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Synthesizing a Cellulase like Chimeric Protein by Recombinant Molecular Biology Techniques

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Abstract

In order to meet the Renewable Fuels Standard demands for 30 billion gallons of biofuels by the end of 2020, new technologies for generation of cellulosic ethanol must be exploited. Breaking down cellulose by cellulase enzyme is very important for this purpose but this is not thermostable and degrades at higher temperatures in bioreactors. Towards creation of a more ecologically friendly method of rendering bioethanol from cellulosic waste, we attempted to produce recombinant higher temperature resistant cellulases for use in bioreactors. The project involved molecular cloning of genes for cellulose-degrading enzymes based on bacterial source, expressing the recombinant proteins in *E. coli* and optimizing enzymatic activity. We were able to generate *in vitro* bacterial expression systems to produce recombinant His-tag purified protein which showed cellulase like activity.

Introduction

Cheap, clean, green energy production is a goal of Department of energy and EPA. Biofuels are made by converting renewable materials--for example, corn kernels, wood chips left over from pulp and paper production, prairie grasses, and even garbage--into fuels and chemicals. Most biofuels used today are made from the fermentation of starch from corn kernels. That process, although simple, is costly because of the high price of the corn kernels themselves.

Agricultural waste, such as corn stover (the leaves, stalks, and stripped cobs of corn plants, left over after harvest), is cheap. These materials are largely composed of cellulose, the chief component of plant-cell walls. Cellulose is far tougher to break down than starch. An additional complication is that while the fermentation reaction that breaks down corn starch needs just one enzyme, the degradation of cellulose requires a whole suite of enzymes, or cellulases, working in concert.

The cellulases currently used industrially, all of which were isolated from various species of plant-decaying filamentous fungi, are both slow and unstable, and, as a result, the process remains prohibitively expensive. Even a two-fold reduction in their cost could make a big difference to the economics of renewable fuels and chemicals; Thermostability is a requirement of efficient cellulases, because at higher temperatures, 70 or even 80 degrees Celsius--chemical reactions are more rapid. In addition, cellulose swells at higher temperatures, which makes it easier to break down. Unfortunately, the known cellulases from nature typically won't function at temperatures higher than about 50°C. Cellulolytic anaerobic bacteria use macromolecular structures known as cellulosomes to hydrolyze recalcitrant cellulosic substrates [1,2]. Within the cellulosome, cellulases and other glycoside hydrolases [3,4] are assembled onto multidomain scaffoldin proteins for efficient degradation of cellulosic substrates [4]. Cellulosome assembly is achieved by binding dockerin domains from enzymes with cohesin domains in scaffoldin, while localization with substrate is mediated by one or more Carbohydrate Binding Modules (CBMs) on the scaffoldin [1,2,5]. The modularity of cellulosomes has spurred interest in 'designer cellulosomes' [6], where different cellulases are synthetically combined for a specific application. Within a given glycoside hydrolase family, a diverse pool of potential cellulases would be beneficial for designer cellulosomes by providing a suite of enzymes with differing properties and an extensive platform for further enzyme engineering. Family 48 cellulases (Cel48) are ideal candidates for designer cellulosomes [3]. As one of the most important families of bacterial cellulases, they are usually a major constituent of bacterial cellulosomes [4,7-12]. Of the 116 bacterial Cel48 genes currently predicted in the CAZy database (http://www.cazy.org/) only 13 have been characterized. We chose SCHEMA recombination to plan to synthesize a diverse set of new family 48 sequences. SCHEMA is a structure-guided, site-directed protein recombination method that has been used to generate thousands of novel P450s, β-lactamases, and fungal cellulases. The chimeric proteins that are made by recombining natural sequences differ. Our objective for this project was to construct chimeric synthetic cellulase genes for production of thermostable cellulases for efficient breakdown of cellulose at high temperature.

