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Production of ethanol from cellobiose by recombinant β-glucosidase-expressing *Pichia pastoris*: Submerged shake flask fermentation

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**Pichia pastoris** strain GS115 was engineered to express a synthetic gene encoding β-glucosidase for its use in ethanol production. β-glucosidase was expressed and secreted either as mature enzyme or N-hexahistidine (His6)-tagged fusion protein, under the control of the strong methanol inducible promoter AOX1. The recombinant β-glucosidase was partially characterized and showed properties comparable to native enzyme. The transformed yeast acquired the ability to assimilate and ferment 1% cellobiose in non aerated batch cultures. Ethanol concentrations were 2.56 g/l and 1.35 g/l which corresponded to 84% and 45% for the non-His-tagged and His-tagged transformants respectively compared to glucose fermentation. A 2.4 fold increase in ethanol production was obtained upon methanol induction of yeast cells in the fermentation medium.

Key words: *Pichia pastoris*; Beta glucosidase; Gene cloning; Cellobiose; Submerged Fermentation; Ethanol.

INTRODUCTION

Production of sustainable bio-energy has provided a focus for research towards renewable resources as an alternative to depletion of fossil resources (Coyle, 2007) as well as growing environmental issues such as emission of green house gases and air pollution by incomplete combustion of fossil fuels. Cellulose, the main component of the abundant lignocellulosic biomass from agricultural residues, grasses, municipal solid waste etc, has the potential to serve as renewable feedstock to chemical commodity and production of fuel, particularly bioethanol. Bioconversion of biomass to fuel ethanol involves enzymatic depolymerization of cellulose to yield cellobiose by means of endoglucanases (cleave the internal bond of cellulose) and exocellulbiohydrolases (hydrolyze cellulose from reducing or non-reducing ends).

In a final step, the enzyme β-glucosidase converts cellobiose to glucose which in turn can further be processed into fuel ethanol. Efficacious hydrolysis needs high conversion rates throughout the depolymerization process. However, cellobiose production appears to be normally more efficient than its hydrolysis, and often accumulates, resulting in severe feedback inhibition of endoglucanase and exoglucanase (Zhao et al., 2004). Then hydrolytic activity of β-glucosidase (BGL) on cellobiose not only relieves the inhibition but also increases the saccharification rate by allowing cellulolytic enzymes to function more efficiently. Besides, considerable research interests have been focused toward BGL because of their potential in various biological processes such as synthesis of oligosaccharides by transglycosylation, aroma-increasing effect in juice and wine; the potential applications of β-glucosidases have been reviewed (Bhatia et al., 2002; Eyzaguira et al., 2005; Sorensen, 2010).

Enzyme cost is considered to be the major impediment in industrial biomass bioprocessing (Howard et al., 2003; Hahn-Hagerdal et al., 2006). Engineering of microorganisms capable of producing desired products by converting cellulosic substrates without added
Saccharolytic enzymes have been suggested as a strategy for a consolidated bioprocess including the production of cellulolytic enzymes, simultaneous hydrolysis of biomass and fermentation in a single and integrated step (Lynd et al., 2005; Inui et al., 2009). In that respect, *Saccharomyces cerevisiae* has served as starting point of number of research aiming at ethanol production from lignocellulosic polysaccharides (Bhatia et al., 2002). However, the methylotrophic yeast *Pichia pastoris* has been developed as an efficient heterologous expression system. This yeast accumulates many advantages over *S. cerevisiae* including high cell density fermentation, strong inducible promoter and high production of foreign protein with low endogenous proteins (Cereghino and Cregg, 2000; Daly and Hearn, 2005).

A β-glucosidase from *Thermoascus aurantiacus* has successfully been expressed in *P. pastoris* KM71H enabling the host to grow on cellobiose (Hong et al., 2007). In our knowledge, nothing has yet been reported about fermentation of cellobiose by this yeast, for ethanol production. This work is part of a broad line research aiming at developing a reliable system for optimal expression and secretion of β-glucosidase enzyme for degrading complex sugars and subsequent fermentation into ethanol. We reported engineering of recombinant *P. pastoris* GS115 strains expressing a β-glucosidase. We evaluated the recombinant β-glucosidase produced in terms of catalytic activity and ability to support growth of the hosts on cellobiose as sole carbon source and energy. In addition, we performed a shake flask anaerobic fermentation to produce ethanol using cellobiose as substrate.

