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Isolation and identification of cellulase producing *Bacillus cereus* from soil samples from Tirupati, India

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Abstract
The aim of the study is to determine the occurrence of cellulase producing *B. cereus* in various soil samples from Tirupati region. In this study, 68 samples were collected from different areas of Tirupati. Out of 68 samples, 58 (85.29%) samples showed characteristic peacock blue colored colonies on selective PEMBA. On Gram staining all these isolates were Gram-positive rods and sometimes spores were seen. Biochemical characterization was done to identify *B. cereus* isolates. Out of 58 isolates 53 isolates were positive for β-hemolysis on sheep blood agar. Fifty three (53) isolates were subjected to various biochemical tests but only 9 (16.98%) isolates showed characteristic biochemical features as that of *B. cereus*. All the 9 isolates were positive for catalase test, motility test, Voges- Proskauer test, citrate utilization test and nitrate reduction test and but negative for oxidase test. The 9 isolates are also positive for starch hydrolysis and gelatin hydrolysis. Out of 9 isolates only 2 (22.22%) isolates were positive for cellulase activity.

Keywords: cellulase, *B. cereus*, PEMBA, BHIB, cellulose-congored agar

1. Introduction
Cellulose is the most abundant biological compound on terrestrial and aquatic ecosystem and is the main component of plant biomass. It is the dominant waste material from agricultural industry in the form of stalks, stems and husk, there has been great interest in utilizing cellulose as an energy resource and feed. The Cellulose is a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages. Cellulose is commonly degraded by cellulase. Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. Cellulolytic enzyme system comprises three classes of soluble extracellular enzymes: 1, 4-β-endoglucanase, 1, 4-β-exoglucanase, and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). Endoglucanase is responsible for random cleavage of β-1, 4-glycosidic bonds along a cellulose chain. Exoglucanase is necessary for cleavage of the nonreducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and β-1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose (Woodward and Wiseman, 1983) [22]. Microorganisms able to producing exo- β-1, 4 glucanase are capable to hydrolyzing native cellulose (filter paper, cotton etc.). Only the synergy of the above three enzymes makes the complete cellulose hydrolysis to glucose (Ryu and Mandels, 1980) [16].

Cellulases potentially used in biotechnology and industrial applications such as, starch processing, alcoholic beverage, malting and brewing, clarify of juice, pulp bleaching, textile industry and animal feed. Many species belonging to *Bacillus* genus have been reported to possess cellulolytic activities, for example, *B. subtilis, B. pumilus, B. megaterium, B. brevis, B. firmis, B. licheniformis, B. agaradbaerens, B. alcalopbilus, B. coagulans and B. amyloliquefaciens* (Maki *et al.,* 2009) [10]. These are gram-positive, spore-forming, aerobic, facultative anaerobic, rod-shaped belonging to the phylum Firmicutes.

The Bacillus species are endemic soil bacteria that occupy diverse ecological habitats. Due to the formation of heat-, UV-, acid- and desiccation-resistant endospores, the bacteria can persist in a dormant state, making it difficult to elucidate their primary ecological niches. In addition to soil, the species have been isolated from fresh and stored foods, invertebrates, and plants (Monnerat *et al.,* 2009 and *Gupta et al.,* 2012) [13, 5].

B. cereus was a highly motile bacterium, generally appearing as single cells but occasionally forming longer threads, and causing rapid liquefaction of gelatin.

Bacillus cereus was found to produce the endoglucanase type cellulase and most of the isolated *B. cereus* / *B. thuringiensis* strains were found to produce extracellular enzymes (Afzal *et al.*, 2012) [1]. The present investigation was designed to isolate and Screen the Cellulase Producing *Bacillus cereus* from Soil.

2. Materials and Methods

A total of 68 soil samples were collected in sterile zipped plastic sachet at 18 areas of Tirupati and near by villages.

