ISOLATION AND CHARACTERIZATION OF ALKALINE CELLULASE BY *BACILLUS SUBTILIS* P15 FROM SOIL

[1]Poovazhagi R, and *Dr. K. Vijaya Ramesh
Research Scholar, Assistant Professor
Department of Plant Biology and Plant Biotechnology
Quaid-E-Millath Government college for Women
Annasalai, Chennai – 600002.
Tamilnadu, India.

Abstract: Cellulose is a long chain polymer, made up of rehashing units of glucose, a simple sugar, combined with β-1, 4 glycosidic linkages. Cellulases influence hydrolysis of the individual cellulose strands to break it into little sugars units and at last creating glucose particles. Alkaline Cellulase producing, *Bacillus subtilis* P15 was isolated from banana orchard soil from Kerala. Among the 27 isolates, 8 strains appearing cellulase activity. From this *Bacillus subtilis* P15 potential for right down to earth utilizes from on carboxymethyl cellulose (CMC) agar plates and distinguished as *Bacillus subtilis* P15 strain by morphological, physiological, and biochemical portrayal and 16S rRNA quality investigation. The impact of various parameters like substrate concentration (1.0%), temperature (37^0), pH (8.0), and incubation time (48 Hrs) was checked with the chosen strain for cellulase production. The purification of cellulase was completed by ammonium sulfate precipitation and dialysis. The molecular weight of the purified carboxymethyl cellulase (CMCase) changed into evaluated to be about 44 kDa with the examination of SDS-page observed by means of staining with Coomassie Brilliant Blue (CBB R-250) providing a simple staining approach and excessive quantitation.

Key Words: Alkaline Cellulase, *Bacillus subtilis* P15, 16s rRNA, SDS-Page, Enzyme characterization.

Introduction

Cellulose, a major constituent of the plant cell wall, is the most abundant natural polymer on earth. It is the essential result of photosynthesis in an earthbound biological system. Cellulose $(C_6H_{10}O_5)n$ is found in crystalline structure with close pressing to frame a reduced structure and comprise of thousands of glucose particles, which are connected together by β-1,4-glycosidic linkage in a direct manner. Cellulose is the important constituent of the cell mass of most earthly plants. The source of cellulose is in plants and it is found as microfibrils (Sethi, S. *et al.*, 2013). This structure the basically solid system in the plant cell wall. Cellulose is commonly degraded by cellulase enzyme which consists of three major components i.e., endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21) (Lugani Y, Singla R, Sooch BS (2015). This enzyme is mainly produced by several bacterial and fungal cultures (Alani B and Zamani 2004). Complete hydrolysis of the enzyme requires synergistic action of 3 types of enzymes, namely, Cellobiohydrolase, Endoglucanase or Carboxymethyl cellulase (CMC-use), and Beta-glycosidase (El-Batal A.I. and Abo-state, M.A.M. (2006). Extensive basic and applied research on cellulases revealed the commercial significance and industrial applicability of this enzyme. Cellulase has a wide range of applications in a variety of sectors such as food, paper/pulp, pharmaceuticals, textiles, alcoholic beverages, malting and brewing, starch processing, biofuel production, and leather, etc. (Bhat M. K. (2000).

However, the difference of applications requires cellulase (s) which is different in particular properties. Several microorganisms; bacteria, yeast, and fungi; have been reported as cellulase producers. Bacterial cellulase is more thermostable than fungal cellulase. Optimal pH for fungal cellulase activity is between 4 and 6 (Zhu, *et al.*, 1982; Yazdi, *et al.*, 1990). While, alkaline pH has been reported as optimal pH for bacterial cellulase activity (Ruttersmith and Daniel, 1993).

Bacteria which have a high growth rate as compared with fungi have good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. The

cellulolytic property of some bacterial genera such as *Cellulomonas*, *Cellulovibrio*, *Pseudomonas* sp. *Bacillus*, and *Micrococcus* (Immanuel, G. Dhanusha, R. Prema, P. Palavesam, A. 2006). Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend upon a complex relationship involving a variety of factors like inoculums size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth (Reddy, K.V. Lakshmi, T.V. Krishna, R .A.V. Bindu, V.H. and Narasu, M. L. 2016).