**MATERIALS AND METHODS**

**Strains and culture conditions**

*Escherichia coli* strain DH5α (Novagen), used for plasmid construction and propagation, was grown on LB medium (5 g/L Yeast extract, 10 g/L Tryptone and 10 g/L NaCl); when necessary, ampicillin was supplemented to 100 µg/ml. The yeast *P. pastoris* GS115 was routinely grown at 30°C in YPD broth (2% Tryptone, 2% Glucose and 1% Yeast extract) or YPD plates (YPD + 2% agar). Minimal Dextrose [1.34% yeast nitrogen base without amino acids (YNB), 0.00004% biotin, and 2% dextrose] was used as selective medium to grow *P. pastoris* transformants. BMGY [Buffered Glycerol-complex Medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.00004% biotin, 1% glycerol)] served for biomass development during protein expression whereas BMMY [Buffered Methanol-complex Medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.00004% biotin, 1% methanol)] was used as induction medium. YPM plates (1% Yeast extract, 2% Tryptone, 5% v/v Methanol, 2% Agar)

Fermentation experiments were performed in MYT medium containing 5 g/L (NH₄)₂SO₄, 5 g/L KH₂PO₄, 0.5 g/L MgSO₄.7H₂O, 5 g/L Yeast extract, 0.05 g/L CaCl₂.2H₂O, 5 mg Thiamine-HCL, 0.3 g/L Citrate-Na and 1 ml trace elements (6 g/L CuSO₄, 0.08 g/L KI, 3 g/L MnSO₄, 0.2 g/L NaMoO₄, 0.5 g/L CoCl₂, 20 mg H₂BO₃, 20 g/L ZnCl₂, 5 ml H₂SO₄, 65 g/L FeSO₄.7H₂SO₄ and 200 mg Biotin). Filtered-sterilized cellobiose or glucose (1% w/v final concentration) was added to autoclaved mixture (above) as sole carbon source.

**Construction of expression plasmids and transformation**

We used a chemically synthesized gene encoding for *Aspergillus niger* β-glucosidase (bgl) available in our laboratory. The bgl gene (2526 bp) was synthesized according to its nucleotide sequence published in DDBJ/EMBL/GenBank databases with accession number A8WE01. The coding sequence was polymerase chain reaction (PCR) amplified from its original host (plasmid pUC19) and subcloned into the vector pPIC9K (Figure 1A) for its overexpression under the control of the strongly inducible alcohol oxidase (AOX1) promoter. Three constructs were made as follows: One DNA fragment was obtained after amplification with primers PBGL1 5'- AATACGTAGATGAATTGGCCTACTCCCCGCCGTATTACC-3' and PBGL2 5'ATAGTTTAGCCGCGCTTATGAGCAGAAGCAGG-3', then digested with SnaBI and NotI and ligated between the same sites on PC90 vector to get a new plasmid pBGKL. Another PCR product resulting from amplification with PPNF 5'- AATACGTACATCATCATTACATCATCATTAGTA GATTTGCCCTACTCC-3' and PPNR 5' ATAGTTTAGCCGCGCTTATGAGCAGAAGCAGG-3' primers was subcloned as above to produce the expression vector pBG-1HIs6s, harboring the bgl gene with the coding region for a polyhistidine (His₆) tag at the N-terminus of the protein. A construct with C-terminal His₆-tag was also made, by amplifying with PPCF 5'- AATACGTAGATGAATTGGCCTACTCCCCGCCGTATTACC-3' and PPCR 5'- ATAGTTTAGCCGCGCTTATGAGCAGAAGCAGG-3' primers and ligated into pPIC9K, digested as above, to produce the plasmid pBG-CHis. In all the constructs, the inserts were fused with the alpha-factor signal sequence. Each PCR reaction mixture (50 µl) contained 20 pmol of each primer, 10 ng template, 0.2 mM each dNTP, 1.25U TAKARA Ex-Tag™ DNA polymerase, 5 µl 10X reaction buffer supplied by the manufacturer, and 31.75 µl of double-distilled water. The recombinant plasmids were used to transform chemically competent E. coli DH5α cells, and the resulting transformants were selected on ampicillin LB plates. Positive transformants were screened by colony PCR and all constructs were further sequenced to rule out possible PCR induced mutation.

