Heterologous expression, purification and enzyme activity of *Neocallimastix sp.* CellulaseA (celA)

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Abstract: *The CellulaseA (celA) encoding gene was isolated from Neocallimastix sp. genomic DNA by PCR using gene specific primers and cloned into pRSETA vector, expressed in E.coli BL21DE3 as a soluble protein. The celA solubility was further improved by optimizing the IPTG concentration and induction temperature. The celA was purified by affinity chromatography using Ni-NTA resins and tested for its cellulolytic activity using CMC substrate by Zymogram. A band of ~47kDa corresponding to celA was observed in Zymogram.*

Keywords: Affinity chromatography, CellulaseA; celA; Carboxy Methyl Cellulose (CMC), Isopropyl β-D-1-thiogalactopyranoside (IPTG); *Neocallimastix sp.*; pRSETA; *E.coli* BL21DE3; Zymogram.

1. INTRODUCTION

The profound utilization of petroleum and petroleum products from unsustainable resources, with reverse effect on minimizing its consumption, in the past few decades has led to increased demand for the development of renewable sources (Kerr, 2007; Stephanopoulos, 2007). In this regard, ethanol from cellulosic source and biodiesel from soy or palm oil source have gained importance due to its low carbon footprint (Hill *et. al.,* 2006). Cellulosic biofuel is considered as a greater energy return on investment in comparison to other sources. However, environmental benefits are not guaranteed as it depends on cellulosic crops grown and practices.

Lignocellulose, a major constituent of plant biomass is the most abundant renewable carbon source on Earth. Lignocellulose consists of cellulose, hemicellulose, and lignin. Cellulose is a linear polymer of glucose that is extensively bonded to each other through strong intra-molecular hydrogen bonds whereas hemicellulose is a complex heteropolymer that comprises a number of polysaccharides such as xylan, galactan, and mannan. Cellulose is protected from hydrolytic enzymes by lignin (Sakuragi *et. al.,* 2011).

The extraction of simple sugars from cellulose involves pretreatment under harsh conditions followed by enzymatic saccharification (Vinuselvi *et. al.,* 2011; Xu *et. al.,* 2009; Mazzoli *et. al.,* 2012). Simple sugars extracted can then be converted to advanced biofuels that resemble petroleum-based fuels by using Cellulase enzymes. The conversion of cellulose to glucose requires sequential co-operative actions by a family of cellulolytic enzymes which consist of at least three classes: endoglucanases (EC 3.2.1 .4), which randomly cleave the internal glucosidic bonds in less ordered regions of cellulose; exoglucanases (mainly cellobiohydrolases : EC 3.2.1 .91), which release

cello-biose from the non-reducing ends of cellulose chains; and glucosidases (EC 3.2.1 .21), which convert cellobiose to glucose.

The ability to develop cellulolytic enzymes under optimum conditions is extremely promising in producing ethanol as an affordable renewable energy source. The aim of this study was to clone and express the Cellulase (celA) encoding gene of *Neocallimastix sp.* in *E.coli* and subsequently, purification and *in vitro* enzyme activity. A genetically recombinant *E. coli* with Cellulase activity would be used as an ideal candidate for biofuel production.

2. MATERIALS AND METHODS

2.1 Strains, plasmid and media

Neocallimastix sp was obtained as a kind gift from Department of Animal Science, Faculty of Agriculture, Kahramanmaras Sutcu Imam University, Kahramanmaras, Turkey. The following expression hosts were used in this study *Escherichia coli* DH5α (Thermo Fisher, Cat. No. 18265017); *Escherichia coli* BL21 (DE3) (Novagen, Cat. No. 69450-3); *Escherichia coli* BL21 (DE3) pLysS (Novagen, Cat. No. 69451-3); *Escherichia coli* BL21 (DE3) pLysS Rosetta-gami (Novagen, Cat. No. 71054-3); *Escherichia coli* JM109 (DE3) (Promega, P9801); *pTZ57R/Tvector and* pRSET A vector (Thermo Fisher, Cat. No. V351-20)

