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Studies about expression of CbhB encoding gene from *Aspergillus niger* into *Saccharomyces cerevisiae*

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Abstract

Celullases are one of the most important industrial enzymes involved in lignocellulosic raw materials degradation for bioethanol production. In the enzymes cocktail, cellobiohydrolases are the key for the total hydrolysis processe. In this study, the ORF sequence of the type I cellobiohydrolase, CbhB fom the fungus Aspergillus niger was fused to the sequence of the glucoamilase Sta1 signal peptide from S. cerevisiae var. diastaticus and expressed under transcriptional control of a galactose inducible promoter into Saccharomyces cerevisiae. Simultaneous expression of Aspergillus niger cellobiohydrolase and Saccharomycopsis fibuligera β-glucosidase was also studied as a second objective for obtaining an Saccharomyces strain with cellobiohydrolase and cellobiase activities. Physical evidence of these two enzymes expression was performed by analysis of the double transformant protein pattern. In order to analyze the relationship between CbhB structure and function, it has also obtained models of the three-dimensional structures of the catalytic domain and of the cellulose binding module (CBM), by using Cel7A of Trichoderma reesei overlapped with the original model of CbhB predicted by I-Tasser.

Keywords: Cellobiohydrolase B, GH7, *Aspergillus niger*, STA1 signal peptide, *Saccharomyces cerevisiae*

Introduction

Cellulases are O-glycoside hydrolases (GH) that acts on the β-1→4 glycosidic bonds in cellulose and were classified into endo- and exoglucanases on the basis of their mode of action. In the cellulose hydrolysis process the sinergic action of the endoglucanases (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4), exoglucanases (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) is essential (J. MEDVE & al. [1], J. HUI & al. [2], R. KUMAR & al. [3]). From this enzymes mixture, exoglucanases or cellobiohydrolases are the most important single enzyme components that acts on the crystalline cellulose liberating cellobiose units from the ends of cellulose chains. All the cellobiohydrolases that are exclusively derived from eukaryotes were included in GH7 family (http://www.cazy.org/GH7.html). From these, Cel7A secreted by *Trichoderma reesei* was the most studied and produces up to 80% of the total secreted proteins as cellobiohydrolase (BK. BARR & al. [4]; JPM. HUI & al. [5]; T. ERIKSSON & al. [6]; I. STALS & al. [7]; YH. ZHANG & LR. LYND [8]; J.TINA & al. [9]).
Because lignocellulose hydrolysis to fermentable sugars is a very complex process which require higher costs, the strategy of degradation was rethinking as a consolidated bioprocessing (CBP) or as a simultaneous saccharification and fermentation (SSF). In both processes are require organisms that can produce active enzymes and are able to produce

glucides fermentation to ethanol. *Saccharomyces cerevisiae* is from this point of view an atractive organism also because of its fermentative capacity and ethanol tolerance. Moreover, it was engineeried for the heterologous expression of the most cellobiohydrolases alongside *E. coli, Pichia pastoris* and *Aspergillus niger var. awamori* (M. ILMEN & al. [10]; WS. ADNEY & al. [11]; J. TINA & al. [9]). Enzyme engineering efforts were made in order to improve the stability of the cellobiohydrolases type I (S.P. VOUTILAINEN & al. [12]; S.P. VOUTILAINEN & al. [13]) or to optimise cellulase expression (Y. RYOSUKE & al. [14]). In the expression of functional enzymes are involved a lot of factors that interfere with the post-translational with the formation of disulfite bridge and glycosylation.

In this work it was examinated the expression into *Saccharomyces cerevisiae* of a new cellobiohydrolase gene amplified from the genomic DNA of *Aspergillus niger* (cbhB), as a preliminary step to obtain yeast strains with improved properties for cellulose fermentation. It was also studied the simultaneous expression of this cellobiohydrolase with β-glucosidase *Saccharomycopsis fibuligera* Bgl1.

Materials and methods

Strains, plasmids and media

The source for celobiohydrolase B encoding gene was *Aspergillus niger CECT 2775***.** *Saccharomyces cerevisiae BY4741* (MATa his3 leu2 met15 ura3) was used as host strain for the expression vectors that were carried out either cellobiohydrolase gene (cbhB) or β-glucosidase gene (BGL1). Genetic manipulations were accomplished in *Escherichia coli XL1Blue* (Stratagene). The plasmid pACT-SPS-Bgl1-KAN (L. GURGU & al. [15]) was used in order to induce celobiase activity in *Saccharomyces cerevisiae* strain that was initially transformed with cbhB gene. Yeast transformation was carried out by the lithium acetate procedure (R.D. Gietz & R.A. Woods [16]).

