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PURIFICATION AND CHARACTERIZATION OF A THERMOSTABLE HALOALKALINE ETHANOL RESISTANT CELLULASE FROM *BACILLUS LICHENIFORMIS* S16

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ABSTRACT

A thermostable strain S16, hydrolysing carboxymethylcellulose (CMC) was isolated from Punjab, Pakistan, where often temperature reaches 50oC during summer. The organism was identified as *Bacillus licheniformis* based on sequence homology. Carboxymethylase enzyme produced by Bacillus licheniformis S16 was purified by 90% ethanol precipitation and microcrystalline affinity chromatography. The purified enzyme gave a single band on SDS PAGE with a molecular mass of 29 kDa. The CMCase enzyme was found to be highly active and stable over broad ranges of temperature (37oC-100oC), pH (3-11) and NaCl concentration (1%-7%) with an optimum at 80oC, pH 7.0 and NaCl 7.0%, which showed excellent thermostable, alkali stable and halo stable properties. Thus purified cellulase from *Bacillus licheniformis* S16 could be greatly useful to industrial utilization.

Key words: *Bacillus licheniformis* S16, CMCase, Ethanol resistant, Haloalkaline, Thermostable.

INTRODUCTION

Extensive consumption of cellulases in industries like fermentation, detergents, paper and pulp, textile and food, agriculture and bioconversion etc., have attracted interest of many labs for the isolation and characterization of cellulase producing organisms with unusual properties (Bhat, 2000; Nsereko *et al*., 2002; Kirk *et al*., 2002; Sukumaran *et al*., 2005; Kuhad *et al*., 2011; Behera *et al*., 2017).

Bacteria inhabit various extreme environmental conditions. Those which can survive at high temperature, pH, salts and various organic solvents have major biotechnological advantages. Cellulases hydrolyse cellulose, one of the three main components of lignocellulose (which also contains lignin and hemicellulose), into sugar molecules. Cellulose is considered the most available biomass around the world and a vital, sustainable, and renewable energy source (De Marco1 *et al*., 2017).

Cellulose is composed of D-glucose units linked together to form linear chain via ß-1, 4-glycosidic linkages. Worldwide industrial enzyme market is contributed to about 8% by cellulases. With the advent of research procedures for the conversion of pre-treated cellulosic material to biofuel and other biobased products on larger scale, cellulases market is expected to expand dramatically (Sadhu and Maiti, 2013; Behera *et al*., 2017).

Cellulolytic enzyme system is a complex mixture of enzyme proteins with different specificities, which act synergistically to hydrolyse glycosidic bonds. The three major cellulase enzyme activities are: Endocellulase or 1, 4-β-D-glucan glucanohydrolases (EC 3.2.1.4.), Exocellulase or 1, 4-β-D-glucancellobiohydrolase (EC 3.2.1.91), β-glucosidase or β-D-glucoside Glucohydrolases (EC 3.2.1.21), Cellobiose Phosphorylase or Cellobiose, Orthophosphate α-Transferase (EC 2.4.1.20), Cellodextrin Phosphorylase or 1, 4-β-D- Oligoglucan Orthophosphate α-D-Glucosyl Transferase (EC 2.4.1.49) and Cellobiose Epimerase (EC 5.1.3.11) (Sadhu and Maiti, 2013; e Oliveira *et al*., 2016; Behera *et al*., 2017).

Cellulases are of different types showing independent folds, and may have diverse domains or modules. Microorganisms hydrolyse and metabolize insoluble cellulose by producing extracellular cellulases, either free or cell associated. Cellulase systems from aerobic and anaerobic bacteria and fungi have been extensively analysed during the past two decades (Henrissat *et al*., 1998; Lynd *et al*., 2002; Sadhu and Maiti, 2013; Behera *et al*., 2017).

High temperatures, pH extremes, organic solvent and halo stability are some of the positive attributes which if present in *Bacillus* strains, made them highly valuable assets for various industrial applications. Thermophilic enzymes are usually optimally active between 60oC to 80oC. With their great stability and activities at high temperatures, thermophilic enzymes have major biotechnological advantages over mesophilic enzymes (Azadian *et*

al., 2016). This study involves the purification and characterization of a cellulase from *Bacillus licheniformis* strain S16, which was isolated from saline soil of Rabwah, Punjab, Pakistan.

MATERIALS AND METHODS

Isolation and identification of strain S16

Luria-Bertani broth (LB) containing carboxymethyl cellulose (CMC) was used to inoculate with soil. The soil containing medium was incubated at 70^{o}C for 72 hours. Media supernatant was diluted and spreaded on 0.25% carboxymethyl cellulose containing Luria agar plates and incubated at 50^{o}C for 24 hours. Colonies grown after 24 hours were overlayed with Luria carboxymethyl cellulose agar (LCA) containing 0.25% carboxymethyl cellulose, 0.7% agar agar, streptomycin and ampicillin (100ug/ml). The plates were again incubated for 16 hours at 50^{o}C. The plates were then flooded with 0.1% Congo red for 15 minutes. After removing the dye, they were soaked in 1M NaCl solution for 15 minutes (Teathers and Wood, 1982). Colonies showing clear zone were selected and further investigated.

