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Study on Cellulase Gene Expressed in *Pichia pastoris* and Analyses of Its Biochemical Characters

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Abstract

Objectives: In order to increase cellulose degradation, cellulase was expressed in this study. **Literature Review:** Cellulose is the most abundant organic carbon source on Earth; its enzymatic hydrolysis will be very useful for bioenergy production and resource recycling. **Methods:** Cellobiohydrlase I (CBH I) gene was amplified from genomic DNA of *Trichoderma koningii* and inserted into pGAPZα A plasmid to construct the vector of pGAPZαA-CBH I. It was linearized and transformed into *Pichia pastoris* by electroporation. The recombinant *Pichia pastoris* was selected and incubated with YPD medium for cellulase secretion. **Results:** The result showed that CMCase and avicelase activity in the supernatant was 1.1798 U/mL and 0.1276 U/mL, the molecular weight of the expressed protein was 53 kDa determined with SDS-PAGE analyses, and the optimal temperature and pH of the expressed cellulase were 45˚C - 50˚C and 4.5 - 5.0, respectively. **Conclusion:** Cellulase gene from *T. koningii* has been successfully cloned and expressed in *Pichia pastoris*.

Keywords

Cellulase Gene, Cloning and Expression, Biochemical Characters, *Pichia pastoris*

1. Introduction

Cellulose, the most abundant organic carbon source, is one kind of recycled biological resource that accounts 1/3 - 1/2 of the dry plant weight on earth. Crop straws are the main resources of cellulose. Due to their low applications, a lot of

them are buried as fertilizer, decayed in the fields or burned on the spot, which cause waste and pollution. If these native resources can be effectively used, it will be conducive to solve environmental pollution, feed shortage and energy crisis. Cellulose is composed of β-1,4-linked glucose units and contains both highly crystalline and amorphous (non-crystalline) regions [1]. A set of enzymes are needed for the complete degradation of cellulose [2]. Depending on their different actions, cellulase family consists of three types such as endo-β-1,4-glucanase, exo-β-1,4-glucanase (cellobiohydrolase) and β-glucosidase [3]. Cellulase is produced by a broad range of organisms including fungi, bacteria, plants and insects. Among these organisms, fungal cellulase has been widely studied.

Although cellulase is widely applied, the large-scale industrial production of cellulase has been restricted by low enzyme activity, high cost and many other factors. In order to improve cellulaseproduction, genetic engineering technologyhas been paid more and more attention. The first cellulase gene was cloned in 1982 [4], and then most of cellulase genes have been expressed in *E. coli*, *S. cerevisiae* and *Pichia pastoris* (*P. pastoris*) [5] [6] [7], but the expressed cellulase activity was low. It is needed to select the different vector and host to produce high levels of cellulase for commercial use.Cellobiohydrlase I (CBH I) is believed to be the most efficient enzyme which can release cellobiose as the main product from highly crystalline cellulose [8]. *Pichia pastoris* has been developed as a widely used host organism for recombinant protein production [9]. It can secrete low levels of its own proteins and high levels of the expressed proteins [10]. In order to increase cellulase production and application, the *CBH* Ⅰ gene from a high cellulase-producing strain of *Trichoderma koningii* was expressed in *Pichia pastoris* in this study to provide the base for cellulose degradation and application.

2. Materials and Methods

2.1. Microorganisms, Plasmid and Incubating Media

Trichoderma koningii (*T. koningii*, CGMCC3.0168) was incubated in PDA medium (0.6% starch soluble, 0.5% trypton, 0.2% yeast extract, 2% glucose, 0.2% K_2HPO4, 0.03% $MgSO_4 \cdot 7H_2O$). PMD19-T plasmid was purchased from TaKaRa Company, *P. pastoris* (X-33) and pGAPZαAplasmid were purchased from Invitrogen Company. *Escherichia coli* (DH5α) was incubated in LB medium (1% trypton, 0.5% yeast extract, and 1% NaCl). *P. pastoris* was incubated in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or YPDS medium (1% yeast extract, 2% peptone, 2% glucose, 1 mol/L sorbitol).

2.2. Genomic DNA and PCR Amplification of CBH I Gene

The genomic DNA of *T. koningii* was prepared according to the former protocol [11]. CBH I gene was amplified with a pair of up-stream and down-stream specific oligonucleotide primers (P1 and P2) designed with CBH I gene sequences from GeneBank at the National Center for Biotechnology Information (NCBI).