2.1 Isolation and identification of *B. cereus*

2.1.1 Isolation

Polymyxin - pyruvate - Egg yolk – Mannitol - Bromothymol blue Agar (PEMBA) media was used for isolation of *B. cereus*. The samples were processed as per the method described by Shinagawa (1990) [19]. The samples were inoculated into brain heart infusion broth (BHIB) containing polymyxin (100 units/ml). The BHIB tubes were then incubated at 37 ºC for 24-48 hours. After enrichment a loopful was streaked on PEMBA plates and incubated at 37 ºC for 24h. The fimbriate peacock blue coloured colonies (3-5mm) surrounded by blue zone of egg yolk hydrolysis against green/greenish yellow back ground were presumed to be *B. cereus*. The presumed isolates were preserved on nutrient agar slants for further characterization.

2.2. Identification and confirmation

All presumptive colonies of *B. cereus* were purified and subjected to morphological and biochemical tests for identification and confirmation as described in Bacteriological Analytical Manual of United States, Food and Drug Administration (Allent *et al.*, 2016) [2], Bergey's Manual of Systemic Bacteriology (Vos *et al.*, 2011) [20] and Veterinary microbiology and microbial disease (Quinn *et al.*, 2011) [15].

2.3 Morphology

Gram stained smears were prepared from slants and examined microscopically. *B. cereus* appeared as large Gram positive bacilli in short to long chains, whereas, spores were ellipsoidal, central to sub terminal and did not swell the sporangium.

2.4 Biochemical characterization of *B. cereus* isolates

2.4.1 Haemolytic activity test

The test was performed on all *B. cereus* isolates for the production of haemolysis on sheep blood agar. A loopful of culture was streaked on blood agar plate and incubated for 24 h at 37 ºC and observed for haemolytic zones around the colonies. *B. cereus* cultures are usually strongly haemolytic and produced zones of complete β-haemolysis surrounding the growth.

2.4.2 Catalase test

The presumptive *B. cereus* isolates were tested for their catalase activity. Briefly, a drop of 3 percent hydrogen peroxide (H_2O_2) was taken on a clean glass slide and the presumed colony was mixed with it. The formation of gas bubbles was taken as positive reaction.

2.4.3 Oxidase test

A loop full of bacterial growth was rubbed using a sterile platinum loop on oxidase disc (Hi Media Ltd., Mumbai) on a sterile microscopic slide. Development of deep purple blue or mauve colour within 10 sec was considered as positive and no color change was taken as negative reaction.

2.4.4 Motility test

The test was performed to observe the motility of *B. cereus* having peritrichous flagella. Nutrient agar medium in a test tube was inoculated by stabbing down the centre with a 24 h culture suspension of presumptive isolate. The tubes were incubated for 18-24 h at 30 ºC and examined for diffuse type of growth away from stab line.

2.4.5 Voges-Proskauer test

In a tube containing 2 ml of sterile glucose phosphate peptone water (Hi Media Ltd., Mumbai) a loopful of young broth culture (18-24 h) was inoculated and incubated at 37 ºC for 48 hrs. After 48 hrs, 0.2 ml of 40 per cent potassium hydroxide was added followed by 0.6 ml of α-napthol solution. The development of pink or crimson colour was recorded as positive reaction while no colour change was the negative reaction.

2.4.6 Citrate utilization test

A loopful of bacterial growth was streaked on Simmon's citrate agar slants and incubated at 37 ºC for 24 hrs. The development of blue colour due to utilization of citrate as a carbon source by the organism was taken as positive reaction while green colour was considered as negative reaction.

2.4.7 Nitrate reduction test

Sterile nitrate broth (Hi Media Ltd., Mumbai) (0.5 ml) was inoculated with a heavy growth of the test organism and incubated at 37 ºC for 24 hrs. Subsequently, one drop each of sulphanilic acid and the α-naphthylamine reagent was added to the test culture in broth.

