down. The 20 mL of sterilized medium was poured into the sterilized petri-dishes containing 0.2 mL of serial dilution of each of the soil sample

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plates and incubated at 37 °C for 24 hours. The bacteria were grown on nutrient agar medium as colonies.

Among the colonies, rod, white-colour colonies were isolated from soil samples and cultured by the streak plate method (Kaur *et al*., 2012), and were transferred into nutrient agar medium with the help of an inoculation loop near the flame of a spirit burner and incubated again at 37 °C for 24 hours to get pure culture.

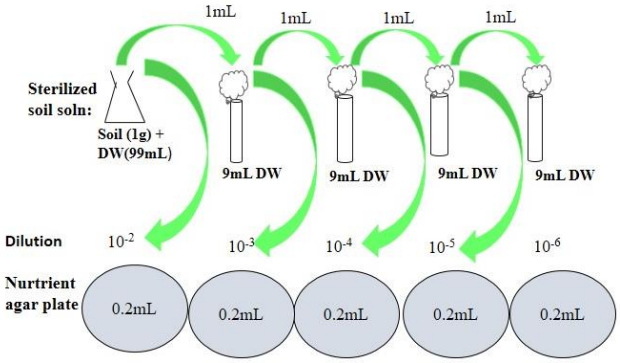

Figure 1. Serial dilution of a soil aqueous solution

Figure 2. Ten types of pure colonies on nutrient agar slants

The isolated bacteria strains were sub-cultured on nutrient agar slant cultures to check their purity and incubated at 37 °C for 24 hours. Then the purified culture was maintained in the refrigerator. The isolates were identified by using biochemical tests such as motility tests, gelatin liquefaction tests, indole tests, Voges-proskaurem tests, urease tests, nitrate reduction tests, starch hydrolysis tests, catalase tests, citrate utilization tests, and methyl red tests (Atlas and Synder, 2006).

Screening of chitosanase activity by the agar plate diffusion method

The plate assay is a rapid determination of the presence of chitosanase activity (Dingle *et al*., 1953). 20 mL of sterile chitosan detection agar (CDA) medium was poured into a petri dish and solidified. Eight bacteria strains **(**A1, A2, A3, A4, A6, A8, A9, and A10) were streaked on (CDA) medium and allowed to grow for 96 hours at 28 °C. After 96 hours incubation, the CDA agar plates that appeared with distinct clear zones were selected. The capability of degradation of chitosan is determined by the clear zone on the agar plates. The most potent isolate with higher chitosanolytic activity was chosen for the determination of chitosanolytic activity. It was subcultured on nutrient agar slants, and maintained at 4 °C for further study.

Results and Discussion

The soil sample was collected from the Htauk-kyant market area, Yangon Region. The soil samples were cultured on nutrient agar (NA) medium. Ten bacterial strains (A1, A2, A3, A4, A5, A6, A7, A8, A9, and A10) were isolated from the collected fresh soil samples.

According to this result data, A5 and A7 gave a red colour and the remaining strains showed a blue colour in the gram staining test. These results indicated that the strains A1, A2, A3, A4, A6, A8, A9, and A10 are gram-positive and A5 and A7 are gram-negative.