To facilitate subsequent cloning of the PCR-derived fragments, *Kpn*I and *Xba*I restriction sites were added to the 5'-ends of P1 and P2 primers, respectively.

P1: 5'-GCG GGTACC CAGTCGGCCTGCACTCTCC-3'

Kpn I.

P2: 5'-CGC TCTAGA CAGGCACTGAGAGTAGTAAGGGTTC-3'

Xba I.

Amplification of the DNA fragments encoding CBH I gene was performed by using polymerase chain reaction (PCR). PCR reactions contained 25 μL 2× Pfu PCR mastermix (Promega), 18.5 μL nuclease-free water, 2.5 μL DNA template, 2.0 μL primers. Reaction conditions for PCR amplification were 25 cycles with 94˚C for 0.5 min, 55˚C for 0.5 min, and 72˚C for 2.5 min, followed by a final extension at 72˚C for 8 min. PCR products were separated by electrophoresis with 1% agarose and purified by High Pure PCR Product Purification Kit (Aidlab). CBH I gene fragment was connected with PMD19-T, and then introduced into competent cells of DH5*α* (Tiangen Biotech). The recombinants were screened by blue white plaque test [12]. After extraction of the recombinant vectors, double endonuclease digestion was used to identify them.

2.3. Construction of Recombinant Expression Vector of pGAPZ*α*A-CBH I

CBH I gene was obtained from the vector of PMD19-T-CBH I through *Kpn*I and *Xba*I double endonuclease digestion, and connected with pGAPZ*α*Aplasmid by ligase which was also digested by *Kpn*I and *Xba*I. The recombinant shuffle vector was introduced into competent cells of DH5*α* by chemical method [12]. Positive cloning strains were selected by low salt LB medium with Zeocin and identified by double *Kpn*I and *Xba*I digestion.

2.4. Transformation of *P. pastoris* and Screening of Recombinant Colonies

Transformation of the recombinant expression vector into *P. pastoris* was performed by electroporation according to the instruction manual for the EasySelect™ Pichia expression system (Invitrogen). Before transformation, the expression cassette pGAPZ*α*A-*CBH*I was linearized with *Bln*I and purified with agarose.

The competent cell of *P. pastoris* was prepared as follow: *P. pastoris* was incubated in 100 mL YPD medium at 30˚C until cells grew to an optical density of 1.1 - 1.3 at 600 nm, then the cells were harvested and kept on ice. The cells were centrifuged at 3000 rounds per min (RPM) for 5 min at 4˚C, washed twice with 100 mL ice-cold ddH_2O, and washed once with 20 mL ice-cold 1 mol/L sorbitol. The cells were finally re-suspended in 0.5 mL of 1 mol/L sorbitol. Eighty μL yeast competent cells were mixed with 5 - 10 μg vector and transferred into an ice-cold 0.2 cm electroporation cuvette. A single pulse of 1.6 kV was applied with a capacitance of 25 μF and resistance of 400 Ω. After electroporation, 1 mL of ice-cold 1 mol/L sorbitol solution was immediately added to the cuvettes, and

the cuvette contents were transferred to 10 mL tube. After incubation at 30˚C for 1 h without shaking, cells were spread on YPDS agar plates containing 100 μg/mL Zeocin (Invitrogen) and incubated at 30˚C for 2 d. YPDS agar plates without zeocin were used as control.

To confirm integration of CBH I gene in the *P. pastoris* genome, two primers (P3 and P4) were designed based on CBH I sequence. The length of PCR products amplified by these two primers should be 1013 bp.

P3: 5'-GCTTCGTCACGCAATCTG-3'.

P4: 5'-GGTCTCGTTTGTCGGGTAGG-3'.

2.5. Cellulase Activity Determination of the Transformed *P. pastoris*

The media used for incubating the transformed *P. pastoris* were YPD medium and YPD medium containing 0.2% Tween 80. The incubation was carried out in 250 mL Erlenmeyer flasks containing 70 mL medium in a rotary shaker of 180 RPM at 30˚C. Incubation lasted for 7 d, during which 2 mL incubation was sampled each day. The supernatant was prepared by centrifugation at 12,000 RPM for 5 min. Carboxymethyl cellulose enzyme (CMCase) activity and avicelase activity in supernatant was determined with a colorimetric method by using a 3,5-dinitrosalicylic acid (DNS) assay with CMC and avicel as the substrates. 0.5% CMC or avicel substrate was prepared by 50 mmol/ L sodium citrate buffer at pH 4.8. The reaction mixture containing 0.5 mL of enzyme solution, 1.5 mL of 0.5% CMC or avicel was incubated at 50˚C for 30 or 60 min. The reaction was terminated by adding 1 mL DNS reagent and boiling at 100˚C for 5 min. The absorption of the reaction mixture was measured at 540 nm by using a UV spectrophotometer [13]. One unit (U) of enzyme activity is defined as the amount of enzyme that produces 1 μmol of D-glucose in one minute under the assay conditions.

