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*Research Paper*

# Isolation and identification of cellulose degrading bacteria and optimization of the cellulase production

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**Abstract**

**The cellulase producing bacteria were isolated from industrial and agricultural areas in Kerala. Potential isolates with cellulase production were identified by Grams iodine dye staining method. The isolate were tentatively identified to be Bacillus species based on cultural, morphological, biochemical analysis and labelled as CB3, CB4 and CB8. Further, the genomic DNA was isolated and amplified with universal primers 27F and 1492s specific for 16S rRNA. The amplified 16S rRNA PCR product of 1500bp was sequenced and the unknown organism was identified using the maximum aligned 16SrRNA sequences available in the GenBank of NCBI through BLAST search. The sample CB3 and CB4 showed (100% and 99% respectively) homology to *Bacillus subtilis* strain. The sample CB8 showed 98% homology to the *Bacillus cereus* strain. To test the evolutionary relationships, phylogenetic analysis was performed with the program MEGA 6.0 using the 16SrRNAsequence. The isolates were then evaluated by submerged fermentation process for maximum cellulase production. The various process parameters like pH, temperature, incubation period, substrate concentration and inoculum volume were then optimized for the maximum production of cellulase by the isolates. The optimum temperature for cellulase production for CB4, CB4 and CB8 was found to be $40^0$C, $30^0$C and $40^0$C respectively. The optimum PH was found to be 7 for the three samples. The incubation period of 48 hours, 72 hours and 96 hours were found to be optimum for CB3, CB4 and CB8 respectively. An inoculum size of 6% was found to be ideal for CB3 and CB4 whereas an inoculums volume of 8% was found to be ideal for CB8 which showed a maximum activity of 4.12U/ml. The CMC concentration of 1.5% was found to be ideal for CB3 and CB4 whereas CB8 showed a maximum activity of 3.21U/ml at a CMC concentration of 1%. Among the three isolates the *Bacillus cereus* strain (CB8) was found to be the most active cellulase producer with maximum activity of 4.12 IU/ml in submerged fermentation.**

**Keywords**: Cellulolytic bacteria, 16srRNA, BLAST, Bacillus species, Carboxy methyl cellulose.

## Introduction

Cellulase is an important and essential kind of enzyme for carrying out the depolymerisation of cellulose into fermentable sugar. Cellulases are commonly used in many industrial applications and the demands for more stable, highly active and specific enzymes are growing rapidly. Further, the value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest[1]. Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources, cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently[2].

Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters. Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. 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. Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium and presence of inducers are important parameters for the optimized production of cellulase enzymes[3,4]. Several previous reports have shown that certain microbes are able to utilize cellulose as a source of energy.

Microorganisms are important in conversion of lignocelluloses wastes into valuable products like biofuels produced by fermentation. These microbes produces extracellular cellulose and hence known as cellulolytic microorganisms. *Bacilli* and fungi are most popular class for commercial production as these cellulases have very high economic value[5-7]. Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production in industries. For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts.

Cellulases are now used in the textile industry for cotton softening and denim finishing, in laundry detergents for colour care, cleaning, in the food industry for mashing, in the pulp and paper industries for drainage improvement and fibre modification, and they are even used for pharmaceutical applications[8]. For understanding the mechanism of cellulose degradation by cellulase, it is necessary to isolate, purify and characterize this enzyme. Therefore, the present investigation was designed to isolate, identify and optimize the fermentation conditions for maximum production of cellulase by the isolates.

## Materials and Methods

### Sample collection

The samples for isolation of microorganisms were collected from various environments where the natural process of cellulose degradation is taking place. Soil and water samples were collected from industrial and agricultural areas in Kerala, and were transported in sterile bottles to the Microbiology laboratory of St.Peter's College, Kolenchery, kerala for bacterial isolation.

