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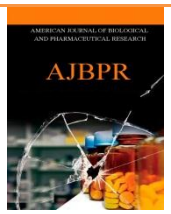

ISOLATION OF POTENTIAL CELLULOLYTIC BACTERIUM, *BACILLUS PUMILUS* DGLB 32 FROM THE VEGETABLE WASTES OF DINDIGUL, SOUTH INDIA AND ITS GROWTH CHARACTERIZATION

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ABSTRACT

The present study aimed to isolate a promising cellulase producing bacterium from vegetable waste of Dindigul district, Tamil Nadu, India. This investigation isolated 34 morphologically distinct bacteria and only one strain that was DGLB32 showed potential cellulase production with 78.9 Units/ml. This strain was identified as *Bacillus pumilus* using 16s rRNA molecular sequencing method. The most potential strain showed maximum growth using the lab conditions of pH 7, 37°C temperature, 2% carboxymethylcellulose and 1% tannic acid. From the above observations, the isolated strain revealed the possibilities of industrial scale production especially for the production and applications of cellulase enzyme.

INTRODUCTION

Sustainable resources, which are in need of human beings, are derived from plant biomass. Cellulose is the major component of plant biomass. Plants produce 4×10^9 tons of cellulose annually [1]. It is a polymer of β-1,4 linked glucose units. Its crystalline structure and insoluble nature represents a big challenge for enzymatic hydrolysis. Microorganisms are important in conversion of lignocellulose wastes into valuable products like biofuels produced by fermentation. Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes [2].

Numerous microorganisms that are able to degrade cellulose have been isolated and identiied.

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However, many studies have put more emphasis on fungi because the cellulases that they produce are abundant and easy to extract, and some of the fungal cellulases have been used as commercial cellulase [3]. Although fungi such as *Trichoderma, Aspergillus, Penicillium, Phanerochaete,* and *Fomitopsis* have beenwidely studied in recent years, researchers have also been paying attention to various bacteria that produce cellulases because of their fast growth, expression of multienzyme complexes, and resistance to extreme environments. Bacteria belonging to the genera *Clostridium, Cellulomonas, Cellulo simicrobium, hermomonospora, Bacillus, Ruminococcus, Erwinia, Bacteriodes, Acetovibrio, Streptomyces, Microbispora, Fibrobacter,* and *Paenibacillus* have been observed to produce different kinds of cellulase when incubated under anaerobic or aerobic conditions [4].

Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium and other nutritional conditions are important parameters for the optimized production of cellulase enzymes [5]. Present study is aimed to isolate bacteria, which can produce more efficient cellulase enzymes which is also optimized for the enhanced production under submerged fermentation conditions.

MATERIALS AND METHODS

Microbial enumeration

One gram of vegetable waste was taken for the microbial analysis which was isolated from two samples collected from Dindigul, South India. The samples were individually grinded using Mortar and pestle. The finely grinded samples were serially diluted on sterile saline water (0.9%) and nutrient agar medium (Himedia, M001) was used for enumeration of microorganisms. The enumeration was done using Quebec colony counter.

Isolation of axenic bacterial cultures

The same plates used for the microbial enumeration was used for the isolation and screening of promising bacterial and fungal strains. Distinct morphological strains were chosen and pure cultured on the same nutrient agar medium as mentioned in the enumeration procedure. Morphologically distinct fungal pure cultures were pure cultured using quadrant strike plate which were maintained as axenic cultures under refrigeration at 0°C

Screening of promising cellulolytic microbes

The axenic cultures were freshly broth cultured on nutrient broth medium using 10ml in 30ml screw cap tubes. After incubation period, the broths were centrifuged at 3000 rpm for 15 min and supernatant was used for the following studies. Cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method [6], through the determination of the amount of reducing sugars liberated from carboxymethylcellulose (CMC) solubilized in 50 mM Tris–HCl buffer, pH 8.0 [7]. This mixture was incubated for 20 min at 50°C and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10 min, cooled in water for color stabilization, and the optical density was measured at 550 nm. The cellulase activity was determined by using a calibration curve for glucose (Sigma–Aldrich). One unit of enzyme activity was defined as the amount of enzyme that released 1^{-1}mol of glucose per minute.

Molecular identification

The molecular identification of the promising strain was done by 16S rRNA partial sequence method. Analysis of 16S ribosomal RNA region using the universal set of the Eubac primers, 27F – Forward (5′- AGAG TTTG ATCM TGGC TCAG -3′) and 1492R – Reverse (5′- GGTT ACCT TGTT ACGA CTT -3′). PCR amplification was done on a thermal cycler (Eppendorf) with 50µl reaction mix. The reaction mixer contained 10× amplification buffer (5µl), 1.5mM MgCl2 (5µl), 1µl of each forward and reverse primer, 1µl dNTP and 0.25µl Taq polymerase. After an initial denaturation at 95°C for 1min, strain for further studies. Similar to the present investigation, an earlier study isolated *Cellulomonas* sp. amplification was carried out with 35 cycles of 35 s at 94 ◦C, 40 s at 55 ◦C, 2min at 72 ◦C followed by a final extension for 8min at 72 ◦C. The PCR product was purified using the Qiagen PCR purification kit and then sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA). The evolutionary distances were computed using the maximum-composite-likelihood method [8] and the evolutionary analyses was conducted using MEGA7 software [9]. The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the neighbor-joining method.

