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Research Article

Heterologous Expression of Exoglucanase from *Trichoderma resei* in *E. coli*

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Abstract

FPases/Exoglucanases are usually produced in lesser quantity from the wild cellulolytic strains in comparison to the quantities of Endoglucanases, Beta glucosidases and Xylanases. In the present work, an intracellular soluble expression of exoglucanase was attempted in a prokaryotic expression host *E. coli* SHuffle. A gene *Cel7a* that codes for Exgolucanases/FPases was isolated from *Trichoderma resei* and ligated into the pRSET B vector. *E. coli* SHuffle strain was transformed with the plasmid vector. His-Tag metal affinity purification was performed after a fermentation that lasted for 3.5 hr using the recombinant *E. coli* Shuffle strain. Around 0.7 g/L of exoglucanases of 58 KDa molecular weight was obtained after the purification. The recombinant Exoglucanase had an activity of around 2.5 FPU/ml when assessed using the standard IUPAC Ghose assay for cellulase.

Keywords: Bioethanol; Fermentation; Cellulase; Vector; Prokaryotic expression.

Introduction

The use of ligno-cellulosic biomass for ethanol production is one of the principal methods to produce an efficient gasoline additive and to circumvent problems of depleting fossil fuels and emission of greenhouse gases [1-5]. The deconstruction of the cellulose polymer to fermentable monosaccharides requires the usage of an enzyme complex called cellulases. Cellulases comprises of endoglucanases/FPases, exoglucanases/CMCases and β-glucosidases/cellobiases.

Endoglucanases/CMCases cleave the internal β (1→4) bonds of cellulose followed by exoglucanases/FPases cleaving the cellulose into cellobiose units [1-4,7,9]. Cellobioses are cleaved to fermentable glucose by β-glucosidases/Cellobiases. Cellulase production is a very expensive process constituting around 50% of the total bioethanol production cost. There are many contemporary techniques to increase cellulase production of the native cellulolytic strains such as adaptation of cellulolytic microbes, protoplast fusion of high yielding fungi, protein engineering of the cellulosome complex, pathway engineering, and expression of heterologous cellulase coding genes in an expression host [4,5].

Genetic Manipulation of the native cellulase producers such as *T.resei* by the deletion of certain inhibitory genes has been performed. Recombinant hosts such as *E.coli* [6,11]. *Saccharomyces cereviseae, Klebisella oxytoca* expressing heterologous cellulases have been developed as well. [1,4,5,9].

The need of a recombinant enzyme arises due to the fact that using a recombinant system yields the product consistently with a higher degree of purity and activity in a lesser time span than the native strain. The production of cellulases usually requires a slightly longer period of cultivation [7]. In a cloned protein production system, the expression vector's choice and the expression strain's potential exerts a strict regulation over the enzyme production ambience. While producing cellulases within a reactor in a laboratory system, catabolite repression is observed. Moreover, the quantity of exoglucanases/FPases is lesser in comparison to the other two enzymes in the cellulase enzyme complex. In this work, we zeroed in on the production of recombinant

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Exoglucanases/FPases due to the mentioned reasons.

E.coli, the Gram-negative bacterium is the widely used recombinant expression host owing to its capability to quickly multiply and produce high density of recombinant proteins on cheaper growth sources [1,3,10,12]. Though *E.coli* has been employed widely, uncertainity prevails in the recombinant gene product expression in its fully folded active form [5,8]. Certain other problems also occur in *E.coli* such as slow multiplication of the host, formation of inclusion bodies (IB's), inactive non-functional protein and no expression at all.

FPase/Exoglucanase, being an eukaroyotic protein has several disulfide linkages which helps several structural and catalytic roles. The lack or non-formation of disulfide linkages may result in the non-folding of proteins or no activity of the expressed recombinant protein [14]. In *E. coli* the reducing nature of the intracellular cytoplasmic compartment provided by glutaredoxin and Thioredoxin pathways makes it impossible for such disulfide bond possessing proteins to fold and perform actively. Hence certain *E. coli* strains with genetic manipulations, such as the presence of an oxidizing environment provided by the disulfide bond isomerase within the cytoplasm permitting disulfide linkages have been developed. One such is the *Shuffle E. coli* expression strain procured from M/s New England Biolabs (NEB) used in the present work.

