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Research Article

Expressed Cellobiohydrolase Enzyme of *Thermobifidia fusca* in *Pichia pastoris* as Host Can Act on Cotton Substrate

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Abstract

Cellulose which is extremely produced by plants, can be used for biofuel production but this function needs chemical or enzymatic digestion. Cellulose hydrolysis of plant wastes for ethanol production requires a mixture of three enzyme groups, including endoglucanases, exoglucanases, and beta-glucosidases. The cellobiohydrolase enzyme (Cel6B) from *Thermobifidia fusca* has been used for cellulase activity extensively. This research aimed to express recombinant Cel6B enzyme in *Pichia pastoris*. For this purpose, *cel*6B gene in control of AOX1 promoter (methanol inducible) was introduced into *Pichia pastoris.* Amplification of *cel6B* gene was performed by PCR technique and then introduced into the Phil-S1 yeast vector. The recombinant construct contained the *cel6B* gene sequence and PHO1 signal sequence as secretion signal was transferred into *Pichia pastoris* GS115 strain. The transformed yeast cells expressed the recombinant Cel6B to yield 2.104 U (µmol/min)/ml of culture medium. Purified recombinant enzyme showed the best activity at 60 °C and pH 4.5 and this was agreed with optimum conditions for recombinant Cel6B enzymes which were produced in other systems. The purity of the enzyme was examined by SDS–PAGE technique, and a single band with a molecular weight about 59.6 kDa was observed. As *cel6B* gene sequence was not optimized for expression in the *Pichia pastoris* yeast, this could be one of the reasons for low level activity of recombinant Cel6B enzyme. This thermostable enzyme can be used for cellulolytic digestion of biomaterials in biofuel production research and other uses.

***Keywords*:** Cellulose, Cel6B, yeast, AOX1 promoter, Expression

Introduction

Cellulose produces by plant photosynthesis and can be used as a renewable biological material. Cellulose biodegradation by cellulase enzymes or cellulolytic microorganisms produces CO_2 and CH_4 in nature. Main cellulase enzymes include endoglucanases (EC 3.2.1.4) that make free ends on cellulose chains; cellobiohydrolases or CBHs (EC 3.2.1.91) that degrade cellulose chains from free ends and produce cellobiose units and β-glucosidase (EC 3.2.1.21) that hydrolyze cellobiose to glucose (Zhang et al. 2009). All of these enzymes are necessary for the hydrolysis of crystalline cellulose (Sun and Cheng, 2002; Kazzaz and Fatehi, 2020; Xiao et al. 2019).

Thermobifida fusca is an anaerobic bacterium and it hydrolyzes the cellulose using synergistic cellulase activities to supply its sole carbon source. Cel6B enzyme (a cellulase enzyme) is necessary for the function of this synergistic hydrolysis, therefore its mobility usually is weak on the substrates. Cel6B is an exocellulase and cuts β-glycosidic bonds from the end of cellulose chains (Vuong and Wilson, 2009; Wu et al. 2013; Gomez del Pulgar and Saadeddin, 2014). More amounts of this valuable enzyme could be obtained by expressing *cel6B* gene in non-original hosts such as yeast cells. *Pichia pastoris* was a desirable host for producing of CBH enzyme from *Trichoderma reesei*. This host can secrete the recombinant enzyme into the culture medium due to secretion signal peptide that attached to the protein, can do protein folding and post translational modification for normal function of recombinant proteins (Fang and Xia, 2015; Fang et al. 2019).

Zhang *et al*. (2018) expressed GHF9 (an endoglucanase enzyme) from *Reticulitermes speratus* microorganism in *P. pastoris* and

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recombinant enzyme showed suitable stability at pHs between 4.0 and 11.0 and temperatures lower than 40℃ in RsEGm mutant (Zhang et al. 2018; Zhang et al. 2020). Cellobiohydrolase II from *Trichoderma reesei* was expressed in *P. pastoris* and exhibited the highest activity at pH 5.0 and 50℃ temperature (Fang and Xia, 2015). Various enzymes for cellulose and hemicellulose degrading such as xyloglucanases, exoglucanases, and xylanases have been produced in *P. pastoris* (Tenkanen et al. 2012; Sun et al. 2016). *P. pastoris* can produce high levels of heterologous proteins. It is possible to obtain up to 12 g/L of recombinant proteins in high biomass *P. pasroris* fermentations (Lindenmuth and McDonald, 2011; Srivastava et al. 2018).

In this study, an exocellulase gene, *cel6B*, from filamentous soil bacterium *Thermobifida fusca*, was expressed in *P. pastoris*. In the following, optimum conditions (pH and temperature) were determined for recombinant enzyme.