2.2 Isolation of genomic DNA Neocallimastix sp

Neocallimastix sp was used as a source of DNA in this study. *Neocallimastix sp.* culture was maintained on potato dextrose agar. The dehydrated medium was weighed and mixed with distilled water (3.9 g /100ml). After adjustment of pH at 7.0 with 0.1N NaOH, the media was autoclaved at 121° C for 20 minutes with 15 lb pressure. After

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autoclaving the media was dispensed into Petri plates, slants were dispensed with media prior to sterilization (autoclaving). After 24 hours of incubation at 28 ° C the plates and slants free of contamination were inoculated with *Neocallimastix sp.* For further experimentations seven days old liquid cultures were used. The genomic DNA was isolated by high salt DNA method (Shaila *et. al.,* 2006).

2.3 PCR amplification of celA gene from Neocallimastix sp genomic DNA

The celA gene sequence was found in GenBank, the NIH genetic sequence database (Accession number HM625672). In order to amplify the celA gene, the primers were designed corresponding to ORF of the gene. To facilitate cloning into pRSETA expression vector, HindIII and BamHI restriction sites were incorporated into the primers. One hundred micrograms of genomic DNA was used as a template for PCR amplification using Thermocycler (Eppendorf) under following conditions; 1 cycle of 95 °C for 5 min; 30 cycles of 95 °C for 1 min, 55 °C for 30 sec, 72°C for 2 min; 1 cycle of 72 °C for 10 min. The expected PCR product (celA) size is ~ 0.9 Kb. The PCR product was purified using QIAquick PCR purification kit (Qiagen, USA).

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2.4 Cloning and sequencing

The cleaned PCR product was ligated into the pTZ57R/T vector, transformed into *E.coli* DH5α chemically competent cells by heat shock method and plated in ampicillin (100ug/ml) containing LB agar supplemented with 0.1M IPTG and 40ug/ml X-gal. Following overnight incubation at 37°C white colonies were picked and propagated in LB broth containing ampicillin (100ug/ml). Also, *E.coli* DH5α-pRSETA was grown in LB broth containing ampicillin (100ug/ml). pTZ57R/T-celA and pRSETA vectors were isolated using QIAprep spin miniprep kit (Qiagen, USA). The plasmids were digested with HindIII and BamHI to release celA insert from pTZ57R/Tvector and to linearize pRSETA vector. The celA insert and pRSETA vector was gel purified. The celA insert was ligated into HindIII and BamHI sites of pRSETA expression vector and transformed into *E.coli* DH5α chemically competent cells by heat shock method and plated in ampicillin (100ug/ml) containing LB agar and incubated at 37°C overnight. The transformed clones, *E.coli* DH5α-pRSETA-celA were grown in LB broth containing ampicillin (100ug/ml) at 37°C at 220 rpm overnight. The plasmid, pRSETA-celA was isolated using QIAprep spin miniprep kit (Qiagen, USA) and digested with HindIII and BamHI. The expected size of ~0.9 Kb insert (celA) was released. The pRSETA-celA vector was sequenced using M13 forward primer. Nucleotide sequence translation and alignment was performed online using NCBI bioinformatics tools.