Escherichia coli was grown at 37°C in LB medium with ampicilin (50 μg/mL) which was the selection marker for all the vectors. Yeast strains were cultured either in YPD media (1% yeast extract, 2% peptone, 2% glucose) or in minimal media suplemented with the strain requirements (20 mg/L histidine, 60 mg/L leucine, 20 mg/L methionine with or without 20 mg/L uracil, or with 50 mg/L Geneticine) at 30°C on a rotary shaker at 250 rpm.

Plasmids construction

The region encoding CbhB was amplified by PCR from purified genomic DNA of *Aspergillus niger* either with the nucleotides JM844/JM846 (CbhB, fragment which include the sequence of the signal peptide) or with the nucleotides JM845/JM846 (ΔCbhB, fragment excluding the sequence of the native signal peptide). Table 1 contain the sequences of the primers that were used for the amplifications.

All the amplifications were made with Phusion High-Fidelity DNA Polymerase (Finnzymes). The amplified fragment, CbhB was inserted into pEMBLyex4 vector under transcriptional control of GAL10/CYC inductible promoter (G. CESARENI & AH. MURRAY [17]) after enzymatic restriction with *Xba*I/*Pst*I. The new plasmid was named, pCbhB.

In order to check in the celobiohydrolase expression pattern the influence of the glucoamilase Sta1 signal peptide, it was generated other plasmid, pSP-CbhB. This one was obtained from the vector pSPS-Bgl1 (J. MARÍN-NAVARRO & al. [18]), which is also an pEMBLYex4 vector that contain the Sta1 sequence for the signal peptide in frame with the coding region of *Saccharomycopsis fibuligera* beta-glucosidase (Bgl1). Using the endonucleases *Nhe*I/*Pst*I in the double digestion of pSPS-Bgl1, the catalitic domain of Bgl1 was deleted and the *Nhe*I/*Pst*I - digested ΔCbhB fragment was ligated in the coresponding sites of the vector with T4 DNA Ligase (Fermentas). These two recombinat vectors and the pEMBLYex4 (for the control) were used in the *Saccharomyces cerevisiae BY4741* transformation.

Table 1. Oligonucleotides used for the cbhB gene amplification showing the sequence of the restriction sites in italics

Primer	Sequence	Characteristics
JM844	CTG***TCTAGA***ATGTCTTCCTTCCAAATCTACCG (*Xba*I)	CbhB (forward with signal peptide)
JM845	ATTCAC***GCTAGC***CAGCAGGTTGGCACCTACAC (*Nhe*I)	CbhB (forward-without signal peptide)
JM846	GTC***CTGCAG***CTACAAACACTGCGAGTAGTACG (*Pst*I)	CbhB (reverse)

Proteins SDS-PAGE analysis

Samples from the YPD cultures of *S. cerevisiae* single and double transformants previously induced with 0.5% galactose were taken at 24h and 48h intervals. The induction was made after 24 h from the culture` initiation. An aliquot of these samples was centrifuged to separate the cell pellet from the culture media and was treated with 8 U/L EndoH (New England Biolabs), including a protease inhibitor mixture (Complete EDTA-free, Roche) and 140 mM β-mercaptoethanol, at 37°C for 16 h.

The control samples were prepared in the same conditions without EndoH treatment. SDS 2× loading buffer was mixed with the samples and then were boiled for 5 min. 10% polyacrylamide gels was performed for SDS-PAGE which was stained with Coomassie R250. Spectra Multicolor from Fermentas (cat# SM1841) was used as protein standard.