Materials and Methods

Vector construction and transformation

We used the CDS of *cel6*B gene presented in plasmid pSZ143 gifted by Dr. Wilson from California University. Gene amplification performed by PCR technique, and primers designed by PrimerBlast software. The following primer pairs were used, Cel6B F (5′-TCCATACGAATTCGCCGGCTGCTCG-3′; the underlined sequence was *Eco*RI site) and Cel6B R (5′-TCACTCCGGGATCCAGAGGCGGGTA-3′; the underlined sequence was *BamH*I site). PCR was performed for 35 cycles by Pfu DNA polymerase (Fermentase Company): Cycles programmed as 95°C for 1 min (denaturation step), 60°C for 40 s (annealing step), and 72°C for 2 min (extension step), and 72 °C for 5 min to final extension. PCR products were digested using *Eco*RI and *BamH*I, and DNA fragment purified by NaOAc (1 M) and ethanol method. Yeast plasmid pHIL-S1 was also digested using of *Eco*RI and *BamH*I, and then purified (Table 1). Cloning of *cel6*B gene in pHIL-S1 plasmid was done by T4 ligase enzyme (Table 2), and the recombinant plasmid was transferred into *Escherichia coli* DH5α. Successful transformation was confirmed by DNA sequencing (Macro Gene Company). Figure 1 showed the recombinant plasmid pHIL-S1 containing *cel6B* gene.

The *cel6B* gene was ligated to the pHIL-S1 vector along with its PHO1 sequence, a signal sequence. The recombinant vector was amplified in the *E. coli* DH5α strain. The expression construct, PhilS1-Cel6B was extracted from *E. coli* and linearized by *Stu*I restriction enzyme according to manufacture protocol (Fermentase). The linearized plasmid was transformed into *P. pastoris* GS115 strain by electroporation technique (Agilent Technologies). The linearized recombinant DNA (2 µg) was used for electroporation using an electroporator Gene PulserXcell™ (BIORAD). The mixture (DNA and yeast cells) was transferred into a pre-chilled electroporation cuvette (2 ml) and incubated on the ice for 5 min. The voltage and time constant were, 2kV and 5 milli-seconds, respectively. After electroporation, 1 mL of ice-cold sorbitol (1M) was added to the cuvette.

Screening of transformed *P. pastoris* colonies

P. pastoris culture media included BMGY, BMMY, MD, MM and, YPD (Invitrogen protocol, Invitrogen | Thermo Fisher Scientific – US). According to the Invitrogen instructions, if the recombinant construct is linearized with StuI enzyme and transferred to *P. pastoris* yeast cells GS115 strain. Although most transformants must be Mut $^{+}$ phenotype in the presence of AOX1, recombination is likely to occur at the AOX1 site and disrupt the wild AOX1 gene and resulting in His^{+} Mut^{S} transformants. Therefore, the test was performed on minimum dextrose (MD) and minimum methanol (MM) media. Mut^{+} phenotype colonies could grow on both culture media, and the Mut^{S} phenotype colonies, would only grow on MD medium and not on MM medium.
After the transformation process, cells were transferred onto MD and MM media. A sterile toothpick was used for picking one colony from MD medium, and streaking or patching on MM and MD media on the same pattern of primary plate. Mut^{+} transformants grew well on both MD and MM plates, but Mut^{S} transformants grew well only on MD plates, and show little or no growth on the MM plates (http://tools.thermofisher.com).

Confirmation of *P. pastoris* transformants

The genomic DNA of *P. pastoris* was extracted using Volossiouk et al. (1995) method. The insertion of gene cassette into the yeast genome was confirmed by PCR technique using AOX1 primers, forward primer (5′-GACTGGTTCCAATTGACAAGC-3′) and, reverse primer (5′- GGCAAATGGCATTCTGACATCCT-3′). PCR was programmed as 94℃ for 5 min, 30 cycles of 94℃ for 1 min, 60℃ for 1 min, 72℃ for 2 min, and final extension at 72℃ for 5 min.

Recombinant soluble protein detection

One recombinant colony was transferred into a 100 mL BMGY medium (http://tools.thermofisher.com) and incubated at 30℃ overnight. Then, the whole culture medium was centrifuged at 3000×g for 5 min, then supernatant removed and pellet transferred into 100 mL BMMY medium (http://tools.thermofisher.com) until OD_{600} = 1. Methanol (0.5% (v/v) was added to the medium every 24 h, the culturing was carried out at 250 rpm and 30℃ for 96 h. The content of soluble protein was determined using Bradford reagent (Sigma Aldrich). Protein standard curve was determined using serial concentrations of bovine serum albumin. Then the standard curve was used for quantifying soluble protein content produced by different transformed yeast colonies. Absorbancies of all samples were read at 595 nm.

