ORIGINAL RESEARCH PAPER

RECOMBINANT VECTORS CONSTRUCTION FOR CELLOBIOHYDROLASE ENCODING GENE CONSTITUTIVE EXPRESSION

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Cellobiohydrolases (EC 3.2.1.91) are important exo enzymes involved in cellulose hydrolysis alongside endoglucanases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21). Heterologous cellobiohydrolase gene expression under constitutive promoter control using *Saccharomyces cerevisiae* as host system is of great importance for a successful SSF process. From this point of view, the main objective of the work was to use Yeplac181 expression vector as a recipient for cellobiohdrolase - *cbh*B encoding gene expression under the control of the actin promoter, in *Saccharomyces cerevisiae*. Two hybrid vectors, YEplac-Actp and YEplac-Actp-CbhB, were generated using *Escherichia coli XLI Blue* for the cloning experiments. Constitutive *cbh*B gene expression was checked by proteine gel electrophoresis (SDS-PAGE) after insertion of these constructs into *Saccharomyces cerevisiae*.

Keywords: cellobiohydrolase I, constitutive promoter, gene expression

Introduction

Microbial degradation of lignocellulosic waste is accomplished by a concerted action of several enzymes, the most important and prominent are cellulases, which are produced by a number of microorganisms that are included in glycoside hydrolases (GH) families. It is already known that, in this complex process of cellulose degradation are involved exoglucanases (1,4-β-D-glucancellobiohydrolase, EC 3.2.1.91), endoglucanases (1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21). Exoglucanases or cellobiohydrolases (CBHs) are one of the fundamental enzyme types involved in cellulose saccharification. According to their specificity of action, two types of CBHs, namely type I (CBH I) and type II (CBH II), are distinguished. CBH I act over reducing ends of cellulose molecules whereas CBH II acts over non-reducing ends. From a structural point of view, there are CBHs with different molecular architecture which are classified as glycoside hydrolases belonging to families

GH6, GH7, GH9 and GH48 (Cantarel *et al* 2009). Family GH7 are CBH I enzymes of fungal origin with β-jelly roll structure.

Studies on cellobiohydrolases are centered on the mechanism of enzymatic action or on the microorganisms that synthezise them. An artificial recipe with celulolitic microorganisms which could sinergically act in the cellulose hydrolysis process, is still an unsettled problem. Gene expression to *Saccharomyces cerevisiae* can be modified, even in the absence of vectors with autonom replication just by changing the native promoter with another one that can offer a higher rate of transcription (Verstrepen and Thevelein, 2004).

Classical systems used for the induction of gene expression in *Saccharomyces cerevisiae* require either expensive inductors as galactose (for the *GAL1-10* promoter) (Sertil *et al*., 2003) or toxic inductors as copper (for the *CUP1* promoter) (Labbe *et al*., 1991). On the other hand, gene expression is blocked if the inductor is absent.

From the biotechnological point of view, there are some advantages that constitutive promoters can offer when they replace the inducible ones: higher rate of gene transcription followed by a higher enzymatic production; enzyme synthesis in the absence of an expensive inductor; offers the possibility to use the transformants in the industrial level.

Considering this information, the objective of the study was to express in *Saccharomyces cerevisiae* one cellobiohydrolase gene under the control of a constitutive promoter. Sometimes, the choice of which constitutive promoter to use is therefore frequently based on the availability of a promoter, rather than the suitability of the promoter for a particular experiment. In this case, the promoter choice had the background of the results that Gurgu et al. in 2011 obtained when they used actin promoter for *BGL*1 expression in different yeast strains from *Saccharomyces* genus.

In order to reach the objective, there were designed two expression vectors by insertion of the actin promoter upstream to the *cbh*B gene, gene that encodes an cellobiohydrolase from *Aspergillus niger*.