The development of red colour within one minute was taken as positive reaction. The tube that did not show red colour within five minutes was treated with zinc powder and allowed to stand for 5 min. In such cases, the development of red colour indicated the presence of nitrate in the medium, as it was not reduced to nitrite by the test organism.

2.4.8 Starch hydrolysis test

A loopful of bacterial growth was streaked on starch agar plate and incubated at 37 ºC for 48 hrs. After 2 days of incubation, the plate was flooded with iodine solution. Iodine turns blue or black in the presence of starch. A clearing around the bacterial growth indicated that the organism has hydrolysed starch.

2.4.9 Gelatin hydrolysis test

Nutrient gelatin stab method is the standard and most commonly employed method for gelatin hydrolysis. In this method, a heavy inoculum of an 18- to 24-hour-old test bacteria is stab-inoculated into tubes containing nutrient gelatin. The inoculated tubes and an uninoculated control tube are incubated at 25 °C, or at the test bacterium's optimal growth temperature, for up to 1 week, and checked everyday for gelatin liquefaction. Gelatin normally liquefies at 28 °C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath for 15 to 30 minutes. Afterwards, tubes are tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin will result in a liquid medium even after exposure to cold temperature (ice bath), while the uninoculated control medium will remain solid. For weak positive results, incubate the inoculated

nutrient gelatin tube longer until complete liquefaction is observed. The hydrolysis of gelatin indicates the secretion of gelatinase by the test organism into the medium.

2.4.10 Cellulose hydrolysis test

Cellulose-degrading ability of *B. cereus* isolates was tested by streaking on the cellulose Congo-Red agar media with the following composition: KH2PO4 0.5 g, MgSO4 0.25 g, cellulose 2 g, agar 15 g, Congo-Red 0.2 g, and gelatin 2 g; distilled water 1 L and at pH 6.8– 7.2. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies (Patagundi *et al.*, 2014) [14].

3. Results

In the present study, a total of 68 soil samples collected from Tirupati and nearby villages were analysed for the presence of *B. cereus*. Out of 68 samples 58 (85.29%) samples showed characteristic peacock blue coloured colonies on selective PEMBA (Fig.1).

All these isolates were Gram positive rods and sometimes spores were also seen (Fig.2 and 3). Biochemical characterization to identify *B. cereus* isolates was done. Fifty three (58) isolates were subjected to β-hemolysis on sheep blood agar but only 53 (77.94%) isolates showed characteristic β-hemolysis (Fig.4). Fifty three (53) isolates were subjected to various biochemical tests but only 9 (16.98%) isolates showed characteristic biochemical features as that of *B. cereus*. All the 9 isolates were positive for catalase test (Fig.5), motility test (Fig.6), Voges- Proskauer test (Fig.7), citrate utilization test (Fig.8) and nitrate reduction test (Fig.9) and but negative for oxidase test. The 9 isolates are also positive for starch hydrolysis (Fig.10) and gelatin hydrolysis (Fig.11). Out of 9 isolates only 2 (22.22%) isolates were positive for cellulase activity (Fig.12)

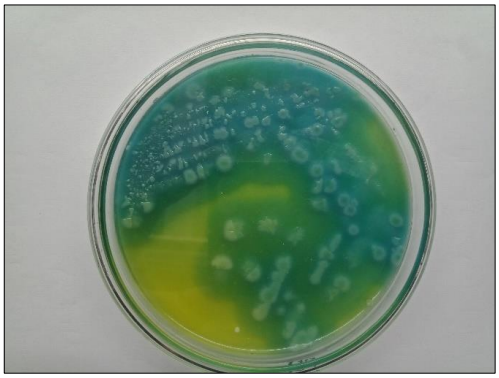

Fig 1: *B. cereus* colonies on PEMBA

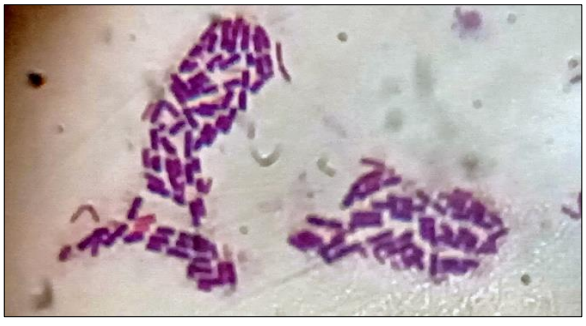

Fig 2: Gram staining of *B. cereus* (Gram positive rods)