Cellobiohydrolase assay

Phosphoric acid-treated cotton (2.5 mg/ml) as PC was utilized as the substrate of enzyme reactions. PC was prepared according to Vuong and Wilson method (Vuong and Wilson, 2009). The reaction mixtures were prepared at one ml volumes (three replications) in 50 mM sodium acetate buffer (pH 5.5) and maintained at 50°C for 16 h. Reducing sugar concentrations were measured using phenol-sulfuric acid reagent (PAS) and a standard curve of glucose. Enzyme activities of different recombinant colonies were determined and expressed as a percentage of activity. One unit of enzyme activity is defined as the content of an enzyme that releases one µmol of reducing sugars per milliliter for one minute.

SDS-PAGE

Recombinant enzyme was detected by SDS-PAGE technique (Laemmli, 1970). SDS–PAGE was performed on the precast 12% polyacrylamide gel (Bio-Rad). Polyacrylamide gel was rinsed twice by water after electrophoresis, and stained by Coomassie blue.

Recombinant enzyme properties

The pH range of 3.5-7.5 was tested for determining the optimum pH of CBH activity. pH adjustment was done by 50 mM sodium acetate buffer (NaOAc buffer pH 5.5) solution (pH 3.5-6) and 50 mM potassium phosphate buffer (pH 6-7.5). The optimum temperature of CBH activity was determined by enzyme assays at the temperature range of 40℃ to 60℃ with 10 ° C intervals using 50 mM NaOAc buffer solution, pH 5.5.

Table 1. Reaction compounds of pHIL-S1 plasmid digestion.

Component	Volume (µl)	Concentration in final reaction
pHIL-S1 vector	6	9 µg
Tango 10X buffer	6 of 10X	2X
*Eco*RI enzyme	1	1 (U/µl)
*Bam*HI enzyme	1	1 (U/µl)
Distilled water	16	Up to 30 µl

Table2. Ligation reaction of *cel6*B gene subcloning into the pHIL-S1 vector

Component	Volume (µl)
digested and purified gene fragment (1673 bp. 15.2 ng / µl)	8
Digested and purified pHIL-S1 vector (20 ng / µl)	2
T4 ligase enzyme buffer (10X)	2
T4 DNA Ligase enzyme (5U / µl)	1
Distilled water	7

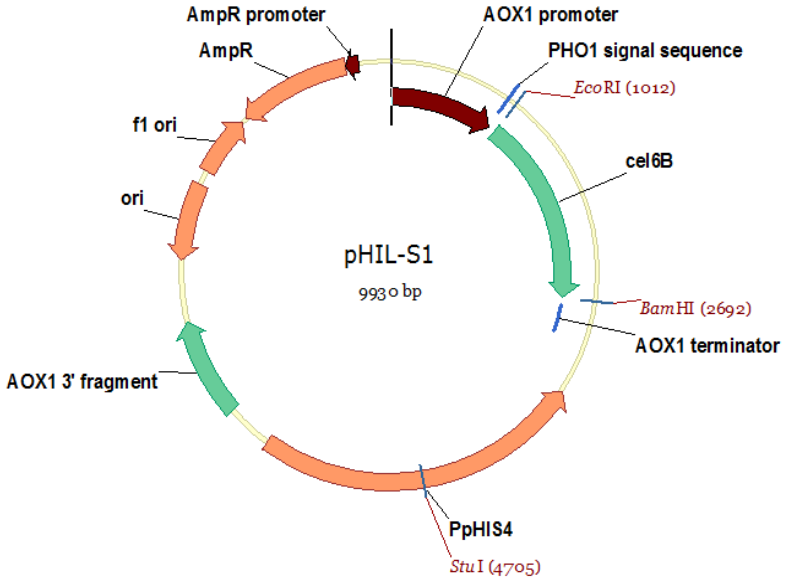

Figure 1. Recombinant pHIL-S1 plasmid containing *cel6B* gene, (pHIL-S1: *P. pastoris* vector for expression of a secreted recombinant protein).

Results and Discussion

Construction of the Cel6B expression vector

The *cel6B* gene was cloned into the vector pHIL-S1 to obtain a good expression. At first, we cut plasmid pHIL-S1 using *EcoR*I and *BamH*I enzymes for transferring CDS of Cle6B gene to this vector. Enzymatic digestion of the vector was confirmed by gel electrophoresis method, and the size of digested and uncut pHIL-S1 vectors was compared on agarose gel (Figure 2). The *cel6B* gene (a 1.6 Kb fragment) was observed on the agarose gel, that indicated the expression vector was correctly constructed. For testing the successful ligation, the expression cassette was digested with *Stu*I restriction enzyme, and linearized and uncut recombinant vectors were compared on the agarose gel (Figure 3).