Materials and Methods

Genomic DNA from bacteria Cellulomonas sp. (ATCC® 21399) was used as a template to do PCR using standard PCR reagents and assay conditions using the primers:

CCELcdCTHEdock+Xbalfwd	GCAATACTCTTCCCAGATTCTAGAATGACAT ATAAAGTACCTGGTACTCCTTCTACT
CCELcdCTHEdock+Xbalrev	AGGTACTTTATATGTCATTCTAGAATCTGGG AAGAGTATTGCATAAACTCCATTTGC

The amplicon was further sequenced and the obtained sequence (Figure 1) was subjected to NCBI-BLAST search and showed homology to *A. thermophillum* celA gene (Figure 2).

The amplicon was then cloned into a Gateway System (Invitrogen, USA) his-tag expression vector and BL-21 *E. coli* bacteria was transformed with this construct. The bacteria was then grown in LB medium and IPTG was used to induce the protein, which was then his-

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CATATGGCCAGCAGCGATGATCCGTATAAGCAACGTTTCTTGGAACTGTGGGAAGA
GTTGCACGATCCGAGCAACGGTTATTTCAGCTCCCATGGTATTCCGTACCACGCGGT
CGAGACGCTGATCGTTGAGGCACCTGATTATGGCCACCTGACCACCAGCGAAGCGA
TGTCTTACTATCTGTGGCTGGAAGCGCTGTACGGCAAATTTACGGGTGATTTTAGCT
ATTTCATGAAGGCCTGGGAAACCATTGAGAAGTACATGATTCCGACCGAGCAGGAT
CAACCGAACCGCTCCATGGCTGGTTACAATCCGGCTAAACCAGCGACCTATGCCCCT
GAATGGGAAGAACCGAGCATGTATCCGTCTCAGCTGGACTTCAGCGCACCGGTGGG
CATTGACCCGATTTACAATGAGCTGGTGTCCACCTATGGTACCAATACGATTTACGG
TATGCACTGGCTGCTGGATGTGGATAACTGGTACGGCTTTGGCCGTCGTGCGGACCG
TATCAGCAGCCCAGCCTATATCAACACCTTCCAACGTGGCAGCCAAGAGTCCGTGTG
GGAGACGATCCCGCAACCGTGCTGGGATGATCTGACCATCGGTGGCCGTAACGGTTT
TCTGGACCTGTTTGTCGGCGATAGCCAGTACTCGGCACAATTTAAGTACACGAATGC
ACCGGACGCGGATGCGCGTGCCATCCAGGCGACGTACTGGGCGAACCAGTGGGCGA
AAGAGCACGGCGTGAATTTGAGCCAGTATGTTAAGAAGGCAAGCCGCATGGGCGAC
TACCTGCGCTATGCAATGTTCGACAAATACTTTCGTAAAATTGGTGATTCCAAACAA
GCAGGTACCGGCTACGACGCAGCCCATTACCTGCTGTCCTGGTACTATGCGTGGGGT
GGTGGCATCACGGCTGATTGGGCATGGATTATTGGCTGTTCCCACGTTCATGCAGGC
TACCAGAATCCGATGACGGCGTGGATTCTGGCCAACGATCCGGAGTTTAAACCGGA
AAGCCCGAACGGTGCTAATGATTGGGCGAAAAGCCTGGAGCGCCAGCTGGAGTTCT
ATCAATGGCTGCAGAGCGCTGAGGGTGCAATCGCAGGTGGTGCGACGAATAGCTAC
AAAGGTCGCTACGAAACCCTGCCAGCAGGTATCAGCACGTTCTATGGCATGGCGTAT
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TGGACGATGCAGCGCGTGGCGGAATACTACTATCTGACCGGTGATACGCGTGCAGA
GCAACTGTTGGACAAATGGGTCGATTGGATCAAGTCCGTTGTTCGTCTGAACAGCGA
CGGCACCTTCGAGATTCCGGGTAACCTGGAGTGGTCGGGTCAACCGGACACCTGGA
CCGGTACTTACACGGGTAATCCGAACCTGCATGTCAGCGTTGTTTCTTATCGTACGG
ACTTGGGTGCAGCGGGTTCTCTGGCAAATGCTCTGCTGTACTATGCCAAAACCAGCG
GTGACGACGAAGCACGTAATCTGGCGAAAGAATTGCTGGACCGTATGTGGAACCTG
TACCGTGACGACAAAGGTTTGTCCGCACCGGAGACTCGCGAAGATTACGTCCGCTTT
TTCGAACAAGAGGTTTACGTTCCACAGGGTTGGTCTGGTACGATGCCTAACGGCGAT
CGTATCGAACCGGGTGTTACTTTCCTGGACATCCGCTCGAAATACCTGAACGACCCG

Figure 1: Nucleotide sequence of the PCR amplified amplicon.