One of the cultures i.e., strain S16 was selected after doing Gram staining and cellulase activity. The culture was identified by performing 16S ribosomal RNA analysis. The genomic DNA was isolated using Wizard® Genomic DNA Purification Kit, Promega Corporation, USA. The genomic DNA was stored at −80 °C and used as a template for 16S rRNA PCR. The following universal primers were used to amplify 16S rRNA gene: (5'-AGAGTTTGATCMTGGCTCAG-3') 27-Forward and (5'-ACGGCTACCTTGTTACGACTT-3') 1492- Reverse. The amplified PCR product was analysed by 1% agarose gel electrophoresis. The amplified fragment was sequenced by sending the samples to MACROGEN, Korea. Sequence similarities were compared using the Basic Local Alignment Search Tool (BLAST) programme on NCBI and 16S rDNA gene sequence homology analysis was done by using Gene Bank data (http://blast.ncbi.nih.gov/Blast). The multiple sequence and phylogenetic analysis was carried out with Clustal X2 (Larkin *et al.*, 2007).

Enzyme Assay

Quantitative analysis of Cellulase was performed using DNS method by Ghose (Ghose, 1987). 100 µL of enzyme was mixed with 100µL of 1% CMC. The reaction was incubated at 50^{o}C for 10 minutes. 200 µL of DNS was added and boiled for 15 minutes, then cooled to room temperature. It was diluted with 1100µL of distilled water with rigorous mixing. The OD was taken at 540nm. One unit of enzyme activity, nkatal (nanokatal), as interpreted by Mawadza *et al*. (2000), was the amount of enzyme that released 1 nmol of glucose per second.

Production and Purification of Cellulase

Bacillus licheniformis strain S16 was grown in 1.0 L of Luria carboxymethyl cellulose broth at pH 7.0 for 72 hours at 50^{o}C at 150 rpm. Biomass was removed by centrifugation at 10,000 rpm for 10 minutes (Beckman coulter). The supernatant was concentrated 10 folds in vacuum rotary evaporator (Eyela). Chilled concentrated broth was precipitated with chilled ethanol to 95% saturation. The precipitated broth was kept at -20^{o}C for 12 hours. The precipitate was removed and stored at -20^{o}C. Ethanol precipitated protein from *Bacillus licheniformis* strain S16 was dissolved in 10mM Tris-HCl buffer, pH 8.0. The salt and protein sample was filtered and passed through G-25 Gel filtration column chromatography. The protein sample was then applied to Carboxymethyl Cellulose Microcrystalline Affinity Gel Column which was pre equilibrated with 10mM Tris-HCl buffer, pH 8.0 containing 0.5M NaCl. After removing the unbound with the same equilibration buffer, bound proteins were eluted with distilled water (Sa´nchez, *et al*., 2003). Unbound and bound fractions were analysed qualitatively on CMC agar plates for cellulase activity.

SDS-PAGE and Zymography

Purified protein (1ml) sample was concentrated on EYELA Rotary Vacuum Evaporator and analysed on 12.5% SDS-PAGE (Laemmli, 1970). The activity of cellulase band was visualized on 12.5% SDS-PAGE containing 0.1% CMC using Biorad Mini Protean II unit. The gel was renatured twice, in 10mM Tris-HCl buffer, pH 8.0, containing 25% isopropanol, at 50^{o}C. The gel was then transferred to 10mM Tris-HCl buffer, with 10mM $CaCl_2$ at 50^{o}C for 3 hours. After removal of the buffer the gel was stained with Congo red and destained with 1 M NaCl two times (Laemmli, 1970; Bischoff and Rooney, 2006).

Estimation of the Optimum Temperature and pH of the cellulase

The effect of temperature (37-100^{o}C) on cellulase activity was investigated and recorded. Cellulase activity was assayed at different pH values, ranging from pH 4 to 12, and optimum pH was estimated. The buffers used were

citrate buffer (pH 4, 5, 6), Phosphate buffer (pH 7), Tris-HCl buffer (8, 9), and Glycine–NaOH buffer (pH 10, 11, 12).

RESULTS AND DISCUSSION

Bacillus licheniformis strain S16, isolated from soil sample, collected from saline soil of Rabwah, Punjab, showed clear zones around the colonies on Luria carboxymethyl cellulose agar plates, following staining with 0.1% Congo red indicating the secretion of cellulase.

On the basis of 16S rRNA analysis the strain was identified as *Bacillus licheniformis* and it was designated as *Bacillus licheniformis* strain S16. The PCR product of 16S rRNA gene of target organism was analyzed using the Basic Local Alignment Search Tool (BLAST) programme on NCBI and sequence homology of the gene using GenBank data (http://blast.ncbi.nih.gov/Blast) (Fig. 1).