Materials and methods

Commercially available kits were used for certain procedures and the protocols followed were as suggested by the manufacturers. A Genomic DNA (34 Mb) isolation of *Trichoderma resei* was performed using Norgen Fungal genomic DNA isolation kit. Suitable Primers were designed and a PCR was performed to amplify the gene of interest *Cel7a* (1.5 Kb) [1,3,9,10]. Double restriction digestion of the gene and the plasmid pRSET B (2.9 Kb) from M/s Invitrogen was carried out using commercial *Xho1*and *EcoR1*enzymes. The prokaryotic expression host used was *E. coli* Shuffle procured from M/s NEB. The advantage of using a shuffle strain is that it permits the folding and disulfide bond formation for a protein within the cytoplasm of *E. coli*. Competent *E. coli* Shuffle from cells were prepared. The cells were transformed with the plasmids with the gene insert by a step of chemical transformation. The cells with the insert were selected on 100 ppm ampicillin agar plate. The insert was confirmed by a Lysate PCR of the culture using the same primers used for gene amplification. After the conformation of the insert, a fermentation of 1L volume was carried out in a Bioreactor (KLF advanced). 1 vvm of aeration was maintained. 1.5% (v/v) of inoculum was used. The media composition in the fermentor was as follows: Yeast Extract – 5 g/l, Soyapeptone – 10 g/l, Sodium chloride – 10 g/l, Dipotassium hydrogen phosphate – 6 g/l, Potassium dihydrogen orthophosphate – 3 g/l, Ammonium chloride – 1 g/l, Dextrose – 20 g/l, Magnesium Sulphate – 0.3 g/l

After 3 hrs when the OD at 600 nm of the culture was around 3, 1 mM of IPTG was used to induce the culture [13]. Samples were collected prior to induction and 3 hrs post induction. SDS PAGE was used to analyze the proteins. After 3 hrs post induction, the fermentor was stopped and the contents were harvested under sterile conditions. The broth was centrifuged and the dry cell pellets were collected and sonicated after resuspension in the M/s Novox 6x His tag sonication buffer. After sonication and centrifugation, the top layer containing the soluble fraction of the total intracellular proteins were collected [1,3,9,10]. A step of His Tag purification was performed using the 10 ml column with affinity resin from M/s Novox for the collected fraction of the proteins. The eluted proteins were the protein of interest. The eluted proteins were concentrated 10 times using a 10 KDa cut off membrane from M/s Sartorius. A buffer exchange was performed using M/s Zeba spin column and the proteins were suspended in Tris buffer pH 8.0.

Primer design and sequence

The primer designed was specific for the isolation of the *CEL7a/CBHI* (as it was formerly called) of *T.resei* (as available in the website of NCBI) gene. The annealing temperature was around 61 degree Celsius.

Forward Primer:

5-AATGCACTCGAGATGTCTAGAGTTGTGAAGTCGCT-3'

Reverse Primer:

5'-TGCGTAGAATTCCATGATGCCAGTCCGCGGTTGAC-3'

The restriction sites were of the enzymes *Xho 1* and *EcoR1*.

Results and discussion

The isolated genomic DNA was around 34 Mb as seen in fig. 1. pRSET B, the 2.9 Kb plasmid seen in fig. 2, was double restricted and digested using *Xho 1* and *EcoR* as shown in fig. 3. *Cel7a* gene was amplified using a PCR as seen in fig. 4 which was later double restricted digested using *Xho 1 and Ecor1* as shown in fig. 5. After PCR purification, the gene was ligated using T_4 DNA ligase. The plasmid along with the gene insert sized up to 4.4 Kb as seen in fig. 6.