Enzymatic assays

For the enzyme activities toward Avicel, in 1.5 mL Eppendorf tube were mixed yeast culture supernatant with 2% Avicel (3:1, v/v), 0.05 M acetate buffer pH 5.0, 0.5 µl Novozyme-188 (Sigma) and were incubated at 30°C with shaking at 200 rpm. At the 24 h and 48 h time intervals were taken 200 µL samples of the enzyme-substrate mixture to determine the amount of sugars released using DNS method (G. MILLER & al. [19]**).** 100 µL of the supernatant obtained after samples centrifugation (6000 rpm x 1min) were mixed with 100 µl DNS solution and heated at 95°C for five min and cooled at 4°C for two min. The absorbance values were read at 540 nm after 900 µL of millliQ water were added on each tubes. Maltose was used to set a standard curve from which the amount of glucose released during the assay was determined. The percentage of Avicel hydrolysed expresses the celobiohydrolase activity. Beta-glucosidase activity was assayed using the substrate analogue p-nitrophenyl glucopyranoside, as previously described (MJ. Arrizubieta & J. Polaina [20]; J. Marín-Navarro & al. [18]; L. Gurgu & al. [15]). One unit of activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute.

Three different colonies obtained from the double transformant were inoculated in YPD with 50 mg/L geneticin and were grown with agitation at 250 rpm at 30 °C for 48 h. Because in all the transformants, CbhB was expressed under the transcriptional control of the GAL10/CYC promoter, after 24h from the culture initiation, the YPD media was supplemented with 0.5% galactose. Samples were collected after 24 h and 48 h, respectivelly from the induction. The free cells supernatants were directly used to assay either cellobiohydrolase activity or beta-glucosidase activity (in case of the double transformant) released from the cell.

Protein structure analysis

The cellobiohydrolase structure prediction was generated by I-Tasser server (http://zhanglab.ccmb.med.umich.edu/ITASSER) (ROY & al. [21]) using as template the Cel7A of *Talaromyces emersonii.* Pymol was used to create the protein structure images (http://www.pymol.org/).

Results and discussion

Recombinant vectors descriptions and CbhB expression

Cellobiohydrolase encoding gene with its own secretion signal peptide or with the fragment that encode Sta1 signal peptide was cloned into pEMBLYex4 yeast expression vector between GAL10/CYC1 inducible promoter and the 2μ FLP terminator (Fig 1). In pSP-CbhB vector, between Sta1signal peptide (SPS) and the ORF sequence of cbhB there is a linker region for four amino acids translation, Gly-Ala-Ser-Gly, most of them are neutral amino acids with the exception of glycine that is an hydrophobic one. In both recombinant constructs Amp^R gene is conferring ampicillin resistance in *Escherichia coli* and URA3 which is the allele encoding the orotidine 5-phosphate decarboxylase for uracil synthesis is the auxotrophic marker for selection of the yeast transformants.

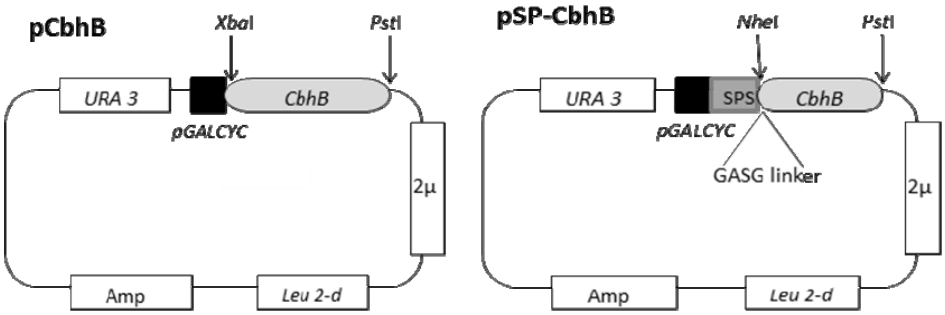

Fig 1. Schematic map representation of the recombinant vectors constructed, pCbhB and pSP-CbhB, with the restriction sites used for the subcloning of CbhB encoding gene.
SPS represents the sequence encoding the signal peptide of Sta1 from *S. cerevisiae var. diastaticus*. pGALCYC represents the GAL10/CYC promoter from pKS2 (L. LATORRE-GARCÍA & al. [22]).