**Yeast transformation and screening**

*P. pastoris* GS115 was transformed with individual recombinant plasmid isolated from positive *E. coli* transformants. Expression plasmids were linearized by digestion with *PmeI* and transformed into *P. pastoris* by electroporation method described by Invitrogen (2010). An overnight culture of a single yeast colony was used to inoculate 50 ml YPD in 250 ml flask to an OD₆₀₀ of 0.005 and allowed to grow for 7 h. Yeast cells were then harvested by centrifugation at 1500 rpm for 5 min at +4°C. The pellet was washed successively in 50 and then 25 ml ice-cold sterile water. Thereafter cells were resuspended in 20 ml ice-cold 1M sorbitol and pelleted as before. Finally, the cell pellet was resuspended in 200 µl ice-cold 1 M sorbitol and kept as aliquot (80 µl). For transformation, 10 µg of each linearized vector was mixed with the above electrocompetent cells, and incubated in ice for 5 min after which each mixture was transferred into a chilled 0.2 cm electroporation cuvette. Cells were pulsed at 1.5 kV for 5 ms using an Eppendorf electroporator (Eppendorf) and immediately, 1 ml 1M ice-cold sorbitol was added to the cuvette. The electroporated cells were then transferred into a sterile 1.5 ml microcentrifuge tube and incubated for 2 h at 30°C without shaking. 130 µl aliquots of transformation mixture were spread on MD plates and cultured at 30°C. Plasmid integration in transformants was verified by PCR.
Figure 1. Plasmid pPIC9K (A) used as host and expression cassettes of recombinant plasmids harboring coding sequence for β-glucosidase. (B) pBGL; (C) pBGL-N-His\textsubscript{6} for expression with N-terminal His-tag; (D) pBGL-C-His\textsubscript{6} for expression with C-terminal His-tag. The gene of interest was PCR amplified using TAKARA Ext-Taq polymerase, and subcloned downstream of the α-factor signal sequence in pPIC9K and expression is under the methanol inducible promoter AOX1.

Analysis of the β-glucosidase expression

To screen for enzyme expression, positive transformants were spotted on YPM plates and allowed to grow three days at 30°C. Plates were then overlaid with 4 ml 0.7% agar containing 0.3 mM 4-methylumbelliferyl-β-D-glucopyranoside (MuGlc) in 50 mM phosphate citric buffer pH 6, and further incubated at 37°C for 1 h. The β-glucosidase activity was identified by luminescence around the colony under UV light.

One positive colony exhibiting strong β-glucosidase activity on YPM plate was chosen for each of the three recombinant yeasts to study the kinetic of the expression. Single colonies were grown separately at 30°C with shaking (230 rpm) in 250 ml Erlenmeyer containing 25 ml of BMGY medium up to OD\textsubscript{600} = 2. Cells collected by centrifugation 3000 rpm for 5 min were used to inoculate 100 ml of BMGY medium to an OD\textsubscript{600} of 0.7; thereafter growth continued for 72 h as described and samples were taken at given time points for analysis. Induction was maintained by adding 1% (final concentration) methanol every 24 h.

Expression of β-glucosidase was checked on 1% agar plates containing 0.3 mM MuGlc using cell-free extract and cell lysates prepared from samples as enzyme sources. The cell-free extracts were obtained by centrifuging the cultures at 12000 rpm for 1 min. The cell pellets were first resuspended in breaking buffer (50 mM NaH\textsubscript{2}PO\textsubscript{4}, 1 mM EDTA, 5% glycerol, 1 mM PMSF) pH 7.4, then vortexed with glass beads for 1 min. The clear supernatants obtained after 1 min of centrifugation at 12, 000 rpm constituted cell-lysates. To screen the β-glucosidase activity, 5 µl of each sample was dropped on MuGlc plates; plates were incubated for 1 h at 50°C and thereafter checked for luminescence under UV light. No activity was associated with cell lysates and as a result, quantitative assay of the β-glucosidase activity was performed on cell-free culture medium.