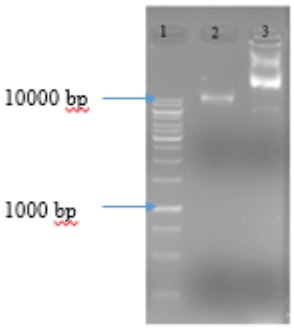

Figure 2. Confirmation of recombinant pHIL-S1 construction. Lane 1: 1 kb DNA size marker (Fermentas), Lane 2: pHIL-S1 vector was cut using *Eco*RI and *BamH*I restriction enzymes, Lane 3: uncut pHIL-S1.

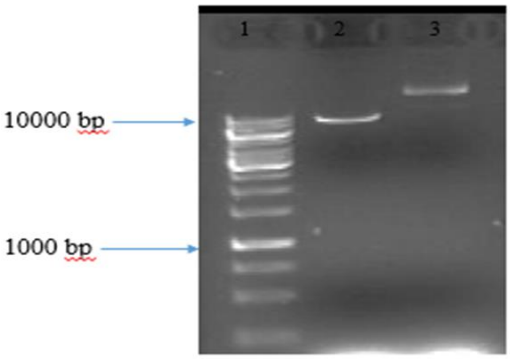

Figure 3. Linearization of recombinant pHIL-S1 vector. Lane 1: 1 kb DNA size marker. Lane 2: linearized recombinant pHIL-S1 containing *cel6*B gene. Vector was linearized by *Stu*I restriction enzyme, Lane 3: Uncut recombinant pHIL-S1 plasmid.

Electroporation and the primary screening

The linearized recombinant DNA, pHIL-S1-Cel6B, was transferred into *P. pastoris* GS115 host using electroporation technique. Then yeast cells were transferred onto the MD plate, and transformed colonies were grown. These colonies were able to produce and secret the Cel6B recombinant protein. These transformants had different capacities for enzyme production because of varied gene

integration modes and gene copy numbers (http://tools.thermofisher.com). It has been shown that increasing the copy numbers up to more than 10 copies, could increase protein expression (Sun et al. 2016). Figure 4 shows *Pichia pastoris* colonies after transformation step on two different MD and MM media. These cultures were prepared to identify rapid-growth yeast colonies and strains.

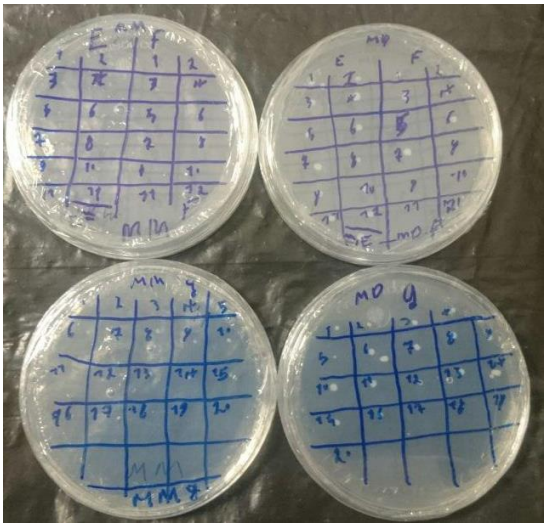

Figure 4. Colonies of *P. pastoris* after transformation step on two different media, MD (right) and MM (left).

Confirmation of transformants

The transformants that had grown on the MM and MD plates were Mut$^+$ GS115 strains. These colonies were used for PCR confirmation (Figure 5). Figure 5, lanes 1 and 2, showed the PCR products amplified from the genomic DNAs of yeast transformants using specific primers of *cel6B* gene and lanes 4 and 5 showed the PCR products amplified from the genomic DNAs of yeast transformants using *AOX1* primers. Figure 5 confirmed the integration and stability of *cel6B* gene into the yeast genomic DNA, sometimes genetic traits might be lost from one generation to the next (Sun et al. 2016).

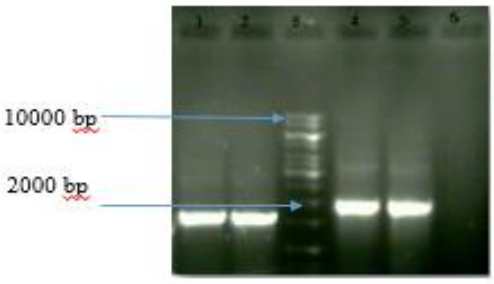

Figure 5. PCR products of *P. pastoris* transformants carried the expression construct containing *cel6*B gene. Lane 1 and 2: PCR products using specific primers of *cel6*B gene, Lane 3: 1kb DNA size marker. Lane 4 and 5 PCR products using AOX1 primers, Lane 6: negative control.