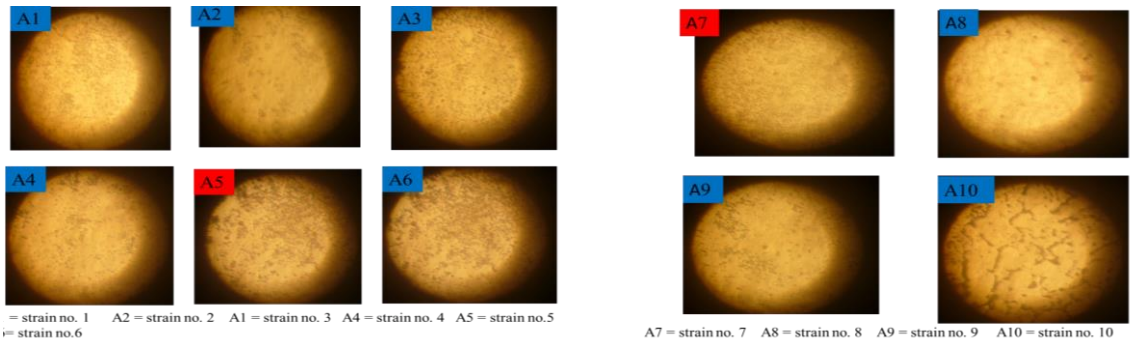

Figure 3. Classification of bacterial strains by the gram staining method

Among ten bacterial strains, only eight (A1, A2, A3, A4, A6, A8, A9, and A10) were found to be gram-positive, and these eight bacterial strains were selected for identification by biochemical tests. Some biochemical tests such as motility tests, gelatin liquefaction tests, Voges-proskaurem tests, urease tests, nitrate reduction tests, starch hydrolysis tests, indole tests, catalase tests, citrate utilization tests, and methyl red tests of selected bacteria (A1, A2, A3, A4, A6, A8, A9, and A10) were studied. The experiment data were summarized and compared with the reported data of Beesely *et al*., (2010), Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957; Buchanam and Gibbon, 1974) for *B.megaterium*, described in Table 2 and figure 4. The result of the sugar fermentation test are shown in Table 3 and figure 5.

The chitosanase activity of the isolated *B. megaterium* was screened by the agar plate diffusion method and the DNS assay method. The intracellular chitosanase activity of selected eight bacterial isolates was screened qualitatively by zone formation on the agar plate using colloidal chitosan. Chitosanase activity was found after 96 hours of incubation of eight bacterial isolates (A1, A2, A3, A4, A6, A8, A9, and A10) (Figure 6 and Table 4). Three bacterial isolates (A2, A4, and A9) were found to produce chitosanase activity. Among these three, A2 (40 mm) produced a clear zone larger than A4 (18 mm), and A9 and A2 (22 mm) might possess the highest activity. Therefore, A2 was selected for further study.

The chitosanase activity of the selected bacteria (A2) was also determined by using the DNS (3, 5 - dinitrosalicylic acid) assay method. The colour changes that occurred in the reaction before and after incubation at 55 ºC were described in Figure 7. It was found that a deep red colour formation developed at the end of the reaction, indicating that *B. megaterium* produced the chitosanase enzyme and possessed the chitosanase activity. Therefore, the selected bacteria A2, *B. megaterium* was confirmed as chitosanase producing bacteria.

Table 1. Characterization of Ten Isolated Bacterial Strains by Gram Staining Test

Strain number	Observed colour	Result
A1	Blue	+ Gram-positive
A2	Blue	+ Gram-positive
A3	Blue	+ Gram-positive
A4	Blue	+ Gram-positive
A5	Red	- Gram-negative
A6	Blue	+ Gram-positive
A7	Red	- Gram-negative
A8	Blue	+ Gram-positive
A9	Blue	+ Gram-positive
A10	Blue	+ Gram-positive

Table 2. Results of Biochemical Tests on Selected Isolated Bacteria

Biochemical tests	Observation of selected bacteria strains on biochemical tests								
	A1	A2	A3	A4	A6	A8	A9	A10	**B. megaterium*
Motility	-	+	-	+	-	-	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-
Urease	+	+	-	-	-	+	-	-	+
Nitrate reduction	+	-	-	+	-	-	+	+	-
Starch hydrolysis	+	+	+	+	-	-	-	-	+
Catalase	-	+	+	+	+	+	+	+	+
Citrate utilization	+	+	-	-	-	-	-	-	+
Methyl red	+	+	+	+	-	-	+	+	+
Indole	-	-	-	-	-	-	-	-	-

(+) = positive, (-) = negative

*(Beesley & Vanner; 2010)