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Query  73    AACGGTTATTTCAGCTCCCATGGTATTCCGTACCACGCGGTCGAGACGCTGATCGTTGAG  132
             |||||  |||||  | |    ||||  ||  ||  ||  ||  ||||  |||||  ||||    ||
Sbjct  3304  AACGGGTATTTTAACCAGGATGGGATACCATATCATTCGGTAGAGACATTGATATGCGAA  3363

Query  133   GCACCTGATTATGGCCACCTGACCACCAGCGAAGCGATGTCTTACTATCTGTGGCTGGAA  192
              ||||||||||||  ||  |||||||  ||  ||  ||  |  ||  ||||||  |  |||  |  ||
Sbjct  3364  CGACCTGATTATGGTCATTTGACCACGAGTGAGGCATTTTCGTACTATGTATGGTTAGAG  3423

Query  193   GCGCTGTACGGCAAATTTACGGGTGATTTTAGCTATTTCATGAAGGCCTGGGAAACCATT  252
                 ||  ||||  ||  ||  ||  ||||||||  |  |||  |  ||  |  ||  ||  ||||| ||  |
Sbjct  3424  GCAGTGTATGGTAAGTTAACGGGTGACTGGAGCAAATTTAAGACAGCATGGGACACATTA  3483

Query  253   GAGAAGTACATGATTCCGACCGAGCAGGATCAACCGAACCGCTCCATGGCTGGTTACAAT  312
              ||||||||  |||||  ||  |  |  |  |  |||||  ||||  |  ||          ||  ||
Sbjct  3484  GAGAAGTATATGATACCATCAGCGGAAGATCAGCCGATGAGGTCA---------TATGAT  3534

Query  313   CCGGCTAAACCAGCGACCTATGCCCCTGAATGGGAAGAACCGAGCATGTATCCGTCTCAG  372
              ||    ||  ||||||||  ||  ||    ||  |||||  ||||  ||  ||||||  ||  |  |
Sbjct  3535  CCTAACAAGCCAGCGACATACGCAGGGGAGTGGGAGACACCGGACAAGTATCCATCGCCG  3594

Query  373   CTGGACTTCAGCGCACCGGTGGGCATTGACCCGATTTACAATGAGCTGGTGTCCACCTAT  432
               ||||  ||  |  |  |||  ||  ||||  ||||||  |  |  |||||  ||  |||  |||  |||
Sbjct  3595  TTGGAGTTTAATGTACCTGTTGGCAAAGACCCGTTGCATAATGAACTTGTGAGCACATAT  3654

Query  433   GGTACCAATACGATTTACGGTATGCACTGGCTGCTGGATGTGGATAACTGGTACGGCTTT  492
              ||||  ||      ||  ||  ||||||||||||  ||  ||||  ||  ||  ||||||||  ||  |  |
Sbjct  3655  GGTAGCACATTAATGTATGGTATGCACTGGTTGATGGACGTAGACAACTGGTATGGATAT  3714

Query  493   GGCCGTCGTGCGGACCGTATCAGCAGCCCAGCCTATATCAACACCTTCCAACGTGGCAGC  552
              |||    |  |  ||||  |  |  ||  |  ||  |  |  |  |||||||||  |||||  |  ||  ||
Sbjct  3715  GGCAAGAGAGGGGACGGAGTAAGTCGGGCATCATTTATCAACACGTTCCAGAGAGG--GC  3772

Query  553   C--AAGAGTCCGTGTGGGAGACGATCCCGCAACCGTGCTGGGATGATCTGACCATCGGTG  610
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Sbjct  3773  CTGAGGAGTCTGTATGGGAGACGGTGCCGCATCCGAGCTGGGAGGAATTCAAGTGGGGCG  3832

