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Construction of whole-cell biocatalyst for xylan degradation through cell-surface xylanase display in *Saccharomyces cerevisiae*

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Abstract

We constructed a yeast-based whole-cell biocatalyst displaying *Trichoderma reesei* xylanase II (XYNII) on the cell-surface and endowed the yeast-cells with the ability to degrade xylan. The fusion gene encoding the mature region of XYNII and the C-terminal half (320 amino acid residues from the C-terminal end) of yeast α-agglutinin (XYNII-α-agglutinin) was constructed and expressed in *Saccharomyces cerevisiae* under the control of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. The expression system of fusion gene encoding XYNII-α-agglutinin tagged with RGSHis6 consisting of arginine, glycine, serine, and histidine hexamer (RGSHis6-XYNII-α-agglutinin) was also constructed. Immunofluorescence labeling to confirm cell-surface display of the RGSHis6-XYNII-α-agglutinin fusion protein, and confirmation of similar xylanase activity in yeast-cells expressing XYNII-α-agglutinin and RGSHis6-XYNII-α-agglutinin but not in the culture medium, indicated that XYNII was displayed on the cell-surface in the active form. The XYNII-displaying yeast-based whole-cell biocatalyst showed highest XYNII activity at pH 5.0 and 40 °C, respectively. This whole-cell biocatalyst is expected to find application not only in the first step of fermentation of xylan to ethanol but also in xylooligosaccharide production.
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Keywords: Xylanase; *Trichoderma reesei*; *Saccharomyces cerevisiae*; Cell-surface display; Xylan

1. Introduction

Many bacterial and fungal species can produce multiple endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) utilizing xylan, a complex polymer which is the major component of hemicellulose in the plant-cell wall and consists of a β-1,4-linked xylopyranose backbone substituted with acetyl, arabinosyl and glucuronosyl side chains. Enzymatic hydrolysis of xylan to xylose is catalyzed by xylanase and β-xylosidase, the former randomly hydrolyzing xylan to xylooligosaccharides and the

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latter producing xylose from xylooligosaccharides [1].

The xylanase genes from *Aureobasidium pullulans* [2], *Cryptococcus albidus* [3], *Aspergillus nidulans* [4], *A. kawachii* [5,6] and *Trichoderma reesei* [7] have been cloned, sequenced and expressed in the yeast *S. cerevisiae*. Meanwhile, the filamentous fungus *T. reesei*, well-known as a producer of cellulolytic and xylanolytic enzymes, has two major xylanases characterized as xylanase I (XYNI) and xylanase II (XYNII), which have different molecular weights, isoelectric points and pH optima [8,9], but which both hydrolyze xylan to form similar hydrolysis end products.

The most abundant renewable resource in the world, cellulosic biomass includes substances such as lignin, cellulose, and hemicellulose, and production of ethanol from these last two has been investigated. However, since the yeast *S. cerevisiae* cannot utilize xylose as a carbon source, yeasts for xylose fermentation have been genetically engineered by integration of xylose reductase and xylitol dehydrogenase genes from *Pichia stipitis* [10,11]. Xylanase also has applications in the foodstuff and feed industry in xylooligosaccharide production and in bleaching processes in the paper and pulp industry.

Recently, genetic engineering techniques have been used to display various heterologous peptides and proteins (enzyme, antibody, antigen, receptor and fluorescence protein, etc.) on the yeast-cell-surface [12–21]. Living cells displaying various enzymes on their surface could be used repeatedly as 'whole-cell biocatalysts' like immobilized enzymes. These cells have novel functions and combine the ability to display enzymes on their surface with intracellular metabolic ability [15,16,21].

In the present paper, we report on the genetic immobilization of *T. reesei* XYNII on the *S. cerevisiae* cell-surface using the C-terminal half of α-agglutinin as an anchor; and on the characterization of XYNII-displaying whole-cell biocatalysts.