With the aim of obtaining transformants with cellobiohydrolase activity, *Saccharomyces cerevisiae* having the genotype MATa his3 leu2 met15 ura3 was transformed with the pCbhB and the pSP-CbhB recombinant vectors, respectively. Recombinant strain abbreviation was: *Sc-pCbhB* and *Sc-pSP-CbhB*. For the expression of *Aspergillus niger* cbhB gene, the GAL promoter has been successfully used and the protein secretion was obtained upon induction with galactose of mid-logarithmic phase transformant cultures grown in YPD (J. MARIN-NAVARRO & al. [18]). The protein pattern of the transformats that has inserted in the extracromosomal DNA the SP-CbhB gene matched that of its corresponding parental strain except for one single band (Fig 2). The differential bands shown in figure 2a which coresponds to SP-CbhB transformants are very likely to represent CbhB if it is taking in account that the molecular mass of *Aspergillus niger* cellobiohydrolase is about 56 KDa (http://www.uniprot.org/uniprot/Q9UVS8). On the other hand, in the SDS-PAGE pattern of CbhB transformants it could not be differentiate any new band comparing with the control protein pattern. This means that, the replacement of the nativ CbhB signal peptide by that of Sta1 (SP-CbhB) helps the protein to be expressed and secreted. Marrin-Navaro et al., reported in 2010 that, the production of Bgl1 β-glucosidase carrying the Sta1 signal peptide increased by about threefold comparing with the production of the enzyme with its native signal sequence. Heterologouse SP-CbhB cellobiohydrolase secretion also increased in 48h compare with the secretion obtained in 24 h after induction (Fig 2a).

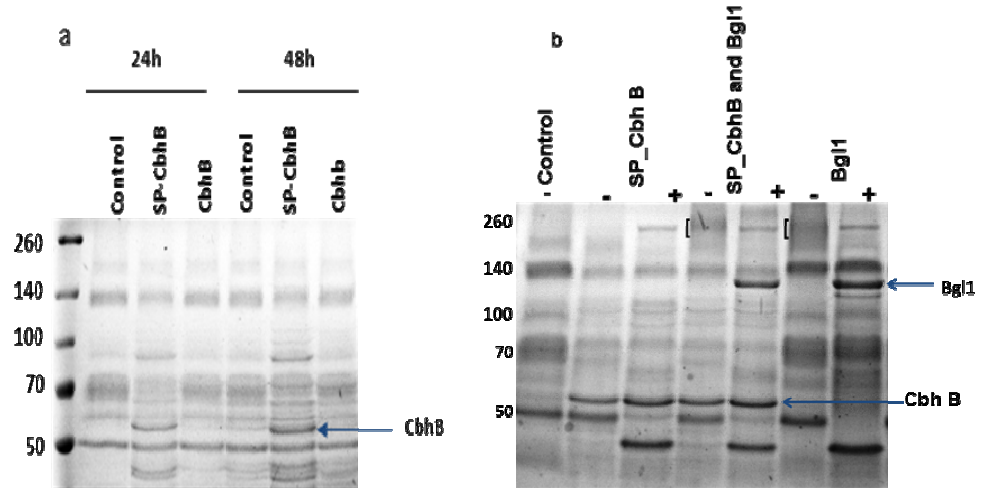

Fig 2. Electroforetic pattern of secreted proteins from *S. cerevisiae* transformants expressing the gene encoding the heterologous protein SP-CbhB (a), Bgl1 (J. MARÍN-NAVARRO & al. [18]) or both proteins in SP_CbhB and Bgl1(b) compare with the control (*S. cerevisiae* transformed with pEMBLYex4).With arrows are indicated differential bands after treatment with endoH (+) and with brackets the bands that appeare without endoH treatment (-)

It is already known that the cellobiohydrolase activity is blocked by the celobiose accumulation in the cellulose hydrolysis processe if no β-glucosidase is added into the cultivation. Following this idea, the second objectiv of the study was to develop a yeast strain with cellobiohydrolase and cellobiase activities. The gene encoding Bgl1 from *Saccharomycopsis fibuligera* was inserted into pACT-SPS-Bgl1-Kan by L. GURGU & al. [15], in 2011 and the cellobiase hydrolysis was proved by a set of industrial yeast strains and one laboratory strain (T500) when they were grown anaerobically on cellobiose.