### Screening and isolation of bacteria

The samples were collected in sterile containers and stored at 4ºC until used. The samples were allowed to grow directly in the CMC agar plates. The microorganisms capable of growing in the cellulose only medium were isolated and checked for the cellulolytic activity by gram's iodine clearing zone assay method. Tenfold serial dilutions of each soil sample were prepared in sterilized distilled water and 0.1 ml of the diluted sample was spread on Carboxymethyl cellulose medium with the following composition (g/l) : Carboxymethylcellulose (CMC), 10, Tryptone, 2, $KH_2PO_4$, 4, $Na_2HPO_4$, 4, $MgSO_4.7H_2O$, 0.2, $CaCl_2.2H_2O$, 0.001, $FeSO_4.7H_2O$, 0.004, Agar, 15 and pH adjusted to 7[9] . Plates were incubated at 37ºC until the visible colonies form. The plates were flooded with Grams Iodine and distilled water to see the cellulolytic activity of isolated strain. The formation of a clear zone of hydrolysis indicated the cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulose producer[10]. The largest ratio was assumed to contain the highest activity. Bacterial colonies were purified by repeated streaking. The purified colonies were preserved at $4^oC$ for further identification and screening for cellulase production.

### Grams Iodine Stain

0.133 g Potassium iodide and 0.067 g Iodine were dissolved in 20 ml distilled water[10].

### Morphological and cultural characteristics

The morphological identification and cultural characteristics of isolates were examined. Colonies were compared for their diameters, overall colors, texture, size, cell arrangement, elevation and pigmentation. All the isolates were also subjected to microscopic analysis for their characterization and identification by the methods given by Bergey's Manual of determinative bacteriology[11].

**Biochemical characterization**

Different biochemical tests were analyzed including Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, starch hydrolysis, Nitrate reduction, Catalase test, Oxidase test, Phenylalanine deteramination and Sugar fermentation test.

**Molecular Identification of Bacteria**

Pure culture of the target Bacteria was grown overnight on nutrient Broth for the isolation of DNA. The DNA was isolated from the bacteria using the Phenol - Chloroform method and 16S rRNA was amplified by Thermocycler (Eppendorff) using the primers, 27F and 1492R.The primers for PCR amplification were obtained from Sigma-Aldrich[12].

**Universal Primer**

**27 forward** 5AGAGTTTCCTGGCTCAG 3
**1492 reverse** 5ACGGCTACCTTGTTACGATT 3

The PCR was performed in 50µl reaction mixture containing 5µl of 10X assay buffer, 5µl dNTP mix of 2.0 mM, 3.0µl of $MgCl_2$, 0.1µl each of forward and reverse primer (0.02µmol), 0.5µl of Taq polymerase, 5µl of template DNA and 31.3µl of HPLC grade water with the following amplification for 16s rRNA initial denaturation at 94ºC for 5 min followed by 38 cycles of denaturation, annealing and extension (94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 1.5 min) and final extension at 72ºC for 10 min followed by hold for infinity at 4ºC.

The amplified 16S rRNA PCR product was sequenced using automated sequencer (SciGenom Labs Pvt. Ltd., Kochi, India.). The unknown organism was identified using the maximum aligned 16SrRNA sequences available in the GenBank of NCBI through BLAST search. The best sequence alignment results were noted. To test the evolutionary relationships, phylogenetic analysis was performed with the program MEGA 6.0 using the 16SrRNAsequence [13].

**Optimization of Culture Conditions on cellulase activity**

In order to determine effects on cellulase production, the selected bacterial isolates were grown in CMC broth and incubated at various parameters. The influence of all factors on enzyme activity was determined by measuring cellulase activity at varying pH values from 6 to 7.5, temperature varying from 30 to 45ºC, incubation period varying from 48 to 120 h at 37°C, inoculums size varying from 1% to 6% and substrate concentration (CMC) varying from 0.2% to 1.5%. Carbon and nitrogen sources have been replaced with various substances. The influence of the factors on enzyme activity was determined by measuring cellulase activity.

**Submerged Fermentation process**

For preparation of standard inoculum, those isolates showed a maximum zone of hydrolysis were cultured in 20 ml inoculation medium [composition (g/l): Carboxymethylcellulose (CMC) 5, Tryptone 2, $KH_2PO_4$ 4, $Na_2HPO_4$ 4, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.001, $FeSO_4.7H_2O$ 0.004 and pH adjusted to 7] individually and incubated at 37ºC for 24 h until an average viable count of 2-3.5x$10^6$ cells /ml culture was obtained. This was used as inoculums for the production medium. The composition of production medium was same as of inoculums medium except the concentration of Carboxymethyl cellulose which was 1% instead of 0.5%. Fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml sterile production medium and inoculated with 5% of standard inoculums (containing 2-3.5x$10^6$ cells /ml). The flasks were incubated at 37ºC on a rotary shaker at 150 RPM for 72h[9].

