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Original Research Paper

Isolation, Characterisation and Enhanced Amylase Activity of a Chlorpyrifos Degrading Bacterial Strain, *Bacillus stearothermophillus*

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ABSTRACT

In the present investigation a bacterial strain was isolated from agricultural soil sample polluted with insecticide chlorpyrifos, and the growth pattern was determined. Characteristic feature of the strain indicates that it belongs to the genus *Bacillus* and species *stearothermophillus*. The amylase activity was studied in the presence and absence of the pollutant and it was found that the bacterium grown in the presence of the pollutant showed a significant increase in the enzymatic activity by 140 µg/g/min.

INTRODUCTION

Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper (Pandey et al. 2000). They are categorized into exo-acting, endo-acting and debranching enzymes. Among the amylases, α-amylase is exo-acting whereas β-amylase is endo-acting enzyme. The α-amylase randomly hydrolyzes alpha 1,4-glucosidic linkages in starch, glycogen and related polysaccharide yielding dextrins, oligosaccharides, maltose and D-glucose (Takeshita & Hehre 1975). Amylases can be obtained from several sources such as plant, animal and microbes (Kathiresan & Manivannan 2006). Amylases are widely distributed in bacteria and fungi. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Microbial amylase has almost surpassed the synthetic sources in different industries (Pandey et al. 2000). Bacterial α-amylases are extensively important in industrial processes such as production of ethanol and high fructose corn syrup, baking, in laundry washing powders and dish washing detergents, textile desizing, and paper recycling (Nigam & Singh 1995). Unusual bacterial amylases are found in acidophilic, alkalophilic and thermoacidophilic bacteria (Boyer & Ingle 1972). Nowadays amylase from these sources is vastly used in amylase production under extreme conditions of pH and temperature. There are various reports on starch degrading microorganisms from different sources and respective amylase activity (Aiba et al. 1983, Tonkova et al. 1993, Kathiresan & Manivannan 2006). But, reports on chlorpyrifos degrading bacteria with respect to the amylase activity are not available. Bacteria belonging to the genus *Bacillus* have been widely used for the commercial production of thermostable α-amylase. These include *B. coagulans, B. stearothermophilus, B. caldolyticus, B. brevis, B. acidocaldarius* and *B. thermoamyloliquefaciens* (Campbell 1954, 1955). The present investigation deals with isolation of a bacterial strain, *Bacillus stearothermophillus* from soil samples collected from agricultural soil polluted with chlorpyrifos insecticide and physiological and biochemical features were determined. *B. stearothermophilus* (or *Geobacillus stearothermophilus*) is a rod-shaped, Gram-positive bacterium and a member of the division Firmicutes. The bacterium is a thermophile and is widely distributed in soil, hot springs, ocean sediment, and is a cause of spoilage in food products. It is commonly used as a challenge organism for sterilization validation studies and periodic check of sterilization cycles (Internet source: http://en.wikipedia.org/wiki/*Bacillus_stearothermophilus*). Its amylolytic activity under different physiological conditions was correlated with the growth kinetics.

MATERIALS AND METHODS

Microbial culture: During the present investigation, *Bacillus* strain was isolated from soil samples collected from a

private agricultural farm on Hosur road, Bangalore. One gram of soil sample was added to 99 mL of 2% starch broth. After 24 h of shake flask culture, fresh inoculum was taken for batch culture at 37°C, which was followed by plating on starch agar medium after 7 days.

Culture Media: Starch broth and starch agar were supplemented with the following components - peptone 0.05%, KCl 0.01 % (w/v), $MgSO_4$.$7H_2O$ 0.05%(w/v), $(NH_4)_2SO_4$ 0.01% (w/v), NaH_2PO_4 0.01% (Srivastava & Baruah 1986) and 2% starch. The starch agar medium was composed of all these components along with 0.8% agar. Colonies of the isolated strain were transferred by replica plating on to starch agar plates and incubated at 37°C for 48 h. Plates with bacterial colonies were then flooded with Gram's iodine reagent (0.01 M I_2-KI solution). If a strain was amylolytic then it started hydrolysing the starch present in the surrounding and in the zone of degradation there was no blue colour formation. Selection was done as per colonies with and without clear and transparent zone as amylase producing (Amy+) and amylase non-producing (Amy-) strain, respectively.

Identification of the isolated strain: Colony characteristics of the isolated organism were studied followed by biochemical tests. The results obtained from various morphological and physiological analyses were used to identify the isolates using the standard methods (Harold Benson 1990).