The pACT-SPS-Bgl1-Kan expression vector (L. GURGU & al. [15]), in which the bgl1 gene was expressed under control of the constitutive actin promoter, was used in the transformation of the strain *Sc-pSP-CbhB*. Colonies that were transformed with both plasmids (pSP-CbhB and pACT-SPS-Bgl1-Kan) were selected on minimal media suplemented with histidine, leucine, methionine and geneticine (G418). Acumulation of the both proteins in the culture medium was proved by SDS-PAGE analysis after Coomassie staining. Figure 2b shows the β-glucosidase band expressed by the *T500* (*Bgl1*) (L. GURGU & al. [15]) and also by the *Sc-SP-CbhB+SP-Bgl1-Kan* with (+) and without (-) endoH treatment. As it was presented before by J. MARIN-NAVARRO & al. [18], Bgl1 β-glucosidase from *Saccharomycopsis fibuligera* is an N-glycosylated protein and without deglycosylation, it appeare as a dispersed band which become a band with lower mass upon digestion (J. MARÍN-NAVARRO & al. [18]) (Fig 2b). Bgl1 band with the same characteristics is observed in the protein pattern of the strain transformed with cbhB and bgl1 gene, respectivelly. Moreover, in the double transformant protein pattern, CbhB cellobiohydrolase is distinguished as a precise band that appeare almost at the same level in the SDS-PAGE gel even after deglycosylation treatment (Fig 2b). This result is backed by the fact that the CbhB from *Aspergillus niger* has one potential N-glycosylation site as it was predicted by the program NetNGly from the Expansy server. The threshold prediction was 0.5 and the asparagine from the sequence 351 NGSS obtained a jury agreement of 9/9.

In order to check the enzymatic activity of CbhB on crystalline cellulose, the *Sc-SP-CbhB* and *Sc-SP-CbhB+SP-Bgl1-Kan* transformant culture supernatants were incubated with Avicel

cellulose. Even when external β-glucosidase was added (just in case of the *Sc-SP-CbhB*) the CbhB activity could not be measured effectively on this insoluble substrate for none of the transformants. In comparation with the CbhB activity, the Bgl1 production in the culture media of the transformant *Sc-SP-CbhB+SP-Bgl1-Kan* was measured by activity on p-nitrophenyl glucopyranoside and it was found to be 63.4±2.12 nmol/(mL·min). However, the activity of *Aspergillus niger* CbhB expressed in *Saccharomyces cerevisiae* has been less successful and this result can not be explained by hyperglycosylation as it was reported previously for the *T. reesei* CBH1 (T. Reinikainen & al.[23]; H. Boer & al.[24]; M. ILLMEN & al. [10]). It is possible that the absence of CbhB activity on Avicel to have the reason of other post-transcriptional events and also in the plasmid copy number which is very important for the level expression.

Structural analysis of *Aspergillus niger* CbhB

Cellobiohydrolase from *Aspergillus niger* is a typical enzyme for glycoside hydrolase family 7 (GH 7) and its architecture is similar to other enzymes from this family, being composed of a signal peptide, one catalytic domain and one carbohydrate binding module (http://www.uniprot.org/uniprot/Q9UVS8). The best template for the predicted CbhB structure using I-Tasser was theCel7A from*Talaromyces emersonii.* Because this enzyme lacks the carbohydrate-binding module compare with CbhB, in order to setting up the 3D model of the CbhB catalytic domainandof thecellulose binding module, the structureofCel7A from *Trichoderma reesei* was overlappedwith the original model of CbhB predicted by I-Tasser (Fig 3).