Materials and methods

Strains and transformation procedure

Aspergillus niger CECT 2775 was the source for the cellobiohydrolase, *cbhB* encoding gene. Cloning experiments for generating different recombinant vectors were carried out in *Escherichia coli XL1Blue* (Stratagene), that was grown at 37°C in LB media suplemented with 50 μg/mL ampicilin, as selection marker. *Saccharomyces cerevisiae BY4741* (MATa his3 leu2 met15 ura3) transformation was carried out by the lithium acetate procedure, as Gietz and Woods (2002) previously described. Parental and transformant strains were grown at 30°C either in minimal media (SD) supplemented with the requirements (20 mg/L histidine, 60 mg/L leucine, 20 mg/L methionine) or in standard complete media, YPD (1% yeast

extract, 2% peptone, 2% glucose). All the liquid cultures were aerated by agitation in an orbital shaker.

Plasmid constructs

The sequence encoding the *cbh*B gene was amplified by PCR from *Aspergillus niger CECT 2775* genomic DNA using Pfu DNA polymerase (Fermentas) with primers JM844 (CTG**TCTAGA**ATGTCTTCCTTCCAAATCTACCG) and JM846 (GTC**CTGCAG**CTACAAACACTGCGAGTAGTACG) which contain the restriction sites for *Xba*I and *Pst*I (in red), respectively. pAct-Kan (Gurgu *et al.*, 2011, Figure 1) was used as a source for the constitutive promoter, Actp. In order to allow the replication of *cbh*B gene in *Escherichia coli XL1Blue* and *Saccharomyces cerevisiae BY4741*, shuttle cloning vector, YEplac181 (Gietz and Sugino, 1988) was used as the recipient vector. The recombinant plasmids obtained were analyzed by enzymatic restriction and also by sequencing.
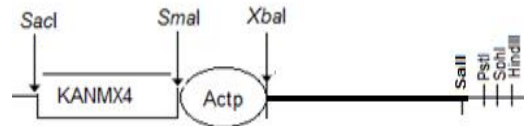
Figure 1. Schematic restriction map of the KANMX4-Actp region from pAct-Kan vector (Gurgu *et al.*, 2011). KANMX4 module conferres resistance to geneticin in yeast; Actp represents the actin promoter from YEpACT-AMY (Rández-Gil *et al.*, 1995)
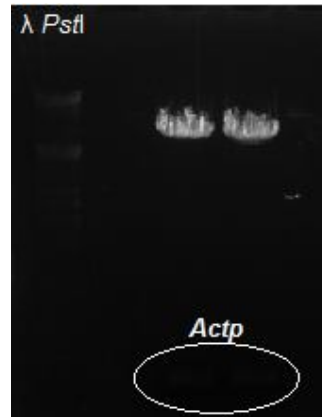
Protein electrophoresis

S. cerevisiae transformants and the corresponding parental strain were grown in 50 mL flasks with YPD liquid medium at 30°C with agitation at 250 rpm. After 48 h of growth, all the cultures were centrifuged (3000 g x 6 min) and then were filtrated in order to obtain free cells culture media. A 23-fold concentrated sample from the culture media was incubated with (+) or without (-) EndoH for 16 h at 37°C in order to deglycosylate all the glycoproteins. Protein electrophoresis, SDS–PAGE was performed using 10% polyacrylamide gel that was stained with Coomassie R250. Standard used for molecular mass determination was Spectra Multicolor from Fermentas (cat# SM184).

Results and discussion

Construction of YEplac-Actp hybrid

In order to promote a constitutive expression for the cellobiohydrolase, *cbh*B encoding gene, the actin promoter, Actp, was cut from pAct-Kan vector (Gurgu *et al.*, 2011) with *Sma*I/*Xba*I restriction endonucleases (Figure 2) and was subcloned in the episomal vector YEplac181 (Gietz and Sugino, 1988) generating YEplac-Actp vector.