**Preparation of crude enzyme*:***

After incubation, the cultures were centrifuged at 1600 RPM for 20 min at 4°C and supernatant was used as a source of crude enzyme. The crude enzyme solution was utilized for determination of enzyme activities [9].

**Cellulase enzyme assay**

Carboxymethylcellulase (CMCase) activity was estimated using a 1% solution of carboxymethlycellulose (CMC) in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution and 1ml of crude enzyme solution. The reaction was carried out at 45°C for 30 min. The amount of reducing sugar released in the hydrolysis was measured by DNSA method. The Enzyme unit (EU) was determined as the amount of CMCase

required to release of 1µmole of reducing sugar per ml per minute under above assay condition[14]. One International Unit (IU) of enzyme activity for endoglucanase was defined as the amount of enzyme releasing 1 µmol of reducing sugar from CMC per minute. The specific activity was determined as the number of units of enzyme activity per milligram of enzyme protein[9].

**Protein determination**
Protein concentrations in a crude sample were determined by using a Folin Lowry method with bovine serum albumin (BSA) as a standard[15].

**Optimization of cellulase production**
The optimum parameters were determined for cellulase production from the efficient isolates. The cellulase fermentation was carried out at different ranges of parameters including temperature, pH, incubation period, substrate concentration and inoculums size. After fermentation at different parameters the crude enzyme sample was collected from each to check the enzyme activity.

**Effect of temperature**
To determine the optimum temperature for cellulase production, fermentation was carried out at various temperatures in the range of 30ºC, 35ºC, 40ºC and 45ºC.

**Effect of pH**
Different values of pH ranged from 6.0, 6.5, 7.0 and 7.5 were chosen for studying their effects on cellulase enzyme production.

**Incubation period**
To obtain maximum cellulase production fermentation was carried out at different incubation periods ranging from 48, 72, 96 and 120 hours.

**Effect of substrate concentration:**
To evaluate the effect of substrate concentration on cellulase production the production medium was supplemented with different concentration of CMC including, 0.2%, 0.5%, 1% and 1.5%.

**Inoculums size**
The inoculum size was optimized for maximal enzyme production. The fermentation medium was inoculated with 1, 2, 4, and 6% of standard young log phase inoculums (containing 2-3.5x$10^6$ cells /ml).

**Results and Discussion**

**Isolation and primary screening for cellulase producing bacteria**
Total 7 samples were collected from 6 different sites and 12 isolates were obtained. From these, 9 out of 12 isolates were removed due to similar colonial and morphological characteristics. The resulting 3 isolates were then tested on CMC agar for cellulase activity. The CMCase activity was assessed based on the formation of zone of hydrolysis or the halo zone formed due to the secretion of cellulase and subsequent hydrolysis of cellulose incorporated in the medium by the bacterial isolates. The ratio obtained by dividing zone diameter by colony diameter gives a measurement of cellulase reactivity (Table: 1). The ratio for an effective cellulase producer will be ≥1.5. Three isolates which showed potent CMCase secreting activity with the ratio greater than 1.5 were selected for further studies. The CMCase activity was found to be in the following order based on the formation of zone of hydrolysis CB8> CB4 > CB3.The isolates were maintained in pure culture in CMC agar slants.

Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes. Habitats that contain these substrates are the best sources to find these microorganisms[16]. So the sites for sample collections were selected as those were rich in cellulosic biomass such as wood furnishing region, paper industry waste, agricultural waste and cow dung, hence there were maximum possibilities to get potential cellulose producing bacterial strain. A rapid primary screening of isolates was carried out for their cellulase activity using media containing 1% CMC as a sole source of carbon and after incubation the plates were flooded with grams iodine and poured off after 10 minutes which showed the cellulase activity by a clear zone formation around the cellulase producing colonies[10]. Around 12 bacterial samples were isolated from the 7 samples out of which, 3 strains were found to be potent cellulase producers.

The three isolates gave the maximum ratio of clear zone diameter to colony diameter on the CMC agar plate as compared to plates cultured with the other strains. Based on the calculation of the ratio of the diameter (mm) of the zone of clearance to the diameter of the colony, it was determined that these bacterial isolates demonstrated potential ability to degrade CMC. The isolated bacterial colonies were characterized for their morphological and biochemical characteristics as described by Cappuccino and Sherman. The stains CB3, CB4 were identified as *Bacillus subtilis and* CB8 as *Bacillus cereus* respectively. The strains (CB3, CB4 and CB8) were further subjected to molecular identification by analysing 16SrRNA amplified PCR product.