2.6. Determination of Cellulase Biochemical Characters

Cellulase thermostability was estimated according to the following protocol: 2 ml sample was added into the pre-warmed (30 min) 10 ml test tubes with lid. The duplicates were 3 for each temperature points. The temperature levels were: 30˚C, 35˚C, 40˚C, 45˚C, 50˚C, 55˚C, 60˚C, 70˚C, 80˚C. The reaction was stopped by putting the tubes into ice immediately after 15 min reaction. The residual cellulase activity was estimated with the above protocol.

The optimal pH was determined with the following 8 levels of pH buffers: 0.2 M sodium citrate-citric acid (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0), and the residual cellulase activity was estimated with the above protocol.

2.7. Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) Analysis

Analytical SDS-PAGE was performed with 12% polyacrylamide gel. The gels were stained with coomassie brilliant blue R-250 (Sambrook and Russell 2001).

Supernatant sample was performed by boiling at 100˚C for 5 min with equal volume of 5 × SDS-PAGE loading buffer (250 mM Tris-HCl, 10%SDS (w/v), 0.5% BPB (w/v), 50% Glycerine (v/v), 5% *β*-mercaptoethanol (w/v)), and centrifuged at 10,000 RPM for 3 min. The electrophoresis was set with 50 V for the first 30 min and then kept at 100 V. Incubation supernatant of native *P. pastoris* was used as control.

2.8. Statistical Analysis

The data were analyzed using the ANOVA procedures of Statistical Analysis Systems Institute, 2004. Duncan's multiple range test was used to evaluate treatment means. The results were considered statistically significance at $P < 0.05$.

3. Results and Discussion

3.1. CBH I Geneexpression in *P. pastoris* and Enzyme Activity Determination

The CBH I gene was amplified from genomic DNA of *T. koningii* and ligated with PMD19-T plasmid to construct PMD19-T-*CBH*I vector, which was confirmed by double endonuclease digestion. The CBH I gene (1626 bp) was sequenced and submitted to the NCBI data base under accession of No.JX103160. Blast analysis indicated that it had a high homology of 99.63% with CBH I gene of *Trichoderma viride* (No. FJ871063.1). Comparison of the cloned sequence with the mRNA sequence in Genebank showed that this gene contained two introns of 66 bp and 69 bp in length. A protein of 497 amino acids should be encoded and the molecular weight was about 52.29 KDa.

CBH I gene fragment (1626 bp) from PMD19-T-CBH I was ligated with pGAPZ*α*A. The recombinant pGAPZ*α*A-CBH I was confirmed by double endonuclease digestion (**Figure 1**). Integration of CBH I gene into *P. pastoris* genome was confirmed with PCR by using gene-specific primers of P3 and P4 (**Figure 2**). From PCR result, it was shown that the positive clones had a bright band at about 1000 bp point, and no band for the native *P. pastoris*. Sequencing result of this PCR product indicated that CBH I gene was integrated into *P. pastoris* successfully.

Cellulase protein from the incubating supernatant of the transformed *P. pastoris* was analyzed with SDS-PAGE. There was a band at 52.29 KDa for the transformed *P. pastoris* and no band for the native *P. pastoris* (**Figure 3**). This result was in agreement with the calculated molecular weight of 52.29 kDa based on the obtained amino acid sequence information. **Figure 4** and **Figure 5** indicated that cellulase activity in the incubation supernatant of transformed *P. pastoris* containing 0.2% Tween 80 was higher than that without Tween 80. Avilase activity reached the peak (0.1276 U/mL) at the third day incubation, and CMCase activity reached the peak (1.1798 U/mL) at the fourth day incubation. Both of them would decrease with incubation prolonging.