Figure 2. Actin promoter DNA fragments after migration in low-melting point agarose gel electrophoresis

DNA fragments of the actin promoter with ~ 500 bp and with a concentration of 9.8 ng/μL, after purification was ligated with the purified DNA fragment of the YEplac181 vector, also cut with *Sma*I/*Xba*I endonucleases in order to generate blunt ends. In the ligation process, T4 DNA ligase (Fermentas) catalyzes the formation of a phosphodiester bond between juxtaposed 5` phosphate and 3` hydroxyl termini in the DNA duplex. The replication of the recombinant vector into *Escherichia coli XLI Blue* cells was possible because of the 2micron2_origin existing in the 1907-743 bp region of the Yeplac181. The ampiciline resistance gene was the marker which allowed the selection of the recombinant *E. coli XLIBlue* clones. Recombinant DNA molecules (YEplac-Actp) extracted from 10 bacterial colonies were analyzed by enzymatic restriction with the same enzymes that were used for the cloning procedure (Figure 3).
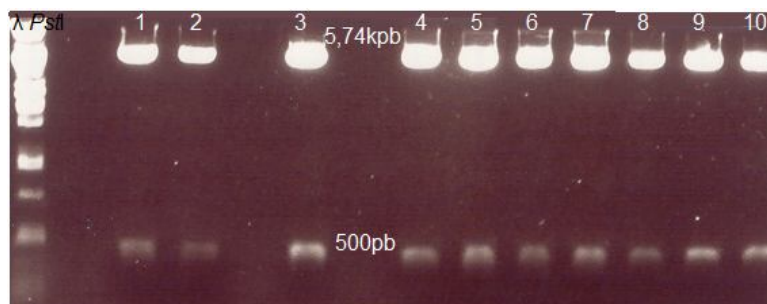

Figure 3. DNA fragments (YEplac181- 5.74 kbp and Actp - 500 bp) migration in 1.0 % agarose gel electrophoresis after enzymatic restriction, with *Sma*I/*Xba*I, of the recombinat construct YEplac-Actp isolated from ten *E. coli XLIBlue* transformants

Electrophoretic DNA pattern after enzymatic treatment proves the right insertion of the Actp promoter to the *Sma*I/*Xba*I sites in all ten recombinant vectors which were

analyzed. The recombinant construct, YEplac-Actp shown in position 1 of the Figure 3 was selected as a host vector for the cellobiohydrolase encoding gene insertion.

### *Construction of YEplac-Actp-cbhB hybrid*

Cellobiohydrolase, CbhB from *Aspergillus niger*, belongs to glicoside hydrolase family 7 (GH 7) and is an enzyme with a typical modular structure for this family, having a signal peptide region with 21 amino acids, one catalitic domain with 437 amino acids, a Ser/Thr-rich linker and one carbohydrate binding module (CBM1) with 37 amino acids (http://www.uniprot.org/uniprot/Q9UVS8). The coding sequence of this enzyme was amplified by PCR from genomic DNA of *A. niger* with the oligonucleotides JM844/JM846 and purified. Figure 4 shows the amplicon pattern after migration in 1.0% agarose gel electrophoresis compared to λ DNA Marker digested with *Pst*I.
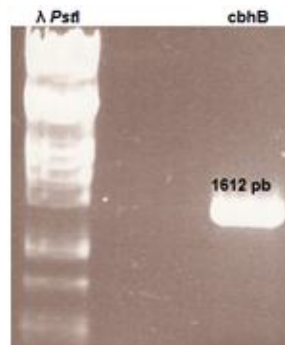
Figure 4. DNA band of the amplified cbh B after migration in 1.0 % agarose gel electrophoresis. Line 1, λ DNA/PstI Marker

The purified amplification product has 1612 bp length and a concentration of 36.7 ng/µL after the purification procedure. For constitutive expression, the amplicon was inserted downstream of the Actp promoter that exists in YEplac-Actp vector. Cellobiohydrolase gene subcloning was made after enzymatic restriction with *Xba*I/*Hind*III of YEplac-Actp. Gene integration was performed following the ligation procedure with T4 DNA ligase enzyme. *E. coli XLI Blue* competent cells were transformed with the ligation product, YEplac-Actp-CbhB and then, the right transformants were assayed for their resistance to ampicilline. Six *E. coli XLI Blue* transformants were randomly selected for recombinant DNA extraction. In Figure 5 is shown the *Xba*I/*Hind*III restriction pattern of the YEplac-Actp-CbhB recombinant DNA extracted from *E. coli XLI Blue* colonies.