**Table 1: Zone of hydrolysis**

| Organism | Clear zone diameter (cm) | Colony diameter (cm) | Zone diameter / colony diameter (cm) |
|---|---|---|---|
| CB3 | 1.5 | 0.9 | 1.67 |
| CB4 | 1.6 | 0.7 | 2.29 |
| CB8 | 1.2 | 0.4 | 3 |

**Morphological, cultural and biochemical characteristics**

A microscopic examination revealed that the three isolates were Gram negative spore forming bacteria. Furthermore, the biochemical analysis of the isolates was performed and identified to be *Bacilli species.* Their colonial, morphological, and biochemical characteristics are tabulated in Table 2 and 3.

**Table 2: Colony and Morphological characteristics**

| Organism | Colony characteristics | Morphology |
|---|---|---|
| CB3 | 3-5 mm colonies, rough, flat, complete, irregular, Spreading type, granular. | Gram Positive, Rod shaped, Arranged in chains, Actively motile, with central spore. |
| CB4 | 4mm colonies, rough, flat, entire, irregular margin, Spreading type, granular, whitish. | Gram Positive, Long to short rod shaped, motile, arranged in a chain of 3 to 4 cells, sporulating. |
| CB8 | 2-5mm colonies, rough, flat, complete, irregular, Spreading type, granular, off white colour. | Gram Positive, Short rods shaped, motile, Arranged in chains, spore producing. |

**Table 3: Biochemical characteristics**

| Biochemical Test | Organism | | |
|---|---|---|---|
| | CB3 | CB4 | CB8 |
| Indole | - | - | - |
| Methyl Red | - | - | - |
| Voges-Proskauer test (VP) | + | + | + |
| Citrate | + | + | + |
| Oxidase | + | - | + |
| Catalase | + | + | + |
| Casein Hydrolysis | + | + | + |
| Starch hydrolysis | + | + | + |
| Urease | - | + | - |

| Sugar Fermentation | | | |
|---|---|---|---|
| Glucose | + + | ++ | ++ |
| Fructose | + + | ++ | ++ |
| Sucrose | + + | -- | ++ |
| Starch | + + | ++ | ++ |
| Lactose | + + | -- | -- |

**Molecular identification of cellulolytic bacteria**

Being highly sensitive and selective, molecular methods are currently used to identify microorganisms. The strains were further subjected to molecular identification by analyzing 16S r RNA sequence. DNA isolated from the three pure cultures of CB3, CB4 and CB8 were amplified with 27F and 1498R primers specific for 16srRNA and generated specific amplicons of 1500 bp. The amplified 16S rRNA PCR product was sequenced at SciGenom Labs Pvt. Ltd., Cochin, Kerala. The Sequence Similarity Search was done for the 16S rDNA sequence using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast/).

The isolates were identified using the maximum aligned sequence through BLAST search. The results have shown that CB3 had highest homology (100%) with *Bacillus subtilis*. CB4 had highest homology (99%) with *Bacillus subtilis* and CB8 had highest homology (98%) with *Bacillus cereus.* The size of amplified PCR product was determined by 2.5% agarose gel electrophoresis using DNA markers of 500bp (Figure 1). The amplified product was sent for sequencing to SciGenom Labs Pvt. Ltd., Cochin, Kerala. The sequencing information was subjected to phylogenetic analysis in order to establish genetic relatives of the isolated organisms (Figure 2, Figure 3 and Figure 4).

The 16S rRNA sequencing appears to have the potential ability to differentiate strains at the subspecies level[17]. When compared to morphological and biochemical characterization methods, 16S rDNA analysis is found to be the novel and accurate method for identifying unknown species. The DNA from the strains CB3, CB8 and CB4 was isolated and the 16S rDNA was amplified using the primers (27F and 1492R) and sequenced. The partial amplification of 16S rRNA confirmed on the agarose gel electrophoresis. The BLAST analysis of the strains using its 16S rDNA sequence data showed that strains had highest homology (100 %) with *Bacillus subtilis* and *Bacillus cereus*.