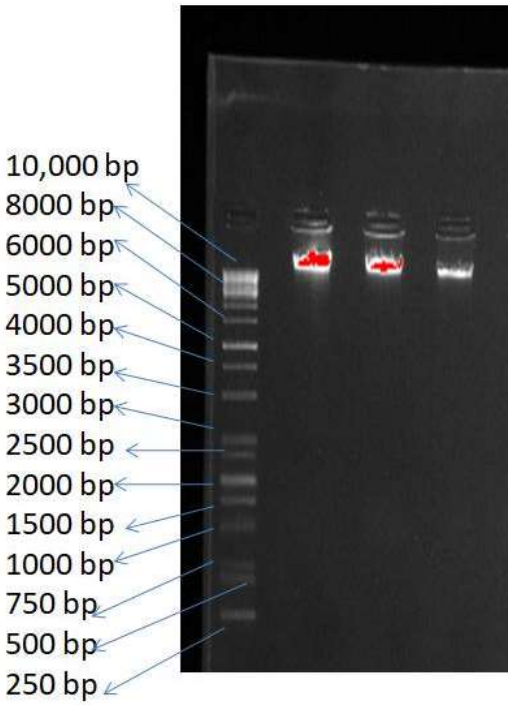

Fig. 1. Isolation of genomic DNA from *Trichoderma resei*: The size of the genomic DNA in lanes 2, 3, and 4 is more than 10 Kb as seen in the figure. DNA ladder was loaded in the lane 1 of the Agarose gel

A step of lysate PCR as seen in fig. 7 was performed using the previously designed primer. The presence of the gene insert was seen when a 0.8% agarose gel electrophoresis was performed and a band at the size of 1.5 Kb was obtained. *E.coli* shuffle strain has the capability to allow a protein to fold and form disulfide bonds within the cytoplasm, which is not the case with other conventional *E. coli* expression systems. The doubling time of the recombinant *E. coli* was around 30-40 minutes [19].

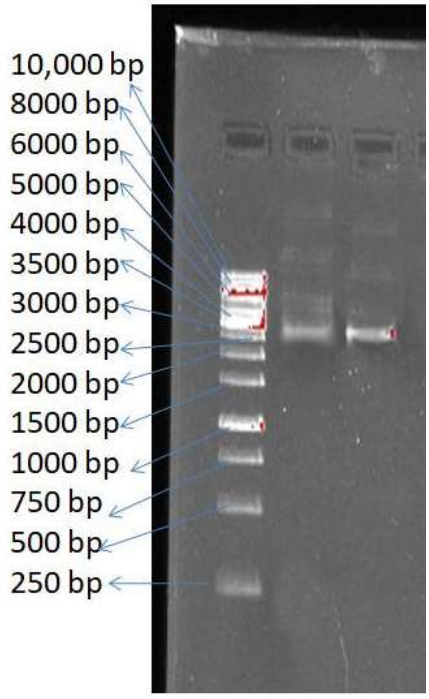

Fig. 2. Isolation of the plasmid 2.9 Kb pRSET B using a Qiagen Plasmid Isolation Kit: Lane 1 contains the DNA Ladder while Lane 2 and 3 contains the isolated pRSET B plasmid

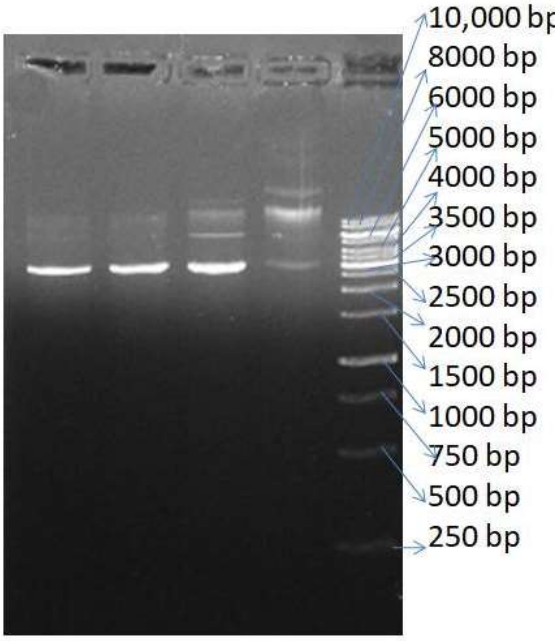

Fig. 3. Restriction and double digestion of the vector pRSET B using *Xho 1* and *EcoR1*.The digested vector is seen at 3.0 Kb: Lane 5 contains the DNA ladder while lanes 1-4 contain the double digested plasmid pRSET B

Fermentation was performed and 3 OD at 600 nm was attained in less than 3.5 hrs. Induction was performed at 3 OD at 600 nm to allow the maximum possible cell population to get induced and produce the required protein. A total cell pellet weight of 6.5 g/l was obtained at

the end of the fermentation process. The total intracellular protein content was around 2.6 g/l. His-tag purification of the soluble fraction of the intracellular protein yielded around 0.7 g/l of recombinant FPases.