Soluble protein content of transgenic yeast cells

After transferring the construct into the yeast cells and confirming it by PCR technique, selected recombinant colonies containing AOX1 must produce recombinant enzyme by inducing the colonies in the induction medium. Since this yeast plasmid has a secretory signal, the protein must be secreted into the yeast culture medium. For this purpose, transgenic colonies were induced in BMMY induction medium for 4 days. Sampling was performed every 24 hours and the best day and strain were determined in terms of protein production, using Bradford method. A BSA standard curve with R^2 = 0.98 and line equation y = 0.1513x + 0.5484 was obtained by Excel software (Figure 6). Then the amount of produced protein was calculated and the results showed that colony 4E, showed the highest protein production among the transgenic colonies, 24 hours after induction. Figure 7 shows the Bradford assay results of two colonies.

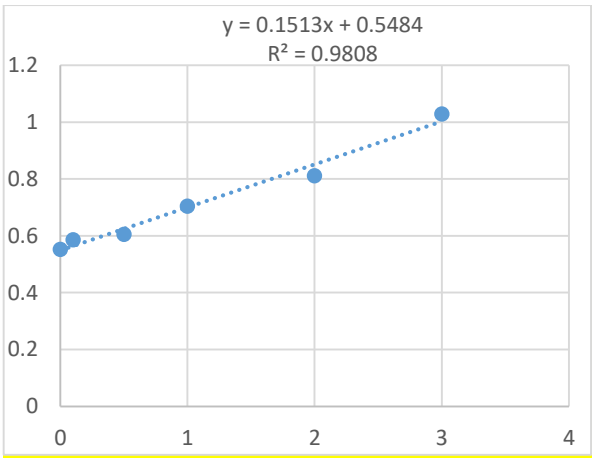

Figure 6. Bovine serum albomin standard curve

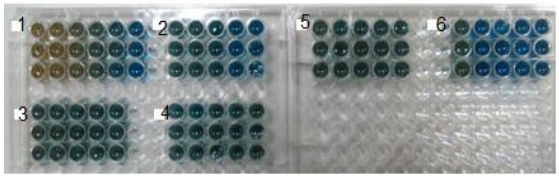

Figure 7. Bradford assay of yeast colonies. (1) BSA, (2) non-recombinant *P. pastoris* GS115 colony, (3) non-induced recombinant colony 7A, (4) non-induced recombinant colony 4E, (5) induced recombinant colony 7A, and (6) induced recombinant colony 4E.

Statistical analysis was performed using JMP 8 software for 4E and 7A strains and their controls which were non-transgenic colonies: 4eni, 7ani, and gs115 non-transgenic colonies. The mean comparison was performed at 1% significance level. As shown in Figure 8, 4E colony had the highest protein production after 24 hours of induction and was significantly different from other colonies.

Screening recombinant yeast colonies with high activity

Multi-copy yeast colonies were screened to determine the expression level of Cel6B enzyme (Figure 8). Three colonies were cultured on a BMMY medium and induced by 0.5% methanol (v/v) for 96 h to detect the Cel6B activity. Colony-4E (Figure 8) showed a slightly higher CBH activity (0.11 U/mL). Therefore, Colony-4E was selected for the next phase of the experiment. SDS-PAGE analysis of the Colony-4E culture medium (a 72 h-culture) confirms the expression of Cel6B protein (Figure 9). A protein band with 59.6 kDa molecular weight can verify the Cel6B expression.

Enzymatic properties of recombinant Cel6B

The effects of different pHs and temperatures were studied on the activity of recombinant Cel6B on microcrystalline cellulose (Sigma Aldrich) (Figures 10 and 11). pH 4.5 and 60℃ temperature were determined as the optimum pH and temperature for Cel6B enzymatic activity, respectively. There were no significant differences in enzyme activity levels at 40 and 50℃, and higher activity was observed at 60℃.

Optimal temperature of fermentation process can play a role in mRNA synthesis regulation of cells. Temperature can affect extracellular enzyme secretion by changing the physical properties of the cell membrane (Abd Rahman et al., 2004).

The level of enzyme secretion can directly affect the amount and activity of the enzyme (Murao et al. 1988). Lu et al. (2003) reported that the optimum temperature for enzyme production varies from one microorganism to another. Rai et al. (2012) reported that the optimal activity of cellulase enzyme in *Candida* yeast is about 50 °C. Taha et al. (2015) has stated that the cellulase enzyme activity of *Trichoderm viride* is at the highest levels at 50 °C.

It has been reported that 45°C and pH 4.5 led to the highest activity of cellulase enzyme (Alami et al., 2017). The effects of pH on the enzyme activity levels is due to changing of ionic charge of the enzyme active site and thus changing the conformation of the active site.

Taha et al. (2015) observed the high activity of cellulase enzyme from *Trichoderma viride* at pH 6. Harshvardha et al. (2013) stated that the highest activity of cellulases could be observed at pH between 3 and 9.