Optimization of growth conditions

The isolate was cultured in a 45 L fermenter (Scigenics, India) with 20 L working volume. The inoculum was prepared using the exponential phase culture of this promising strain. The other important basal fermentation conditions used during growth standardization were agitation at 150 rpm and aeration at 1.0 vvm. At 48 hours for bacterial strains, broth medium were withdrawn and centrifuged at 2700 ×*g* for 15 min and the cell pellets were used for estimation of cell biomass on dry weight basis [10]. The growth parameters studied for the isolates were different pH conditions at 6, 7 and 8, different carboxy methyl cellulose concentration at 0.5%, 1%, 1.5%, 2 %and 2.5%, three different temperatures at 28°C, 37°C and 45°C and different concentration of tannic acid ranged within 0.5 – 2.5ml/L with the intervals of 0.5 ml/L was determined.

RESULTS AND DISCUSSION

Isolation, screening and identification of potential cellulase producing bacterium

After incubation period, the nutrient agar plates were inspected for total number of heterotrophic bacteria which was ranged between 187×105 - 81×106 CFU and observed for distinct morphological colonies which were isolated and pure cultured on same agar medium. Totally, 43 axenic strains were isolated which were named as DGLB 1 – 34. These isolates were screened for the most potential cellulase producer using cellulolytic biochemical assay. Only 11 % of the isolates showed activity screening assay. In these isolates, only one strain showed potential activity which was DGLB32. The strain showed maximum cellulase activity of 78.9 Units/ml. The molecular identification of the strain was done by amplifying the 16S rRNA region and sequence was examined by BLAST analysis. The results showed that the strain belongs to the genus Bacillus of the family Bacillaceae. Based on phylogenetic comparisons with their closest NCBI strain, the strain DGLB32 was identified as *Bacillus pumilus* (Fig. 1). Based on the potential activities, the study chosen this

ASN2 from soil samples of Pakistan which was identified as the best microbes for the significant production of cellulase [11].

Growth optimization

Optimization plays an important role in the production process of any metabolite. Experiments were carried out to optimize the pH based on *B. pumilus* growth ranging from 6 to 8. Increasing and decreasing the pH effects the growth of the bacterium, the optimal condition was found to be at pH 7 with 3.01 g/L (Fig. 2). The optimal pH for the growth of *Bacillus* isolated from artificial manure was reported as 6.0 [12]. Temperature also has a significant effect on growth the isolated bacterium. Increase in temperature from 28 to 37°C showed an increase in dry weight of cell biomass from 2.91 to 3.207 g/L (Fig. 3). On further increase of temperature, decreased the growth may be due to denaturation of growth enzyme at higher temperature. However, an earlier study observed that the *Bacillus subtilis* CY5 was gradually increased with the increase in temperature and highest growth was found to be at 50°C. In the present study, different concentrations of CMC were examined to study their effects on *B. pumilus* growth. The results showed that maximum growth was observed at 2% CMC with 3.98 g/L cell biomass while at 2.5% CMC, the bacterium showed decreased growth (Fig. 4). Similarly, studying the effect of tannic acid on growth, 1% tannic acid showed enhanced growth of 4.46 g/L cell biomass however the increased concentration of 1.25% showed only 4.42 g/L cell biomass (Fig. 5). The present study highlight that the isolated bacterium *B. pumilus* from the vegetable waste showed the promising production of cellulase enzyme and was also studied for the optimal growth parameters of the bacterium. These observations proved that the cellulase enzyme from this bacterium could be applicable for many biotechnological industrial applications.

Strain *Bacillus pumilus* DGLB32 with their closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences. This analysis was done based on Maximum-likelihood method using MEGA 7 [9]. Numbers at nodes indicate levels of bootstrap support (%) and the black dot indicates the potential strain of this study.

Figure 1. Neighbor joining phylogenetic tree [8] was constructed for the potential

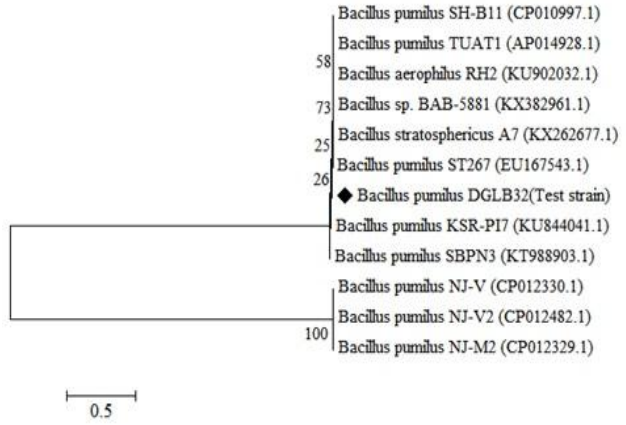


Figure 2. Growth characteristics of *Bacillus pumilus* at various pH conditions.