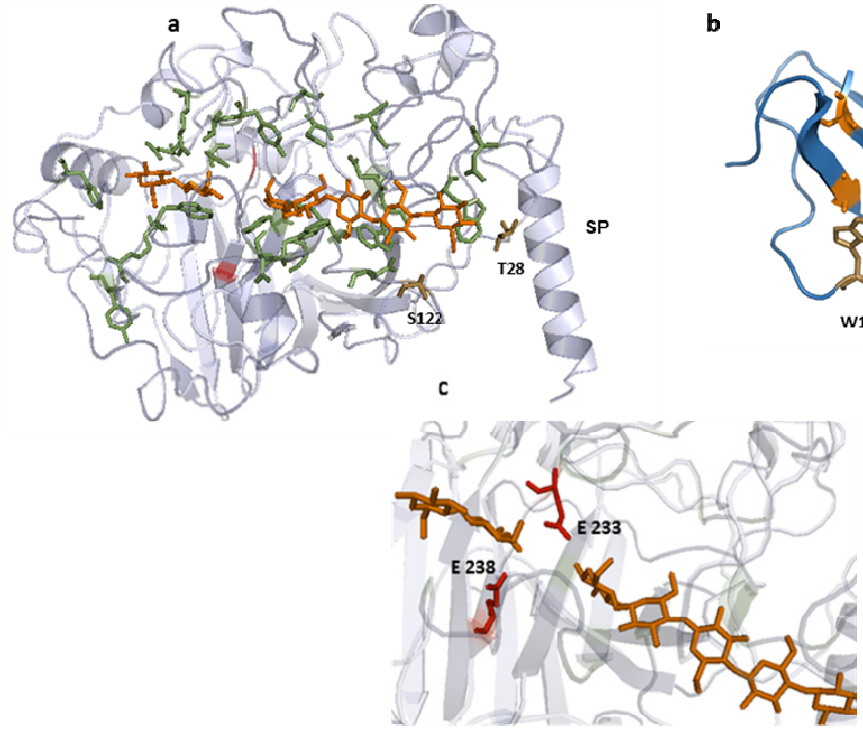

Fig 3. Structural analysis of CbhB from *Aspergillus niger*. a) The location of the binding subsites (green and brown) to the cellohexaose (orange) showing the two aminoa acids, Thr 28 and Ser 122, that are specific for CbhB; SP- CbhB signal peptide structure, b) CBM1 model indicating the hydrophobic residues that may serve as a hydrophobic platform for carbohydrate binding (green and brown); c) Active site representation with the orientation of the catalytic residues (red) and the cellohexaose (orange)

In this way was identified the location of all binding subsites to the cellohexaose (Fig 2a and 2c). Most of them, (N58, W59, W61, N70, N124, R128, Y166, D199, K202, S229, H249,

T267, Y268, R274, D282, Y289, F367, S395, D398, R424) are conserved with *Trichoderma reesei*, with a few exceptions which are Thr 28 (at -7) and Ser 122 (at -6) (Fig 3a). In the GH-7 family was been developed the crystal structure just for four cellobiohydrolases and from these only *T. reesei* Cel7A (C. DIVNE & al. [25]) has been crystalyzed with such a long substrate as it is cellohexaose. The active site of cellobiohydrolase CbhB is also a tunnel-shaped with a loop which coveres the catalytic center as it was found for Cel7A (I. VON OSSOWSKI & al. [26]). The catalytic residues in the active sites of CbhB are represented by E233 and E238 (Fig 2c), two residues, a proton donor and a base, both being necessary in the acid catalysis of the glycosidic bond.

Cellulose binding module of *Aspergillus niger* CbhB is a type I module and has 36 amino acids in size that are arranged in a triple-stranded β-sheet arhitecture at the C-terminus. The 3D structure of *A. niger* CbhB CBM is presented in figure 3b. Three aromatic residues are involved in cellulose binding function: two Tyr (green) which are also conserved in *T. reesei* Cel7A CBM while the third one (W13) is different (a Trp instead of a Tyr). The disulfite bridges are important and offers stability to the cellobiohydrolase structure. Two cysteine residues, C8 and C35, which may form a disulfide bridge are also indicated in orange in figure 3b. There are other two at C19 and C25. For the cellulose binding module were proposed variouse functions: to bind and concentrate the cellobiohydrolase on the cellulose surface and also to disrupt the crystalline surface (L. WANG & al. [27]). Furthermore, M. ILLMEN & al. [10] had improved Avicel hydrolysis by attaching to the C-terminus of the *Talaromyces emersonii* Cel7A, in different constructs the linker and the CBM1 from *H. grisea*, *T. reesei* or *C. thermophilum*. It is obviously that, the presence of the cellulose binding module in the cellobiohydrolases structure is significant for their function on a such insoluble substrat as it is cellulose.

Conclusions

Overall, the results obtained indicates that, the expression of *Aspergillus niger* cellobiohydrolase CbhB took place due to the secretion signal peptide of STA1 glucoamilase from *Saccharomyces cerevisiae var. diastaticus*. Even the CbhB activity could not be proved on cristaline cellulose, which is more exactly a limitation for the initial goal to obtain yeast strains with cellobiohydrolase properties, future experiments are required in order to find a proper substrate for this cellobiohydrolase.

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