The current research work was once examination to isolate a potential alkalophilic cellulase producing bacteria (*Bacillus subtilis* P15) from banana orchard soil. After that, the cellulase enzyme was carried out for purification. The physical process parameters including initial pH, temperature, substrate concentration, and incubation time were also optimized.

Materials and Methods

Isolation of bacteria

Bacteria were isolated from the banana orchard soil sample collected from Kerala, India. The soil sample was serially diluted using sterile distilled water and spread plated on nutrient agar (NA) medium. The plates were incubated at 37°C for 24 – 48hrs.

Screening of cellulase-producing bacteria.

For the screening of cellulase-production, the bacterial isolates were streaked on CMC agar medium and incubated at 37^0C for 24 hours. To visualize the hydrolysis zone, the plates were overflowed with a solution of 0.1% congo red for 15min and washed with 1N NaCl (Apun K. *et al.*, 2000). To reveal the cellulase activity of the organisms, the diameter of the clear region around colonies on CMC agar was measured. A bacterial isolate with the best activity was chosen for optimization of cellulase production.

Identification of cellulase-producing bacteria

Phenotypic characterization

Cells grown on Nutrient Agar (NA) medium were tested for their morphological and cultural traits, which include cell form, colonial appearance, and endospore formation after incubated at 37 °C for 2 days. For biochemical characterization, Catalase and oxidase test reactions, indole production, citrate utilization, Gelatin hydrolysis, and Hydrolysis of starch were determined. All tests were carried out by incubating the cultures at 37 °C.

16s rRNA Gene Sequencing and Phylogenetic Analysis

DNA was extracted and purified by using the method of (Murmur, J. 1961). The 16S rRNA gene sequencing was done by using the universal primer, 27F (5'-AGAGTTTGATCMTGGCTCAG-three'). The PCR product was refined and sequenced as defined at one time (Tanasupawat *et al*., 2009). The 16S rRNA gene sequences were compared with different 16S rRNA gene sequences available in (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with selected sequences received from GenBank by way of the use of CLUSTAL_X model 1.83 (Thompson *et al.*, 1997). The alignment changed into edited manually to eliminate gaps and ambiguous nucleotides previous to the construction of phylogenetic trees. The phylogenetic tree was built through making use of the neighbor-joining method using MEGA 6.0 software (Saitou and Nei, 1987).

Enzyme Production Medium.

Production medium contained Na NO_3 0.2 %, yeast extract 0.3 %, $CaCl_2$ 0.01 %, $FeSO_4$ 0.001 %, K_2HPO_4 0.05 %, $MgSO_4$ 0.05 %, and $(NH)_4SO_4$ 0.005 %. Ten milliliters of medium were taken in a 50mL conical flask. The flasks were sterilized in an autoclave at 121^0C for 15 min, and after cooling, the flasks were inoculated with overnight grown bacterial (P15) culture. The inoculated medium was incubated at 37^0C in shaker incubator for 24 h. At the end of the fermentation period, the culture medium was centrifuged at 10,000 rpm for 15min to obtain the crude extract, which served as an enzyme source.

Enzyme Assay.

Cellulase activity was measured following the method of (Miller, G. L. *et al*., 1959). Briefly, a reaction mixture composed of 0.5 ml of crude enzyme solution in addition 1.5 mL of 0.3 % carboxymethyl cellulose (CMC) in 50 mM phosphate buffer (pH 7.2) was incubated at 37^0C in a shaking incubator for 30 min. The

reaction was ended by adding 3mL of DNS reagent. The contents were boiled for 15 min and after cooling, the color developed was read at 540 nm using UV -Vis spectrophotometer. One unit of cellulase activity is defined as the amount of enzyme required to liberate 1 µmol of glucose per minute under the assay conditions.

Determination of protein concentration.

Protein concentrations were determined by the method of Bradford (1951), using Bovine Serum Albumin as the standard. The absorbance of the protein concentration was measured at 595nm using UV -Vis spectrophotometer.