>AB776696.1 Bacillus licheniformis gene for 16S ribosomal RNA, partial sequence, strain S16:
TTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTG
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAC
AAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGA
ACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCACGAGAGATTGTAACACCCGAAGTCGGTGAGGTAACCTTTGG
AGCCAGCCGCCGAAGGTGGA

Fig.1. 16S Ribosomal RNA Sequence of *Bacillus licheniformis* S16

Multiple sequence alignment was done by online tool Clustal X2 (Fig.2). The analysis showed 99% resemblance to *Bacillus licheniformis* 414-2 (FN298317.1), *Bacillus licheniformis* EMBSO26 (JQ267798.1), *Bacillus licheniformis* TS-17 (KJ842640.1), *Bacillus licheniformis* RA5UN (KU057000), *Bacillus licheniformis* MV/NIGEB4 (LT669759.1), *Bacillus licheniformis* MEA-01 (KY938044.1) *Bacillus haynesii* NRRL B-41327 (MRBL01000076.1) and *Bacillus licheniformis* 183 (EU730933.1). Under study *Bacilli* was named as *Bacillus licheniformis* S16 and sequence of its 16S rRNA gene was submitted to GenBank with accession number of AB776696.1.

By examining the multiple sequence alignment of 16S rRNA of *Bacillus licheniformis* S16, it showed 99% similarity to about 100s of strains of *Bacillus licheniformis*. S16 showed a single dissimilarity with other 8 studied sequences discussed in previous paragraph. At position '579', where 'T' in other sequences is replaced by 'A' in AB776696.1. 16S rRNA gene with an accession number MRBL01000076.1 from *Bacillus haynesii* NRRL B-41327. Phylogenetic tree was constructed using Clustal X2 (Fig.3).

The enzyme was precipitated from the supernatant with ethanol to 95% saturation. The activity of enzyme was reduced many folds when we tried to precipitate it with ammonium sulfate $[NH_4(SO_4)_2]$. The enzyme was purified through Carboxymethyl Microcrystalline Cellulose Affinity Chromatography. The enzyme bounded with the affinity gel with 10mM Tris-HCl buffer with 0.5 M NaCl, pH 8. And then it was eluted in a very unusual pattern. Instead of eluting with a salt or pH gradient, it was eluted with simple distilled water (Fig.4). This was also reported by Sanchez, *et al*, for Cellobiohydrolase Cel48C from *Paenibacillus* species BP-23 (Sa´ nchez *et al*., 2003). The CMCase from the culture broth of *Bacillus sonorensis* HSC7 was purified through multistep purification, ammonium sulfate precipitation, Q-Sepharose, and gel filtration chromatography (Azadian *et al*., 2017).

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                                        10        20        30        40        50
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  1   TTCCGCCCTT TAGTGCTGCA GCAAACGCAT TAAGCACTCC GCCTGGGGAG  50
KY938044.1 Bacillus lichenifor  1   .......... .......... .......... .......... ..........  50
LT669759.1 Bacillus lichenifor  1   .......... .......... .......... .......... ..........  50
KU057000.1 Bacillus lichenifor  1   .......... .......... .......... .......... ..........  50
KJ842640.1 Bacillus lichenifor  1   .......... .......... .......... .......... ..........  50
JQ267798.1 Bacillus lichenifor  1   .......... .......... .......... .......... ..........  50
FN298317.1 Bacillus lichenifor  1   .......... .......... .......... .......... ..........  50
EU730933.1 Bacillus lichenifor  1   .......... .......... .......... .......... ..........  50
MRBL01000076.1 Bacillus haynes  1   .......... .......... .......... .......... ..........  50

                                        60        70        80        90       100
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  51  TACGGTCGCA AGACTGAAAC TCAAAGGAAT TGACGGGGGC CCGCACAAGC  100
KY938044.1 Bacillus lichenifor  51  .......... .......... .......... .......... ..........  100
LT669759.1 Bacillus lichenifor  51  .......... .......... .......... .......... ..........  100
KU057000.1 Bacillus lichenifor  51  .......... .......... .......... .......... ..........  100
KJ842640.1 Bacillus lichenifor  51  .......... .......... .......... .......... ..........  100
JQ267798.1 Bacillus lichenifor  51  .......... .......... .......... .......... ..........  100
FN298317.1 Bacillus lichenifor  51  .......... .......... .......... .......... ..........  100
EU730933.1 Bacillus lichenifor  51  .......... .......... .......... .......... ..........  100
MRBL01000076.1 Bacillus haynes  51  .......... .......... .......... .......... ..........  100

                                       110       120       130       140       150
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  101 GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC GCGAAGAACC TTACCAGGTC  150
KY938044.1 Bacillus lichenifor  101 .......... .......... .......... .......... ..........  150
LT669759.1 Bacillus lichenifor  101 .......... .......... .......... .......... ..........  150
KU057000.1 Bacillus lichenifor  101 .......... .......... .......... .......... ..........  150
KJ842640.1 Bacillus lichenifor  101 .......... .......... .......... .......... ..........  150
JQ267798.1 Bacillus lichenifor  101 .......... .......... .......... .......... ..........  150
FN298317.1 Bacillus lichenifor  101 .......... .......... .......... .......... ..........  150
EU730933.1 Bacillus lichenifor  101 .......... .......... .......... .......... ..........  150
MRBL01000076.1 Bacillus haynes  101 .......... .......... .......... .......... ..........  150