Various hosts can be used for expression of recombinant proteins. *P. pastoris* is a better host than bacteria, fungi or plants. This species has advantages such as a strong AOX1 promoter and the ability of protein secretion. In this study the codon optimization was not done for *cel6B* gene, so the expression was low. Therefore, in following this change should be done. Moreover, Cel6B of *T. fusca* was expressed in *E. coli* but its product was limited to the outer membrane and unable to be excreted (Zhang et al. 2018). As a result, *P. pastoris*, could be a good alternative for producing enzymes such as CBH due to simple separation and purification.

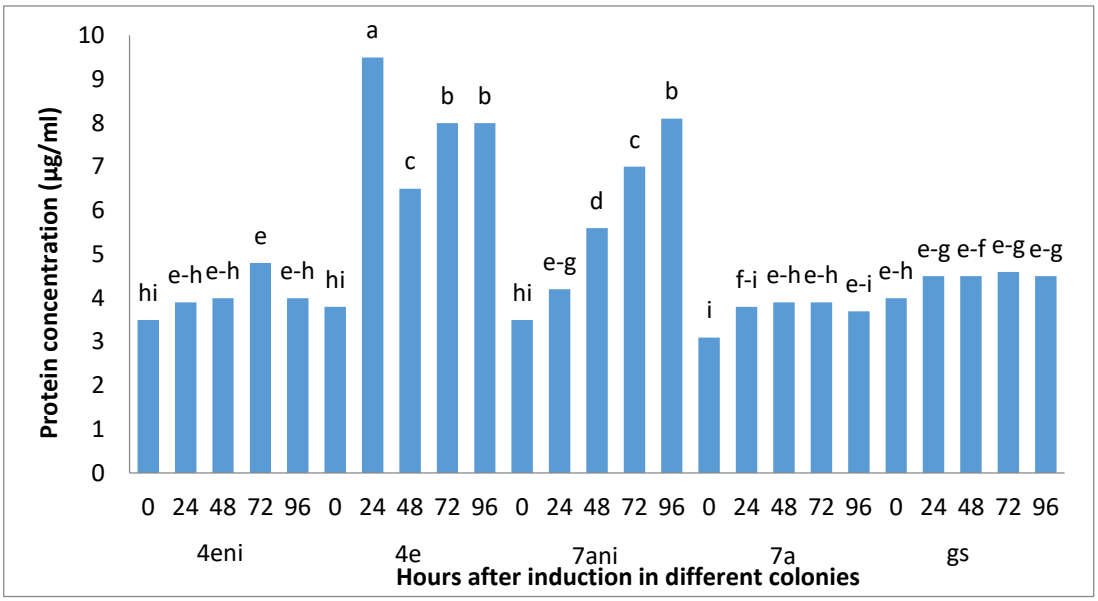

Figure 8. Protein production (µg/mL) by *P. pastoris* strains 4E, 4eni, 7A, 7ani, and GS115 (gs) during 96 hours of induction, columns with the same letters showed no significant differences (α = 5%).

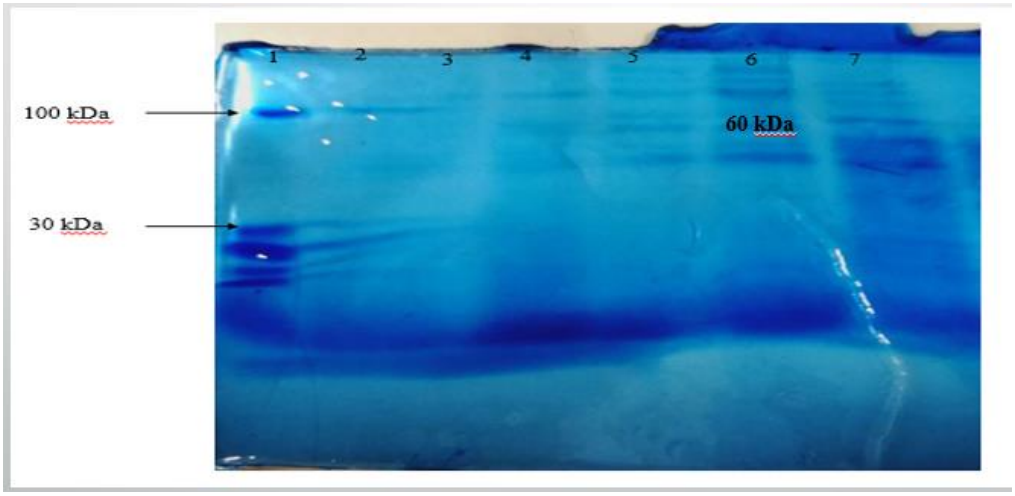

Figure 9. SDS-PAGE analysis of the culture medium of recombinant *P. pastoris*. Lane 1: protein size marker, Lane 2 and 3: culture medium of control *P. pastoris* colonies, Lane 4 to 7: Cel6B protein band. All samples were collected at 72 hours during fermentation.