Liquid assays were performed to determine the optimal induction time for the secretion of the β-glucosidase enzymes using cellobiose as substrate. 50 µl of the substrate (10 g/L) in 50 mM sodium phosphate buffer pH 5 was mixed with 50 µl enzyme. The reactions were carried out at 50°C for 30 min, and stopped by...
boiling for 5 min and the glucose released was assayed using glucose-oxidase kit (Rongsheng Biotech Co., Nanjing China). Activities were expressed as units, with one enzyme unit defined as the amount of enzyme necessary to release one micromole of glucose per minute under assays conditions specified above.

**SDS-PAGE and zymogram analysis**

Proteins were electrophoresed on 10% gels under denaturing conditions as described by Laemmli (1970) and non-denaturing conditions according to Toonkol et al. (2006). Following electrophoresis, gels were stained for protein with Coomassie blue R-250. For Native PAGE, running and sample buffers were prepared without SDS. The gel was washed twice (15 min each) in 1% triton-X, and recovered by another washing 10 min in 0.1 M sodium acetate buffer pH 4.5. Thereafter, the gel was stained by incubating with 1 mM MuGlc, in 50 mM citrate phosphate buffer pH 5, at room temperature in the dark for 5 min. The active protein bands were observed under UV light.

**Ammonium sulfate purification**

Crude extracts were obtained from induced cultures grown for 60 h in BMMY medium as described above. Proteins in the cell-free extracts were fractionated by precipitation at 4 °C with ammonium sulfate precipitation. Enzyme activity in fractions was monitored on MuGlc. The 30 to 70% saturation showed maximal activity. The precipitates were recovered by centrifugation at 12,000 rpm for 25 min at 4 °C, and then resuspended in 50 mM citrate-phosphate buffer pH 5, followed by dialysis overnight at 4 °C against 10 mM phosphate buffer pH 5. Protein contents were determined using a Coomassie (Bradford) protein assay kit.

**Substrate specificity**

Enzyme substrate specificity was determined by screening activities towards various native oligosaccharides (cellbiose, salicin, lactose, sucrose, (1% w/v)) and analog substrate [p-Nitrophenyl-β-D-glucopyranoside (pNPG, Sigma), 3 mM in 50 mM sodium acetate buffer pH 5].

**Effect of metal ions on enzyme activity**

Metal ions Fe³⁺, Cu²⁺, Ca²⁺, Mg²⁺, Mn²⁺ (10 mM final concentration) were incubated in 50 mM sodium acetate buffer pH 5 with 15 μg of enzyme for 30 min at room temperature and the residual activity was measured using cellbiose as substrate.

**Effect of inhibitors and chelators agent**

The partially purified enzyme was first preincubated at room temperature for 30 min with EDTA 1%, SDS 1%, ethanol (0 to 50% v/v) thereafter the substrate cellobiose was added and the relative activity was determined as described.

**Shake-flask fermentation of cellbiose**

Anaerobic fermentation was performed in 100 ml rubber stoppered glass bottles containing 40 ml of MYT supplemented with 1% w/v carbon source either cellobiose or glucose. As inoculums, a stationary phase cell culture was obtained by culturing one colony of P. pastoris harboring respective expression plasmid (pBGL or pBGL-N-His₆) in MYT+2% glucose. Then cells were pelleted at 3000 rpm for 5 min, and washed twice with sterile and carbon-free MYT medium and resuspended in a sugar containing medium to the desired OD. Bottles were vented using a 25-gauge needle inserted through the stopper during growth at 30°C in a rotary shaker at 150 rpm. Bottles were removed sequentially at 12 h intervals for analysis.

**Analytical method**

Cell growth was monitored as the function of OD₆₀₀, and values were converted to dry cell weights (DCW) using the relation 1 OD₆₀₀= 0.27g DCW/l (Zhang at al., 2007). Ethanol was first extracted from the fermentation broth using tributyl phosphate ([TBP, density = 0.98, solubility in water = 0.1% [chemically pure grade, Shanghai lingfeng Chemical Reagent Company, China]) as solvent, then subjected to oxidation by 34 mM (final concentration) potassium dichromate in 5 M sulfuric acid (Seo et al., 2009). This reaction developed a blue-green solution in which the optical density was measured at 630 nm. Amounts were calculated from a standard curve constructed by extracting serial ethanol dilutions from a sugar-free MYT medium. Results were expressed as means of three independent measurements. Cellobiose levels in the fermentation broth were measured according to Tokuhiro et al. (2008). Glucose was assayed using glucose-oxidase kit. Fermentation parameters were calculated as previously described (Lawford and Rousseau, 1993).