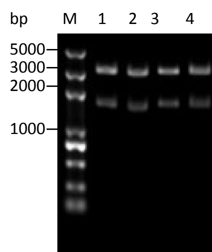

Figure 1. The double endonuclease digestion results of pGAPZ*α*A-CBH I. M: Trans2K Plus DNA Marker; Lane 1 - 4: recombinant pGAPZ*α*A-CBH I digested by *Kpn*I and *Xba*I.

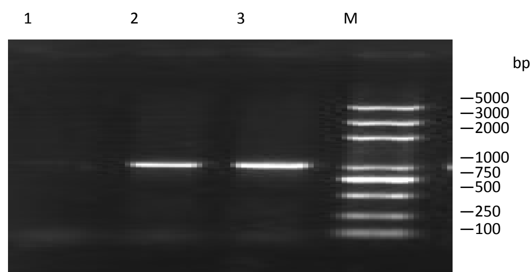

Figure 2. The PCR results of genomic DNA of *P. pastoris*. M: Trans2K Plus DNA Marker; Lane 1: PCR identification of native *P. pastoris*; Lane 2 - 3: PCR identification of recombinant *P. pastoris*.

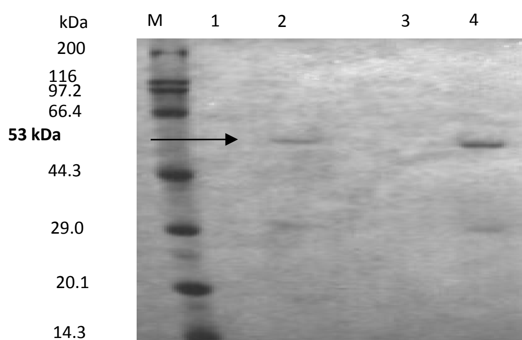

Figure 3. The SDS-PAGE of incubation supernatant. M: Protein molecular weight of marker; Lane 1 and 3: incubation supernatant of native *P. pastoris*; Lane 2 and 4: incubation supernatant of recombinant *P. pastoris*.

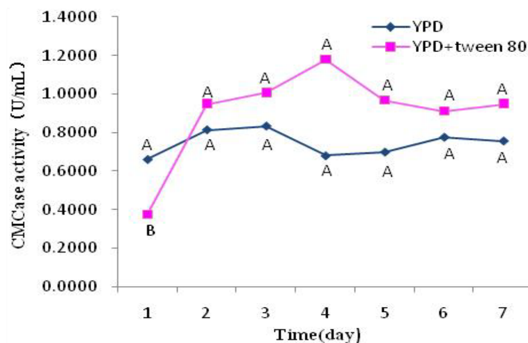

Figure 4. The CMCase activity of recombinant *P. pastoris*. Note: The different letters mean significant difference (P < 0.05); while the same letters mean insignificant difference (P > 0.05).

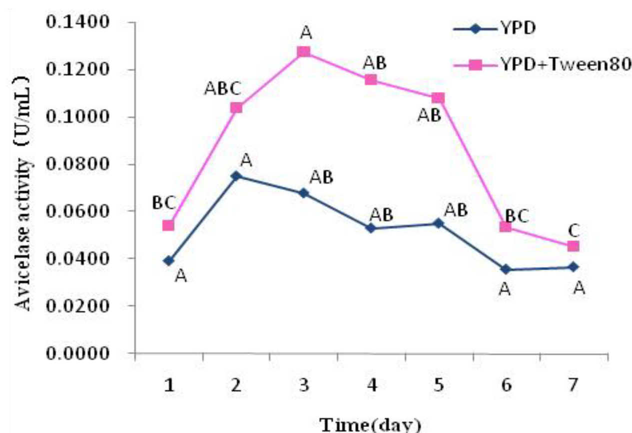

Figure 5. The avicelaseactivity of recombinant *P. pastoris.* Note: The different letters mean significant difference (P < 0.05); while the same letters mean insignificant difference (P > 0.05).

CBH I gene has been previously expressed in *E. coli* and *S. cerevisiae* [5] [6]; however, the expressed cellulase activity is not very high due to the following two problems. The first one is that the enzyme is not secreted and remained as inclusion bodies in cytoplasm when it is expressed in *E. coli* [5]. The second one is that cellulase secreted by *S. cerevisiae*is hyper-glycosylated resulting in reduced substrate-binding capacity and catalytic activity [6]. Even though CBH II gene has been expressed in *P. pastoris* [7], CBH I gene has not been studied and expressed in *P. pastoris*. *P. pastoris* represented an appropriate host for the heterologous expression of functional fungal endoglucanase [14], but the cellulase activity of recombinant yeast was only 0.0670 U/mL [15], which was lower than that in this study (1.1798 U/mL CMCase activity and 0.1276 U/mL avicelase activity).