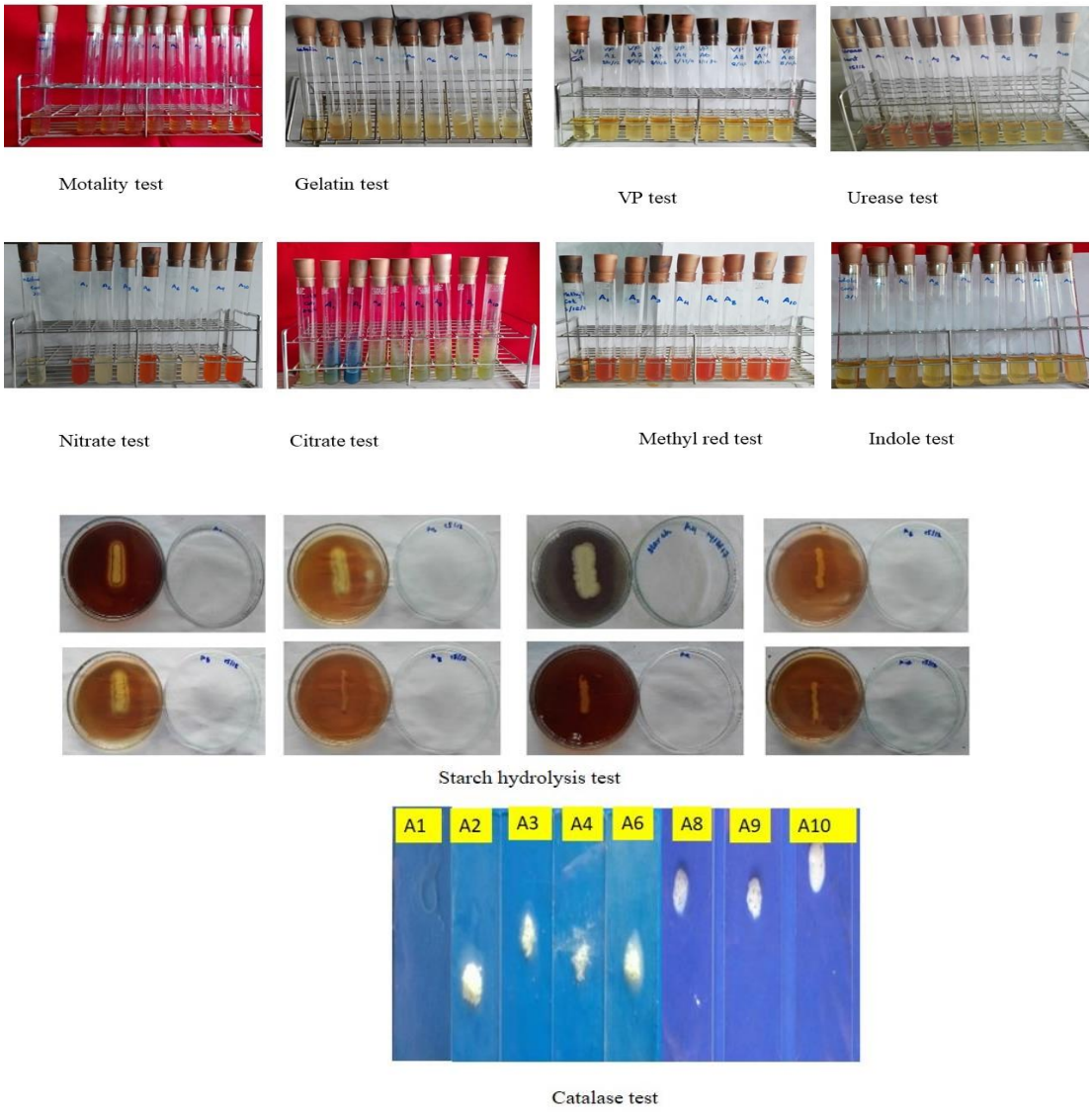

Figure 4. Screening of biochemical tests on selected isolated bacteria

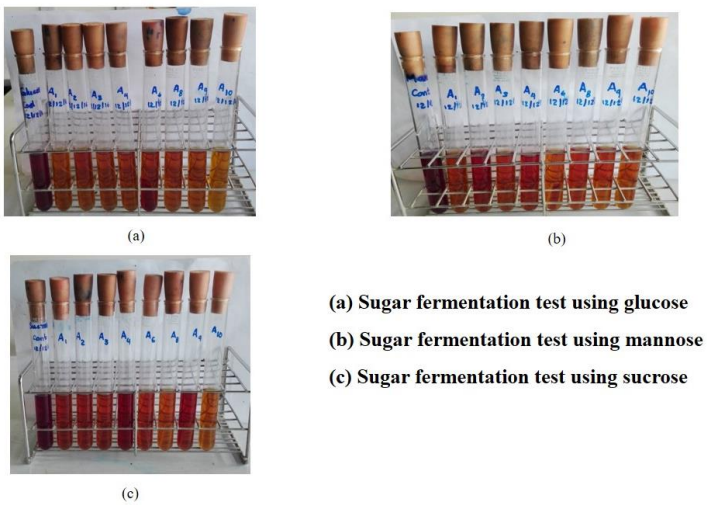

Figure 5. Screening of sugar fermentation tests on four isolated bacteria

Table 3.Results of the Sugar Fermentation Test on Selected Isolated Bacteria

Sugar fermentation tests	Observation of selected bacteria strains on sugar fermentation tests								
	A1	A2	A3	A4	A6	A8	A9	A10	* *B. megaterium*
