# Characterization of thermostable cellulase produced by *Bacillus* strains isolated from solid waste of carrageenan

IOP Conf. Series: Earth and Environmental Science **131** (2018) 012043 doi:10.1088/1755-1315/131/1/012043

# Characterization of thermostable cellulase produced by *Bacillus* strains isolated from solid waste of carrageenan

**N P Listyaningrum[1,2], A Sutrisno[1] and A K Wardani[1]**

[1]Department of Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia
[2]Fisheries Product Processing Technique, Marine and Fisheries Polytechnic, Sidoarjo, Indonesia

E-mail: agustinwardani@ub.ac.id, nikenprawesti@apsidoarjo.ac.id

**Abstract.** Cellulase-producing bacteria was isolated from solid waste of carrageenan and identified as *Bacillus licheniformis* C55 by 16S rRNA sequencing. The optimum condition for cellulase production was obtained at pH and temperature of 8.0 and 50°C, respectively in a medium containing glucose as carbon source and 1.0% carboxymethyl cellulose (CMC) to stimulate the cellulase production. Most remarkably, the enzyme retained its relative activity over 50% after incubation at 50°C for 90 minutes. Substrate specificity suggested that the enzyme is an endoglucanase. The molecular mass of *Bacillus licheniformis* C55 crude cellulase was found about 18 kDa by SDS-PAGE analysis. This thermostable enzyme would facilitate development of more efficient and cost-effective forms of the process to convert lignocellulosic biomass into high-value products.

## 1. Introduction

Cellulose is the most abundant compound in the world and is a renewable source that can be easily utilized by humans. Cellulase is an enzyme that hydrolyzes the cellulose bond β-1,4-glycosidic and is generally divided into endoglucanase and selobiohydrolase. Cellulase has potential in several industries such as food industry, textile and laundry industry, pulp and paper industry, as well as agricultural industy [1]. Thermostable cellulase produced by thermophilic microorganisms are useful for improvement the efficiency of laundry detergents, paper removal and improving pulp drainage, because most of these processes operate at high temperatures.

Cellulose is also found in solid waste of carrageenan processing. Seaweed from *Euchemacottonii* types is a raw material for the manufacture of carrageenan flour. Based on data of Directorate General of Aquaculture Ministry of Marine Affairs and Fisheries (MMAF) Indonesia, seaweed production in Indonesia in 2010 reached 3.91 million tons and increased to 10.23 million tons in 2014. In Indonesia, carrageenan processing industries are only able to process 20% from the total production of seaweed (540,000 dry tons per year), it will generate solid waste of 10,800 tons per year. This leads to very serious pollution problems because the wastes contain cellulose which is very difficult to be degraded. Cellulose is commonly degraded by cellulose which is produced by several microorganisms, commonly by bacteria and fungi. Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production. For this application, the utilization of thermophilic cellulolytic microbes is more advantageous than the mesophilic one because the cellulase enzyme produced remains stable at high temperature and still has optimum activity [2]. Thermostable cellulase

enzymes with high stability can be obtained by isolating from thermophilic bacteria. Most of these bacteria are isolated from soil, compost, animal waste or haystack and sugar cane. Isolation of thermophilic cellulase-producing bacteria isolated from waste of carrageenan processing has not been studied further. This aim of the present study is to isolate and identify cellulase-producing bacteria from solid waste of carrageenan processing and to characterize the thermostable cellulose produced by bacteria.

## 2. Materials and Methods

### *2.1. Materials*

Waste of carrageenan processing was obtained from PT. Kappa Carrageenan Nusantara, Pasuruan, East Java. Waste was obtained aseptically using a sterile spatula, to prevent exogenous microbial contamination during sampling. Samples were brought to the laboratory in polypropylene tubes and stored at temperature of -20°C until analyzed. Carboxy Methyl Cellulose (CMC), Tryptone Soya Agar (TSA), Tryptone Soya Broth (TSB) and glucose were used as growth media.

### *2.2. Enrichment and isolation of thermophilic cellulose-degrading bacteria from carrageenan waste*

The growth media used for enrichment of cellulose-producing bacteria from waste of carrageenan processing were TSB and 1% CMC sodium salt as the carbon source. Before autoclaved, the pH of growth medium was adjusted to 8.0 using NaOH 1 M and then 100 g of culture was inoculated and incubated at 50°C for 72 hours in a shaker waterbath. After enrichment, the mixture of culture was used to isolate thermophilic cellulose-producing bacteria by dilution method using sterile NaCl (0.85%). One hundred (100) µL of each dilution was streaked on media TSA containing 1% CMC sodium salt pH 8.0 and incubated at 50°C for 24 hours. Screening of cellulase-producing bacteria was performed on TSA pH 8.0 containing 1% CMC sodium salt and incubated at 50°C for overnight, then flooded with congo red, continued by washing with 0.1% NaCl. Isolates that produce the largest clear zone were considered as potential isolates.