                                       160       170       180       190       200
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  151 TTGACATCCT CTGACAACCC TAGAGATAGG GCTTCCCCTT CGGGGGCAGA  200
KY938044.1 Bacillus lichenifor  151 .......... .......... .......... .......... ..........  200
LT669759.1 Bacillus lichenifor  151 .......... .......... .......... .......... ..........  200
KU057000.1 Bacillus lichenifor  151 .......... .......... .......... .......... ..........  200
KJ842640.1 Bacillus lichenifor  151 .......... .......... .......... .......... ..........  200
JQ267798.1 Bacillus lichenifor  151 .......... .......... .......... .......... ..........  200
FN298317.1 Bacillus lichenifor  151 .......... .......... .......... .......... ..........  200
EU730933.1 Bacillus lichenifor  151 .......... .......... .......... .......... ..........  200
MRBL01000076.1 Bacillus haynes  151 .......... .......... .......... .......... ..........  200
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Fig.2. cont'd…

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                                        210        220        230        240        250
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  201 GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA GATGTTGGGT  250
KY938044.1 Bacillus lichenifor  201 .......... .......... .......... .......... ..........  250
LT669759.1 Bacillus lichenifor  201 .......... .......... .......... .......... ..........  250
KU057000.1 Bacillus lichenifor  201 .......... .......... .......... .......... ..........  250
KJ842640.1 Bacillus lichenifor  201 .......... .......... .......... .......... ..........  250
JQ267798.1 Bacillus lichenifor  201 .......... .......... .......... .......... ..........  250
FN298317.1 Bacillus lichenifor  201 .......... .......... .......... .......... ..........  250
EU730933.1 Bacillus lichenifor  201 .......... .......... .......... .......... ..........  250
MRBL01000076.1 Bacillus haynes  201 .......... .......... .......... .......... ..........  250

                                        260        270        280        290        300
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  251 TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA GCATTCAGTT  300
KY938044.1 Bacillus lichenifor  251 .......... .......... .......... .......... ..........  300
LT669759.1 Bacillus lichenifor  251 .......... .......... .......... .......... ..........  300
KU057000.1 Bacillus lichenifor  251 .......... .......... .......... .......... ..........  300
KJ842640.1 Bacillus lichenifor  251 .......... .......... .......... .......... ..........  300
JQ267798.1 Bacillus lichenifor  251 .......... .......... .......... .......... ..........  300
FN298317.1 Bacillus lichenifor  251 .......... .......... .......... .......... ..........  300
EU730933.1 Bacillus lichenifor  251 .......... .......... .......... .......... ..........  300
MRBL01000076.1 Bacillus haynes  251 .......... .......... .......... .......... ..........  300

                                        310        320        330        340        350
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  301 GGGCACTCTA AGGTGACTGC CGGTGACAAA CCGGAGGAAG GTGGGGATGA  350
KY938044.1 Bacillus lichenifor  301 .......... .......... .......... .......... ..........  350
LT669759.1 Bacillus lichenifor  301 .......... .......... .......... .......... ..........  350
KU057000.1 Bacillus lichenifor  301 .......... .......... .......... .......... ..........  350
KJ842640.1 Bacillus lichenifor  301 .......... .......... .......... .......... ..........  350
JQ267798.1 Bacillus lichenifor  301 .......... .......... .......... .......... ..........  350
FN298317.1 Bacillus lichenifor  301 .......... .......... .......... .......... ..........  350
EU730933.1 Bacillus lichenifor  301 .......... .......... .......... .......... ..........  350
MRBL01000076.1 Bacillus haynes  301 .......... .......... .......... .......... ..........  350

                                        360        370        380        390        400
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  351 CGTCAAATCA TCATGCCCCT TATGACCTGG GCTACACACG TGCTACAATG  400
KY938044.1 Bacillus lichenifor  351 .......... .......... .......... .......... ..........  400
LT669759.1 Bacillus lichenifor  351 .......... .......... .......... .......... ..........  400
KU057000.1 Bacillus lichenifor  351 .......... .......... .......... .......... ..........  400
KJ842640.1 Bacillus lichenifor  351 .......... .......... .......... .......... ..........  400
JQ267798.1 Bacillus lichenifor  351 .......... .......... .......... .......... ..........  400
FN298317.1 Bacillus lichenifor  351 .......... .......... .......... .......... ..........  400
EU730933.1 Bacillus lichenifor  351 .......... .......... .......... .......... ..........  400
MRBL01000076.1 Bacillus haynes  351 .......... .......... .......... .......... ..........  400
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Fig.2. Cont'd…..