2.5 celA expression and solubility improvement, purification and enzyme assay

The confirmed recombinant plasmid, pRSETA-CelA was transformed into chemically competent *Escherichia coli* BL21 (DE3) (Novagen, Cat. No. 69450-3); *Escherichia coli* BL21 (DE3) pLysS (Novagen, Cat. No. 69451-3); *Escherichia coli* BL21 (DE3) pLysS Rosetta-gami (Novagen, Cat. No. 71054-3); *Escherichia coli* JM109 (DE3) (Promega, P9801) BL21 (DE3)-pRSETA-celA, *E. coli* BL21 (DE3) pLysS-pRSETA-celA, *E. coli* BL21 (DE3) pLysSRosetta-gami2-pRSETA-celA and *E. coli* JM109 (DE3)-pRSETA-celA clones with 1mM IPTG at absorbance OD_{600}=0.5 and further incubated at 37°C at 220 rpm overnight. The cultures were tested for celA expression by SDS-PAGE analysis. *E.coli* BL21 (DE3)-pRSETA-celA was chosen to improve the celA solubility by varying IPTG (0.125mM, expression hosts by heat shock method and incubated at 37°C overnight. The transformed colonies were screened for celA expression. Freshly, inoculated *E.coli* cells harboring celA were grown in LB broth containing ampicillin (100ug/ml) at 37°C at 220 rpm overnight. The overnight grown cultures were subcultured. The celA expression was induced in *E.coli* 0.25mM, 0.5mM, 0.75mM & 1.0mM) concentration and temperature (18 °C, 24 °C & 37 °C). The optimized conditions were used to express celA followed by its purification by affinity chromatography using Ni-NTA resins (Qiagen, USA).

2.6 The Purified celA cellulolytic activity was performed by zymogram method

The purified celA samples (100µg and 200µg each) were mixed with the sample buffer without b-

mercaptoethanol or any reducing agent. The sample without heating was subsequently loaded on 5% stacking gel. Sodium carboxymethyl cellulose (CMC) with a final concentration of 0.1% (w/v) was incorporated into 12% separating gel during gel preparation. After electrophoresis, SDS gel was soaked in 0.1 M sodium acetate buffer, pH 5.2 containing 0.1% Triton X-100 for 30 min to remove SDS from the gel. To allow the proteins to renature, the gel was incubated in the same buffer without Triton X-100 for 1 h. The gel was then stained in 0.2% Congo red for 1 h and destained with 2 M NaCl until the clear zone against red background was observed. A duplicate gel with the same loading pattern was stained with Coomassie brilliant blue R-250 (Methanol: Acetic acid: Water, 40:20:10 + 0.02% w/v Coomassie brilliant blue R-250) and destained with the destaining solution (Methanol: Acetic acid: Water, 40:20:10). The position of the CellulaseA enzyme on the both SDS gels was compared.

3.0 RESULTS AND DISCUSSION

3.1 *Isolation of genomic DNA Neocallimastix sp*

The genomic DNA isolated from *Neocallimastix sp* was found to be intact without shearing and degradation (Fig. 1). The genomic DNA thus obtained is amenable for PCR.

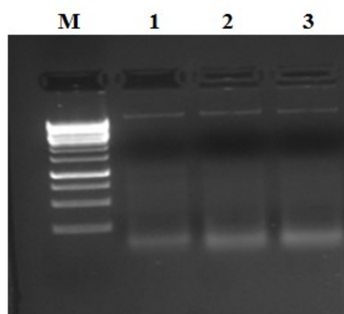

Fig. 1: ***Neocallimastrix sp* genomic DNA isolated by high salt method.** Lane M: 1Kb DNA ladder (NEB, Cat. No. N 3232S); Lanes 1-3: *Neocallimastrix sp* genomic DNA

3.2 *PCR amplification of celA gene from Neocallimastix sp genomic DNA*

The gradient PCR was performed ranging from 55 °C to 65 °C annealing temperatures to amplify CellulaseA (celA) encoding gene using genomic DNA template. The CellulaseA (celA) encoding gene (1.9 Kb) was amplified in all the annealing temperatures tested (Fig. 2).

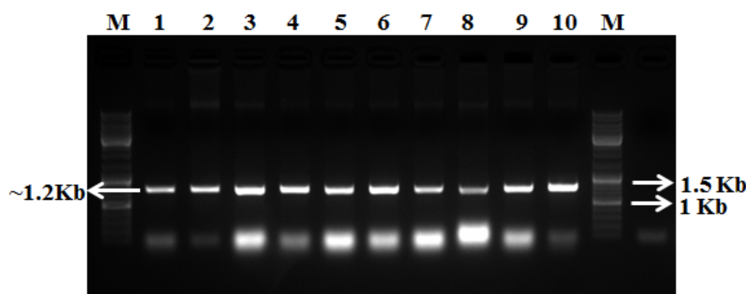

Fig. 2: *PCR amplification of CelA gene from Neocallimastrix sp genomic DNA*. Lane M: 1Kb DNA ladder (NEB, Cat. No. N 3232S); Lanes 1-10: Ta 55°C to 65°C