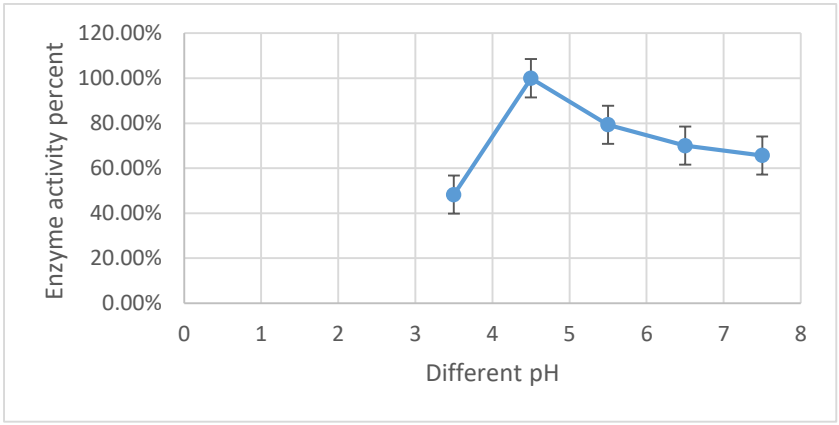

Figure 10. Determination of optimum pH for Cel6B enzyme activity expressed in P. pastoris yeast cells. The activity of recombinant Cel6B enzyme was assayed at 50℃ and various pHs (3.5-7.5). Cel6B enzyme showed the highest activity at pH 4.5.

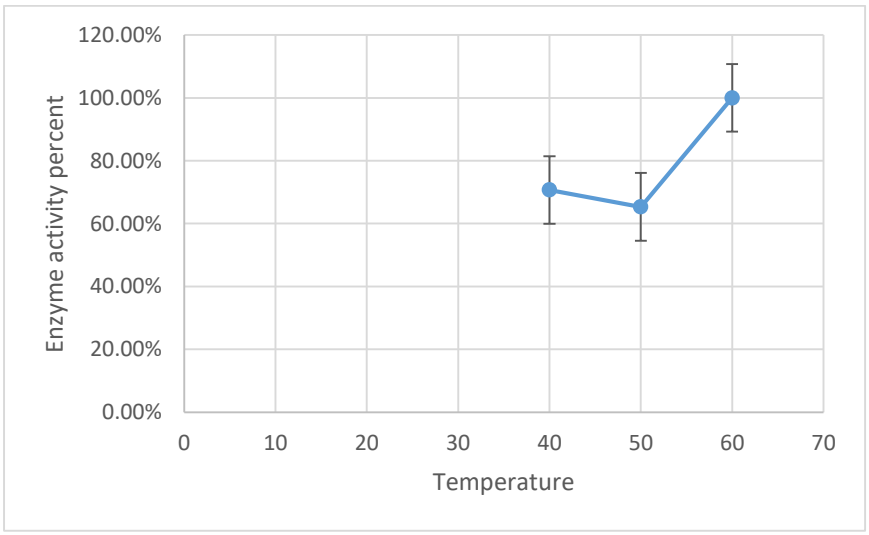

Figure 11. Determination of optimum temperature for Cel6B enzyme activity expressed in *P. pastoris* yeast cells. The activity of recombinant Cel6B enzyme was assayed at pH 4.5 and various temperatures (40-60℃). Cel6B enzyme showed the highest activity at 60℃.

Conclusion

Here we report the production of *T. fusca* cellobiohydrolase in *P. pastoris* yeast cells. The strain GS115 (high methanol-utilizing) showed the highest yield of Cel6B as 2.104 U(µmol/min)/ml culture medium. The optimum temperature and pH were 60℃ and 4.5, respectively, which coincide with similar research. The enzyme purity was studied using SDS–PAGE technique, and a single band was observed with a molecular weight of 59.6 kDa. AOX1 promoter is inducible by methanol, therefore we could have a rapid and easy cloning selection and controlled expression. This thermostable enzyme can be used in future research after codon optimization and other optimal conditions.

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Conflict of Interest: The authors declare that they have no conflict of interest.

References

Abd Rahman R. N. Z., Geok L. P., Basri M. and Saleh, A. B. (2004) Physical factors affecting the production of organic solvent-tolerant protease by

Pseudomonas aeruginosa strain K. Bioresource Technology 96:429-43

Alami N. H., Kuswytasari N. D., Zulaika E. and Shovitri M. (2017) Optimization of cellulase production by Candida G3.2 from the rhizosphere of gunung anyar mangrove surabaya. Proceeding of International Conference on Green Technology 8 (1):399-406.

Fang H. and Xia L. (2015) Heterologous expression and production of *Trichoderma reesei* cellobiohydrolase II in *Pichia pastoris* and the application in the enzymatic hydrolysis of corn stover and rice straw. Biomass and Bioenergy 78:99-109.