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                                      410         420         430         440         450
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  401 GGCAGAACAA AGGGCAGCGA AGCCGCGAGG CTAAGCCAAT CCCACAAATC  450
KY938044.1 Bacillus lichenifor  401 .......... .......... .......... .......... ..........  450
LT669759.1 Bacillus lichenifor  401 .......... .......... .......... .......... ..........  450
KU057000.1 Bacillus lichenifor  401 .......... .......... .......... .......... ..........  450
KJ842640.1 Bacillus lichenifor  401 .......... .......... .......... .......... ..........  450
JQ267798.1 Bacillus lichenifor  401 .......... .......... .......... .......... ..........  450
FN298317.1 Bacillus lichenifor  401 .......... .......... .......... .......... ..........  450
EU730933.1 Bacillus lichenifor  401 .......... .......... .......... .......... ..........  450
MRBL01000076.1 Bacillus haynes  401 .......... .......... .......... .......... ..........  450

                                      460         470         480         490         500
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  451 TGTTCTCAGT TCGGATCGCA GTCTGCAACT CGACTGCGTG AAGCTGGAAT  500
KY938044.1 Bacillus lichenifor  451 .......... .......... .......... .......... ..........  500
LT669759.1 Bacillus lichenifor  451 .......... .......... .......... .......... ..........  500
KU057000.1 Bacillus lichenifor  451 .......... .......... .......... .......... ..........  500
KJ842640.1 Bacillus lichenifor  451 .......... .......... .......... .......... ..........  500
JQ267798.1 Bacillus lichenifor  451 .......... .......... .......... .......... ..........  500
FN298317.1 Bacillus lichenifor  451 .......... .......... .......... .......... ..........  500
EU730933.1 Bacillus lichenifor  451 .......... .......... .......... .......... ..........  500
MRBL01000076.1 Bacillus haynes  451 .......... .......... .......... .......... ..........  500

                                      510         520         530         540         550
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  501 CGCTAGTAAT CGCGGATCAG CATGCCGCGG TGAATACGTT CCCGGGCCTT  550
KY938044.1 Bacillus lichenifor  501 .......... .......... .......... .......... ..........  550
LT669759.1 Bacillus lichenifor  501 .......... .......... .......... .......... ..........  550
KU057000.1 Bacillus lichenifor  501 .......... .......... .......... .......... ..........  550
KJ842640.1 Bacillus lichenifor  501 .......... .......... .......... .......... ..........  550
JQ267798.1 Bacillus lichenifor  501 .......... .......... .......... .......... ..........  550
FN298317.1 Bacillus lichenifor  501 .......... .......... .......... .......... ..........  550
EU730933.1 Bacillus lichenifor  501 .......... .......... .......... .......... ..........  550
MRBL01000076.1 Bacillus haynes  501 .......... .......... .......... .......... ..........  550

                                      560         570         580         590         600
                                ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor  551 GTACACACCG CCCGTCACAC CACGAGAGAT TGTAACACCC GAAGTCGGTG  600
KY938044.1 Bacillus lichenifor  551 .......... .......... ........T. .......... ..........  600
LT669759.1 Bacillus lichenifor  551 .......... .......... ........T. .......... ..........  600
KU057000.1 Bacillus lichenifor  551 .......... .......... ........T. .......... ..........  600
KJ842640.1 Bacillus lichenifor  551 .......... .......... ........T. .......... ..........  600
JQ267798.1 Bacillus lichenifor  551 .......... .......... ........T. .......... ..........  600
FN298317.1 Bacillus lichenifor  551 .......... .......... ........T. .......... ..........  600
EU730933.1 Bacillus lichenifor  551 .......... .......... ........T. .......... ..........  600
MRBL01000076.1 Bacillus haynes  551 .......... .......... ........T. .......... ..........  600
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Fig.2. con'd…..

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                                          610         620         630         640         650
                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AB776696.1 Bacillus lichenifor   601 AGGTAACCTT TGGAGCCAGC CGCCGAAGGT GGA                          633
KY938044.1 Bacillus lichenifor   601 .......... .......... .......... ...CAAGAAA CTGAA          645
LT669759.1 Bacillus lichenifor   601 .......... .......... .......... ...CAGATTT GTT            643
KU057000.1 Bacillus lichenifor   601 .......... .......... .......... ...CCAGAAA GGGA           644
KJ842640.1 Bacillus lichenifor   601 .......... .......... .......... ...CCGAGTG               640
JQ267798.1 Bacillus lichenifor   601 .......... .......... .......... ...CAGAGAT GG            642
FN298317.1 Bacillus lichenifor   601 .......... .......... .......... ...CAGATAT GG            642
EU730933.1 Bacillus lichenifor   601 .......... .......... .......... ...                      633
MRBL01000076.1 Bacillus haynes   601 .......... .T.GAG.CAG .CG.CG.A.G T.GGACAGAT GATTGGGGTG   650
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Fig.2. Multiple Sequence Alignment of 16S rRNA of *Bacillus licheniformis S16*

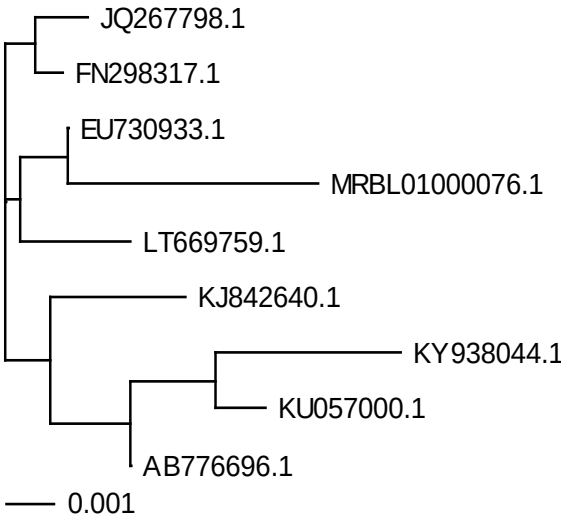


Fig.3. The phylogenetic tree for *Bacillus licheniformis* S16 (AB776696.1) and related strains based on the 16S rRNA sequence. The tree was constructed using Clustal X2.