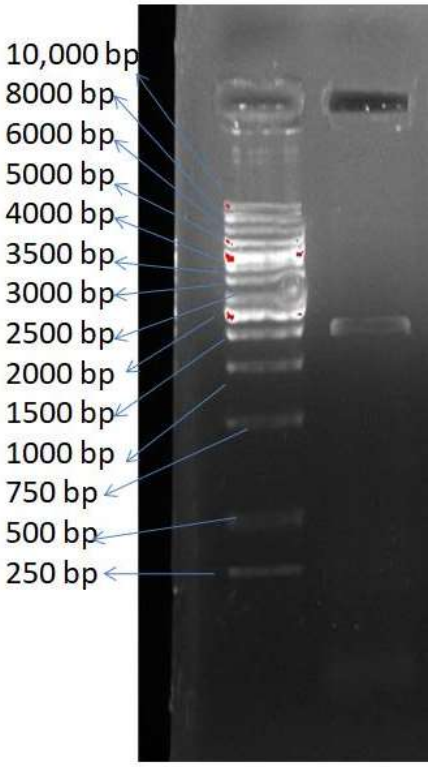

Fig. 4. The size of the amplified gene in lane 2 is around 1.5 Kb as seen in the figure. DNA ladder was loaded in the lane 1 of the Agarose gel

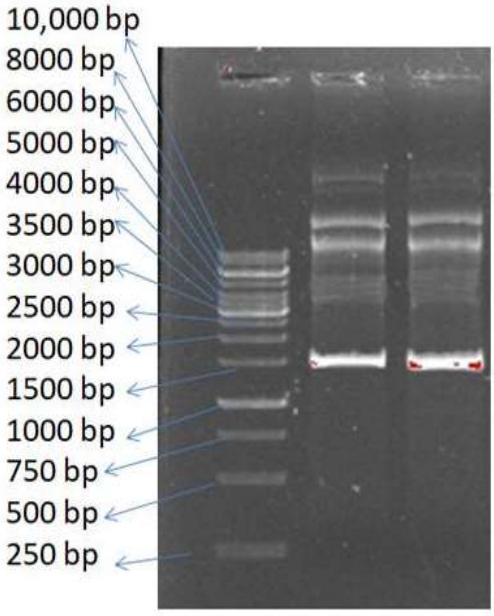

Fig. 5. Restriction double digestion of the gene *Cel 7a* amplicon using *Xho 1* and *EcoR1*: Lane 1 contains the DNA ladder while Lanes 2 and 3 contain the restricted and double digested gene product at 1.5 Kb size

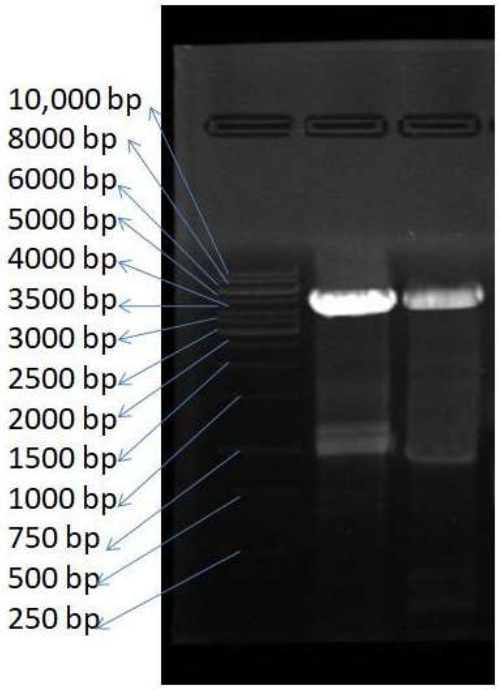

Fig. 6. Ligation of the vector pRSET B and the gene using DNA ligase: The ligated plasmid construct is seen having a size of 4 Kb. Lane 1 contains the DNA ladder while the lanes 2 and 3 contain the ligated vector product ready to be transformed into competent cells of *E.coli Shuffle*