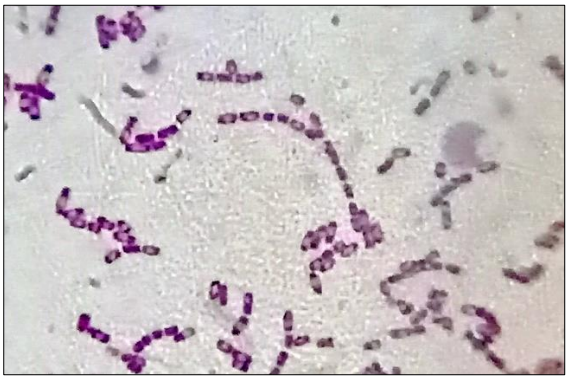

Fig 3: Spores of *B. cereus*

Fig 4: *B. cereus* showing β-haemolysis on sheep blood agar

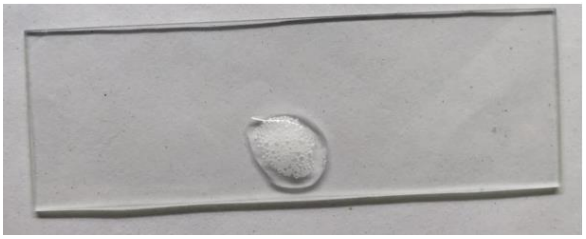

Fig 5: Air bubble formation in Catalase test indicative of positive test

Fig 6: Motility test (haze around stab by motile bacteria)

Fig 7: Voges-Proskauer test (Crimson red ring is indicative of positive test)

Fig 8: Citrate utilization test (blue colour change indicates positive test)

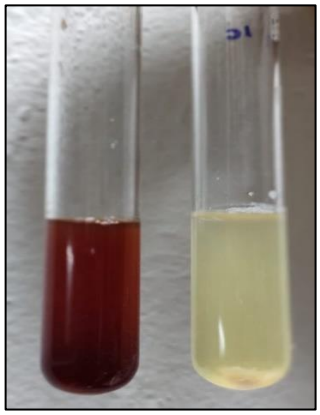

Fig 9: Nitrate reduction test (brick red color indicates positive test)

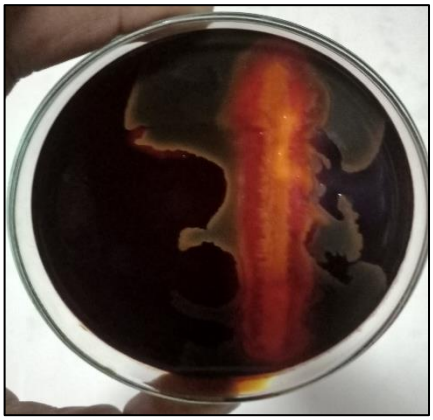

Fig 10: *B. cereus* showing starch hydrolysis (zone around streak)