2. Experimental

2.1. Strains and media

Escherichia coli NovaBlue [*endA1 hsdR17*(r_{K12}^- m_{K12}^+) *supE44 thi-1 gyrA96 relA1 lac recA1/F′* {*proAB*$^+$ *lacI*^{q}*ZΔM15::Tn10* (*tet*^{r})}] (Novagen Inc., Madison, WI, USA) was used as the host strain for the recombinant DNA manipulations. The yeast *S. cerevisiae* MT8-1 (*MATa ade his3 leu2 trp1 ura3*) [22] was used for cell-surface expression and fermentation. *E. coli* was grown in LB medium (1% trypton, 0.5% yeast extract, 0.5% NaCl) containing 100 μg/ml ampicillin. Following precultivation, the yeast was aerobically cultivated at 30 °C in synthetic (S) medium (0.67% yeast nitrogen base without amino acid (Difco Laboratories, Detroit, MI, USA) with appropriate supplement) containing 2% glucose and 2% casamino acids (Difco Laboratories) (SDC medium).

2.2. Construction of expression plasmids

The plasmid for the cell-surface expression of the *T. reesei XYNII* gene was constructed as follows: the mature region of the *T. reesei XYNII* gene and its fusion with the gene encoding RGSHis6 were prepared from the *T. reesei* first-strand cDNA [23] by polymerase chain reaction (PCR) with two primers: XYN2-5′-Bgl II (5′-TCCGTGGCTGTGGAGATCTCCCAGACGATT-3′) and XYN2-3′-Bgl II (5′-GAAGATCTCCGCTGACGGTGATCGAAGCAG-3′); and XYNII-5′-RGSHis6 (5′-CGGGCGTCTTGGCCGCTCCCAGATCTCCAGAGGATCCCATCACCATCACCATCACCAGACGATT-3′) and XYN2-3′-Bgl II, respectively. The two amplified fragments (601 and 635 bp) were digested by *Bgl* II (underlined) and introduced into the *Bgl* II site of the cell-surface expression plasmid pCAS1 [19] containing the secretion signal sequence of the *Rhizopus oryzae* glucoamylase gene and the 3′-half of the α-agglutinin gene (the gene encoding C-terminal 320 amino acid residues and 446 bp of the 3′-flanking region) [24]. The resulting plasmids were named pCAS1-XYNII and pCAS1-RGSHis6-XYNII, respectively (Fig. 1).

2.3. Yeast transformation

Transformation of the plasmid pCAS1-XYNII, pCAS1-RGSHis6-XYNII and the control plasmid pCAS1 to *S. cerevisiae* MT8-1 was carried out through the lithium acetate method using the YEAST MAKER^{TM} yeast transformation system (Clontech Laboratories Inc., Palo Alto, CA, USA). The resulting transformants were named MT8-1/pCAS1-XYNII,

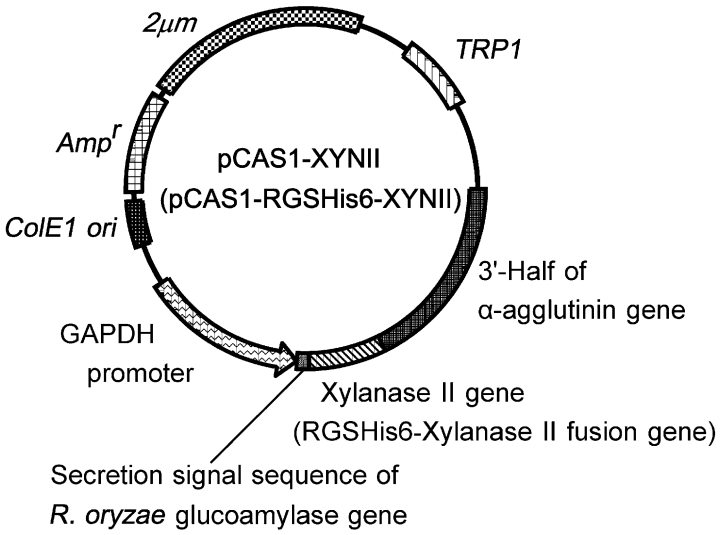

Fig. 1. Plasmid constructed for expression of XYNII-α-agglutinin and RGSHis6-XYNII-α-agglutinin fusion proteins on cell-surface.

MT8-1/pCAS1-RGSHis6-XYNII and MT8-1/pCAS1, respectively.

2.4. Enzyme assay

The transformants were screened for xylan-degrading activity using S medium plates containing 2% glucose and 0.2 % 4-*O*-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB xylan, Nacalai Tesque Inc., Kyoto, Japan) [7,23].