Tween 80 is a surface active agent.It can reduce the surface tension between the microbial cells and medium surface, stimulate the production of a variety of extracellular enzymes, impact cell membrane permeability, and promote nutrients entering cells and metabolites excreted out of cells [16]. The higher cellulase production with Tween 80 addition in this study proves that Tween 80 is able to stimulate cellulase excretion.

3.2. The Optimum Temperature and pH of Cellulose

Figure 6 showed that CMCase and avicelase activity was higher when temperature was below 50˚C, which decreased quickly as temperature was above 60˚C. **Figure 7** indicated that the optimal pH was 4.0 - 6.0 for CMCase and 5.0 - 5.5 for avicelase. The result was consistent with the former research [17]. The relative cellulase activity decreased significantly when temperature was above 60˚C due to enzyme protein structure changing at high temperature. At pH ranges of 3.0 - 3.5 and 6.0 - 7.0, the relative cellulase activity was very low due to the protein structure of cellulase changed under the strong alkali or acid conditions to make cellulase lose its activity.

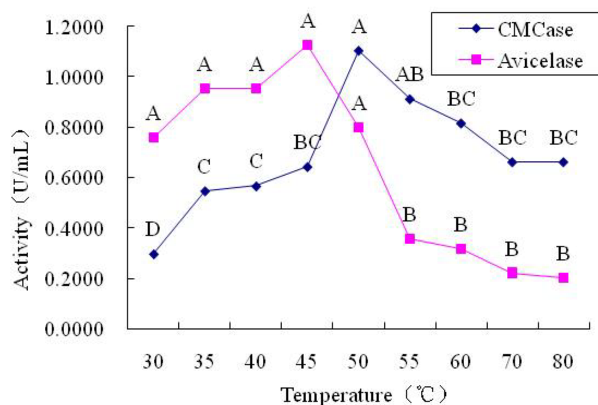

Figure 6. Effect of temperature on cellulase activity of recombinant *Pichia pastoris*. Note: The different letters mean significant difference (P < 0.05); while the same letters mean insignificant difference (P > 0.05).

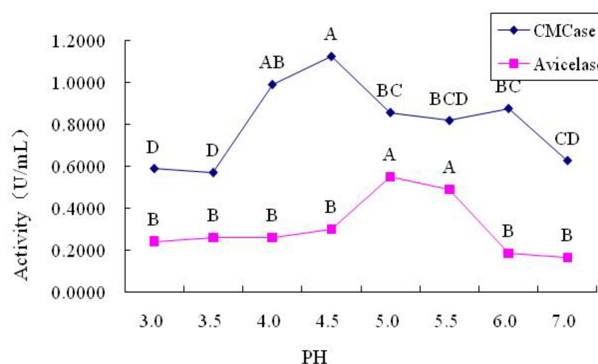

Figure 7. Effect of pH on cellulase activity of recombinant *Pichia pastoris.* Note: The different letters mean significant difference (P < 0.05); while the same letters mean insignificant difference (P > 0.05).

4. Conclusion

The CBH I gene from *T. koningii* has been successfully cloned and expressed in *P. pastoris*, and cellulase biochemical characters were also analyzed. Otherwise, the complete degradation of cellulose is a result of the synergistic effect of cellulase family, so co-expressing a series of cellulase genes in a host would have great significance for cellulose degradation.

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References

[1] Zhang, Y.H.P. and Lynd, L.R. (2004) Toward an Aggregated Understanding of Enzymatic Hydrolysis of Cellulose: Noncomplexed Cellulase Systems. *Biotechnology and Bioengineering*, **88**, 797-824. https://doi.org/10.1002/bit.20282

[2] Bhat, M.K. and Bhat, S. (1997) Cellulose Degrading Enzymes and Their Potential

Industrial Applications. *Biotechnology Advances*, **15**, 583-620.
https://doi.org/10.1016/S0734-9750(97)00006-2

[3] Beguin, P. and Lemaire, M. (1996) The Cellulosome: An Exocellular, Multi-Protein Complex Specialized in Cellulose Degradation. *Critical Reviews in Biochemistry and Molecular Biology*, **31**, 201-236. https://doi.org/10.3109/10409239609106584