Optimization conditions for cellulase production

The optimization of medium and process parameters like substrate concentration, temperature, pH, and incubation time were carried out on the premise of stepwise adjustments for governing the cellulase production by the chosen and distinguished bacterial isolate P15.

Impact of pH

pH is another calculate influencing the microbial growth as well as catalyst production. Subsequently, the tests were carried out by utilizing the optimized media of diverse pH values (4.0, 5.0, 6.0, 7.0, 8.0) to check their impact on cellulase production.

Impact of temperature

Temperature is a vital factor in a bioprocess for the production of the extracellular enzyme. For the choice of optimum temperature for cellulase production by bacterial isolate P15, the inoculated media in separate flasks was incubated at different temperature (30°C, 32°C, 37°C, 45°C, 55°C) for 72 hours.

Impact of substrate (CMC) concentration

Substrate concentration to examine their impact on enzyme activity. The effect of diverse concentrations (0.5%, 1%, 1.5%, w/v) was also studied on the production of cellulase by bacterial isolate P15.

Impact of incubation time

The impact of incubation time was observed on cellulase activity produced by bacterial isolate P15 by incubating the optimized media at 37°C for various time intervals (16, 24, 48, and 72, hours).

SDS-PAGE analysis

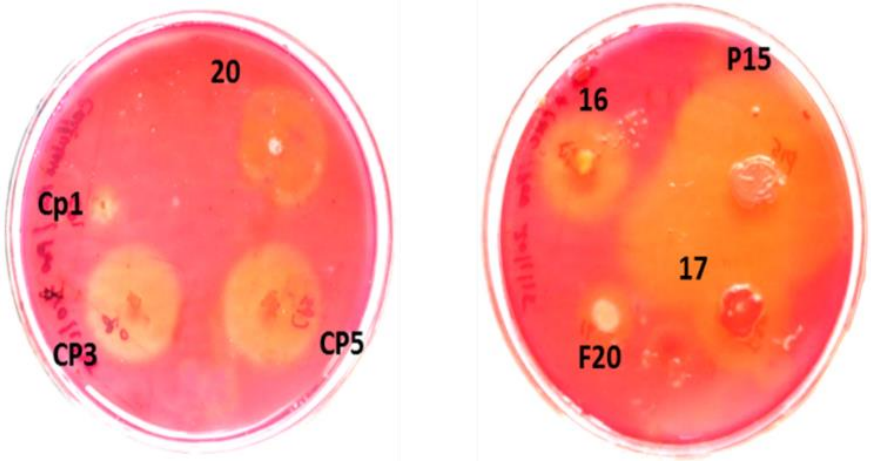
Denaturing sodium dodecyl sulfate/polyacrylamide gel electrophoresis SDS/page (10%) was carried out to determine the molecular mass of the cellulase following the methods of Laemmli (1970). The molecular weight of the enzyme was envisioned with a molecular weight marker in addition to a standard cellulase enzyme. The partially purified enzyme was loaded into the gel. The molecular weight of the purified cellulase determined.

Results and Discussion

Isolation and Screening of bacterial isolates

Twenty-seven bacterial isolates were isolated from banana orchard soil samples collected in Kerala, Tamilnadu. For examined the cellulolytic activity of the isolates on plates, plates were stained with congo-red and destained with NaOH solution. The zones of clearance by isolates reflect their extent of cellulolytic activity. Out of these strains, 8 isolates showed significant hydrolyzing zones on agar plates containing CMC as substrate, after Congo-red staining. Among the 8 isolates (16, 17, 20, CP1, CP3, CP5, F20, and P15), isolate P15 (Figure 1.) showing maximum clearance zone diameter (47mm) was selected for further studies. Sethi S. *et al*., 2013 was to isolate and identify a high cellulase producer from the soil. The use of *Bacillus* sp for CMC production has been reported by various workers (Singh, J. *et al.*, 2013 and Mohamed *et al.*, 2010).