### *2.3. Molecular identification*

Potential cellulase-producing isolates were identified morphologically, physiologically and biochemically and confirmed of gene sequencing 16S rRNA. Extraction and purification of DNA was performed using bacteria kit *(The Presto™ Mini kit gDNA Bacteria geneaid).* Extraction and purification of DNA in accordance with manufacturer protocol. Primer used for sequencing is a universal primer of 16S rRNA27F/1492R as follows: forward primer 27F:AGAGTTTGATCMTGGCTCAG and reverse primer 1492R:TACGGYTACCTTGTTAACGACTT. The PCR condition of 35 cycles covering of denaturation at 95°C for 10 seconds and annealing at 54°C for 30 seconds then elongates for 1 minute at 72°C. The similarity search of sequence was carried out using BLAST on the site http://blast.ncbi.nlm.nih.gov/Blast.cgi, to determine the level of homology species of bacteria selected by bacteria in *Genebank.* The phylogenetic tree was designed using the sequences ratio of 16S-rRNA of other bacteria in the database tracking program *Basic Local Alignment Search Tool* (BLAST) on the site http://blast.ncbi.nlm.nih.gov/Blast.cgi. Alignment was carried out using ClustalW program. The phylogenetic tree was constructed using neighbor-joining method by MEGA7 program.

### *2.4. Enzyme production*

Isolates C55 were grown in a liquid medium TSB contain 1% CMC sodium salt. Isolates that were inoculated as much as two-ose into 10 ml of medium of liquid TSB-CMC and incubated at 50°C in *waterbath shaker*, 120 rpm, for 24 hours. After a phase of exponential growth, the cells from cultures were inoculated back into 90 mL of liquid medium TSB contain 1% CMC sodium salt, then incubated in waterbath shaker at 50° with 120 rpm agitation. Cellulase activity and *Optical Density* ($OD_{600\ nm}$) were measured every 3 hours for 24 hours incubation. The cellulase was harvested at the optimum

incubation time with the value of the highest cellulase activity. The crude extract of the cellulase enzyme was separated from bacterial pellets with 10000 × g for 15 minutes at 4°C.

*2.5. Cellulase activity assay*

The measurement of cellulase activity was performed by incubating the mixture of 100 µL enzyme and 100 µL of 1% (b/v) CMC in 50 mM Tris-HCl (pH 8.0) at 50°C for 30 minutes. The reaction was stopped by adding 3,5-dinitrosalicylic acid solution, boiled for 15 minutes, then cooled in ice for color stabilization. Optical absorbance was measured at 540 nm and the amount of reducing free sugar (glucose equivalent) was estimated on the glucose standard curve. One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol glucose per minute under the test conditions. The control of enzyme and appropriate substrate were included in all tests. Each experiment was repeated three times and the mean value plotted for each experiment.

*2.6. Substrate specificity*

The specificity of the crude cellulase substrate was determined by testing different substrates, namely Carboxy Methyl Cellulose (CMC), filter paper No.1 (Whatman), carrageenan flour, carrageenan waste. Cellulase activity was measured by incubating 200 µL diluted enzyme solution (100 µL enzyme + 100 µL substrates 1% in 50 mM Tris-HCl, pH 9.0) at 50°C for 30 minutes. The reaction was stopped by adding 3,5-dinitrosalicylic acid solution, boiled for 15 minutes, then cooled in ice for color stabilization. The amount of reducing sugar produced was measured by the method of DNS [3].

*2.7. Effect of temperature on activity and stability of crude enzyme*

The optimum temperature of the crude enzyme activity was determined by incubating the mixture of enzyme reactions as much as 100 µL added with 100 mL of 1% (b/v) CMC for 30 minutes at various temperatures between 30°C to 90°C. The stability of temperature was done by incubating crude enzyme on buffer Tris-HCl 50 mM (pH 8) at the optimum temperature for 15, 30, 45, 60, 90, 120 and 240 minutes and residual activity was assayed by standard DNS method [3].