The purity was established by SDS-PAGE showing a single protein band of molecular mass of about 29.12 kDa (Fig.5). The molecular mass of cellulase S16 was found to be smaller than cellulases from other *Bacillus* strains such as *Bacillus subtilis subsp. subtilis* A-53 (56 kDa) (Kim *et al.*, 2009), *Bacillus circulans* (43 kDa) (Hakamada, et al., 2002), *Bacillus flexus* (97 kDa) (Trivedi, et al., 2011), *Bacillus licheniformis* AU01 (37 kDa) (Annamalai *et al*., 2011).

Bacillus licheniformis strain S16 was found to be moderately thermophile. It was grown at 50^{o}C. Biochemical characterization revealed it to be Gram positive, aerobic, spore former (central), catalase positive and motile.

It was reported that during the bioethanol fermentation, ethanol concentration continues to increase. High concentration of ethanol decreased the activity and hydrolysis efficiency of common cellulase. Ethanol tolerant cellulase is desired to bioethanol production by simultaneous saccharification and fermentation (SSF). Ethanol tolerant cellulase could remain highly active and thermostable in high concentration of ethanol. Ethanol tolerant cellulase is valuable for bioethanol production (Narra *et al.*, 2017). Cellulase of *Bacillus licheniformis* strain S16 was found to be ethanol resistant. It remained active in ethanol precipitate to 90% saturation.

Bacillus licheniformis strain S16 was found to tolerate 1-7% NaCl (Fig. 6). Halostabilty is usually a quality ascribed to marine bacteria and their enzymes like a novel thermostable, halostable carboxymethylcellulase (CMCase) from a marine bacterium *Bacillus licheniformis AU01* enzyme was optimally active at 20-30% NaCl concentration and temperature stability ranges from 50–60^{o}C (Annamalai *et al*., 2011). CMCase from *Bacillus licheniformis* strain S16

was stable from 37-100°C, optimal at 80-90°C (Fig.7). Cellulase from *Bacillus licheniformis* 2D55 was reported as highly active over broad temperature range of 50-80°C with optimum of 65°C (Kazeem *et al*., 2016).

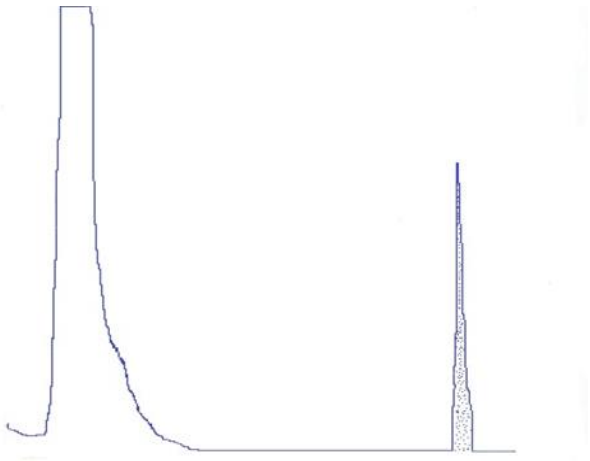


Fig.4. Carboxymethylcellulose Microcrystalline Affinity Gel Chromatography Cellulase from *Bacillus licheniformis* S16 eluted from CMC Microcrystalline cellulose Affinity Chromatography with distilled water