All the recombinant vectors from the colonies that were analyzed show the insertion of the cellobiohydrolase gene downsteam of the actin promoter to the *Xba*I/*Hind*III restriction sites.
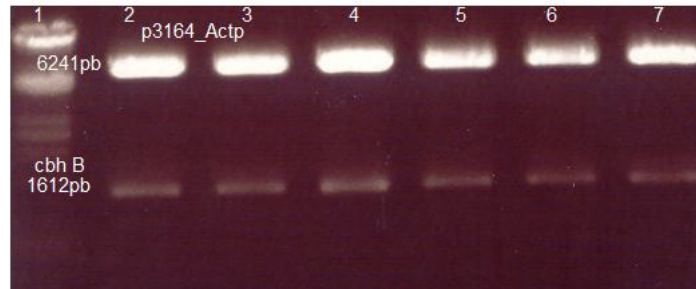
Figure 5. DNA bands of the recombinant vector, YEplac-Actp (6241bp, lines 2-7) and the DNA bands of the inserted gene, cbhB (1612 bp, lines 2-7) after enzymatic restriction with *XbaI/HindIII*. Lambda DNA/PstI Marker, line 1

Cellobiohydrolase gene constitutive expression

In order to create an yeast strain with cellobiohydrolase activity, *Saccharomyces cerevisiae BY4741* (MATa his3 leu2 met15 ura3) was transformed with the two recombinant vectors that were constructed: YEplac-Actp and YEplac-Actp-CbhB, respectively. Transformants were selected by their ability to grow in a minimal medium containing histidine, leucine and methionine. In order to check the pattern of secreted proteins, one transformant clone containing the *cbhB* gene and one clone containing the control plasmid (YEplac-Actp) were grown in YPD media for 48 hours at 30°C and, after centrifugation, the supernatant was 23 fold concentrated using a ultrafilter with a molecular mass cutoff membrane of 10.000 kDa (Millipore). The proteins in the culture media of the *S. cerevisiae* transformants were treated with or without EndoH and analyzed by SDS-PAGE and Coomassie staining (Figure 6).
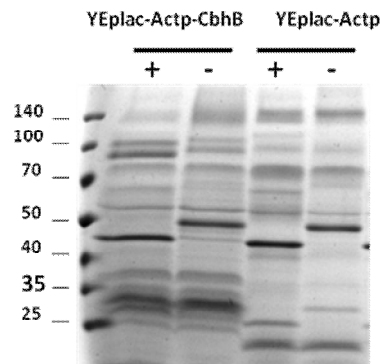
Figure 6. Electrophoretic pattern of secreted proteins from *S. cerevisiae* transformants containing the Yeplac-Actp-CbhB plasmid or the Yeplac-Actp vector (control). The migration of protein standards and their sizes in KDa are shown on the left.

No differential band was found between the clone containing the CbhB gene and the control transformant, suggesting that cellobiohydrolase is not released to the culture media.

Of course that this result is a limitation for the initial objective but it offers an interesting answer about the constitutive expression, more exactly about the selection of a strong promoter that can become a limitative factor for the expression. In the literature, it has been evidenced that heterologous expression of proteins is connected to several stress reactions.

Mattanovich *et al*. in 2004 brought more information about the problems in the expression of heterologous proteins under stress conditions. It is already known that the heterologous protein production is restricted by cellular stress reaction caused by the accumulation of the unfolded proteins in the endoplasmic reticulum (ER) lumen. As a response to ER stress it is induced the unfolded proteine response (UPR) that made part of the intracellular signal transduction patways (Schröder, 2006). Following this idea, it is possible for the actin promoter, which is a strong constitutive promoter, to induce a high transcription rate of cellobiohydrolase gene that can create crucial problems during the translation process.