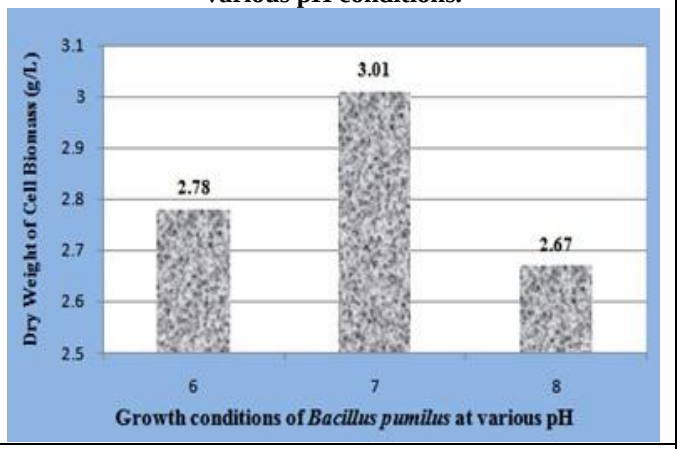


Figure 3. Growth characteristics of *Bacillus pumilus* at different temperature conditions

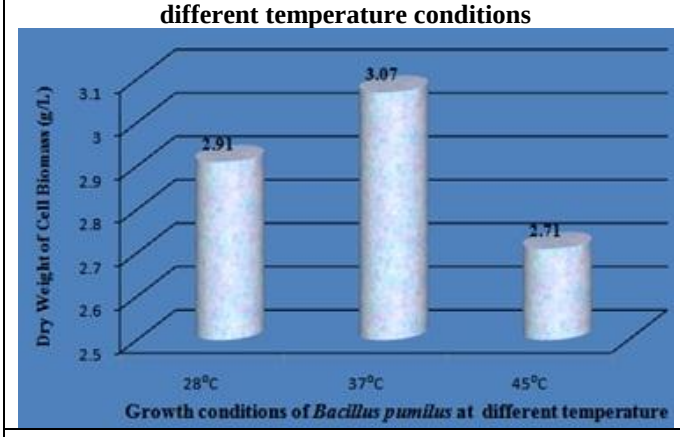


Figure 4. Growth characteristics of *Bacillus pumilus* at different carboxy methyl cellulose concentrations

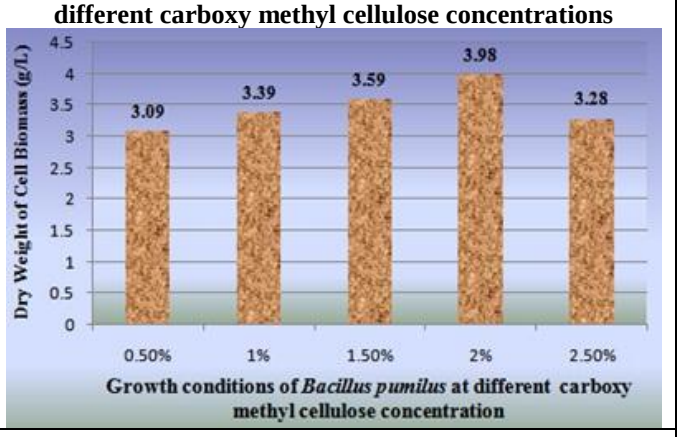
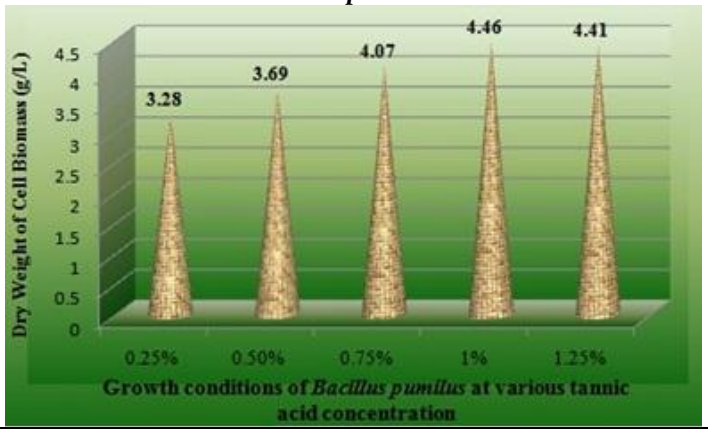


Figure 5. Growth characteristics of *Bacillus pumilus* at various tannic acid concentrations



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