3.3 *Cloning and sequencing*

CellulaseA (celA) PCR product was ligated into pTZ57R/T using T4 DNA ligase at 16°C overnight and subsequently, transformed into *E.coli* DH5α by heat shock method. The

transformed cells were spread onto LB+Amp (100µg/ml) and incubated at 37 ^{o}C overnight. The putative transformants were observed. The white colony (positive clone) was grown in LB+Amp (100µg/ml), isolated plasmid and restriction digested with HindIII and BamHI. The expected insert of celA (~ 0.9 Kb) was released. The insert was ligated into HindIII and BamHI sites of pRSETA expression vector and transformed into chemically competent *E.coli* DH5α cells. The putative transformants were obtained. The positive clones were grown in LB+Amp (100µg/ml), plasmid isolated and restriction digestion was performed with HindIII and BamHI enzymes. The expected insert of celA with few pRSETA nucleotides (~ 1.1 Kb) was released.

The clone was sequenced with M13 forward primer which is as follows

TTG TTT ACT TTA AGA AGG AGA TAT ACA TAT GCG GGG TTC TCA TCA TCA TCA TCA TCA TGG TAT GGC TAG CAT GAC TGG TGG ACA GCA AAT GGG TCG GGA TCT GTA CGA CGA TGA CGA TAA GGA TCG ATG GGG ATC CTT AGG TAA TAC CTT AGA TGC TCA ATG TAT TGA ATA CTT AAA TTA TGA TAA GGA TCA GAC TGC TTC TGA AAC TTG CTG GGG TAA TCC AAA GAC TAC TGA AGA TAT GTT CAA GGT TTT AAT GGA TAA CCA ATT TAA TGT TTT CCG TAT TCC AAC TAC TTG GTC TGG TCA CTT CGG TGA AGC TCC AGA TTA CAA GAT TAA TGA AAA ATG GTT AAA GAG AGT TCA TGA AAT TGT TGA TTA TCC ATA CAA GAA TGG AGC TTT CGT TAT CTT AAA TCT

TCA CCA TGA AAC
TTG GAA CCA TGC
TTT CTC TGA AAC
TCT TGA CAC TGC
CAA GGA AAT CTT
AGA AAA GAT TTG
GTC TCA AAT TGC
TGA AGA ATT TAA
GGA TTA TGA TGA
ACA CTT GAT TTT
TGA AGG ATT AAA
CGA ACC AAG AAA
GAA TGA TAC TCC
AGT TGA ATG GAC
TGG TGG TGA TCA
AGA AGG ATG GGA
TGC TGT TAA TGC
TAT GAA TGC CGT
TTT CTT AAA GAC
TGT TCG TAG TTC
TGG TGG TAA TAA
TCC AAA GCG TCA
TCT TAT GAT CCC
TCC ATA TGC TGC
TGC TTG TAA TGA
AAA TTC ATT CAA
GAA CTT TAT TTT
CCC AGA AGA TGA
TGA CAA GGT TAT
TGC TTC TGT TCA
TGC TTA TGC TCC
ATA CAA CTT TGC
CTT AAA TAA TGG
TGC AGG AGC TGT
TGA TAA GTT TGA
TGC TGC TGG TAA
GAA AGA TCT TGA
ATG GAA CAT TAA
ACT TAA TGA AGA
AGA GAT TGT CGA
TCA AGG TAT TCC
AAT GAT TCT TTG
GTG AAT ATG GTG
CCA TGA ACC GTG
ATA ATG AA

The cloned sequence (~800 bp read) was subjected to NCBI nucleotide BLAST (Basic Local Alignment Search Tool) and found to have 99% homology with *Neocallimastix sp.*