YEp plasmids are ussually used because of the high gene copy number per cell that results in high expression levels of the desired proteins, but sometimes happens it that these expression vectors have a higher segregation instability that results in plasmid loss, especially in the rich medium (Bärbel *et al*., 2005; Nacken *et al*., 1996; Janes *et al*., 1990). This could be another possible explanation for the lack secretion of cellobiohydrolase in the culture medium.

Overall, the results obtained in the present study leave an open door for future experiments concerning to the relation between promoter system, plasmid stability and expression level of the cbhB gene in *Saccharomyces cerevisiae*.

Conclusions

For a higher proteinexpression, the gene of enzyme should be placed under the control of a strong promoter. In this study, cellobiohydrolase encoding gene from *Aspergillus niger* was inserted downstream of the action promoter in Yeplac181 expression vector but heterologous protein expressed was not detected in the *Saccharomyces cerevisiae* transformants culture media compared to the control strain. Of course, gene expression is a complex multi-step process and problems can arise at numerous stages, from transcription to protein stability. Future studies are needed in order to eliminate all the factors that affect protein expression before evolving to a conclusion about the cbhB expression under the constitutive promoter control.

Acknowledgments

References

Bärbel Hahn-Hägerdal, Kaisa Karhumaa, Christer U Larsson, Marie Gorwa- Grauslund, Johann Görgens and Willem H van Zyl. 2005. Role of cultivation media in the development of yeast strains for large scale industrial use. Microbial Cell Factories, **4**, 31.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Research, **37**, D233-238.

Gietz, R.D., Woods, R.A. 2002. Transformation of yeast by lithium acetate/singlestranded carrier DNA/polyethylene glycol method. Methods Enzymology, **350**, 87– 96.

Gietz, R.D., Sugino, A. 1988. New yeast - *Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene, **74**, 527–534.

Gurgu L, A Lafraya, J Polaina and Marín-Navarro J. 2011. Fermentation of cellobiose to ethanol by industrial *Saccharomyces* strains carrying the β-glucosidase gene (*BGL1*) from *Saccharomycopsis fibuligera*. Bioresource Technology, **102**, 5229-5236.

Janes M, Meyhack B, Zimmermann W, Hinnen A. 1990. The influence of *GAP* promoter variants on hirudin production, average plasmid copy number and cell growth in *S. cerevisiae*. Current Genetics, **18**, 97-103.

Labbe, S., and D. J. Thiele. 1999. Copper ion inducible and repressible promoter systems in yeast. Methods Enzymology, **306**, 145–153.

Mattanovich, D., Gasser, B., Hohenblum, H., Sauer, M. 2004. Stress in recombinant protein producing yeasts. Journal of Biotechnology, **113**, 121-135.

Nacken V, Achstetter T, Degryse E. 1996. Probing the limits of expression levels by varying promoter strength and plasmid copy number in *Saccharomyces cerevisiae*. Gene, **175**, 253-260.

Rández-Gil, F., Prieto, J.A., Murcia, A., Sanz, P. 1995. Construction of baker´s yeast strains that secrete Aspergillus oryzae alpha-amylase and their use in bread making. Journal of Cereal Science **21**, 185–193.

Sertil, O., R. Kapoor, B. D. Cohen, N. Abramova, and C. V. Lowry. 2003. Synergistic repression of anaerobic genes by Mot3 and Rox1 in *Saccharomyces cerevisiae*. Nucleic Acids Research, **31**, 5831–5837.

Schröder M. 2006. The unfolded protein response. Molecular Biotechnology **34**(2), 279-290.

Verstrepen, K., Thevelein, J. 2004. Controlled expression of homologous genes by genomic promoter replacement in the yeast Saccharomyces cerevisiae, Methods in Molecular Biology, **267**, 259-66.