Glucose	+++	+++	+++	+++	++	+++	+++	+++	+++
Sucrose	++	++	++	+	++	++	+	++	+++
Mannose	++	+	++	+	++	++	++	++	+

(+) = positive, (-) = negative

*(Beesley & Vanner; 2010)

Table 4. Comparison of Diameter of Clear Zone Produced by Eight Isolated Chitosanolytic Bacteria

Strain	Clear zone diameter (mm)
A1	-
A2	40
A3	-
A4	18
A6	-
A8	-
A9	22
A10	-

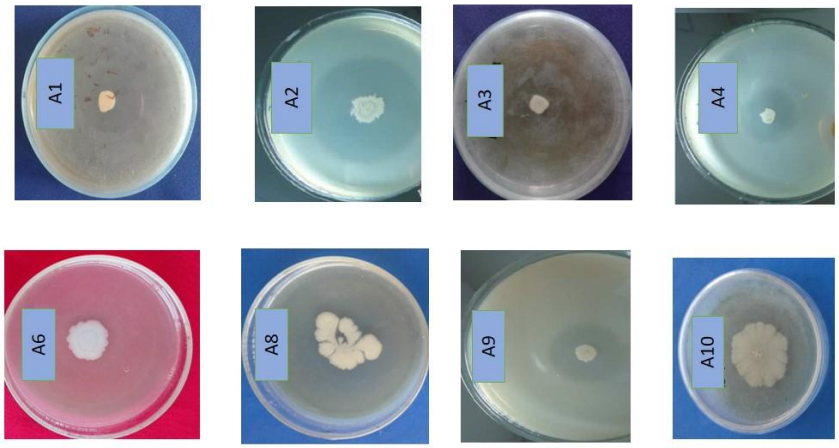

Figure 6 Diameter of clear zone produced by eight isolated chitosanolytic bacteria