Xylanase activity was assayed in 100 mM sodium acetate buffer (pH 5.0) at 30 °C with 0.1% birchwood xylan (Sigma) as substrate. The amount of reducing sugar released was determined as xylose equivalent using the Somogyi–Nelson method [25]. Assay at different pH levels and temperatures was carried out as described before, except that the buffers used were 100 mM acetate buffer in the pH range 3.0–6.0 and 100 mM phosphate buffer at pH 7.0.

2.5. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously [26]. Immunostaining was performed as follows: cells were cultivated in SDC medium at 30 °C for 48 h, collected by centrifugation at 8000 rpm for 5 min and washed with phosphate buffered saline (PBS; 50 mM phosphate, 150 mM NaCl, pH 7.4). Cell suspension was adjusted to $OD_{600} = 10$ with PBS and 200 μl of the cell suspension was collected in a microtube. After incubation in PBS containing 1% bovine serum albumin (BSA), the cells and 200 μl of the primary antibody were further incubated in PBS containing 1% BSA at room temperature for 1.5 h. The primary antibody used was mouse immunoglobulin G (IgG) against RGSHis4 (Qiagen Inc., Valencia, CA, USA) at a dilution rate of 1:500. Cells were then washed with PBS and incubated at room temperature for 1 h at a dilution rate of 1:300 with 200 μl of the secondary antibody, Alexa Fluor 546-conjugated goat anti-mouse IgG (H+L) (Molecular Probes Inc., Eugene, OR, USA). After washing with PBS, the cells were then observed by microscopy.

2.6. Thin layer chromatography (TLC)

TLC analysis of the end products was performed using the method reported by Weill and Hanke [27]. Aliquots (10 μl) of the reaction mixture were spotted on a silica gel 60F254 thin layer chromatography plate (Merck), which was developed with 1-butanol–pyridine–water (70:15:15, v/v/v). Sugars were detected using the detection reagent of Murai

et al. consisting of 4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone, and 30 ml of 85% phosphoric acid [16]. The plate was sprayed with the detection reagent and heated at 105 °C for 30 min.

2.7. High performance liquid chromatography (HPLC)

HPLC analysis of the end products was perfor- med using a refractive index detector (Model RID-10A, Shimadzu, Kyoto, Japan). The column used for separation was a Cosmosil 5NH_2-MS packed column (Nacalai Tesque Inc., Kyoto, Japan). The HPLC was operated at 30 °C with 1.0 ml/min of acetonitrile: water = 60:40 (v/v) as mobile phase.

3. Results

3.1. Expression of XYNII gene

To determine whether the transformants had xylanase activity, a plate assay was carried out using RBB-xylan as substrate. The cells harboring the plasmid pCAS1-XYNII and pCAS1-RGSHis6-XYNII hydrolyzed the substrate and formed a clear halo around the colony, while no halo formation was observed around the cells harboring the control plasmid pCAS1 (Fig. 2). This result indicates that the XYNII-α-agglutinin and RGSHis6-XYNII-α-agglutinin fusion proteins are produced in the active form.

3.2. Xylanase activity

To determine whether the XYNII-α-agglutinin and RGSHis6-XYNII-α-agglutinin fusion proteins were secreted into culture medium or retained on the cell-surface, XYNII activities of MT8-1/pCAS1-XYNII and MT8-1/pCAS1-RGSHis6-XYNII were examined in both the culture supernatant and cell pellet fractions. Cells were aerobically cultivated in S medium containing 0.5% glucose and 2% casamino acids at 30 °C for 24 h until the glucose was consumed, and then separated into culture supernatant and cell pellet. XYNII activities of both fractions were then measured using 0.1% birchwood xylan as substrate at 30 °C. XYNII activities of the cell pellet are shown in Fig. 3. Both MT8-1/pCAS1-XYNII and MT8-1/pCAS1-RGSHis6-XYNII showed high activity (initial reaction rate: 1.78 and 2.01 μmol/min/g-dry cell), whereas, no XYNII activity was detected in the culture supernatant (data not shown). This result indicates that the XYNII-α-agglutinin and RGSHis6-XYNII-α-agglutinin fusion proteins are retained on the cell-surface in the active form. The activity of XYNII tagged with

