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A food-grade industrial arming yeast expressing β-1,3-1,4-glucanase with enhanced thermal stability*

Qin GUO[1], Wei ZHANG[2], Liu-liu MA[1], Qi-he CHEN[1], Ji-cheng CHEN[1], Hong-bo ZHANG[1], Hui RUAN[†‡1], Guo-qing HE[†‡1]

([1]*Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310029, China*)

([2]*Wenzhou Medical College, Wenzhou 325035, China*)

[†]E-mail: ruanhui98@yahoo.com.cn; gqhe@zju.edu.cn

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Abstract: The aim of this work was to construct a novel food-grade industrial arming yeast displaying β-1,3-1,4-glucanase and to evaluate the thermal stability of the glucanase for practical application. For this purpose, a bi-directional vector containing galactokinase (*GAL1*) and phosphoglycerate kinase 1 (*PGK1*) promoters in different orientations was constructed. The β-1,3-1,4-glucanase gene from *Bacillus subtilis* was fused to α-agglutinin and expressed under the control of the *GAL1* promoter. α-galactosidase induced by the constitutive *PGK1* promoter was used as a food-grade selection marker. The feasibility of the α-galactosidase marker was confirmed by the growth of transformants harboring the constructed vector on a medium containing melibiose as a sole carbon source, and by the clear halo around the transformants in Congo-red plates owing to the expression of β-1,3-1,4-glucanase. The analysis of β-1,3-1,4-glucanase activity in cell pellets and in the supernatant of the recombinant yeast strain revealed that β-1,3-1,4-glucanase was successfully displayed on the cell surface of the yeast. The displayed β-1,3-1,4-glucanase activity in the recombinant yeast cells increased immediately after the addition of galactose and reached 45.1 U/ml after 32-h induction. The thermal stability of β-1,3-1,4-glucanase displayed in the recombinant yeast cells was enhanced compared with the free enzyme. These results suggest that the constructed food-grade yeast has the potential to improve the brewing properties of beer.

Key words: α-agglutinin, Food-grade selection marker, β-1,3-1,4-glucanase, α-galactosidase, Thermostability
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1 Introduction

β-glucan is one of the major components of the cell wall in the main cereal grains such as barley and oats (Vis and Lorenz, 1997). During the brewing process, high concentrations of β-glucan resulting from improper malting or poor quality barley lead to high viscosity of beer, the formation of gelatinous precipitate, a reduction of the extract yield, and decreasing run-off of wort (Bamforth, 1994). Problems associated with β-glucan in beer can be alleviated by the application of commercial β-1,3-1,4-glucanase during malt production, fermentation, or lagering.

β-1,3-1,4-glucanase belongs to the glycosyl hydrolases family 16 with strict cleavage specificity for β-1,4-linkages adjacent to β-1,3-linkages, yielding 63.5% cellotriose and 29.5% cellotetraose as the major products when β-glucans are used as substrate (Bielecki and Galas, 1991; Tsai *et al*., 2005). Traditionally, β-1,3-1,4-glucanase is made by separation and purification from natural sources, such as *Penicillium emersonii*, *Aspergillus niger*, *Bacillus subtilis*, and *Trichoderma reesei* (Harman and Kubicek, 1998). Recently, a recombinant β-1,3-1,4-glucanase gene has been successfully expressed in a number of

‡ Corresponding authors

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microorganisms, including *Lactococcus lactis* (Li *et al*., 2009), *Escherichia coli* (Teng *et al*., 2006; Qiao *et al*., 2009), *Pichia pastoris* (Teng *et al*., 2007), and *Saccharomyces cerevisiae* (Hinchliffe and Box, 1984; van Rensburg *et al*., 1997; Zhang Q. *et al*., 2008).

However, the free enzyme preparation involving multiple processing steps is time-consuming and costly. In the brewing industry, a less-costly solution is to use yeast strains producing β-1,3-1,4-glucanase. Considering the lower fermentation performance and stability of laboratory haploid yeasts, industrial yeast strains are preferred as hosts for expression of β-1,3-1,4-glucanase genes (John, 1995). However, with industrial yeast strains it is difficult to choose a suitable selection marker for transformation because the introduction of auxotrophic mutations into polyploid yeast strains is neither practicable nor desirable. Also, the presence of auxotrophic markers in transformants has strong deleterious effects on the production levels of the desired heterologous proteins (Pronk, 2002). While antibiotic resistance markers can provide good selection independent of yeast genotypes, their presence is an undesirable trait in beer products. Therefore, a new dominant marker, safe and selective against a wild-type polyploid background, is required.