3.4 *celA expression and solubility improvement, purification and enzyme assay*

3.4.1 *celA expression*

The CellulaseA expression was induced in *E.coli* BL21 (DE3)-pRSETA-celA, *E. coli* BL21 (DE3) pLysS-pRSETA-celA, *E. coli* BL21 (DE3) pLysSRosetta-gami2-pRSETA-celA and *E. coli*JM109 (DE3)-pRSETA-celA clones with 1mM IPTG at absorbance OD600=0.5 and further incubated at 37 °C at 220 rpm overnight. The CellulaseA expression was observed in all the hosts. The CellulaseA expression was good in *E. coli* BL21 (DE3)-pRSETA-celA and *E. coli* JM109 (DE3)–pRSETA-celA but poor in *E. coli* BL21 (DE3) pLysS-pRSETA-celA and *E.coli*BL21(DE3)pLysSRosetta-gami2-pRSETA-celA expression hosts (Fig 3). The growth rate and biomass (wet cell weight) was relatively better in *E.coli* BL21 (DE3) in comparison to *E.coli* JM109DE3. All the cells were normalized and expression was quantified by densitometry (data not shown).

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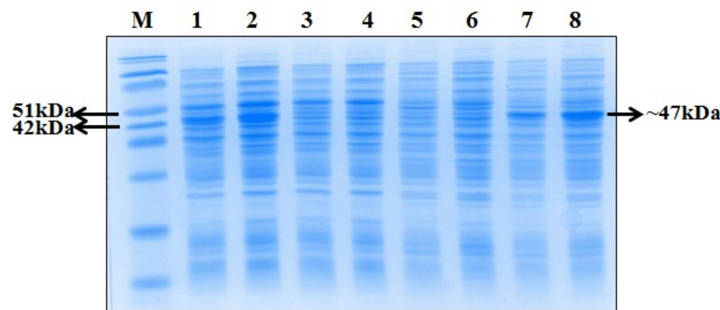

Fig. 3: Heterologous expression of CelA encoding gene in different *E.coli* expression hosts. Lane M: Protein Marker; Lane1: *E.coli*BL21DE3-pRSETA-CelA-Uninduced; Lane2: *E.coli*BL21DE3-pRSETA-CelA-Induced; Lane3: *E.coli*BL21DE3pLysS-pRSETA-CelA-Uninduced; Lane4: *E.coli*BL21DE3pLysS-pRSETA-CelA-Induced; Lane5: *E.coli*BL21DE3pLysSRosettaGami2-pRSETA-CelA-Uninduced; Lane6: *E.coli*BL21DE3pLysSRosettaGami2-pRSETA-CelA-Induced; Lane7: *E.coli*JM109DE3-pRSETA-CelA-Uninduced; Lane8: *E.coli*JM109DE3-pRSETA-CelA-Induced

3.4.2

3.4.3 *Solubility improvement*

E. coli BL21 (DE3)-pRSETA-celA was chosen to improve the celA solubility at different temperatures and IPTG concentrations. At 1mM IPTG concentration, the celA solubility was good at 24 °C and 18 °C in comparison to 37 °C (Fig. 4).