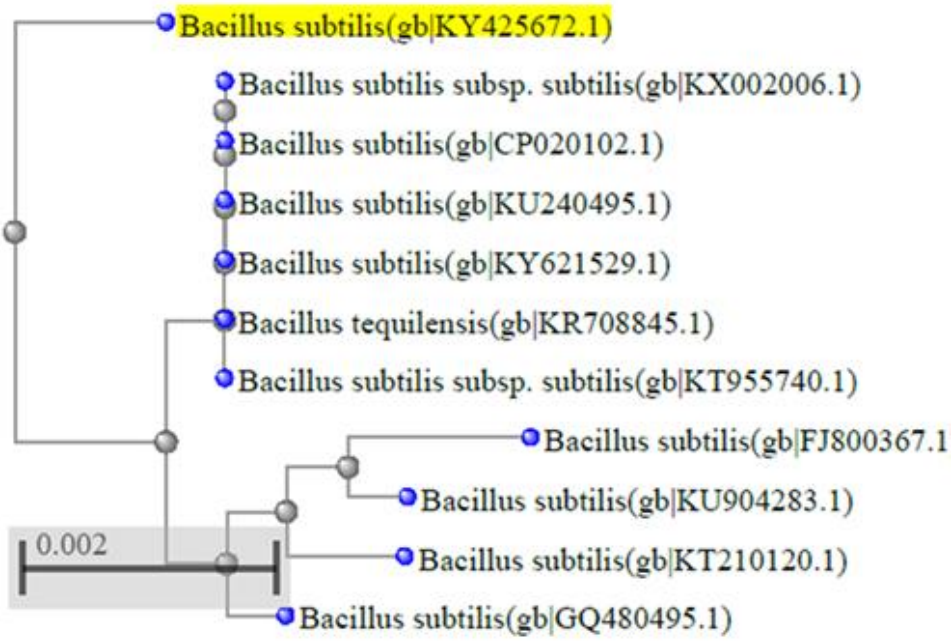
Figure 1. Zone of cellulase-producing bacteria



Identification of isolates

Selected cellulolytic bacteria were Gram-positive, facultatively anaerobic, spore-forming rod-shaped bacteria. The molecular identification of *Bacillus* species was performed by 16s rRNA sequence analysis. The nucleotide sequences were submitted in Gene bank with the accession number KY425672. The obtained sequence was subjected to NCBI BLAST and the results revealed that the identified bacterial strain, had 100% similarity with the *Bacillus subtilis* P15 species. The phylogenetic tree was constructed using MEGA 6 software and designated as *Bacillus subtilis* P15. (Figure. 2). From the phylogenetic tree, it can be observed that the isolate *Bacillus subtili* subspp, *subtilis* (gb/KX002006.1) are very closely related to each other.

Figure 2. Phylogenetic tree of *Bacillus subtilis* P15.



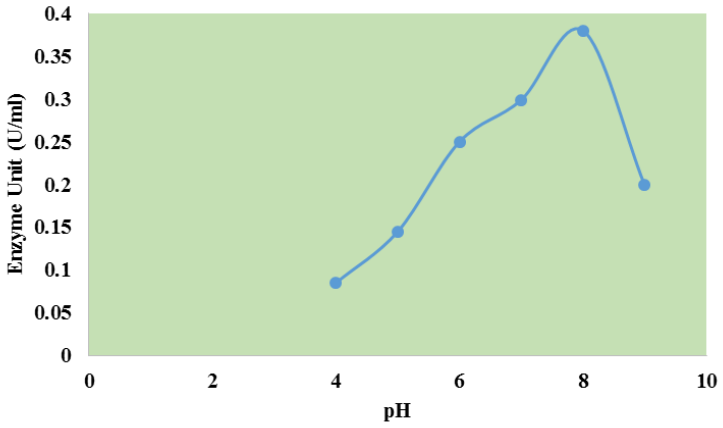
Optimization conditions for cellulase production