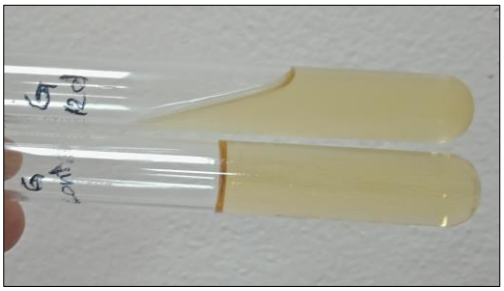

Fig 11: *B. cereus* showing gelatin hydrolysis

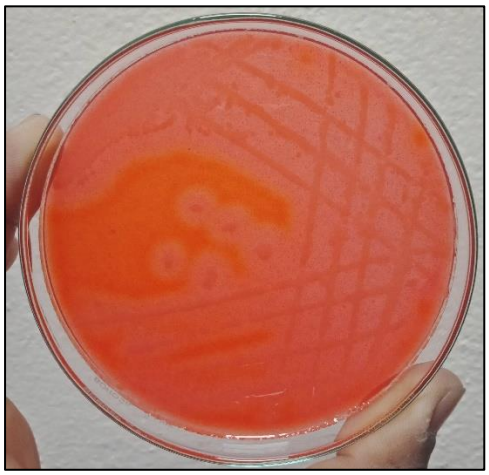

Fig 12: Cellulose Hydrolysis test (Discolouration of Congo red dye is indicative of positive test)

4. Discussion

Cellulose is an abundant natural biopolymer on earth and most dominating agricultural waste. Cellulosic biomass is a renewable and abundant energy source with great potential for bioconversion to value-added bio-products. Cellulose is a crystalline polymer of D-glucose residues connected by β-1, 4 glucosidic linkages. Cellulose can be degraded by cellulase producing bacteria which is most.

important biocatalysts for the industrial process. Cellulases are inducible enzymes which are synthesized by large number of microorganisms either cell-bound or extracellular during their growth on cellulosic materials (Lee and Koo, 2001) [8].

Over the years, culturable, cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material from forestry or agricultural waste, the feces of ruminants such as cows, soil and organic matter, and extreme environments like hot-springs, to name a few (Doi, 2008) [4]. Screening for cellulase production can be done by enrichment growth on microcrystalline cellulose as a sole source of carbon. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria (Lu *et al.*, 2004) [21]. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies. Behera *et al.* (2014) [3] isolated cellulose degrading bacteria from mangrove soils of Mahanadi delta using CMC agar medium and identified five *Bacillus* species based on morphological and biochemical characterization.

In comparison to other microorganisms, *B. cereus* is well versed with diversified characteristics like aerobic and facultative anaerobic growth, motility, psychrotrophic (4 ºC) and thermophilic (50 ºC) nature. *B. cereus* makes its presence either in the form of vegetative cells or spores and is hence,

anticipated from almost all foods of domestic consumption owing to its ubiquitous nature and adaptation to the environmental changes, thus posing a great public health threat. Apart from clinical significance of *B. cereus*, it posses an ability to produce various extra cellular enzymes such as amylase, gelatinase, lecithinase, DNase, Protease, Lipase and xylanase (Molva *et al.*, 2009) [12]. Few strains of *B. cereus* species also possess cellulase enzyme which have endoglucanase activity. The present study focuses mainly on the extracellular enzyme cellulase of *B. cereus*. The bacterial origin of cellulase have added advantage compared to enzymes derived from fungal origin because of high growth rate (Shanmugapriya *et al.*, 2012, Sethi *et al*., 2013 and Patagundi *et al.*, 2014) [18, 17, 14]. Patagundi *et al.* (2014) [14] reported that *Bacillus cereus* showed maximum cellulolytic activity compared to *B. subtilis* and *B. thuringiensis.* Khianngam *et al*. (2014) [7] isolated the cellulase producing *B. cereus* from oil palm meal.

Cellulase from *Bacillus* species are used in many applications such as animal foods and a feed stock for production of valuable organic compounds. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production (Mandels and Weber. 1969) [11] and as a possible food supplement or in pharmaceutical industry. In the present study, two isolates was found to possess cellulolytic activity out of 9 *B. cereus* isolates. These findings helps to produce cellulase in commercial scale by using *B. cereus* strains. Further study was needed to scale up the feasibility of the strains and how much quantity of cellulase to be produced in industrial scale.

5. Acknowledgment

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