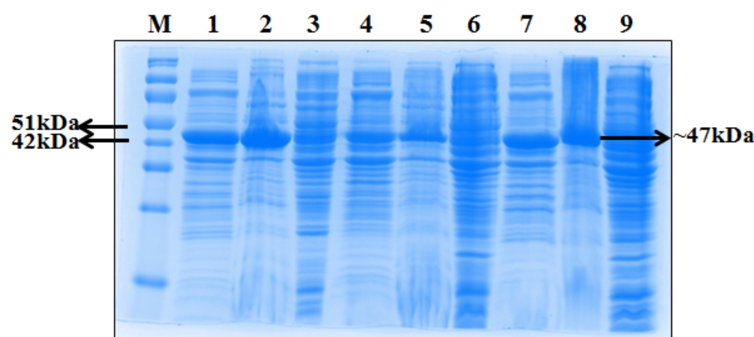

Fig. 4: Solubility improvement of CelA encoding gene in *E.coli*BL21DE3 **at different temperatures.** Lane M: Protein Marker; Lane1: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-24°C Supernatant; Lane2: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-24°C Pellet; Lane3: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-24°C UnInduced; Lane4: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-37°C Supernatant; Lane5: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-37°C Pellet; Lane6: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-37°C UnInduced; Lane7: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-18°C Supernatant; Lane8: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-18°C Pellet; Lane9: *E.coli*BL21DE3 -pRSETA-CelA-1mM IPTG Induced-18°C UnInduced;

The celA solubility was relatively better in 24 °C and 18 °C than 37 °C at 0.5mM IPTG and 0.25mM IPTG concentrations (Fig. 5 and Fig. 6).

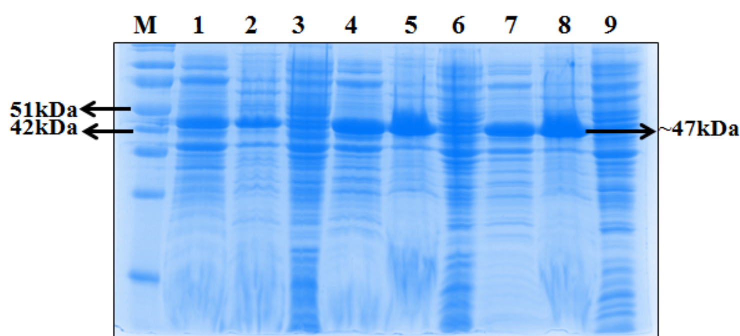

Fig. 5: Solubility improvement of CelA encoding gene in *E.coli*BL21DE3 **at different temperatures.** Lane M: Protein Marker; Lane1: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-37°C Supernatant; Lane2: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-37°C Pellet; Lane3: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-37°C UnInduced; Lane4: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-24°C Supernatant; Lane5: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-24°C Pellet; Lane6: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-24°C UnInduced; Lane7: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-18°C Supernatant; Lane8: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-18°C Pellet; Lane9: *E.coli*BL21DE3 -pRSETA-CelA-0.5mM IPTG Induced-18°C UnInduced;

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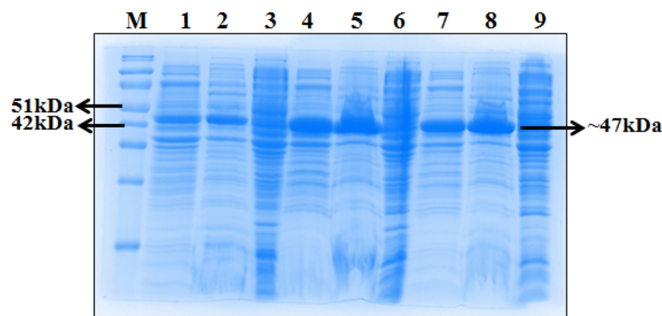

Fig. 6: Solubility improvement of CelA encoding gene in *E.coli*BL21DE3 **at different temperatures.** Lane M: Protein Marker; Lane1: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-37°C Supernatant; Lane2: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-37°C Pellet; Lane3: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-37°C UnInduced; Lane4: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-24°C Supernatant; Lane5: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-24°C Pellet; Lane6: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-24°C UnInduced; Lane7: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-18°C Supernatant; Lane8: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-18°C Pellet; Lane9: *E.coli*BL21DE3 -pRSETA-CelA-0.25mM IPTG Induced-18°C UnInduced;

The celA expression and solubility was 0.5 fold higher (densitometry data not shown) at 24°C at 0.25mM IPTG concentration when compared to all other IPTG concentrations and temperatures tested. The shake flask culture was performed with aforesaid optimal conditions to express soluble celA. This data suggests that *E. coli* system could be used a robust cell factory for expressing soluble recombinant CellulaseA for biofuel production. celA, celB and celC encoded cellulases have been previously isolated and characterized from *P. carotovorum* LY43 (Park *et. al.,* 1998). Enormous data are available on advantages of using E. coli as expression system, its cell biology, fermentation process

development and its ability to produce large quantities of recombinant proteins in an inexpensive way. This process can be In terms of recombinant expression, *E. coli* has always been the preferred microbial cell factory. *E. coli* is a suitable host for expressing stably folded proteins. The role of these cellulases in sugar uptake systems inside the cell was described in the cloned cellobiose phosphotransferase system operon from *Bacillus stearothermophilus* (Lai and Ingram, 1993).