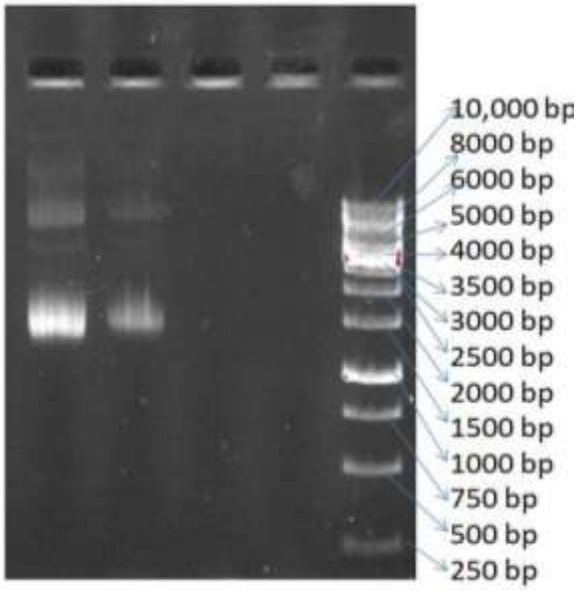

Fig. 7. Lysate PCR of the plasmid with the insert was performed: Lanes 1 and 2 show the presence of a 1.5 Kb within the plasmid vector confirming the presence of *Cel 7a*/ Exoglucanase gene within the plasmid. Lane 5 has the DNA ladder

SDS PAGE analysis showed that there were a lot of proteins in the *E. coli* apart from the induced FPases as shown in fig. 8. The

FPases constituted a major chunk of the produced proteins [6]. After His tag purification, a protein with a molecular weight of around 58 KDa was obtained as seen in fig. 9. The slight increase in the molecular weight in comparison to the actual molecular weight of FPases could be attributed to the presence of an uncleaved Histidine tag. The tag removal was not concentrated as the tag does not hinder the protein activity. IUPAC Ghose assay for cellulase yielded an activity of around 2.5 FPU/ml which is slightly higher than the activity usually obtained per mL using *T. resei* in a submerged fermentation [17].

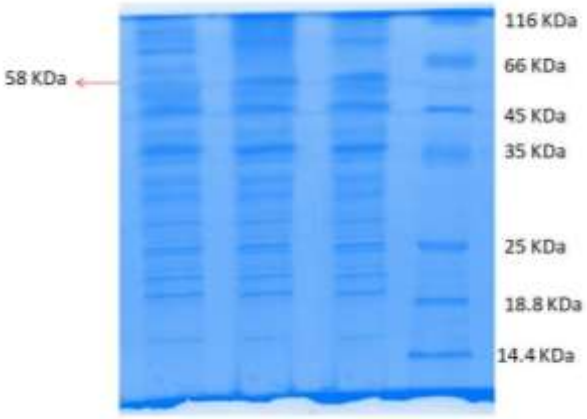

Fig. 8. Lanes 2 and 3 shows a distinct band at 58 KDa which show that 2nd and 3rd hour post induction, the protein is expressed well. A faint band at 58 KDa in Lane 1 shows that a minute fraction of protein is produced just after induction. Protein molecular weight marker is seen in Lane 4

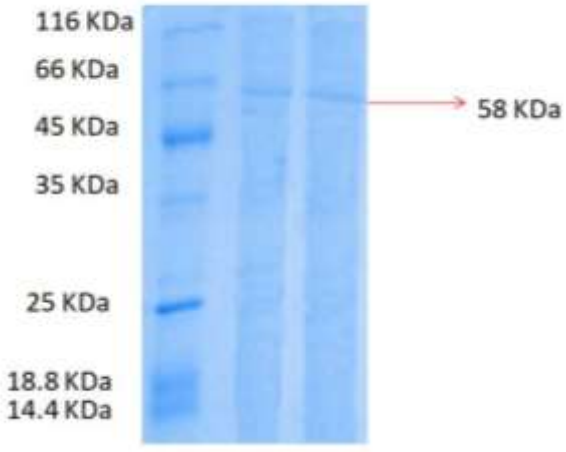

Fig. 9. After a procedure of His Tag purification, single bands of 58 KDa are seen in the lanes 2 and 3, Lane 1 contains the molecular weight marker

The necessity to produce a recombinant enzyme may have several reasons that make the cultivation and harvest of the enzyme tougher using the native enzyme producing strain. The major reason with respect to cellulases is the cultivation time. Using native cellulase producing fungi, it takes around 3-5 days on an average to produce cellulases [7]. In our process employing *E. coli Shuffle* a time of 3-3.5 hours is required to produce cellulases with activities similar to the activity produced using native strains. To separate the enzymes from the rest of the extracellular proteins, it takes a tedious downstream process which is in contrast to the recombinant *E. coli Shuffle* where a single step of His tag purification gives proteins that are relatively pure. Another major concern while cultivating the cellulolytic fungus is the mass transfer limitation that occurs due to the broth's dilatants characteristic after a 48-72 hrs period. The mycelia of the fungi grow to enormous proportions making agitation and aeration an issue. If agitation is increased to force oxygen to reach the bottom layers of the mycelia, the impellers tend to break the mature mycelia thereby hampering enzyme production [18].