*2.8. Effect of pH on activity and stability of crude enzyme*

The optimum pH of crude enzymes secreted by isolates was estimated range from pH 4.0 - 10.0 by using a variety of different solvents. Enzyme activity test performed by incubating the reaction mixture at a temperature of 50°C for 30 minutes. As many as 100 µL of enzyme was added with 100 µL 1% (b/v) CMC dissolved in 50 mM acetate buffer (pH 4, 5, 6); 50 mM phosphate buffer (pH 6, 7, 8); 50 mM Tris-HCl buffer (pH 7 and 8); 50 mM glycine buffer-NaOH (pH 8, 9, 10). The stability of the pH of enzyme were determined by incubating enzyme as much as 100 µL added with 100 µL 1% (b/v) CMC dissolved in buffer mentioned above for one hour at the temperature of 50°C and residual activity was assayed by the DNS standard methods [3].

*2.9. SDS-PAGE and zymogram analysis*

The molecular weight of the crude cellulase was determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with Bio-Rad system using 4% stacking and 12% resolving gel. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie blue, 30% (v/v) methanol, and 10% (v/v) acetic acid. Zymograms of cellulase activity performed in 12% SDS-polyacrylamide gels were polymerized with 0.2% (w/v) carboxymethyl cellulose according to a previously described protocol. Renaturation of proteins following electrophoresis was performed by incubating the gels in 10 mM Tris–HCl buffer (pH 8.0), containing 1% (w/v) Triton X-100, and the incubation period was 2 h at 50°C. To identify protein bands with cellulase activity, the gels were stained with 0.2% (w/v) congo red for 30 minutes and washed with 1 M NaCl until cellulase bands became identified.

## 3. Results and Discussion

### *3.1 Microorganisms*

The cellulase-producing isolate was designated as isolate C55. On the basis of the morphological, physiological, and biochemical characteristics, C55 is gram-positive and endospore-forming *Bacillus* with catalase, which grows in both aerobic and anaerobic environments. The 16S rRNA gene sequencing analysis evidenced that C55 showed high homology (100%) with strain of *Bacillus licheniformis* (Figure 1). Based on the evolution range and phylogenetic tree, this C55 strain was identified as *Bacillus licheniformis* and designated as *Bacillus licheniformis* C55 (accession of GenBank number KY392939.1).