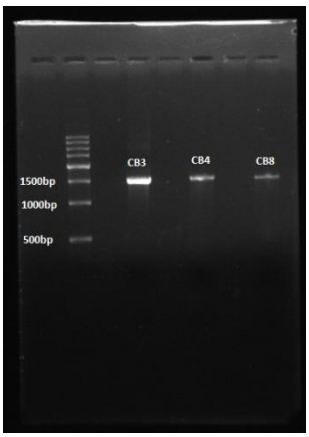


**Figure 1: Polymerase Chain reaction amplification**

Lane 1: 500 bp marker**,** Lane 2: sample CB3**,** Lane 3: sample CB4**,** Lane 4: sample CB8

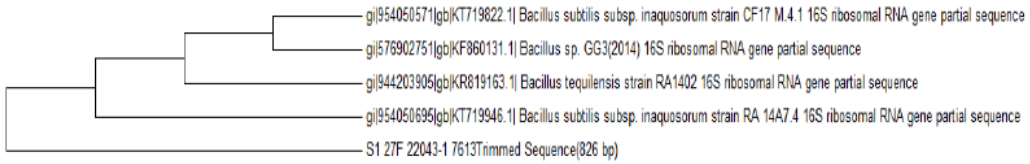


**Figure 2: Phylogenetic tree *Bacillus Subtilis* (CB3)**

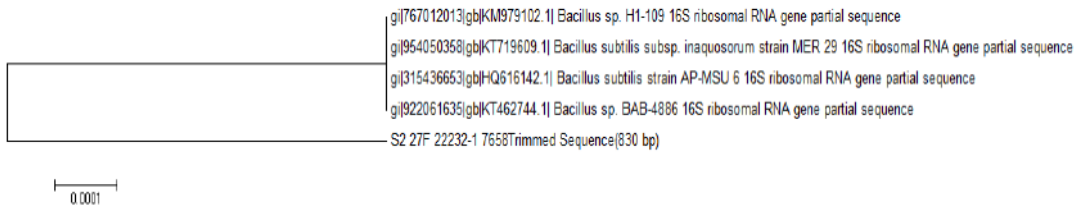


**Figure 3: Phylogenetic tree *Bacillus Subtilis* (CB4)**


**Figure 4: Phylogenetic tree *Bacillus Cereus* (CB8)**

**Optimization of cellulase production**

The optimum parameters were determined for cellulase production from the efficient isolates. After fermentation at the different parameters the crude enzyme product was collected for determination of enzyme activity. Enzyme activity was determined by DNSA method.

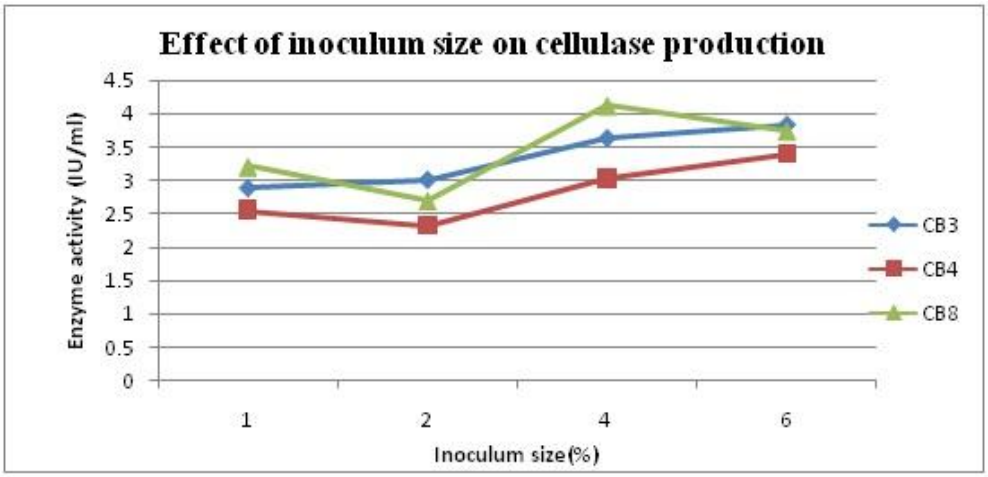


**Figure 5: The effect of inoculums volume on the activity of cellulase**

The enzyme activities of CB3, CB8 and CB4 at the different parameters are tabulated in Table 4 and figure 5. The 4.0% inoculums volume results in maximum cellulase activity of 4.12U/ml (figure 5). These data indicated that the isolate CB8 is a more efficient cellulase producer when compared to CB3 and CB4.