Culture condition growth kinetics: The starch nutrient medium was inoculated with a single isolated colony and cultured for 48h at 37°C with continuous shaking on a rotary shaker at 200 rpm. From this, 1 mL of inoculum was transferred to 100 mL Erlenmeyer flask with sterile nutrient medium incubated at 37°C at 200 rpm for different time intervals (2-14 h). Growth kinetics was obtained by measuring the cell density at 540 nm in different time intervals. Growth pattern under these conditions was observed by measuring absorbance of cells at 540 nm.

Crude enzyme preparation and enzyme assay: To obtain crude enzyme, 48h old cultures were transferred to microcentrifuge tubes and centrifuged at 4000 rpm for 15 min. Cells were discarded and resultant supernatant was used as the crude enzyme for various enzyme assays. Amylase assay was done by standard method (Miller 1959). Optical density of each sample with reaction mixture was taken at 620 nm in a spectrophotometer.

RESULTS

The Amy+ colonies exhibited clear and wide transparent zone due to the production of amylase (Fig. 1). Also, it was observed that colonies were translucent and creamy-whitish in colour. All colonies were with entire regular margin. The strain closely resembled to *Bacillus* species. For the present investigation, one of these colony was selected and taken for all physiological and biochemical analysis (Table 1). Further, the bacterium was identified as *Bacillus stearothermophillus* by a set of experiments (Table 2). Growth kinetics of the presently isolated *Bacillus* strain started log phase right after inoculation. Stationary phase started from late 4 h which continued till 6 h and after that growth declined at 14 h (Fig. 2 and Table 3).

Amylolytic activity from the sample taken from crude enzyme varied in the presence and absence of the pollutant. The activity increased by 140 µg/g/min in the presence of chlorpyrifos. The results for the enzyme activity are given in Table 4.

Table 1: Colony characteristics and biochemical tests of the isolate.

Test	Results
No. of colonies	9
Size in mm	10 (0.5)
Margin	Entire
Elevation	Raised
Optical feature	Translucent
Texture	Smooth
Colour	Off white
Form	Bacillus
Gram's reaction	Gram positive
Endospore	Present,central position
Motility	Motile
Indole Production Test	+
Methyl Red Test	+
Voges-Proskeaur test	-
Citrate utilization test	-
Catalase test	+
Oxidase test	-
Nitrate test	+
Glucose fermentation test	+
Amylase test	+

Table 2: Identification of the bacterial species.

Growth at 50°C	+
Growth at 60°C	+
Growth at 7% NaCl	-
Growth at pH 6	+
Identification	*Bacillus stearothermophillus*

Table 3: Growth kinetics of the isolated *Bacillus* strain from contaminated soil.

S. No.	Time (h)	Mean O.D
1	2	0.29 (0.01)
2	4	0.60 (0.01)
3	6	0.67 (0.01)
4	8	0.54 (0.01)
5	10	0.47 (0.01)
6	12	0.41 (0.01)
7	14	0.31 (0.01)

Figures in parenthesis refer to standard deviation

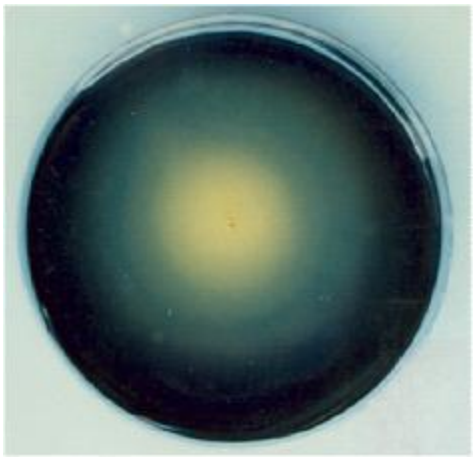

Fig. 1: A starch agar plate showing amylase activity with clear white zone at the center surrounding the single colony of *Bacillus* isolated from the soil sample.

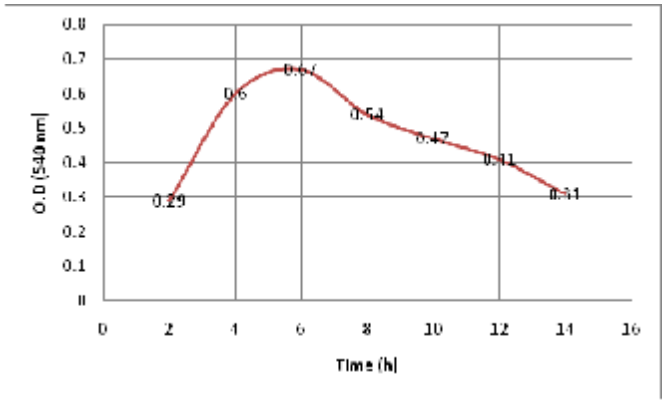

Fig. 2: Growth kinetics of the isolated *Bacillus* strain from contaminated soil. Readings taken at every 2 h intervals till 14 h.