Query  611   GCCGTAACGGTTTTCTGGACCTGTTTGTCGGCGATAGCCAGTACTCGGCACAATTTAAGT  670
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Sbjct  3833  GACCGAATGGATTTTTAGATTTGTTTATTAAGGATCAGAACTATTCGAAGCAGTGGAGAT  3892

Query  671   ACACGAATGCACCGGACGCGGATGCGCGTGCCATCCAGGCGACGTACTGGGCGAACCAGT  730
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Sbjct  3893  ATACGGATGCACCAGATGCTGATGCGAGAGCTATTCAGGCTACTTATTGGGCGAAAGTAT  3952

Query  731   GGGCGAAAGAGCACGGCGTGAATTT---GAGC----CAG-TATGTTAAGAAGGCAAGCCG  782
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Sbjct  3953  GGGCGAAGGAGCAAGG--TAAGTTTAATGAGATAAGCAGCTATGTAGCGAAGGCAGCGAG  4010

Query  783   CATGGGCGACTACCTGCGCTATGCAATGTTCGACAAATACTTTCGTAAAATTGGTGATTC  842
                 ||||| |||||  |  | ||||| ||||| ||||| || ||||     ||| | |||
Sbjct  4011  GATGGGAGACTATTTAAGGTATGCGATGTTTGACAAGTA-TTTCAAGCCATTAG-GATGT  4068

Query  843   CA----AACAA-------GCAGGTACCGGCTACGACGCAGCCCATTACCTGCTGTCCTGG  891
              ||    || ||       | ||| || || || |||   || ||||| ||||| || |||
Sbjct  4069  CAGGATAAGAATGCGGCTGGAGGAACGGGGTATGACAGTGCACATTATCTGCTATCATGG  4128

Query  892   TACTATGCGTGGGGTGGTGGCATC--ACGGCTGATTGGGCATGGATTATTGGCTGTTCCC  949
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Sbjct  4129  TATTATGCATGGGGTGGAG-CATTGGATGGAGCAT-GGTCATGGAAGATAGGGAGCAGCC  4186

Query  950   ACGTTCATGCAGGCTACCAGAATCCGATGACGGCGTGGATTCTGGCCAACGATCCGGAGT  1009
                | || ||    || || |||||||||||| |||| |||    | || || |||   ||
Sbjct  4187  ATGTGCACTTTGGATATCAGAATCCGATGGCGGCATGGGCATTAGCGAATGATAGTGATA  4246

Query  1010  TTAAACCGGAAAGCCCGAACGGTGCTAATGATTGGGCGAAAAGCCTGGAGCGCCAGCTGG  1069
              | || ||| |    ||||| || || | ||| ||||| || ||  || || | ||| | |
Sbjct  4247  TGAAGCCGAAGTCGCCGAATGGAGCGAGTGACTGGGCAAAGAGTTTGAAGAGGCAGATAG  4306

Query  1070  AGTTCTATCAATGGCTGCAGAGCGCTGAGGGTGCAATCGCAGGTGGTGCGACGAATAGCT  1129
              | || ||    ||| | |||   || ||||| || || ||||| || ||||| |||   |
Sbjct  4307  AATTTTACAGGTGGTTACAGTCAGCGGAGGGAGCGATAGCAGGAGGCGCGACAAATTCAT  4366

Query  1130  ACAAAGGTCGCTACGAAACCCTGCCAGCAGGTATCAGC-ACGTTCTATGGCATGGCGTAT  1188
                 || ||  | || || |     |||||||| | |||| || || ||||| ||||| |||
Sbjct  4367  GGAATGGCAGATATGAGAAGTATCCAGCAGGGA-CAGCAACATTTTATGGAATGGCATAT  4425
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Query  1189  GAAGAACATCCGGTGTACCTGGATCCGGGTAGCAACACGTGGTTTGGCTTTCAGGCGTGG  1248
             |||    ||||||| || |  ||||| || |||||||| |||||||| || ||||| |||
Sbjct  4426  GAACCGAATCCGGTATATCATGATCCTGGGAGCAACACATGGTTTGGATTCCAGGCATGG  4485