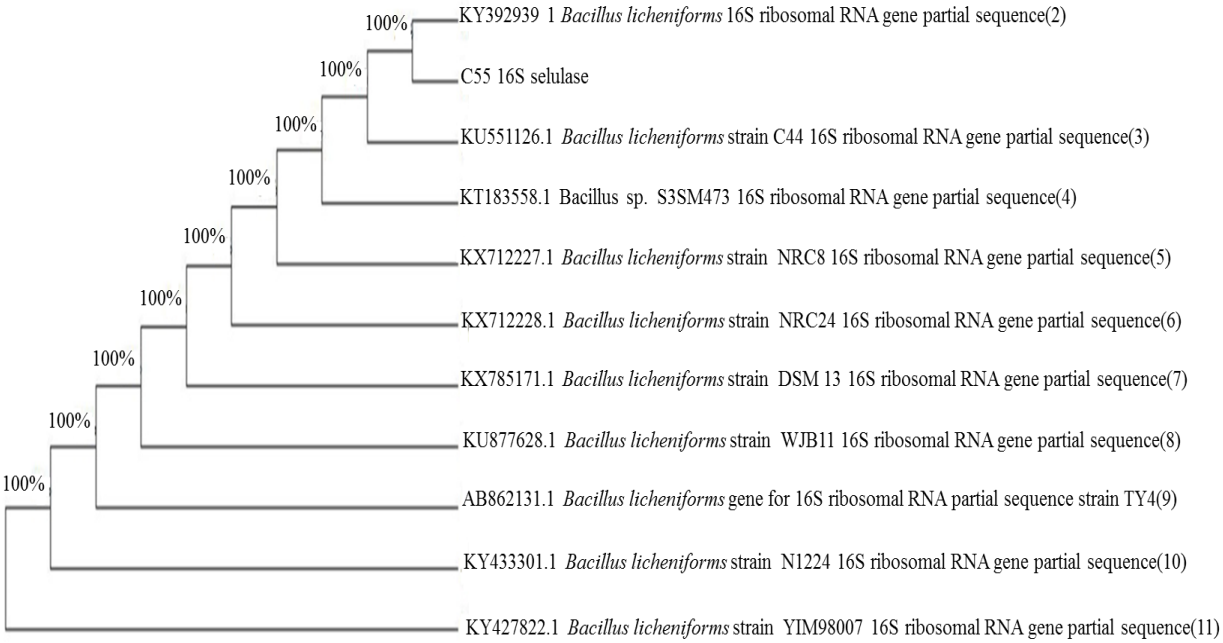



**Figure 1**. Phylogenetic tree of isolate C55 16S rRNA gene sequence with other *Bacillus* species/strain

### *3.2 The effect of temperature and pH on enzyme activity and stability*

The effect of temperature on the activity and stability of cellulase was determined on various temperature ranges from 30 and 70°C. The optimum temperature for cellulase activity of isolate C55 were found at 50°C in pH 9.0 (Figure 2A). Thermostability studies revealed that crude enzyme was 100% stable in optimum temperature at 50°C for 30 minutes and it retained relative activity more than 50% for 90 minutes (Figure 2B). The optimal cellulase temperature and thermostability which was obtained by isolate C55 was the same as obtained by *B. licheniformis* WBS1 and *Bacillus* sp. WBS2 [4], *B. cereus* JD0404 [5], *B. amyloliquefaciens* DL-3 [6] and *B. subtilis* subsp. *Subtilis* A-53 [7] and *Bacillus* sp. HSH-810 [8].

The effect of pH on crude cellulase activity produced by isolate C55 was examined at various pHs ranged from pH 4.0 until pH 10.0. The optimal pH for cellulase activity was 8.0 and it exhibited more than 60% relative activity between pH 7.0 and 8.0 (Figure 2C). The Optimum pH of crude cellulase from isolate C55 was higher than cellulase produced by *B. amyloliquefaciens* DL-3 [6], *Bacillus sphaericus* JSI [9] and *B. subtilis* subsp. *Subtilis* A-53 [7] which exhibited optimum activity between pH 6.0 and 7.5. The pH stability studies showed that crude cellulase from isolate C55 was active in the range of pH between 7.0 and 8.0. Crude cellulase was 100% stable in pH 8.0 and it retained 50% of its original activity even at pH 7.0 (Figure 2D). The pH stability of crude enzyme of cellulase from

isolate C55 was the same with cellulase produced by *Bacillus spherical* JS1 [9] and *Bacillus* sp. HSH-810 [7] which were stable at pH 8.0.