The different parameters for submerged fermentation were optimized at lab scale for both isolates to improve the performance and for reducing the cost of production process. Submerged fermentation was carried out at different pH, temperature, and incubation period, different concentration of CMC and at a different inoculums size. Many efforts were taken to generate microorganisms with high ability to produce cellulase that can degrade native cellulose[18]. From the present study among all isolated strains, the three cellulolytic bacterial strains the maximum enzyme activity were showed in *Bacillus cereus* (4.12 IU/ml) followed by *Bacillus subtilis* (3.83 IU/ml) at 4% and 6% inoculum volume respectively. Nakamura et al[19] reported cellulase activity of 66 U/ml from *Bacillus cereus* and the strain was confirmed by 16s rDNA method.

The optimum temperature for cellulase production for CB4, CB4 and CB8 was found to be $40^0$C, $30^0$C and $40^0$C respectively. The optimum pH was found to be around neural for the three strains. The incubation period of 48 hours, 72 hours and 96 hours were found to be optimum for CB3, CB4 and CB8 respectively. An inoculum size of 6% was found to be ideal for CB3 and CB4 whereas an inoculums volume of 8% was found to be ideal for CB8 which showed a maximum activity of 4.12U/ml. CMC was selected as a substrate which gave the highest yield of enzyme because it was assumed that this was due to the less complexity and hence easy assimilation of it by the isolated microbe[20]. The CMC concentration of 1.5% was found to be ideal for CB3 and CB4 whereas CB8 showed a maximum activity of 3.21U/ml at a CMC concentration of 1%.

**Table 4: Optimization of cellulase production**

| Different Parameters | Different Values | Enzyme activity of isolates (U/ml) | | |
|---|---|---|---|---|
| | | CB3 | CB4 | CB8 |
| Incubation Time (hours) | 48 | **2.56** | 1.87 | 2.971 |
| | 72 | 2.50 | 2.01 | **3.154** |
| | 96 | 2.012 | **2.09** | 2.487 |
| | 120 | 1.871 | 1.60 | 2.814 |
| Temperature ($^{o}$C) | 30 | 1.235 | **1.713** | 1.173 |
| | 35 | 1.45 | 1.36 | 1.301 |
| | 40 | **1.812** | 1.21 | **1.832** |
| | 45 | 1.471 | 1.11 | 1.636 |
| pH | 6 | 2.669 | 1.85 | 2.689 |
| | 6.5 | 2.68 | **3.07** | 2.891 |
| | 7 | **3.52** | 2.97 | **2.96** |
| | 7.5 | 3.41 | **2.64** | 2.63 |
| Inoculum Size (%) | 1 | 2.887 | 2.56 | 3.2 |
| | 2 | 3.011 | 2.33 | 2.69 |
| | 4 | 3.63 | 3.03 | **4.12** |
| | 6 | **3.83** | **3.40** | 3.74 |
| Concentration of CMC (%) | 0.2 | 2.211 | 2.014 | 2.45 |
| | 0.5 | 2.487 | 2.403 | 3.01 |
| | 1.0 | 2.70 | 2.9 | **3.21** |
| | 1.5 | **2.76** | **3.15** | 2.98 |

## Conclusion

Habitats that contain these substrates are the best sources to find these microorganisms [19]. Several microorganisms have been discovered for decades which have capacity to convert cellulose into simple sugars but the need for newly isolated cellulose degrading microorganism still continues [3].The present work was carried out to isolate and identify potential cellulose producing bacteria from areas rich in cellulosic biomass as well as optimize the fermentation conditions in order to achieve the maximum production of cellulase by the isolated microorganisms. Three bacterias were isolated from the paper industry waste based on its ability to grow in the CMC coated plates. The potential cellulase producing activity of the strains were confirmed by the ability to form clear zone in the presence of Grams iodine. The strains CB3, CB4 and CB8 were identified by its morphological, cultural and biochemical characteristics as *Bacillus subtilis* and *Bacillus cereus* respectively. The isolates were subjected to molecular identification by analysing 16s r RNA sequence. The 16s rRNA sequencing makes it possible to identify and distinguish closely related bacterial species. Further the phylogenetic tree constructed from the sequence analysis gives the evolutionary relatives of the isolates. The Optimum parameters for maximum cellulase activity and stability was also studied. From the determination of enzyme activity maximum enzyme activity was shown by *Bacillus cereus* (4.12 IU/ml) followed by *Bacillus subtilis* (3.83 IU/ml) at 4% and 6% inoculum volume respectively. Further studies are in progress to get cellulase with high yield, purity and stability.