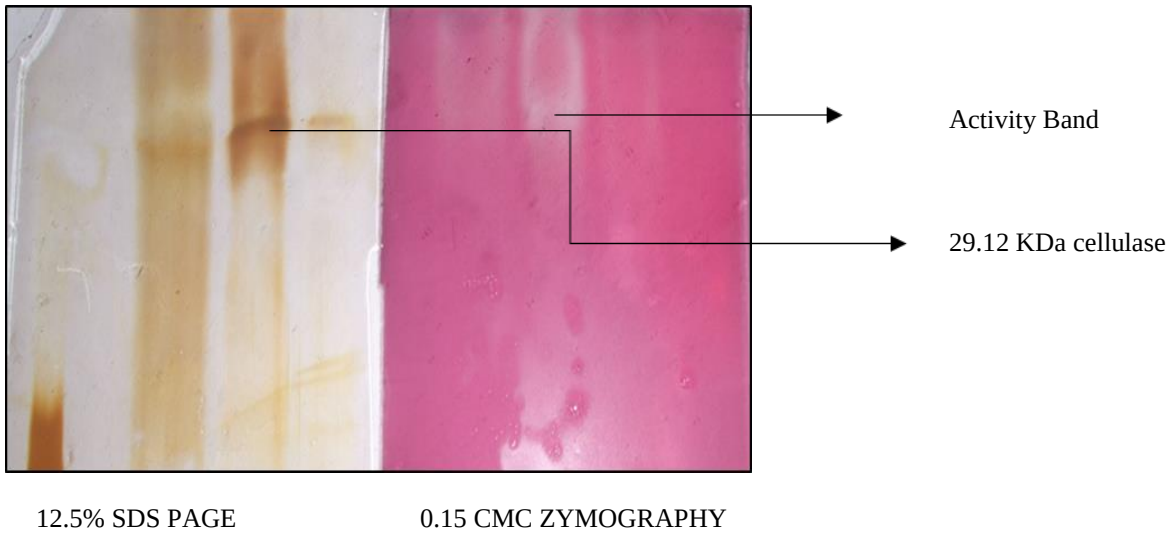
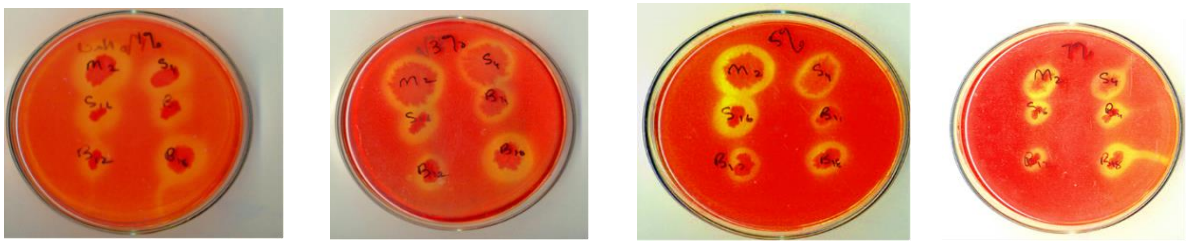


Fig. 5. SDS PAGE and zymographh (Silver Staining)

It has been reported that cellulase from *Bacillus sonorensis* HSC7 was optimally active at 70°C and pH 4.0. It also remained stable at pH 5.0 (Azadian *et al*., 2017). Cellulase-producing thermophilic bacteria from West Coast hot spring from Maharashtra was identified as *Bacillus licheniformis* NCIM 5556 which can grow at 43°C (Shajahan, *et al*., 2017). Thermophilic Geobacillus sp. HTA426 was isolated from hot spring. 40 kDa CMCase had been found to have optimal activity at pH 7.0 and temperature of 60°C (Potprommanee *et al.*, 2017.

Carboxymethylcellulase (CMCase) from a marine bacterium *Bacillus licheniformis* AU01 had molecular weight of 37 kDa. The enzyme was found to be optimally active at pH 9–10 and shown temperature 50–60°C and it was most stable up to pH 12 and 20–30% NaCl concentration (Annamalai *et al*., 2011). CMCase from *Bacillus licheniformis* S16 was found to be active from pH 3-11. In citrate buffer best activity was shown in pH 5.0 at 80°C (Fig.8). In phosphate buffer at pH 8.0 at 80°C (Fig. 9). It showed best activity in Tris HCl buffer at pH 7.0 at 80°C (Fig.10) and in Glycine NaOH buffer it showed highest activity at pH 10.0 at 80°C (Fig.11).



1% NaCl 3% NaCl 5% NaCl 7% NaCl

Fig. 6. Various Concentrations of NaCl in Luria agar over layered with Luria cellulose soft Agar