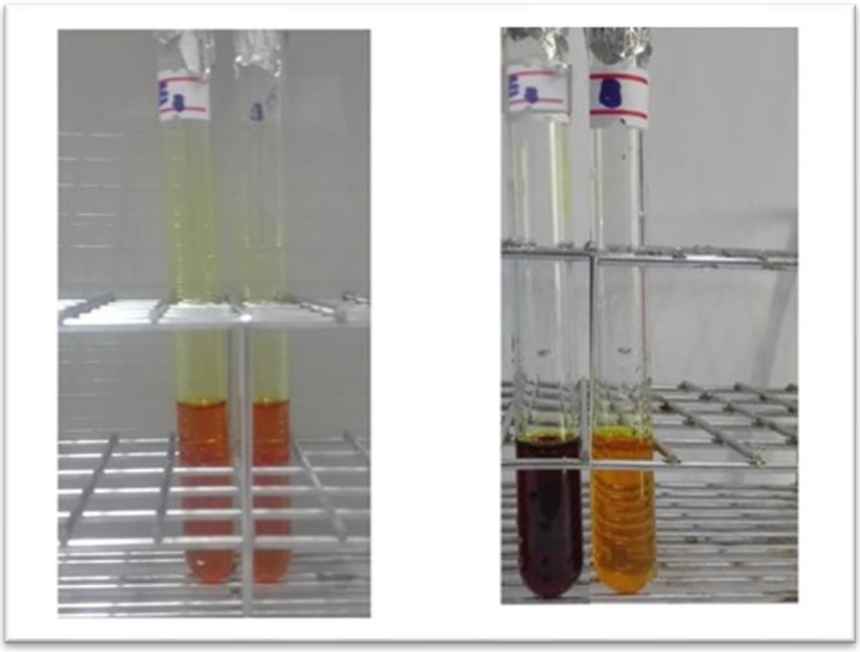

Figure 7 Qualitative screening of chitosanase activity

(a) Before incubation (b) After incubation

According to the morphological and biochemical characteristics, the A2 bacterial strain was identified as *Bacillus megaterium* and it possessed maximum chitosanolytic activity.

Conclusion

In gram staining method, the observed background colour of strains A1, A2, A3, A4, A6, A8, A9, and A10 gave blue, indicating that the isolated bacterial strains were found to be gram-positive, and the observed background colour of strains A5 and A7 gave red, indicating that the isolated bacterial strains were found to be gram-negative. The biochemical characteristics of the (A1, A2, A3, A4, A6, A8, A9, and A10) selected bacteria were identified by biochemical tests.

According to biochemical tests among the eight bacterial strains, positive results for A2 were motility, catalase, methyl red, gelatin, urease, citrate utilization, starch hydrolysis, and sugar fermentation tests, and negative results were observed in indole and, Voges-Proskauer, and nitrate reduction tests. The results of biochemical tests and sugar fermentation tests of bacterial strain A2 agreed almost exactly with the reported data of *B. megaterium.*

The chitosanase activities of the eight selected strains were confirmed by using chitosanase detection agar, and A2 produced a larger clear zone than the rest of the seven bacterial strains. In addition, in the DNS assay method, A2 also showed colour changes from yellow to a deep red colour at the end of the reaction. Therefore, A2 has maximum chitosanase activity.

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References

Atlas, R. M. and J. W. Snyder. (2006). "*Handbook of Media for Clinical Microbiology*". New York: 2nd Ed., CRC Publisher, 278-339

Beesley, C. A., C. L. Vanner, and L. O. Helsel. (2010). "Isolation and Characterization of Clinical *Bacillus* Spp. Isolates Phenotypically Similar to *Bacillus anthracis*." *Federation of Europen Micrological Scocieties Microbiol Lett*., **313**, 47-53

Breed, R. S., E. G. D. Murry and N. R. Smith. (1957). *Bergey's Manual of Determinative Bacteriology***.** 7th Ed. The Willians and Wilkins Company, Baltimore, 505-552

Buchanam, R. E. and N. E. Gibbon. (1974). *Bergey's Manual of Determinative Bacteriology***.** United States of America**:** 8th Ed, The Willams Wilkins Company, Baltimore, 532-533

Dingle, J., W. W. Reid, and E. L. Solomons. (1953). "The Enzymatic Degradation of Pectin and Other Polysaccharides. Application of the Cup Plate Assay to the Estimation of Enzymes." *J. Sci. Food Agric*., **4**,146-149

Kaur, K., V. Dattajirao, and U. Bhardwaji. (2012). "Isolation and Characterization of Chitosan- Producing Bacteria from the Beaches of Chennai, India." *Enzyme Res.*, **20**(12), 1-6