[4] Whittle, D.J., Kilburn, D.G., Warren, R.A.J. and Miller, J.R.C. (1982) Molecular Cloning of a *Cellulomonas fimi* Cellulase Gene in *Escherichia coli*: Recombinant DNA; Plasmid pBR322; Immunoassay. *Gene*, **17**, 139-145.
https://doi.org/10.1016/0378-1119(82)90066-X

[5] Laymon, R.A., Adney, W.S., Mohagheghi, A., Himmel, M.E. and Thomas, S.R. (1996) Cloning and Expression of Full-Length *Trichoderma reesei* Cellobiohydrolase I cDNA in *Escherichia coli*. *Applied Biochemistry and Biotechnology*, **57/58**, 389-397. https://doi.org/10.1007/BF02941718

[6] Penttila, M., Andre, L., Lehtovaara, P., Bailey, M., Teeri, T.T. and Knowles, J. (1988) Efficient Secretion of Two Cellobiohydrolases by *Saccharomyces cerevisiae*. *Gene*, **63**, 103-112. https://doi.org/10.1016/0378-1119(88)90549-5

[7] Zahri, S., Zamani, M.R., Motallebi, M. and Sadeghi, M. (2005) Cloning and Characterization of CBH II Gene from *Trichoderma parceramosum* and Its Expression in *Pichia pastoris*. *Iranian Journal of Biotechnology*, **3**, 204-215.

[8] Koivula, A., Ruohonen, L., Wohlfahrt, G., Reinikainen, T., Teeri, T.T., Piens, K., Claeyssens, M., Weber, B., Vasella, A., Becker, D., Sinnott, M.L., Zou, J., Kleywegt, G., Szardenings, M., Stahlberg, J. and Jones, T.A. (2002) The Active Site of the Cellobiohydrolase *Cel6A* from *Trichoderma reesei*: The Roles of the Aspartic Acids D221 and D175. *Journal of the American Chemical Society*, **124**, 10015-10024.
https://doi.org/10.1021/ja012659q

[9] Cereghino, J.L. and Cregg, J.M. (2000) Heterologous Protein Expression in the Methylotrophic Yeast *Pichia pastoris*. *FEMS MicrobiologyReviews*, **24**, 45-66.
https://doi.org/10.1111/j.1574-6976.2000.tb00532.x

[10] Romanos, M.A., Scorer, C.A. and Clare, J.J. (1992) Foreign Gene Expression in Yeast: A Review. *Yeast*, **8**, 423-428. https://doi.org/10.1002/yea.320080602

[11] Zhang, X.H., Guo, C.H., Jiang, X.X. and Luo, H. (2007) Studies on Extraction of Genomic DNA from *Trichoderma koningii*. *Biotechnological Bulletin*, **5**, 128-130.

[12] Sambrook, J. and Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual. 3rd Edition, Cold Spring Harbor.

[13] Coleman, D.J., Studler, M.J. and Naleway, J.J. (2007) A Long-Wavelength Fluorescent Substrate for Continuous Fluorometric Determination of Cellulase Activity: Resorufin-β-D-Cellobioside. *Analytical Biochemistry*, **371**, 146-153.
https://doi.org/10.1016/j.ab.2007.08.027

[14] Ding, S.J., Gea, W. and Buswell, J.A. (2002) Secretion, Purification and Characterization of a Recombinant *Volvariella volvacea* Endoglucanase Expressed in the Yeast *Pichia pastoris*. *Enzyme and Microbial Technology*, **31**, 621-626.
https://doi.org/10.1016/S0141-0229(02)00168-0

[15] Ding, X.L., Wang, T.H., Zhang, G.T. and Lu, Y. (2005) Study of the Expressions of Cellulase from *Trichoderma reesei* in *Saccharomyces cerevisiae*. *Liquor-Making Science and Technology*, **9**, 28-35.

[16] Zhuang, X.L., Zhang, H.X., Ma, G.R. and Kong, J. (2000) Effect of Tween80 on the Growth of *Lactococcus lactis* SM526 and Nisin Activity. *Engineering Chemistry of Metallurgy*, **4**, 145-148.

[17] Quay, D.H.X., Baker, F.D.A., Rabu, A., Said, M., Illias, R.M., Mahadi, N.M., Hassan, O. and Murad, A.M.A. (2011) Overexpression, Purification and Characterization of the *Aspergillus niger* Endoglucanase, EglA, in *Pichia pastoris*. *African Journal of Biotechnology*, **10**, 2101-2111.