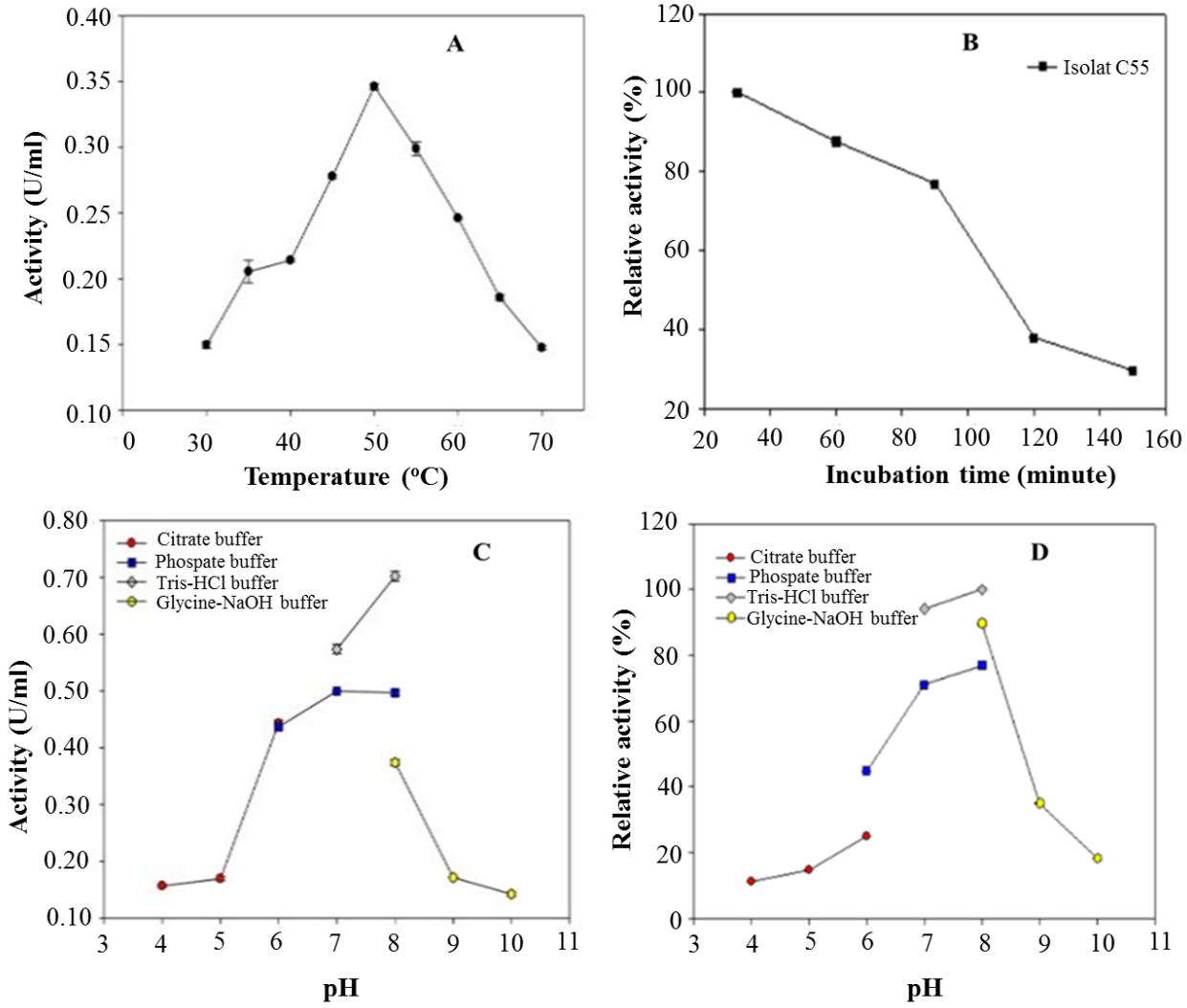



**Figure 2.** The effect of temperature and pH on activity of crude cellulase of isolate C55. (A). Effect of temperature on activity of crude cellulose; (B). Effect of temperature on stability relative activity of crude cellulose; (C). Effect of pH on activity of crude cellulose; (D). Effect of pH on stability relative activity of crude cellulase

*3.3 Substrate specificity*

Substrate specificity assayed revealed that crude cellulase which was produced by *Bacillus licheniformis* C55 is shown in Figure 3. This result indicated that the enzyme is an endoglucanase. It displayed high activity against CMC, low activity against filter paper, carrageenan and waste of carrageenan. The most *Bacillus* has high enzyme activity in hydrolyzing carboxymethyl cellulose [10].

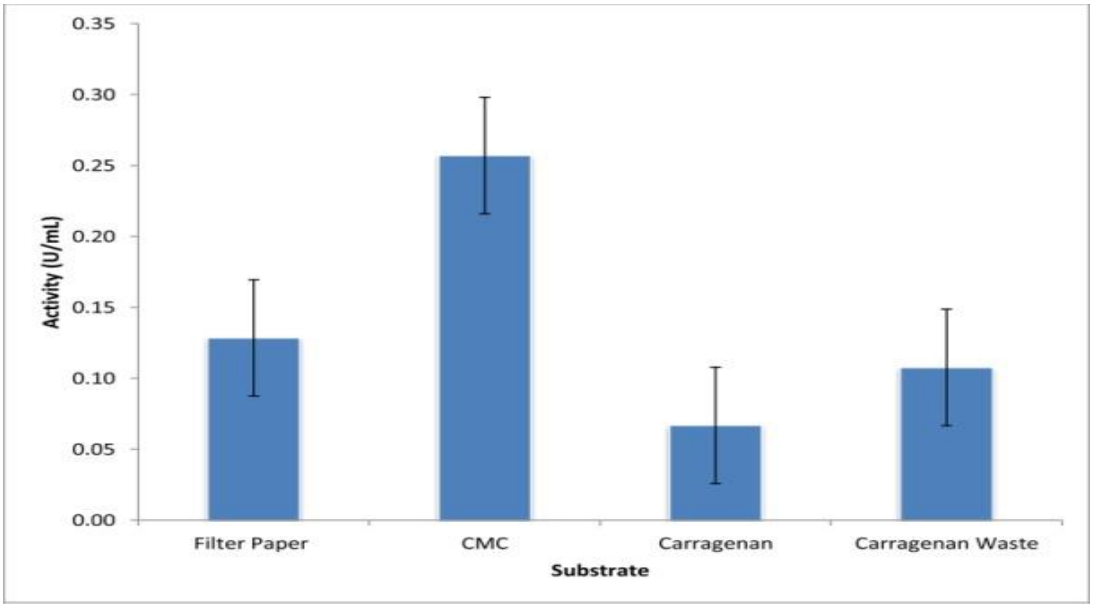
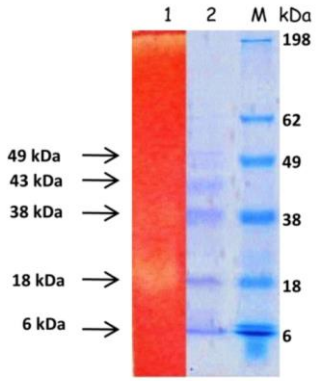