Impact of pH

optimum pH for cellulase activity was determined by incubation of the purified cellulase with its substrate(CMC) at exceptional pH values ranging from pH 4.0 to pH 9.0. results in (figure. 3) showed that cellulase was active over an extensive range of pH (4.0-9.0) with a most desirable activity of 6.28 U/ml at pH 8.0, whereas any similar growth in pH from the best value (pH 8.0) cellulase showed reducing trends in its activity. Our results also confirmed that medium ph is a vital factor affecting cellulase activity. The optimum pH 8 for maximum cellulase production was found in this study. A similar finding was also reported by (Mukesh Kumar, *et al*., 2012) and (Chittoor, J. *et al*., 2015) for cellulase enzyme production. The effect of pH on enzyme activity showed very good activity at pH range 6 - 9 with optimal activity and stability at pH 9

(Ekwealor C. C. *et al.*, 2017). The pH of the growth medium influences many enzymatic reactions by affecting the transport of chemical products and enzymes across the cell membrane (Liang Y. *et al.*, 2010).

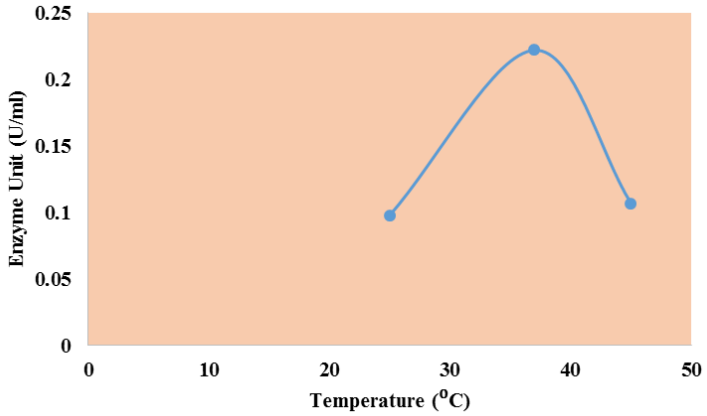
Figure 3. Impact of pH



Impact of temperature

The optimum temperature for cellulase activity was examined at different temperatures ranging among 30°C, 32°C, 37°C, 45°C, 55°C. Results showed (figure. 4) that 37 °C was the most desirable temperature for cellulase activity (8.80 U/ml). Temperature is a critical parameter for the success of the fermentation reaction. It controls the growth and production of metabolites by microorganisms and usually differs from one organism to another (Banerjee R. *et al.*, 1992). other researchers recorded varied optimum temperatures for different species of *Bacillus*. Activity decreased after enzyme incubation at values less or more than 40°C. Above a certain temperature, enzyme activity decreases with an increase in temperature because of enzyme denaturation (Rajesh *et al.*, 2012). comparable results of maximum cellulase production of 0.5851 ± 0.006 IU/mL has achieved after 72 hours of incubation at 37°C from *Bacillus pumilus* EWBCM1 (Shankar, T. *et al.*, 2011).

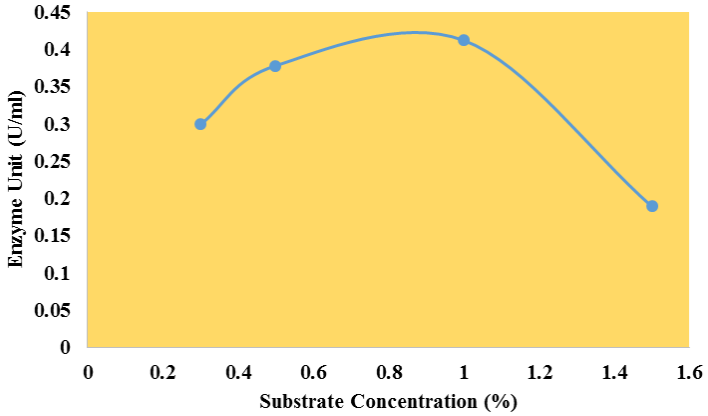
Figure 4. Impact of temperature



Impact of substrate (CMC) concentration

The best carbon source with maximum cellulase production from *Bacillus subtilis* P15 was observed to be CMC at a concentration of 1%. Therefore to analyze the effect of different concentrations of CMC on cellulase production by the isolate, different concentrations of CMC (0.5%, 1%, 1.5%, w/v) were used in the media. The maximum cellulase production with CMCase activity of 6.95 U/ml was observed with 1% (w/v) concentration of CMC (Figure 5). Similar observations were also made by some other workers in the past. Lugani Y. *et al.*, (2015) showed similar results with maximum cellulase production from Bacillus sp Y3 supplemented with 1% CMC as a substrate at 40°C after 72 hours of incubation. Very high cellulase production was additionally found from *Bacillus* sp. BSS3 in the optimized media containing 1% (w/v) CMC at 150 rpm and 37°C (Sreedevi S. *et al.*, 2013).