Query  1249  ACGATGCAGCGCGTGGCGGAATACTACTATCTGACCGGTGATACGCGTGCA-GAGCAACT  1307
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Sbjct  4486  TCGATGCAGAGGGTAGTGGAGTATTACTATGTGACAGGAGATAAGGACGCAGGAGC-ACT  4544

Query  1308  GTTGGACAAATGGGTCGATTGGATCAAGTCCGTTGTTCGTCTGAACAGCGACGGCACCTT  1367
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Query  1368  CGAGATTCCGGGTAACCTGGAGTGGTCGGGTCAACCGGACACCTGGACCGGTACTTACAC  1427
              | ||| |||   |  || || |||  | | ||||| || || |||| |||  | || ||
Sbjct  4605  TGCGATACCGTCGACGCTTGATTGGAAGCGACAACCTGATACATGGAACGGGGCGTATAC  4664

Query  1428  GGGTAATCCGAACCTGCATGTCAGCGTTGTTTCTTATCGTACGGACTTGGGTGCAGCGGG  1487
               || |||   ||| | ||||| |  || ||    ||| |||| ||||| ||   | | |
Sbjct  4665  AGGGAATAGCAACTTACATGTTAAGGTAGTGGACTATGGTACTGACTTAGGAATAACAGC  4724

Query  1488  TTCTCTGGCAAATGCTCTGCTGTACTA---TGC-------CAAAACCAGCGG-----TGA  1532
              ||  |||| |||||  || |||||||   |||        | ||  |  ||     |||
Sbjct  4725  GTCATTGGCGAATGCGTTGTTGTACTATAGTGCAGGGACGAAGAAGTATGGGGTATTTGA  4784

Query  1533  CGACGAAGCACGTAATCTGGCGAAAGAATTGCTGGACCGTATGTGGAACCTGTACCGTGA  1592
              || | |||    ||| | ||||| |||||||||||| | ||||||||  ||||| | ||
Sbjct  4785  TGAGGGAGCGAAGAATTTAGCGAAGGAATTGCTGGACAGGATGTGGAAGTTGTACAGGGA  4844

Query  1593  CGACAAAGGTTTGTCCGCACCGGAGACTCGCGAAGATTACGTCCGCTTTTTCGAACAAGA  1652
              || || || ||||| || || ||||   | |  || |||    | || || || |||||
Sbjct  4845  TGAGAAGGGATTGTCAGCGCCAGAGAAGAGAGCGGACTACAAGAGGTTCTTTGAGCAAGA  4904

Query  1653  GGTTTACGTTCCACAGGGTTGGTCTGGTACGATGCCTAACGGCGATCGTATCGAACCG-G  1711
              ||| ||  | ||    || |||   || | |||||| || || ||| |||   ||  | |
Sbjct  4905  GGTATATATACCGGCAGGATGGATAGGGAAGATGCCGAATGGAGAT-GTAATAAAGAGTG  4963

Query  1712  GTGTTACTTTCCTGGACATCCGCTCGAAATACCTGAAC--GACCCGGACTACCCGAAGCT  1769
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Sbjct  4964  GAGTTAAGTTTATAGACATAAGGAGCAAGTA--TAAACAAGATCCTGATTGGCCGAAGTT  5021

Query  1770  GCAGCAGGCGTATAACGAAGGCAAAGCGCCAGTGTTCAACTATCACCGTTTCTGGGCTCA  1829
              ||  ||| || ||   |||  | || || | |||||  |||||| | |||||||| ||
Sbjct  5022  AGAGGCGGCATACAAGTCAGGGCAGGCACCTGAGTTCAGATATCACAGGTTCTGGGCACA  5081