**RESULTS AND DISCUSSION**

**Gene cloning and characterization**

Three recombinant plasmids were constructed (Figure 1B) for the expression of the β-glucosidase under the control of the AOX1 promoter in the yeast P. pastoris. pBGL was made to express the mature β-glucosidase; pBGL-N-His₆ and pBGL-C-His₆ were produced for expression with an N-terminal and C-terminal polyhistidine-tag respectively. We chose genomic rather than episomal integration of the plasmid in view of the mitotic stability; transformation by electroporation was appropriate for that purpose. The recombinant plasmids were linearized by Pmel to enable homologous recombination between the AOX1 region on the plasmid and its homolog in the yeast genome. Correct integration of the constructed plasmids, following electroporation was confirmed by PCR. Results show that transformation with pBGL and pBGL-NHis generated only Mut⁺ (Methanol utilization fast) recombinants, as a result of gene insertion, whereas pGBGL-CHis in addition yielded some Mut⁺ (Methanol utilization slow) phenotypes indication gene replacement at the AOX1 region, according to INVITROGEN (data not shown). Transformation events in P. pastoris can happen either by gene insertion or gene replacement (Higgins and Cregg, 1998). Thus, the two events occurred when transforming with pBGL. Transformants carrying expression vector were re-streaked on YPM plates for preliminary enzyme expression assays. Results clearly indicate the expression of β-glucosidase for several transformants harboring pBGL and pBGL-N-His₆, upon
observation under UV light, whereas only a faint halo could be observed around positive strains transformed with the pBGL-C-His6 compared to the non-transformed yeast used as control. This result suggests that the recombinant enzyme was successfully produced and secreted out of the yeast. Transformants identified as GLuO and NHis68 which showed the strongest activity on YPM plates from pBGL and pBGL-N-His6 respectively were selected for further experiments.

Expression and identification of the recombinants β-glucosidases

The expression of the enzyme was monitored by screening its activity. High β-glucosidase activity was detected in the culture supernatants from GLuO and NHis68 transformants, following methanol induction as previously described in Materials and Methods. This is evidence that an active β-glucosidase was successfully being processed and secreted correctly in the culture medium, driven by the Saccharomyces cerevisiae alpha-factor signal peptide. Figure 2 shows a time course enzyme activity of the extracellular β-glucosidase. The highest activities of the crude extracts (11.02 and 8.38 U/ml) towards cellobiose were obtained after 48 h of methanol induction with protein concentrations of 0.46 and 0.43 g/L for enzyme produced by GLuO (rec-gluO) and NHis68 (rec-nhis68) respectively. A native gene from A. niger has been cloned and expressed in P. pastoris with approximately the same yield (Dan et al., 2000). The two strains did not grow at equal OD therefore, the slightly difference in β-glucosidase production could arise from divergence in cell growth. The control (non-transformed yeast strain) showed no β-glucosidase activity; therefore, we decide to use the partially purified enzyme prepared as described earlier. Ammonium sulfate precipitation resulted in 1.6 and 1.85 fold purification for rec-gluO and rec-nhis68, respectively.

The gene bgl encoded a polypeptide of 842 amino acids. We deduce a protein sequence from the nucleotide sequence of bgl by using Gene Runner software and a motif search performed throughout this sequence showed a glycosyl hydrolase family 3 catalytic domain (LLKAELGFQGFVMSDWAA) at amino acid 247 – 264, suggesting that the recombinant β-glucosidase belongs to this family of enzymes. Native-PAGE analysis (Figure 3A) of the partially purified enzyme showed a single active protein band with an apparent molecular weight of 90-91 kDa estimated by denaturing SDS-PAGE (Figure 3B). This was consistent with the expected size of the protein, and accordingly, the secreted β-glucosidase was considered to be a monomer.