Melibiose can be hydrolyzed into galactose and glucose by α-galactosidase (EC 3.2.1.22) encoded by the *MEL1* gene (Ruohola *et al*., 1986). Various food-grade expression systems based on melibiose fermentation have been reported for *L. lactis* (Boucher *et al*., 2002; Jeong *et al*., 2006) and *Streptococcus thermophilus* (Labrie *et al*., 2005). However, melibiose is not commonly used as a substrate for the industrial yeast *S. cerevisiae*, because only a few *Saccharomyces* strains, such as *Saccharomyces bayanus* var. *uvarum*, *carlsbergensis*, and *oleaginosus*, are *Mel*-positive. Therefore, the construction of a novel food-grade vector harboring a dominant marker based on the phenotype of fermenting melibiose is an attractive option for the industrial yeast *S. cerevisiae*.

The lack of thermal stability of the recombinant β-1,3-1,4-glucanase is also a bottleneck for its application in the beer industry. As a consequence, the surface-display production of β-1,3-1,4-glucanase in yeast becomes more attractive. Unlike secreted enzymes, such displayed enzyme is readily produced at a low cost and is "naturally" immobilized onto the cell surface. Therefore, no tedious purification or immobilization processing is required (Murai *et al*., 1997). Moreover, such immobilization offers enzymes a physical support that often improves thermostability and facilitates reusability (Shusta *et al*., 1999; Park *et al*., 2006; Tanino *et al*., 2006; Gai and Wittrup, 2007).

In this paper, we report the development and evaluation of a novel food-grade industrial arming yeast displaying β-1,3-1,4-glucanase using α-galactosidase as a selection marker. The thermostability of β-1,3-1,4-glucanase displayed on the cell surface of yeast cells was successfully improved. This work is of value to the beer industry and to biocatalysic processes involving the expression of other related enzymes.

2 Materials and methods

2.1 Strains, plasmids, and growth conditions

E. coli DH5α was used for the maintenance and manipulation of plasmids. Industrial brewer's yeast MS-1 (preserved in our laboratory) was used as the host strain for the cell-surface display system. *B. subtilis* mutant ZJF-1A5 and wild-type *S. bayanus* var. *uvarum* (preserved in our laboratory) were the donor strains for the β-1,3-1,4-glucanase gene and α-galactosidase gene, respectively. Plasmid YEPlac181 (Gietz and Sugino, 1988) was a generous gift from Prof. R. Daniel Gietz (University of Manitoba, Canada) and was used as a backbone vector.

E. coli was grown in Luria-Bertani (LB) medium (0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, and 1% (w/v) tryptone; pH 7.0) at 37 °C with 100 μg/ml ampicillin when necessary. Yeast cells were cultured in YP medium (1% (w/v) bacto-peptone and 2% (w/v) yeast extract) supplemented with either 2% (w/v) glucose (YPD) or 3% (v/v) glycerol and 2% (v/v) lactic acid (YPGL) (Tuan, 1997). Using α-galactosidase as a selection marker, yeasts harboring the vector were selected on MSD medium (0.17% (w/v) yeast nitrogen base without amino acids and 0.5% (w/v) ammonium sulfate) supplemented with 2% (w/v) melibiose or on the YPD plate containing X-α-gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside; 4 mg/ml) at 30 °C. For solid media, 2% (w/v) agar was added.

2.2 Polymerase chain reaction (PCR) and DNA technique

The *PGK1* promoter (*PGK1*$_P$), the *GAL1* promoter (*GAL1*$_P$), the prepro-secretion signal sequence of the mating factor *α* 1 (*MFα1*), the α-agglutinin gene containing the 3′ half of the region encoding 320 amino acids and a 238 bp flanking region, and the alcohol dehydrogenase (*ADH1*) terminator (*ADH1*$_T$) were amplified by PCR using MS-1 genomic DNA as the template (their corresponding primers shown in Table 1), with the relevant restriction enzyme recognition sites added to the primers in 5′-ends and 3′-ends. The α-galactosidase and β-1,3-1,4-glucanase genes were amplified using *S. bayanus* var. *uvarum* genomic DNA and *B. subtilis* ZJF-1A5 genomic DNA as the templates (Table 1), respectively.

