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Isolation of *Bacillus megaterium* and its Commercial Importance

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Abstract : Isolation of *Bacillus megaterium*, majorly an aerobic spore forming bacterium that is found in widely diverse habitats. The research paper gives a brief account about the bacteria, bacterial sources and its uses for environment and human welfare in brief. The methodology undertaken for the cultivation and isolation of the bacteria by the HiCrome Bacillus Agar medium preparation method is well-reasoned. The physiology and commercial products of *B. megaterium* like amylase, glucose dehydrogenase, *Penicillin amidase*, vitamin B_{12}, oxetanocin and other antimicrobial agents and bioremediation are provided with thoughtful clarification. The interpretation part contains the addendum of the bacteria and its application in the market and its potential future.

Keywords : *Bacillus megaterium*, Hydroxy Citric acid (HCA), Amylase, Vitamins B12, Glucose Dehydrogenase and Oxetanocin.

Introduction

Bacillus megaterium (*Bacillus megaterium*) was first identified over 100 years ago and is a curio in the present, due to its physiology and unusually useful enzymes. It has the capacity of sporulation and a simple cell differentiation cycle, which helps in understanding gene regulation during temporal and morphological developments. *Bacillus megaterium* has large size vegetative cell and spore. Though considered a soil organism, it may also be found in diverse environment like rice paddies, dried food, seawater, sediments, fish, and normal flora and even in bee honey.Since *B. megaterium* is able to grow on a wide variety of carbon sources, they are found in many ecological niches such as waste from meat industry or petrochemical effluents.

Bacillus megaterium has economic importance because of production of commercially important enzymes like penicillin amidase, steroid dehydrolase, amylase, and products like hydroxyl citric acid (HCA), during its growth cycle.It is the major aerobic producer of vitamin B, and is one of the organisms involved in fish spoilage. An extensive review emphasizing its commercial applications have recently been published (Vary, 1992); major research areas and commercial applications are summarized in the illustration that appears

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on the cover of this issue of *Microbiology* [140(5)]. During the 1980s, genetic techniques of transduction, plasmid transformation, protoplast fusion and transposition became developed enough in *B. megaterium*to apply them to the study of many of its metabolic and developmental functions. Moreover, it is increasingly used as a host to produce foreign genes since it has been found to express, secrete and process foreign proteins without degradation.

Sporulation:

There has been a long-term effort invested in *Bacillus megaterium* to study sporulation for two principal reasons. First, for the better understanding of the process in order to circumvent the devastating effects of spore-mediated diseases such as food poisoning, anthrax, botulism and tetanus. Many of the seminal biochemical studies on sporulation were done using *B. megaterium* because of its large size, its ability to sporulate very efficiently on diverse media and to germinate synchronously on a wide range of germinant (Setlow & Kornberg, 1969; Vary & Kornberg, 1970). Much progress has been made since in understanding the regulation and temporal expression of sporulation genes. There are over 150 genes involved in sporulation and germination that have been mapped in *B. subtilis*, and many loci have been cloned (Hoch, 1993).

Results and Discussion

Collection of the sample:

The Garcenia Samples were collected from Western Ghats region of Karnataka, The infected fruits , leaves were collected for the studies. The root and soil samples of Garcenia samples were taken. The Samples were collected from two different trees of Garcenia. And so collected fruits and leaves are stored under cool conditions (40C).

Isolation and identification of *B.megaterium:*

Bacillus megaterium is isolated by enrichment culture techniques. 10gms of samples collected from different regions of Western Ghats is suspended in 100ml of distilled water and serial dilutions were made.From the serial dilution, 100µL of soil suspension at different dilutions were drawn and poured onto the surface of HiCrome Bacillus Agar plates, HiCrome Bacillus Agar plates were prepared according to the composition given in Table-1, which is a selective medium for isolating Bacillus megaterium.The inoculated plateswere incubated at 28-30ºC for 48 hrs. Colonies were observed on plates.

Table 1: composition of HiChrome Bacillus Agar Medium

Composition Hicrome bacillus agar medium	**Ingredients Gms/litre**
Peptic digest of animal tissue	10.000
Meat extract	1.000
D-mannitol	10.000
Sodium chloride	10.000
Chromogenic mixture	3.200
Phenol red	0.025
Agar	15.000
Final pH(at 25^{o}C)	7.1 ± 0.2

Identification of Isolates:

Cells from typical colonies were scraped from the agar surface and suspended in sterile water, after observation at 40x magnification, with a light microscope. The cultures so isolated were characterized through a number of microbiological and biochemical tests to confirm their identity as Bacillus megaterium as shown in Figure 1.

Fig-1: Bacillus megaterium streaked on agar plate

Biochemical Tests

Gram Staining:

The Gram staining is done in initial stages of the study to confirm the type of bacteria in the inoculums (Gram+ve or Gram-ve) and to characterize the initial features of the organism present in the inoculums. The samples are heat fixed on glass which was treated with Crystal violet then treated with an decolorizer 70%Ethanol after suitable incubation with dye after the treatment with an alcohol it is treated with Saffron dye and is washed after suitable incubation as shown in Figure 2.