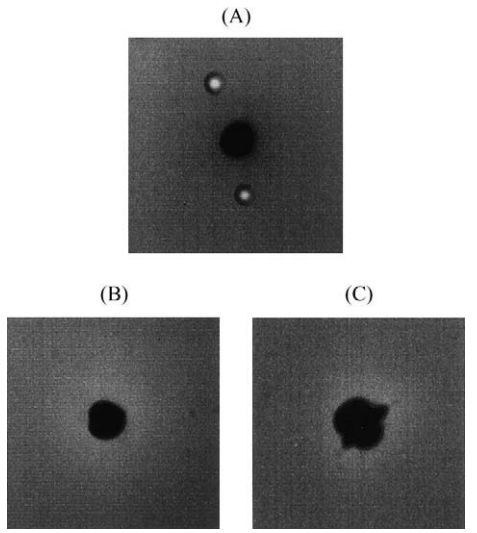

Fig. 2. Detection of xylanase activity on plate. (A) MT8-1/pCAS1 (control); (B) MT8-1/pCAS1-XYNII; (C) MT8-1/pCAS1-RGSHis6-XYNII.

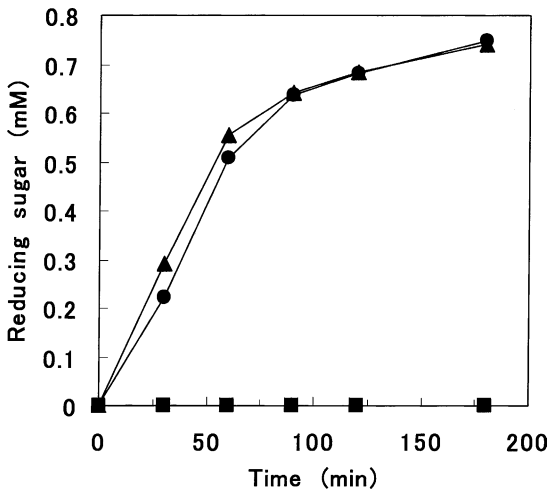

Fig. 3. Time course of hydrolysis of birchwood xylan by transformants. ■: MT8-1/pCAS1 (control); ●: MT8-1/pCAS1-XYNII; ▲: MT8-1/pCAS1-RGSHis6-XYNII. The cell concentration in reaction mixture was $A_{600} = 5.0$.

RGSHis6 at the N-terminus (MT8-1/pCAS1-RGSHis6-XYNII) was nearly equal to that of non-tagged XYNII (MT8-1/pCAS1-XYNII). The RGSHis6 tag therefore does not affect cell-surface XYNII activity.

3.3. Immunofluorescence microscopy

To confirm the presence of the RGSHis6-XYNII-α-agglutinin fusion protein on the cell-surface, immunofluorescence labeling of the cells was performed with mouse anti-RGSHis4 IgG as the primary antibody and Alexa Fluor 546-conjugated goat anti-mouse IgG as the secondary antibody. Cells were aerobically cultivated in SDC medium at 30 °C for 48 h. As shown in Fig. 4, MT8-1/pCAS1-RGSHis6-XYNII was labeled by Alexa Fluor 546-conjugated goat anti-mouse IgG and red fluorescence was observed on the cells, while no red fluorescence was observed on the cells harboring the control plasmid pCAS1, MT8-1/pCAS1, indicating that the RGSHis6-XYNII-α-agglutinin fusion protein is displayed on the cell-surface.

3.4. Effects of pH and temperature

To evaluate the features of the enzyme displayed on the cell-surface, the effect of pH (3.0–7.0) and temperature (20–60 °C) on XYNII activity of MT8-1/pCAS1-XYNII, which was aerobically cultivated in SDC medium at 30 °C for 48 h, was determined. The optimal pH for XYNII activity on the cell-surface was pH 5.0 (Fig. 5A), with the XYNII-α-agglutinin fusion protein stable in the pH range 3.0–7.0 (data not shown), and the optimal temperature 40 °C (Fig. 5B).