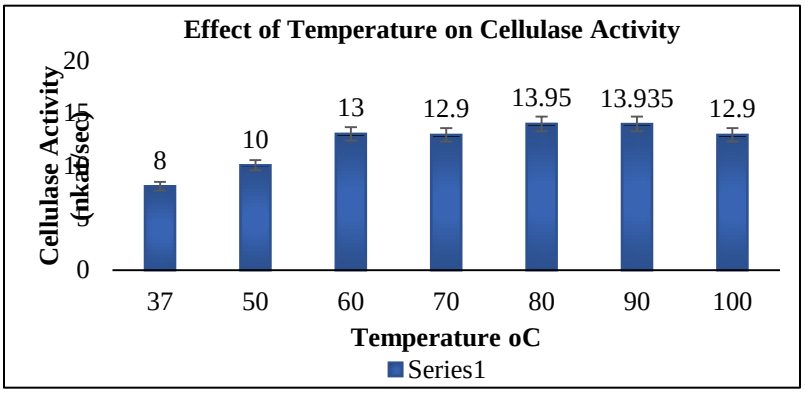


Fig. 7. Temperature Stability of Cellulase of *Bacillus licheniformis* S16

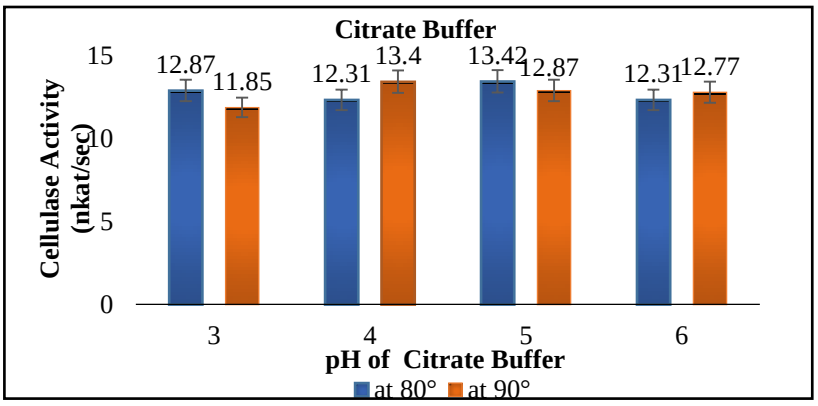


Fig. 8. pH Stability of Cellulase in Citrate Buffer at 80°C and 90°C

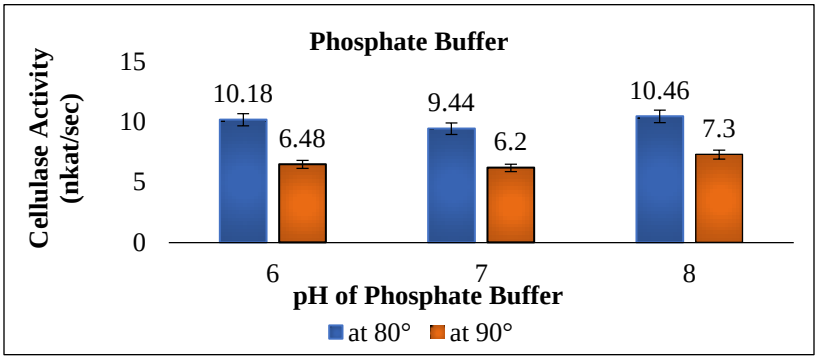


Fig. 9. pH Stability of Cellulase in Phosphate Buffer at 80°C and 90°C

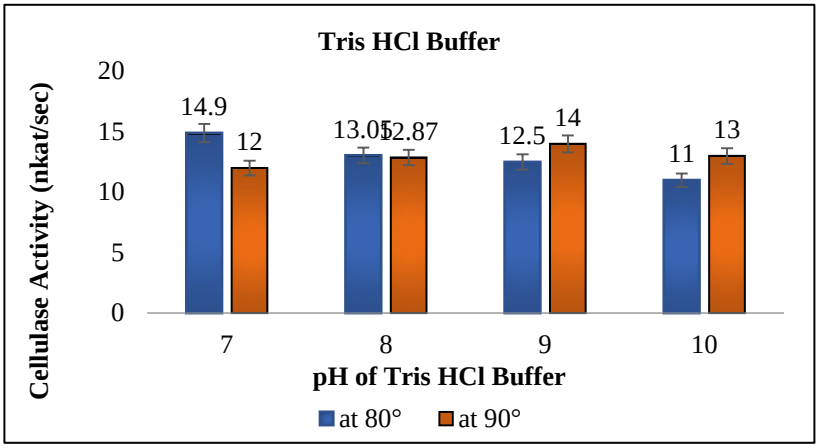


Fig. 10. pH Stability of Cellulase in Tris HCl Buffer at 80°C and 90°C

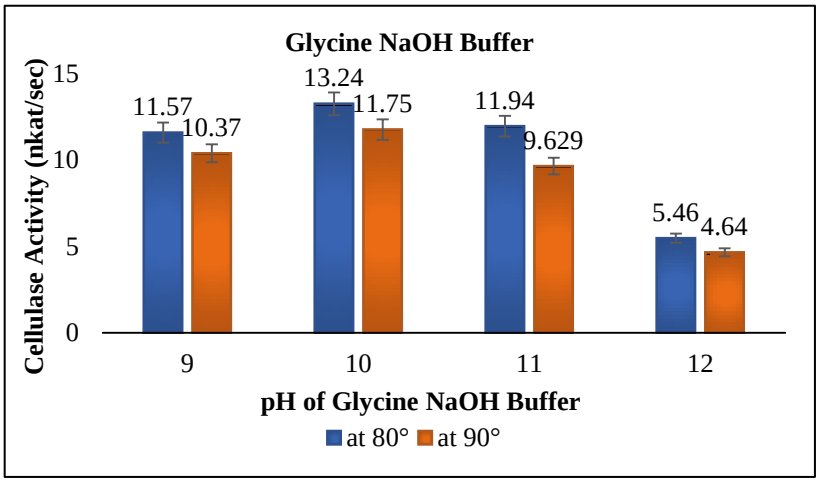


Fig. 11. pH Stability of Cellulase in Glycine NaOH Buffer at 80°C and 90°C

Furthermore, it has been reported that recombinant Cel12A from *Bacillus licheniformis* 429 was found to be active from pH 5-10 optimally at pH 9.0 (Prajapati *et al*., 2018).

CONCLUSION

We have isolated and identified cellulase producing thermotolerant *Bacillus licheniformis* strain S16. The enzyme purified was found to be heat resistant, halostable and has versatile pH stability. These excellent attributes makes it a promising candidate for industrial utilization.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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