Fang H., Zhao R., Li C. and Zhao C. (2019) Simultaneous enhancement of the beta–exo synergism and exo–exo synergism in *Trichoderma reesei* cellulase to increase the cellulose degrading capability. Microbial Cell Factories 18(1):1-14.

Gomez del Pulgar E.M. and Saadeddin A. (2014) The cellulolytic system of *Thermobifida fusca*. Critical Reviews in Microbiology 40(3):236-247.

Harshvardhan K., Mishr A. and Jha B. (2013). Purification and characterization of cellulase from a marine Bacillus sp. H1666: a potential agent for single step saccharification of seaweed biomass. Journal of Molecular Catalysis B: Enzymatic 93:51-56.

Kazzaz A. E. and Fatehi P. (2020) Technical lignin and its potential modification routes: a mini-review. Industrial Crops and Products 154:112732.

Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680-685.

Lindenmuth B. E. and McDonald K. A. (2011) Production and characterization of *Acidothermus cellulolyticus* endoglucanase in *Pichia pastoris*. Protein Expression and Purification 77(2):153-158.

Murao S., Sakamoto R. and Arai M. (1988) Cellulase of *Aspergillus aculeatus*. In methods in Enzymology. Academic Press. London, 274-299 pp.

Lu W., Li D. and Wu Y. (2003) Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solid-state fermentation by *Aspergillus sulphurous*. Enzyme and Microbial Technology 32:305-311.

Rai P., Tiwari S. and dan Gaur R. (2012). Optimization of process parameters for cellulase production by novel thermotolerant yeast**.** BioResources 7:5401-5414.

Srivastava N., Srivastava M., Mishra P. K., Gupta V. K., Molina G., Rodriguez-Couto S., et al. (2018) Applications of fungal cellulases in biofuel production: advances and limitations. Renewable and Sustainable Energy Reviews 82:2379-2386.

Sun F. F., Bai R., Yang H., Wang F., He J., Wang C., et al. (2016). Heterologous expression of codon optimized *Trichoderma reesei* Cel6A in *Pichia pastoris*. Enzyme and Microbial Technology 92:107-116.

Sun Y. and Cheng J. (2002) Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technology 83(1):1-11.

Taha, A. S. J., Taha, A. J., & Faisal, Z. G. (2015). Purification and kinetic study on cellulase produced by local Trichoderma viride. Nature and Science, 13(1), 87-90.

Taha, A. S. J., Taha, A. J. and Faisal, Z. G. (2015) Purification and kinetic study on cellulase produced by local *Trichoderma viride.* Nature and Science 13:87-90.

Tenkanen M., Vršanská M., Siika-aho M., Wong D. W., Puchart V., Penttilä M., et al. (2012) Xylanase XYN IV from *Trichoderma reesei* showing exo- and endo-xylanase activity. FEBS Journal 280(1):285-301.

Volossiouk T., Robb E. J. and Nazar R. N. (1995) Direct DNA extraction for PCR-mediated assays of soil organisms. Applied and Environmental Microbiology 61(11):3972-3976.

Vuong T. V. and Wilson D. B. (2009). Processivity, synergism, and substrate specificity of *Thermobifida fusca* Cel6B. Applied and Environmental Microbiology 75(21):6655-6661.

Vuong T. V. and Wilson D. B. (2009). The absence of an identifiable single catalytic base residue in *Thermobifida fusca* exocellulase Cel6B. The FEBS Journal 276(14):.3837-3845.

Wu M., Bu L., Vuong T.V., Wilson D.B., Crowley M.F., Sandgren M., et al. (2013). Loop motions important to product expulsion in the *Thermobifida fusca* glycoside hydrolase family 6 cellobiohydrolase from structural and computational studies. Journal of Biological Chemistry 288(46):33107-33117.

Xiao W., Li H., Xia W., Yang Y., Hu P., Zhou S., et al.(2019) Co-expression of cellulase and xylanase

genes in *Sacchromyces cerevisiae* toward enhanced bioethanol production from corn stover. Bioengineered 10(1):513-521.

Zhang N., Wright T., Wang X., Savary B. J. and Xu J. (2020) Production of thermostable endo-1, 5-α-L-arabinanase in *Pichia pastoris* for enzymatically releasing functional oligosaccharides from sugar beet pulp. Applied Microbiology and Biotechnology 104(4):1595-1607.

Zhang P., Yuan X., Du Y. and Li J. J. (2018). Heterologous expression and biochemical characterization of a GHF9 endoglucanase from the termite *Reticulitermes speratus* in *Pichia pastoris*. BMC Biotechnology, 18(1):1-9.

Zhang Y.P., Hong J. and Ye X. (2009). Cellulase assays. In Biofuels. Humana Press, Totowa, NJ, 213-231 pp.