Substrate specificity

Rec-gluO and rec-nhis68 preferentially hydrolyzed cellobiose 28.12 and 19.31 U/ml respectively. They did not cleave pNPG, the most used substrate for assaying β-glucosidase activity; this is unusual property compared to most of the known β-glucosidases. Family 3 hydrolase enzymes are broadly classified as aryl-β-glucosidases or true cellobiases (Bhatia et al., 2002; Eyzaguira et al., 2005). Thus, accordingly, the recombinant β-glucosidase expressed here may be considered as cellobiases. This result suggests potential application of the β-glucosidase produced in the treatment of cellulosic materials to convert cellulose to glucose, in conjunction with cellulolytic enzymes. Furthermore, no activity was detected towards lactose and sucrose.

Effect of ions and other reagents

The enzyme rec-nhis68 showed enhanced activity in the presence of almost all the metallic ions tested; only Cu²⁺ caused a 36% loss in activity. In contrast, only Mn²⁺ positively influenced the activity of rec-gluO, others showed slight inhibition (Table 1). Activation by Mn²⁺ may be explained by stabilization of the enzyme. 1% EDTA did not greatly affect the enzyme activity, indicating that β-glucosidases are not metalloproteins. In the presence of 1% SDS, rec-nhis68 retained only 27% of it activity. In the presence of ethanol, the activity of both enzymes decreased with increasing amounts; at 20% v/v, a loss of about 50% of the activity was observed (data not shown).

Fermentation of cellobiose

Batch submerged fermentation was carried out, under anaerobic conditions using shake flasks to investigate our recombinant P. pastoris strains GluO and NHis68 for their ability to produce ethanol from a minimal medium supplemented with 1% (w/v) cellobiose as a single carbon source. Figure 4 illustrates the rate of substrate consumption and correspondingly ethanol production. The concentrations of sugars decreased with time and accordingly the ethanol concentration increased. The highest ethanol concentrations were 2.56 and 1.35 g/L (Figure 4B) which corresponded to 84 and 45% for GluO and NHis68 respectively compared to 3.05 and 3.0 g/L (Figure 4A) obtained from fermentation of glucose (used as positive control) in the same conditions.

The fermentation profiles in terms of rate and productivity for both the two strains are summarized in Table 2. Total consumption of cellobiose was observed for GluO, yielding 0.25 g ethanol/g cellobiose, and volumetric production rate of 0.09 g/L during 60 h period. The non transformed P. pastoris showed no substantial growth in cellobiose-supplemented medium. However, this yeast have been reported to possess a cytosolic β-glucosidase, not secreted into the culture medium and therefore might not support the growth of the yeast in a
Figure 2. Time-curse (0-72 h) of β-glucosidase activity. *P. pastoris* transformants, GluO and NHis68, were induced with 1% (v/v) methanol in BMMY medium to express non-tagged (black) and His-tag (white) enzyme respectively. Samples were taken at given time points; activity assayed using cellobiose as substrate and the liberated glucose was determined. One unit of activity represents the amount of enzyme catalyzing the release of 1 micromole glucose per minute at 30°C.

Table 1. Effect of cations on the activity of the recombinant β-glucosidases. Enzyme was preincubated 30 min at room temperature with different ions in solutions. Residual enzyme activity was measured at 50°C using cellobiose as substrate in 0.1M sodium acetate buffer pH 5.0).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Rec-gluO</th>
<th>Rec-nhis68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>93</td>
<td>120</td>
</tr>
<tr>
<td>CuSO₄</td>
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<td>64</td>
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<td>174</td>
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<tr>
<td>FeSO₄</td>
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<td>124</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>94</td>
<td>133</td>
</tr>
<tr>
<td>EDTA</td>
<td>86</td>
<td>95</td>
</tr>
</tbody>
</table>

A mixture of enzyme in acetate buffer served as a control, activity towards cellobiose in absence of any metal ion was considered as 100%.

medium containing cellobiose as sole carbon source (Turan and Zheng, 2005). We successfully expressed synthetic *bgl* gene in *P. pastoris* and as a result, this strain acquired the ability to utilize cellobiose as sole carbon source. Moreover, the recombinant strains could produce ethanol by fermenting cellobiose (Figure 4B). However, as shown in Table 2, maximum substrate consumption rates, in media containing initially 10 g of sugar/L, were 0.44 g (2.44 mmol/L h) and 0.54 g (0.3 mmol/L h) in glucose-grown cultures while cellobiose-grown cultures fermented a maximum of 0.1 g (0.292 mmol/l h and 0.09 g (0.175 mmol/l h) for GluO and NHis68, respectively.