3.4.4 *celA purification*

The N-Terminal hexahistidines from pRSETA vector aid in celA purification by affinity chromatography using Ni-NTA resins. The celA eluted with 250mM imidazole was good with meager host proteins and ideal to perform celA assay. N-Terminal hexahistidines ensures that the transcription and translation always encounter 5′ and N-terminal sequences that are compatible with robust RNA synthesis and protein expression, respectively. Histidine tags do not have any effect on the protein characteristics and largely does not interfere with its enzyme activity.

3.4.5 celA enzyme assay

celA enzyme activity in polyacrylamide gel was examined using Zymogram (Schwarz et al., 1987) staining technique. The Zymogram analysis revealed a band of ~47 kDa in the region of CellulaseA activity. It is likely

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that overlapping activities explain the single activity signature observed on zymograms. In this procedure, we have taken 30µl (100µg and 200µg) of purified sample with lamelli buffer loaded in CMC substrate contain Poly acrylamide gel to just check and confirm the cellulolytic activity, molecular weight determination and any quantification was not done for cellulase enzyme (Machenko, 2002).

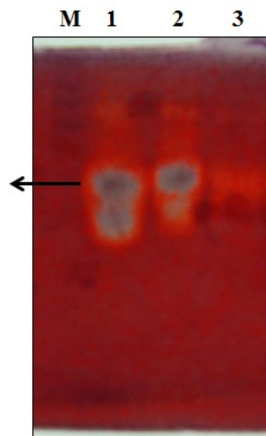

Fig. 7: Zymogram of CelA analyzed on an 10% SDS-PAGE gel containing CMC substrate. Lane M : Protein marker; Lane 1: Ni-NTA Purified CelA (200µg); Lane 2: Ni-NTA Purified CelA (100µg); Lane 3: Buffer control; Proteolytic zone corresponding to ~ 47kDa is indicated by arrow mark

Zymography is an electrophoretic technique that includes a substrate copolymerised with the polyacrylamide gel for the detection of enzymes and their activity. This technique used for detection of hydrolytic enzymes on the basis of substrate degradation (Vandooren 2013; Manchenko, 2002). Zymography has been applied to a variety of enzymes including xylanases, cellulases, proteases, lipases and chitinases (Howard *et. al.*, 2003; Choi et. al.,2009; Cheng and Chang, 2011; Kwon *et. al.*, 2011).

Endoglucanase rapidly reduces the chain length of

Cellulose and are the most sensitively detected by the Congo Red-CMC-staining technique (Laskowski and Kato, 1980). The advantages of using Congo red assay include a much better contrast of clearance zones against the background than that obtained by precipitation with hexadecyltrimethylammonium bromide. Contrast can be further improved by acidification, which turns the background blue, and the low pH, being sufficient also to prevent any further enzyme action. (Beguin, 1983) has described a system for the detection of endoglucanase activity in polyacrylamide gels using Congo red staining of agar overlays. This offers much greater sensitivity than earlier published methods. CMC, treated at a comparatively low concentration (0.1%, w/v) in the overlay gel, complexes with Congo red, except in areas where the CMC has been degraded, which appear as colourless bands.

4.0 CONCLUSION

We have reported the expression of CellulaseA (celA) encoding gene in *E.coli* as a soluble form. Further, the purified celA was active as evident from the cellulolytic activity using CMC substrate by Zymography. The production of active CellulaseA (celA) in *E.coli* is promising and scalable at industrial scale.

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