3.5. Analysis of end products

To analyze the end products released from birchwood xylan by XYNII displaying yeast strain MT8-1/pCAS1-XYNII, which was aerobically cultivated in SDC medium at 30 °C for 48 h, TLC

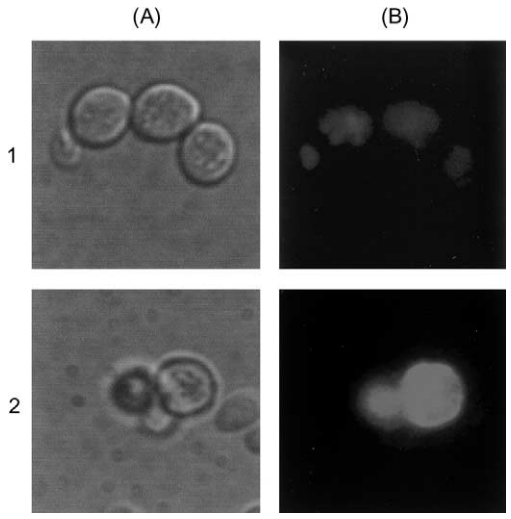

Fig. 4. Immunofluorescence labeling of transformants. Nomarski differential interference micrographs (A) and immunofluorescence micrographs (B). (1) MT8-1/pCAS1 (control); (2) MT8-1/pCAS1-RGSHis6-XYNII.

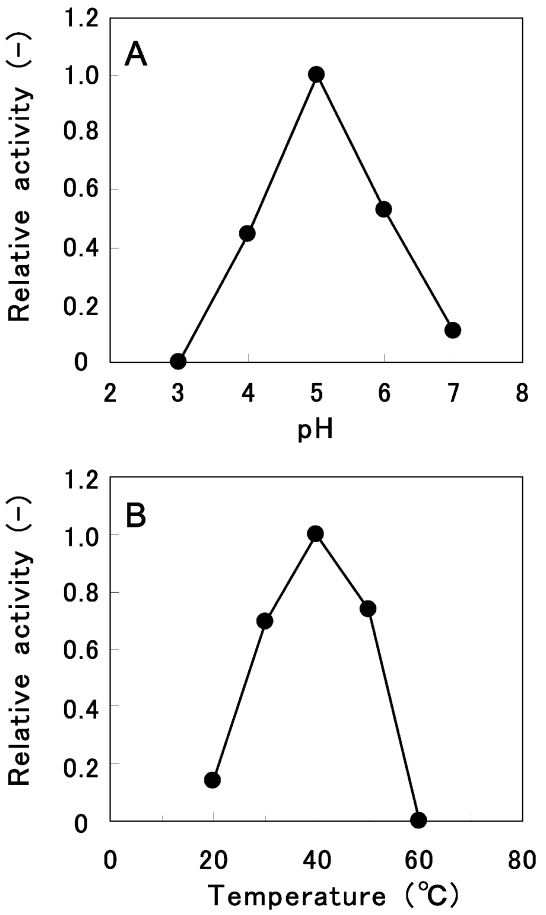

Fig. 5. Effect of pH (A) and temperature (B) on XYNII activity. Buffers used were 100 mM acetate (pH 3.0–6.0) and 100 mM phosphate (pH 7.0). The cell concentration in reaction mixture was $A_{600} = 5.0$.