No glucose released from cellobiose hydrolysis accumulated during the anaerobic fermentation in the culture broth. Therefore, utilization process of sugar by the yeast could account for one reason for the difference observed. *P. pastoris* is considered as crabtree-negative species (Cereghino and Cregg, 2000) but uptake of sugar by this yeast is yet to be clarified. In some yeast, disaccharides such as cellobiose are cleaved outside the yeast cell prior to transport through glucose transporters...
**Figure 3.** Polyacrylamide gel electrophoresis analysis: Proteins were separated on a 10% gel according to Laemmli. Gels were either stained by Coomassie blue (B) or overlaid with 1mM Muglc (A) for β-glucosidase activity. Lane 1: rec-GluO, Lane 2: rec-NHis68, Lane 3: Commercial β-glucosidase from Almond (Sigma), M: molecular weight marker.

**Figure 4.** Submerged fermentation by GluO (filled symbols) and NHis68 (open symbols). Bath cultures were carried out at 30°C in MYT medium supplemented either with glucose (A) or Cellophase (B) as sole carbon source. Ethanol produced (dash) from 10 g/l substrate was determined through oxidation by dichromate potassium after solvent extraction (n=3) using Tributyl phosphate.
Table 2. Fermentation parameters for growth of recombinant *P. pastoris* strains GluO and NHs68 in batch culture with cellobiose and glucose as carbon substrate (10 g l$^{-1}$)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GluO</th>
<th>NHs68</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose</strong></td>
<td>0.88</td>
<td>0.78</td>
</tr>
<tr>
<td><strong>Cellobiose</strong></td>
<td>1.01</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Q_{s_{\text{max}}}(g/l h)</strong></td>
<td>0.44</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>q_{s}(g/g cell h)</strong></td>
<td>0.5</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Q_{p_{\text{max}}}(g/l h)</strong></td>
<td>0.175</td>
<td>0.157</td>
</tr>
<tr>
<td><strong>q_{p}(g/g cell h)</strong></td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Y_{p/s}(g/g)</strong></td>
<td>0.28</td>
<td>0.29</td>
</tr>
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</table>

Parameters were calculated as described (Lawford and Rousseau, 1993). DCW: Dry cell weight; $Q_{s_{\text{max}}}$: Volumetric rate of sugar consumption; $Q_{p_{\text{max}}}$ Volumetric rate of ethanol production, $q_{s}$: Specific volumetric ethanol productivity; $q_{p}$: Specific rate of sugar utilisation; $Y_{p/s}$: Ethanol yield.

for subsequent catabolism, and thus slowing down the fermentation process, whereas others transport both the disaccharide and the resulting monosaccharide (Mortberg and Neujahr, 1986; Barnett, 1981). Thus, the quality of the carbohydrate used as substrate may affect the rate of fermentation. This could be applied here, assuming that cellobiose (disaccharide) must be hydrolyzed to glucose before entering the catabolic pathway in *P. pastoris*. Moreover, if this is true, then the amount of β-glucosidase present in the culture medium could further be a rate-limiting step for the fermentation process.

To assay this hypothesis, we conduct another anaerobic fermentation, in almost similar conditions as described before for GluO but with a 0.5% (v/v) methanol supplementation every 24 h in the fermentation medium (MYT), to induce the expression of β-glucosidase. Methanol was added aseptically through the gauge needle. A cellobiose-free medium containing 0.5% (v/v) methanol served as the control. The β-glucosidase activity in the supernatant of both the induced and non-induced cultures was checked on MuGlc plates and quantifies using cellobiose as substrate as described.
yeast efficiently ferment cellobiose to produce ethanol in shake flask culture, thus suggesting potential exploitation of this new construct in simultaneous saccharification and fermentation process. \textit{P. pastoris} is well characterized by the ease with which its cultures can be scaled up from shake flask to large fermentor (Higgins and Cregg, 1998). Our next attempts are currently directed in that respect.