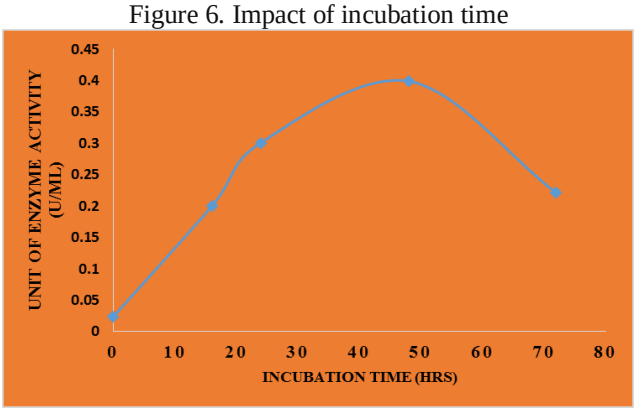
Figure 5. Impact of substrate (CMC) concentration



Impact of incubation time

The optimized media was incubated for different time durations i.e., 12, 24, 48, hours after inoculating with *Bacillus subtilis* P15. To analyze the effect of incubation time on cellulase production. The optimum time for cellulase production with maximum CMCase activity (6.67 U/mL) was found at 48 hours of incubation (Figure. 6). The cellulase activity was significantly reduced after 48 hours because of depletion of nutrients or accumulation of other byproducts in the fermentation media which result in lower in cellulase activity. Vipul Verma V. *et al.*, (2012) recorded maximum CMCase activity took place at 48hrs of incubation period by *Bacillus subtilis*.

Figure 6. Impact of incubation time

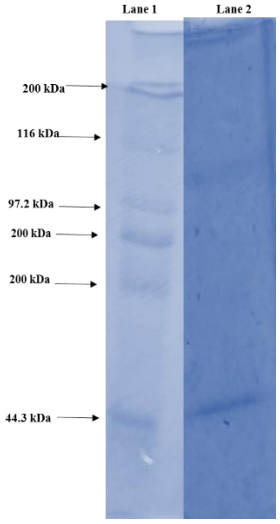


From the results, the optimized parameters pH 8.0, 37ºC temperature with 1% substrate concentration was taken for further studies.

SDS-PAGE analysis

The purity of the enzyme was confirmed by the presence of a single band on SDS-page and its molecular weight was about 44 kDa. who reported a molecular weight range of 23 - 65 kDa for cellulases produced by *Bacillus* sp. However, larger sizes (100 - 185 kDa) of CMCase enzyme have also been reported for other *Bacillus* strains (Rawat, R. 2012).

Figure 7. The SDS-PAGE analysis of alkaline cellulase from *Bacillus subtilis* P15 (Lane 1-Molecular weight Marker, Lane 2 – Alkaline cellulase from Bacillus subtilis P15)



Conclusion

Several microorganisms able to converting cellulose into simple carbohydrates were discovered for a long time. however, desires for newly isolated cellulolytic microbes nevertheless remained. In this, we have isolated and identified efficient cellulase producing bacteria from the cellulose-rich environment. The bacterial isolate was characterized based on 16s rRNA sequence and was identified as *Bacillus subtilis* P15. The isolate *Bacillus subtilis* P15 showed the potential to produce cellulase using CMC as a substrate and its enzyme production efficiency was increased by optimization of cultural conditions and media components. Isolation, characterization, and optimization of cellulose producing, bacteria may also provide a great starting point for the discovery of such beneficial enzymes for plant cell wall degrading enzyme.

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