Query  1830  ATGCGACATCGCTATCGCGAACGGCTTGTATAGCATTCTGTTTGGCA  1876
              |||||||| || || || || |     |||   || ||||||||||
Sbjct  5082  GTGCGACATAGCAATAGCTAATGCAACATATGAAATACTGTTTGGCA  5128
```

A. thermophilum celA gene and manA pseudogene
Sequence ID: emb|Z86105.1| Length: 5513 Number of Matches: 1
Related Information
Range 1: 3304 to 5128 GenBankGraphics Next Match Previous Match

Alignment statistics for match #1				
Score	**Expect**	**Identities**	**Gaps**	**Strand**
369 bits (408)	2e-97	1202/1847 (65%)	65/1847 (3%)	Plus/Plus

Figure 2: NCBI-BLAST search result of the sequenced amplicon DNA.

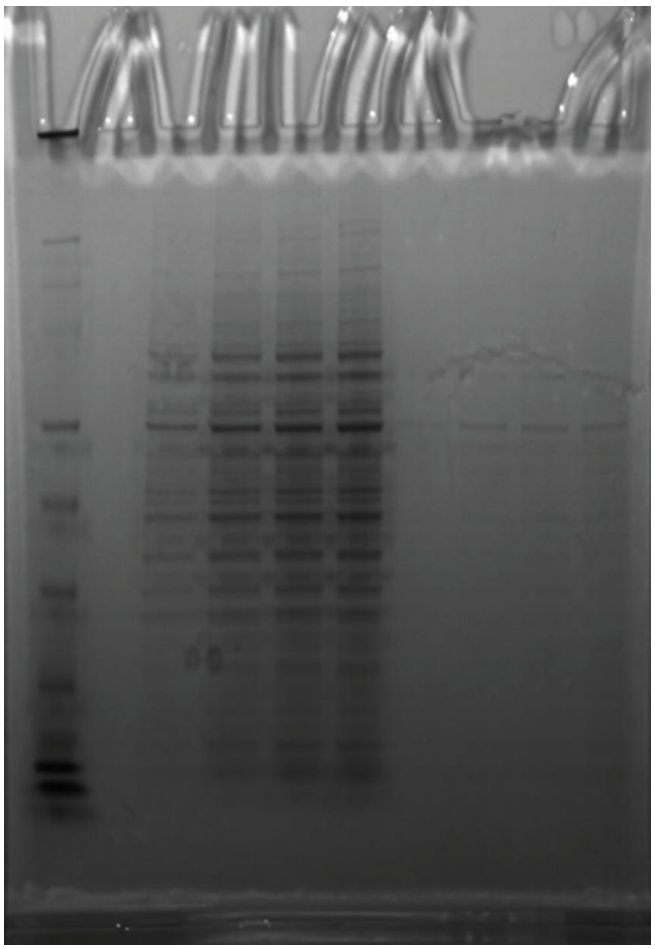

Figure 3: Lane 1=Protein marker, Lane 3-6=Different fractions of bacterial protein expressed, Lane 7-10=His-tag purified recombinant cellulase like Chimeric protein.

Enzyme Concentration	Bioactivity
100 µg/µl	0.50
50 µg/µl	0.25
25 µg/µl	0.15
10 µg/µl	0.05

Table 1: Showing cellulase bioactivity of the novel recombinant chimeric protein by Park Johnson Assay.

tag purified using a nickel column (please see the gel picture in Figure 3), protein concentration was measured by using standard Bradford method (Sigma, USA).

Cellulase Assay

Method

A standard assay for cellulase activity was performed with a reaction mixture containing 0.52% carboxymethyl cellulose in 10 mM sodium phosphate (pH 7.0) at 30°C. Reduced sugar produced by the reaction was determined using the method described by Park and Johnson [13] using a standard BioRad (USA) spectrophotometer.

Results and Discussion

We were interested to synthesize a chimeric synthetic cellulase gene from the different cellulases DNA sequence that are there in the gene bank to produce a thermostable cellulose, our initial bioinformatics analysis by using the CAZy database and SCHEMA recombination to design gene sequences which will fulfill those conditions resulted in production of a chimeric protein. We derived the following full length DNA sequence (Figure 1) which showed homology to Cel A gene of *A. thermophillum* (Figure 2) and we expressed and purified the recombinant protein by His-tag method (Figure 3). The activity of this novel chimeric protein was determined to be cellulase when tested for activity by standard Park Johnson assay (Table 1). Thus our recombinant chimeric proteins have definite Cellulase enzyme characteristics. We look forward to scaling up productions and temperature and pH stability testing for its usefulness for bioremediation.

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