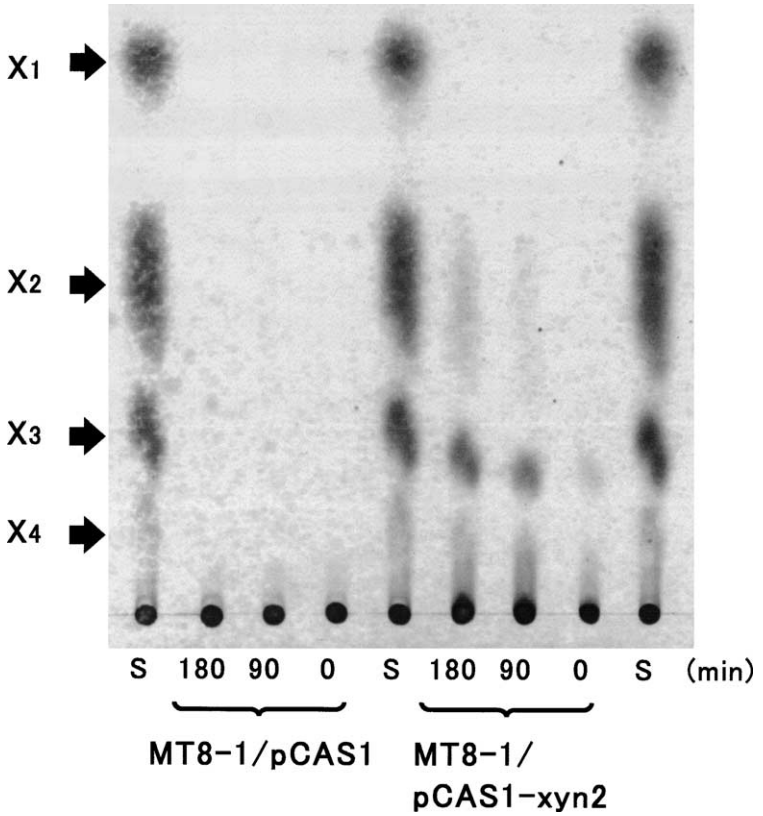

Fig. 6. TLC analysis of end products released from birchwood xylan. Aliquots (10 μl) of hydrolysate after hydrolysis for 0, 90 and 180 h by MT8-1/pCAS1 (control) and MT8-1/pCAS1-XYNII and authentic sugars (S, composed of X_1, X_2, X_3 and X_4) were spotted on silica gel plates. The cell concentration in reaction mixture was $A_{600} = 5.0$. The sugars detected were as follows: X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose.

and HPLC analysis was performed. As shown in Fig. 6, xylobiose, xylotriose and xylotetraose were detected as hydrolysates of birchwood xylan by MT8-1/pCAS1-XYNII, while no hydrolysate was observed in the reaction mixture of MT8-1/pCAS1. Formation of xylooligosaccharides was also confirmed by HPLC analysis (data not shown).

4. Discussion

We constructed a yeast-based whole-cell biocatalyst endowed with xylanolytic ability on the cell-surface. The *T. reesei XYNII* gene was isolated from the *T. reesei* first strand cDNA by PCR and expressed on the yeast-cell-surface as a fusion protein with the C-terminal half of α-agglutinin under the control of a strong constitutive GAPDH promoter. XYNII activity was detected by plate assay (Fig. 2) and was confirmed in the cell pellet with no leakage into the culture medium by birchwood xylan hydrolyzation experiment (Fig. 3). Immobilization of XYNII on the cell-surface was also confirmed by immunofluorescence microscopy (Fig. 4). These results indicate that the active XYNII-α-agglutinin and RGSHis6-XYNII-α-agglutinin fusion proteins are displayed on the cell-surface. Since enzyme was localized on the cell-surface, the resulting whole-cell biocatalyst was able to directly hydrolyze polymeric substrates such as xylan. While the preparation of enzyme powder and immobilized enzyme is laborious and costly, whole-cell biocatalyst can be prepared easily by cultivation.

The pH and temperature dependence of cell-surface XYNII activity are shown in Fig. 5. The optimal pH and temperature for XYNII activity on the yeast-cell-surface were pH 5.0 and 40 °C, similar to those for authentic *T. reesei* XYNII [8,9]. XYNII activity on the cell-surface was stable over a wide pH range (data not shown). Although authentic *T. reesei* XYNII showed high stability below 45 °C and retained its activity for 24 h (pH 4.5), enzyme activity was rapidly lost under incubation at over 55 °C [8]. The displayed XYNII was also stable at below 40 °C at pH 5.0 (data not shown). Surface display of XYNII thus did not affect its molecular characteristics.

The main products of birchwood xylan hydrolyzation by XYNII-displaying yeast-cells were xylobiose and xylotriose (Fig. 6). These xylooligosaccharides were identical to the products of authentic *T. reesei* XYNII [9]. XYNII-displaying yeast-based whole-cell biocatalysts would thus be effective in production of xylooligosaccharides and pulp bleaching.

Many researchers are engaged in extensive studies of ethanol production from cellulose and from hemicellulose, the main component of which is xylan, which in turn consists of xylose. Although *S. cerevisiae* has been used as an ethanol producer, it cannot utilize xylose. However, fermentation of xylose has recently become possible using genetically engineered yeast strains into which xylose reductase and xylitol dehydrogenase genes from *P. stipitis* are integrated [10,11]. Since XYNII displayed on yeast-cells is stable and active at pH 5.0 and 30 °C, suitable conditions for yeast growth and ethanol production, direct ethanol production from xylan will be possible through xylanase and β-xylosidase co-display on the cell-surface of xylose-utillizing recombinant yeast-cells. Further studies are required to develop a yeast strain which can produce ethanol directly from hemicellulose.

Acknowledgements

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