DISCUSSION

In the present investigation a pure strain of *Bacillus stearothermophillus* was isolated from the polluted agricultural soil. The soil was found to be polluted with a large number of pesticides and was rich in one particular insecticide, chlorpyrifos. Literature indicates that agricultural soil pollutants include large number of pesticide compounds and their derivatives. Interestingly, chlorpyrifos insecticide was found in abundance in the soils that show occurrence of *Bacillus* species (Vidyalakshmi et al. 2009).

Optimization of growth conditions is a prime step in using microorganisms in fermentation technology (Kathiresan & Manivannan 2006). In the present study, we observed 37°C as the optimum growth temperature for the presently reported *Bacillus* strain and higher temperature (50°C-60°C) also supported the growth of colonies. This could be due to the thermophilic nature of the species. Among the physiological parameters, optimum temperature, substrate concentration and pH range are the most important for enzyme production by microbes (Bose & Das 1996, Gupta et al. 2003). Most of the starch degrading bacterial strains revealed a pH range between 6.0 and 7.0 for normal growth and enzyme activity (Gupta et al. 2003). The presently isolated *Bacillus* strain also showed optimum growth in this range. The composition and concentration of media greatly affect the growth and production of extracellular amylase production in bacteria (Chandra et al. 1980, Srivastava & Baruah 1986). Starch is ubiquitous and is an easily accessible source of energy (Ryan et al. 2006). In past studies, a number of carbon and nitrogen sources have been examined for amylase production in several *Bacillus* species (Bose & Das 1996, Srivastava & Baruah 1986, Ryan et al. 2006). The present study also showed that the presence of starch in the media increased the amylase production.

The growth curve of *Bacillus stearothermophilus* is shown in Fig. 2. Growth is not completely inhibited and the organism is able to complete its life cycle. *Bacillus* species found in the mercury contaminated lake along with other bacteria have been reported to show high resistance to mercury (Kafilzadeh & Mirzaei 2008). Since, mercury is one of the most commonly found components in many pesticides, the above report can be considered in favour of our findings. Further, this is the first report of bacterial growth curve in *Bacillus stearothermophilus* in the presence of chlorpyrifos (Savitha 2009). Results of our study show that the organism is able to grow well in the media with insecticide and also co-exists with chlorpyrifos.

The results of the α-amylase activity in *Bacillus stearothermophilus* in the presence and absence of chlorpyrifos are presented in Table 4. A significant increase is evident in the enzymatic activity of the bacteria in presence of the pollutant. The α-amylase activity is found to be higher by 140µg/g/min in the presence of chlorpyrifos. Directly or indirectly pollutants influence the cell metabolism including that of the changes in the activity of enzymes. The enzymatic activities of *Bacillus stearothermophilus* in the presence of chlorpyrifos in our study also showed significant changes. An increase in the α-amylase activity may be due to the uptake and degradation of the insecticide. *Bacillus stearothermophilus* showed a 14% increase in the amylase activity in presence of chlorpyrifos as compared to that of control. The findings of this study reveals that *Bacillus stearothermophillus* is involved in taking up chlorpyrifos, further chlorpyrifos is found to enhance the α-amylase activity. These findings are of great significance as it is first of its kind. The increase in the enzymatic activity shows that the bacteria must have involved in the uptake and biodegradation of the pollutant.

Table 4: The α-amylase activity in bacteria.

Sample	Phosphate buffer (mL)	1% Starch (mL)	Incubated at RT for 5 min	Enzyme (mL)	Incubated at RT for 20 min	DNS (mL)	Incubated in boiling water bath for 10 min	D.W. (mL)	Mean O.D (620nm)	α-amylase activity of bacteria (μg/g/min)
Blank	1.0	0.5		0.0		0.5		2.5	0	0
Sample A	1.0	0.5		0.5		0.5		2.5	0.17(0.01)	660
Sample B	1.0	0.5		0.5		0.5		2.5	0.20(0.01)	800

Figures in the parenthesis refer to standard deviation; P value equals 0.0213; This difference is considered to be statistically significant.
Sample A: *Bacillus stearothermophilus*; Sample B: *Bacillus stearothermophilus* in the presence of chlorpyrifos.

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