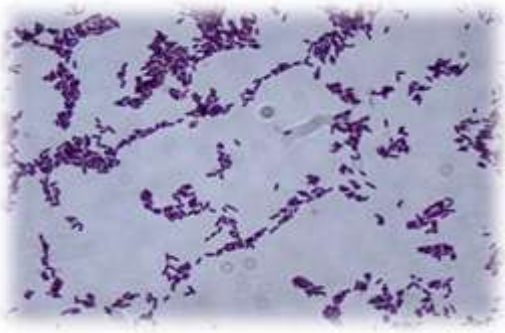

Fig-2: The bacteria staining purple blue color (Gram +ve)

Catalase Test:

The purpose of doing the Catalase test is to see if the microbe has Catalase, a protective enzyme capable of destroying the dangerous chemical hydrogen peroxide. The Catalase activity is determined by the growth from an overnight culture of the microbe is spread on a microscope slide. A droplet of 3% hydrogen peroxide was added. If copious bubbles are observed, the microbe is positive for Catalase as shown in figure 3.

Fig-3: slide showing positive catalase test

Mannitol Fermentation Test:

Phenol-Red Mannitol Broth is used for this test. It is a nutrient broth to which 0.5-1% mannitol is added and the pH indicator used here is Phenol-Red which is red at neutral pH and turns to yellow on change of pH<6.8. It would also change to pink on increase in from neutral pH. An overnight incubated culture is streaked on the medium. An isolated single colony was picked from HiCrome Bacillus Agar plates and suspended in medium. Suspension was streaked on tubes and incubated overnight at 35-37ºC. Later, results were checked for color change from red to yellow, would indicate positive result. The medium contains peptic digest of animal tissues and meat extract, which provide nitrogenous compounds. Mannitol serves as the fermentable carbohydrate, fermentation of which can be detected by phenol red. Mannitol fermenting organisms like *B. megaterium* yield yellow coloured colonies as shown in Figure 4.

Fig-4: Test tube having mannitol red agar showing positive result

Urease Test:

The Urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urease-positive Protease from other Enterobacteriaceae. Urease is a constitutively expressed enzyme that hydrolyzes urea to carbon dioxide and ammonia.

$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$

Urease test media contain 2% urea and phenol red as a pH indicator. An increase in pH due to the production of ammonia, results in a color change from yellow (pH 6.8) to bright pink (pH 8.2). Urea broth (Stuart's urea broth) is a highly buffered medium requiring large quantities of ammonia to raise the pH above 8.0 resulting in a color change. Urea agar (Christensen's urea agar) has reduced buffer content and contains peptones and glucose. This medium supports the growth of many enterobacteria allowing for the observation of urease activity. The procedure for urease test is to Streak the surface of a urea agar slant with a portion of well isolated colony. Alternatively, slant can be incubated with 1-2 drops of overnight brain-heart infusion broth. Leave the cap on loosely and incubate the test tube at 35ºC in ambient air for 48 hours to 7 days. If organism produces urease enzyme, the color of the slant changes from light orange to magenta. If organisms do not produce urease the agar slant and butt remain light orange (No color change

Voges–Proskauer(VP):

VP test was developed to detect the presence of acetone in the bacterial culture. The test was performed by adding potassium hydroxide and alpha-naphtholto the V-P broth which has been inoculated with bacteria. On inoculation, if cherry red color is seen then the result is positive, while negative result is yellow-brown color.

It depends on the conversion of glucose to acetyl methyl carbinol. If glucose was broken down, it would react with alpha-naphthol (VP reagent I) and potassium hydroxide (VP reagent II) to give a red color change.

Methyl Red (MR) Test:

The microbe could ferment mixed acids on supply of glucose. Proportions and types of acids produced by anaerobic fermentation of glucose is a key characteristic which helps to differentiate various genera of enteric bacteria. On fermentation, culture would give us 1mol of CO_2, 1 mol of H_2 per mol and 4mol of acidic products. The large amounts of acid results significant decrease in the pH of the medium below 4.4. This is visualized by using pH indicator, methyl red (p-dimethylaminoaeobenzene-O-carboxylic acid), which is yellow above pH 5.1 and red at pH 4.4 as discussed in **Table 2**

Table 2: Biochemical test result and characteristic of *Bacillus megaterium.*

Tests	**Results**
Gram Test	Gram +ve
Motility	Present
Length in micrometer	2 to 4
Width in micrometer	>= 0.9
Spores(under aerobic conditions)	Oval or Cylindrical sporangia
Methyl red Test	Positive
Catalase	Positive
Manitol Fermentation test (With Manitol)	Positive
Manitol Fermentation (Without Manitol)	Positive
Starch Hydrolysis	Positive
Urease	Positive
Growth with Glucose	Positive
VogesProskauer	Negative