Aeration increased by increasing the inlet air in the sparger may cause excessive foaming which would hamper the reactor operation. In the present work using a recombinant cellulase producing strain, foaming and aeration problems don't cause a major drawback as bacterial fermentation systems such as this require standardized simple parameters to maintain optimum productivities. Inconsistencies may be observed between various batches of the same organism. Cellulases are produced by certain native cellulolytic fungi such as *Trichoderma*, *Aspergillus* specie. The problem arises when we try to cultivate them in a reactor using an insoluble cellulose substrate. When cellulose is converted to cellobiose and glucoses by the produced cellulases within the submerged fermentation reactor [1-4,7,9,10] there occurs a phenomenon called catabolite repression, where the organism ceases cellulase production or shows a very minute increment in the production of cellulases. In this phenomenon, the fungus chooses to utilize the easily metabolizable substrate glucose or cellobiose instead of the tough insoluble cellulose thereby showing no or very little cellulase secretion. To produce cellulases of sufficient quantity from the fungi, a

stress level needs to be maintained within the reactor to enable a higher level of circulating cAMP's [5].

However, in the recombinant *E. coli Shuffle* strain developed in this work there are no such catabolite repression issues that hamper enzyme production and cause batch to batch variability. The production is defined and varies very minutely among consecutive batches. Contemporary Researchers have been looking to develop a strain that yields higher cellulases irrespective of the environmental and substrate conditions [20]. Among the three significant enzymes of the cellulase complex, Exoglucanase/FPase seems to be the least produced. The Exoglucanases are principal in hydrolyzing the longer cellulose polysaccharide to cellobioses which are then acted upon by cellobiases or beta-glucosidases to get hydrolyzed to individual beta-D-glucose units [1-4,7,9,10]. Our focus laid in producing exoglucanses in a recombinant host with a slightly higher enzyme activity than the native host.

When a eukaryotic protein is attempted to be produced in a prokaryotic host, there are possibilities that the protein may not fold properly within the cytoplasm due to the reducing nature [3,14]. Moreover certain important bonds such as disulfide bonds may not form within the cytoplasm of the host [14]. Though there are reports showing successful expression of such eukaryotic functional proteins within the cytoplasm [9,10,15] the activity of such recombinant proteins may be lesser. Signal sequences may be used in some cases of prokaryotic expression where a signal sequence may direct the protein to the periplasm of the expression host. However, the usage of a strain such as Shuffle helps in assisting the folding of the protein within the cytoplasm compromising the reducing environment within the expression host *E. coli*. The strain has a chromosomally integrated signal sequences-devoid DsbC, an oxido reductase chaperone which permits proper disulfide bond formation [14]. Researchers who compared the periplasmic expression of the proteins and cytoplasmic expression with the Shuffle strain showed that expression in the Shuffle strain is slightly higher. Glycosylation of the cellulases confers stability and defense to proteolytic degradation. However, the expression of an eukaryotic cellulase in a prokaryotic expression host without glycosylation may be prone to proteolytic degradation, nevertheless the activity being conserved as observed in the analysis the doubling time of the recombinant *E. coli* is very less around 3 hours when compared to the native strain that produces FPases with similar activities. It takes 3-4 days to produce FPases with activities around 1.9 FPU/ml.

Conclusions

The present work was performed with an attempt to produce a recombinant FPase producing strain. The FPase coding gene isolated from *T. resei* was cloned and expressed in a prokaryotic expression host *E. coli* Shuffle. The protein was produced as a soluble and functional protein. The total enzyme activity of the protein was around 2.5 FPU/ml. The time taken to produce the 0.7 g/l of 58 KDa molecular weight proteins was around 3 hrs in comparison to the production of the same protein from a wild *T. resei* strain. Hence the method is time saving and the operation is economical in comparison to the expensive substrate and processes used in the production of FPases from the wild strain.

Conflicts of Interest

The authors declare no conflict of interest.

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