## REFERENCES


Inui M, Vertes, AA, Yukawa H (2009). Advanced fermentation Technology In: Biomass to Biofuels: strategies for global industries,

earlier in materials and methods. Results are summarized in Table 3. Fermentation in the presence of methanol as inducer produced 17.78 ± 0.3 U/ml β-glucosidase activity after 12 h. A maximum amount of 6.19 ± 0.22 g ethanol/l accumulated after 36 h; this amount was 8 times higher than that produced in the absence of inducer in the same time interval. The yeast grew well in the medium containing only methanol but no ethanol was produced. Cultures in non-induced medium produced from 1.22 ± 0.2 to 22.07 ± 0.6 U/ml of β-glucosidase activity (Table 3); this production could be attributed to cellobiose induction. In fact, cellobiose when used as substrate has been shown to induce the production of β-glucosidase in yeast during culture (Strauss and Kubicek, 1990; Moussa and Tharwat, 2007). Ethanol concentration in induced culture decreased after 36 h, suggesting its metabolism by the yeast cells, as a result of complete consumption of the cellobiose available (data not shown). Overall, earlier secretion of the enzyme β-glucosidase in the fermentation medium following methanol induction enhanced substrate consumption and subsequent ethanol production. Therefore, it appears obviously that continuous expression and enzyme secretion in the reactor could be of great importance in industrial applications like simultaneous saccharification and fermentation. The yeast \textit{P. pastoris} is well known for its suitability for large-scale production of heterologous intracellular and secreted proteins in fermentor cultures (Koganesawa et al., 2002; Reddy and Dahms, 2002). In addition, fermentation medium for this yeast, containing salts and trace elements are economical and well defined, rendering this yeast potential candidate for its exploitation in biomass saccharification.

We reported integration and expression of a synthetic gene encoding β-glucosidase into the genome of the yeast \textit{P. pastoris} strain GS115. The engineered yeast produced an enzyme with properties comparable to known native β-glucosidases. This result demonstrates the use of chemical synthesis as an alternative for conventional gene cloning. In addition, the recombinant

### Table 3. Effect of methanol supplementation in the fermentation of cellobiose (10 g/l) in shake flask by GluO strain. Methanol 0.5% (final concentration) was added each 24 h in the fermentation medium (40ml) to induce expression and secretion of β-glucosidase; cultures were carried out at 30°C under agitation 150 rpm.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Biomass (g/l)</th>
<th>β-glucosidase activity (U/ml)</th>
<th>Ethanol (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.254 (0.196)</td>
<td>17.78 ± 0.3 (1.22±0.2)</td>
<td>3.34 ±0.3 (0.76 ±0.04)</td>
</tr>
<tr>
<td>24</td>
<td>1.22 (0.245)</td>
<td>1.38 ± 0.15 (2.15±0.1)</td>
<td>3.97 ±0.47 (0.81 ±0.15)</td>
</tr>
<tr>
<td>36</td>
<td>1.528 (0.902)</td>
<td>4.29 ± 0.00 (22.07±0.6)</td>
<td>6.19 ±0.22 (0.77±0.08)</td>
</tr>
<tr>
<td>48</td>
<td>1.540 (1.012)</td>
<td>-d (8.54±0.04)</td>
<td>4.25 ±0.24 (1.85 ± 0.12)</td>
</tr>
</tbody>
</table>

Values in parenthesis were obtained in the absence of methanol induction. a\text{OD}_{600} values were transformed to g biomass/l according to Zhang et al. (2007). bActivity was determined at 50°C for 30 min using cellobiose as substrate as previously described (materials and methods). One enzyme unit (U) corresponds to the amount of enzyme necessary to liberate 1 micromole glucose per min. cEthanol was extracted from the fermentation broth using tributyl phosphate as solvent and oxidized by potassium dichromate (Seo et al., 2009). Amount were determined from a standard curve constructed by extracting serial ethanol dilutions from a sugar-free MYT medium. dNo activity was detected.