The biochemical tests show the presence of *Bacillus magaterium* &*Bacillus stublis* in the mixed inoculums. It was shown by Hida.et.al that *Bacillus magaterium* is the excellent source of HCA production the Enrichment media for the *B.magateium* is designed and are cultured in the enrichment media as mentioned earlier. And Growth are assessed at different pH

Physiology:

B. megaterium can grow on many carbon sources including waste from the meat industry and corn syrups as well as a wide range of sugars; it may be found in petrochemical effluents and can oxidize thiosulfate compounds (Priest e t al., 1988; Vary, 1992). Only a few examples of the extensive physiological studies on B.megaterium can be mentioned in this review. Currently, one of the best characterized carbon source operons is the xylose operon (Rygus & Hillen, 1992). The biochemical pathways of many amino acids have also been studied in depth. Some of the most interesting proteins of B.megaterium are a family of P-450 cytochrome monooxygenases. These have been of great interest since they have considerable similarity to eukaryotic P-450 important in many disease conditions.

Commercially important products of Bacillus megaterium

Enzymes secreted by B. megateritlm, including a-amylase, P-amylase, penicillin amidase,neutral protease, P-glucanase, megacins (the phospho –lipase MegA), glucanotransferase and chitosanases (Vary,1992). The species also synthesizes unusual enzymes such asan epoxide hydrase, an isomerase for maleylpyruvate, a α-glucanase used as a probe for structural analysis of yeastcell walls, and the enzyme alphostatin, which inhibits calfalkaline phosphatase.Some of the commercial applications and products of *B. megaterium* are listed in**Table3** and some of the enzymes are discussed below:

Table 3: Different types of enzymes produced by *B.megaterium* and its application

Product	Use
Alphastatin production	Inhibitor of alkaline phosphatase
α amylase	Can replace pullulanase
β amylase	Bread industries
chitosanases	Yeast cell wall analysis
fungicidal toxin	For Rbizoctonia, one isolated
Glucose dehydrogenase	Generator of NADH, Immobilization, Biosensors
Oxetanocin production	Inhibits HIV,hepatitis B,herpes
Phosphate solubilization	Phosphate fertilizer
Penicillin amidase	Construction of synthetic penicillin
Vitamin B12 production	Only acrobic producer

- **Amylase:** Different forms of amylases are produced by Bacillus megaterium, α-amylase and β-amylases. In bread industries Bacillus megaterium amylases are used to convert branched saccharides to a form that is easily hydrolysed by glucoamylases .

- **Glucose dehydrogenase :** Laboratories in both Japan and Germany have described multiple glucose dehydrogenase (gdh) genes from species of B. megaterium. It is easily immobilized and is often used both to generate NADH in industrial processes and as a biosensor (Kittsteiner-Eberle e t al., 1989).

- **Penicillin amidase :** Another important enzyme is penicillin acylase, or amidase, which is used to cleave the side-chain of penicillins to generate new synthetic antibiotics.

- **Vitamin B12 :** B. megaterium is the major aerobic source for vitamin B12 or cobalamin, which is synthesized from the haem-biosynthetic pathway.

- **Oxetanocin and other antimicrobial agents :** A few antibiotics such as emimycin are produced by B. megatrium (Vary, 1992), but by far the most exciting is a unique analogue antibiotic, oxetanocin, that has been shown to be effective against a number of important pathogenic viruses, even ones that do not produce thymidine kinase (Kohlbrenner e t al., 1990).

- **Bioremediation :** B. mrgaterium is found in unusual and sometimes toxic environments and may have potential as a detoxifying agent. Quinn e t al. (1989) described a carbon-phospholyase in both Pseudomonas and B. megaterium that cleaves C-P bonds so that both species were able to degrade 14 of 15 C-P herbicide compounds tested.

Conclusion

Bacillus megaterium is isolated by enrichment culture techniques. Soil suspension at different dilutions were drawn and poured onto the surface of HiCrome Bacillus Agar medium being a selective medium for isolating *Bacillus megaterium*.Organisms like *B. megaterium* yield yellow coloured colonies.The cultures isolated were characterized through a number of microbiological and biochemical tests. The colonies identified as catalase-positive, Gram-positive, endospore-forming rods.

Bacillus megaterium has many physiology and commercial uses.they secrete enzymes like a-amylase, P-amylase, penicillin amidase, neutral protease, P-glucanase, megacins, glucanotransferase and chitosanase. They also synthesizes unusual enzymes such asan epoxide hydrase, an isomerase for maleylpyruvate, a α-glucanase used as a probe for structural analysis of yeastcell walls, and the enzyme alphostatin, which inhibits calfalkaline phosphatase. There are many other commercials that have been mentioned above.

Bacillus megaterium is a very useful organism due to all the above properties mentioned and its isolation is relatively simple and can done on an industrial level.

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