**Figure 3.** Effect of substrate specificity on crude cellulase of isolate C55

3.4 *SDS-PAGE and zymogram analysis*

The crude cellulase protein was observed as a single band with a molecular weight of about 18 kDa on SDS-PAGE gel, and the protein band was visualized by colloidal Coomasie blue staining (Figure 4). In zymogram gel, the appearance of proteins with carboxymethylcellulase activity was observed with supplementation of 1 % carboxymethyl cellulose (Figure 4). The molecular mass of *Bacillus licheniformis* C55 crude cellulase was found about 18 kDa by SDS-PAGE analyses, which was similar size to *Chryseobacteriumindologenes* McR-1.




Notes: 1. Zymogram, 2. Crude protein, M. Molecular weight standards)

**Figure 4**. SDS-PAGE and zymogram analysis of the crude cellulose from *Bacillus licheniformis* C55

## 4. Conclusion

In this research, isolate C55 has the similarity 100% with *Bacillus licheniformis*. Enzyme that was produced by isolate C55 worked optimum in pH 8.0 and at 50°C. The enzyme can retain relative activity over 50 % at 50°C for 90 minutes. The molecular mass of *Bacillus licheniformis* C55 crude cellulase was found about 18 kDa. Substrate specificity indicated that cellulase enzyme which is produced by isolate C55 is endoglucanase.

## References

[1] Bhat M K 2000 Cellulases and related enzymes in biotechnology *Biotechnol Adv.* **18** 355–383
[2] Baharuddin A S, Razak M N A, HockL S, Ahmad M N, Aziz S A, Rahman N A A, Shah U K M 2010 Isolation and Characterization of Thermophilic Cellulase Producing Bacteria from Empty Fruit Bunches-Palm Oil Mill Effluent Compost *Am J Appl Sci*. **7** 1 56-62
[3] Miller G L 1959 Use of dinitrosalicyclic acid reagent for determination of reducing sugar *Anal. Chem*. **31** 426-428
[4] Acharya S and Anita C 2011 Effect of nutritional and environmental factors on cellulase activity by thermophilic bacteria isolated from hot spring *J. Sci. and Ind Res*. **70** 142-148
[5] Chantarasiri A 2015 Aquatic *Bacillus cereus* JD0404 isolated from the muddy sediments of mangrove swamps in Thailand and characterization of its cellulolytic activity *Egypt J Aquat res*. **41** 257-264
[6] Lee Y J, Kim B K, Lee, B H, Jo K I, Lee N K, Chung C H, Lee Y C, Lee J W 2008 Purification and characterization of cellulase produced by *Bacillus amyloliquefaciens* DL-3 utilizing rice hull *Bioresour Technol*. **99** 378–386
[7] Kim B K, Lee B H, Lee Y J, Jin I H, Chung C H, Lee J W 2009 Purification and characterization of carboxymethylcellulase isolated from a marine bacterium, *Bacillus subtilis* subsp. subtilis A-53 *Enzyme Microb Technol*. **44** 411–416
[8] Kim J Y, Hur S H, Hong J H 2005 Purification and characterization of alkaline cellulase from a newly isolated alkalophilic *Bacillus sp.* HSH-810 *Biotechnol Lett.* **27** 313–316
[9] Singh J, Batra N, Sobti R C 2004 Purification and characterization of alkaline cellulose produced by a novel isolate *Bacillus sphaericus* JS1 *J Ind Microbiol Biotechnol*. **31** 51–56
[10] Ladeira S A, Cruz E, Delatorre A B, Barbosa J B, Martins M L L 2015 Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compability *Electron J Biotechn*. **18** 110-115