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**References**


1. Bhat M.K.., Cellulases and related enzymes in biotechnology, Biotechnol. Adv.,18, 355-383 **(2000).**

2. Sethi S., Datta A.B., Gupta L. and Gupta S., Optimization of Cellulase production from bacteria isolated from soil, ISRN Biotechnology , 1-7, **(2013)**

3. Immanuel G., Dhanusha R., Prema P and Palavesam., A Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment, Int. J. of Environmental Sci. and Technol., 3(1), 25- 34 **( 2006)**

4. Angsana R., Warinthorn S., Annoop N and Pawinee C., Combination effect of pH and acetate on enzymatic cellulose hydrolysis, J. of Environmental Sci., 21(7), 965- 970 **(2009)**

5. Gerardi M., The Microbiology Of Anaerobic Digesters, John Wiley & Sons Inc. Publication, New York **(2003)**

6. Karan R., Capes M.D. and Dassarma S., Function and biotechnology of extremophilic enzymes in low water activity, Aquatic Biosystems, 8, 4, **(2012)**

7. Gopinath S.C.B., Anbu P. and Hilda A., Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. Mycoscience, 46, 119 -126, **(2005)**

8. Sazci A., Erenler K., Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method, J. of Applied Bacteriol., 61, 559-562, **(1986)**

9. Shaikh N.M., Patel A.A., Mehta S.A. and Patel N.D., Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulase production, Univer. J. Env. Res. Technol., 3(1), 39-49, **(2013)**

10. Hardik R., Gohel**,** A comparative study of various staining techniques for determination of extra cellular cellulase activity on carboxy methyl cellulose (CMC) agar plates, Int. J. Curr. Microbiol. App. Sci*.,* 3(5), 261-266, **(2014)**

11. Buchana R.E., Gibbons N.E., Bergey's manual of determinative bacteriology, Baltimore: The Williams and Wilkins Co., **(1974)**

12. Lane D.J., Stackebrandt E., M. Good fellow B, 16S/23S rRNA sequencing, In Nucleic Acid Techniques in Bacterial Systematics, Wiley, New York, 115 - 175, **(1991)**

*13.* Tamura K., Dudley J.,Nei M., Kumar S.,MEGA4: molecular evolutionary genetics analysi (MEGA) software version 4.0*,* Mol. Biol. Evol., 24, 1596-1599, **(2007)**

14. Miller G.L., Use of Dinitrosalicyclic acid reagent for determination of reducing sugar, Analytical chemistry, 31, 426-429, **(1959)**

15. Lowry O.H., Rosenberg W.J., Farr A.L. and Randell R.J., Quantification of protein using Folin Ciocalteu reagent, J Biol Chem, 193, 265-275, **(1951)**

16. Ariffin H., Abdullah N., Umi K., Shirai Y., Hassan MA Production and characterization by *Bacillus pumilus* EB3, Int. J. Eng. Technol., 3, 47-53, **( 2006)**
17. Huang X.P., Monk C., Purification and characterization of a cellulase from a newly isolated thermophilic aerobic bacterium *Caldibacillus cellulovorans*gen, nov. *sp*. World J. of Micro and Biotech., 20(1), 85-92, **(2004)**

18. Saraswati Bai, Ravi kumar M., Mukesh kumar D.J., Balashanmugam P., Bala kumaran M.D., Kalaichelvan P.T., Cellulase Production by *Bacillus subtilis* isolated from Cow Dung, Archives of Applied Science Research, 4(1), 269-279, **(2012)**

19. Nakamura K., Kappamura K., Isolation and identification of crystalline cellulose hydrolyzing bacterium and its enzymatic properties, J. Ferment. Technol., 60(4), 343, **(1982)**

20. Teeri T.T., Crystalline cellulose degradation: New insights into the function of cellobiohydrolases, Trends Biotechnol*.,* 15, 160-167, **(1997)**