The PCR mixture (50 μl), prepared according to the instructions of the manufacturer of PrimeSTAR HS DNA polymerase (Takara, Kyoto, Japan), was incubated at 94 °C for 5 min and the reaction was then run at 98 °C for 10 s, 58 °C for 15 s, and 72 °C for 3 min for 31 cycles. All PCR products were purified and sequenced (Shanghai Sangon Co., Shanghai, China).

2.3 Plasmid construction

The fragment *GAL1*$_P$ was fused to the *MFα1* signal peptide sequence containing a multiple cloning site (MCS; *Nhe*I, *Nco*I, *Sac*II, and *Kpn*I) in vitro after the *Sph*I digestion. The fused fragment was digested with *Hin*dIII and *Kpn*I and then inserted into the *Hin*dIII/*Kpn*I site of YEp181, forming the plasmid YGM. The α-agglutinin and *ADH1*$_T$ gene fragments were digested with *Kpn*I and ligated to produce the fusion fragment NA. YGMNA was constructed by cloning the fragment NA into *Sac*II/*Eco*RI site of YGM. The *MEL1* gene fragment encoding α-galactosidase was amplified, digested with *Sal*I, and was then ligated with *PGK1*$_P$ fragments digested with the same enzyme to obtain the fusion fragment PM. The PM fragment was digested with *Aat*II and *Cla*I, and inserted into the same site of YGMNA, resulting in the plasmid YGMNA-PM. The gene encoding β-1,3-1,4-glucanase derived from *B. subtilis* mutant ZJF-1A5 was digested with *Nhe*I and *Sac*II, and then introduced into the YGMNA-PM vector to generate plasmid YGMPNA-PM-a. Finally, the Amp^R gene of YGMPNA-PM-a was removed by double digestion of *Aat*II and *Hin*dIII. The final construct of YGMPNA-PM was obtained (Fig. 1).

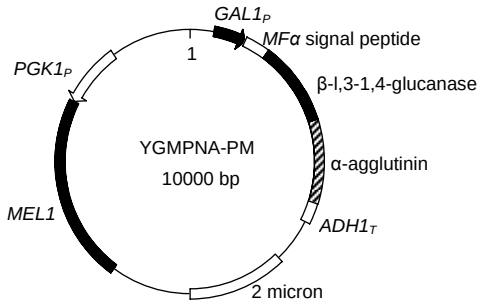

Fig. 1 The constructed plasmid YGMPNA-PM for expression of *MEL1* and the β-1,3-1,4-glucanase/ α-agglutinin fusion gene

2.4 Yeast transformation

Yeasts were transformed by electroporation (Thompson *et al*., 1998). Selection and maintenance of the transformants were carried out on MSD medium.

Table 1 Primers used in this study

Primer	Sequence (5′→3′)[a]	Restriction site
GAL1$_P$-S1	GGCAAGCTTTAGTACGGATTAGAAGCC	*Hin*dIII
GAL1$_P$-AS1	GGCGCATGCTCTCCTTGACGTTAAAGT	*Sph*I
MFα1-S1	GCGGCATGCAGAATGAGATTTC CTTC	*Sph*I
MFα1-AS1	TAGGTACCCCGCGGCCATGGGCTAGCTTCAGCCTCTCTTTTA	*Kpn*I, *Sac*II, *Nco*I, *Nhe*I
α-agglutinin-S1	TCCCCGCGG AGCGCCA AAAGCTCTTTTATC	*Sac*II
α-agglutinin-AS1	GCGGTACCTAATGAAACGAGCGGTAACG	*Kpn*I
ADH1$_T$-S1	GGGGTACCGCGAATTTCTTATG	*Kpn*I
ADH1$_T$-AS1	GGGAATTCGCATATCTACAATTGGG	*Eco*RI
PGK1$_P$-S1	CGGAAGCTT GACGTCCTTCAACTCAAGACGCACAG	*Aat*II
PGK1$_P$-AS1	GGCGTCGACTGTTTTATATTTGTTGTAAAAAGTAG	*Sal*I
Mel1-S1	GGCGTCGACATGTTTGCTTTATACTTTCTCA	*Sal*I
Mel1-AS1	GGCGGTACCATCGATCGAGTTTCTCAGAGTGCTTGGT	*Cla*I

[a] Underlines indicate the restriction sites for the fragments

2.5 α-galactosidase assay

α-galactosidase activity in the supernatants was determined by the method of McCleary (1988). *p*-nitrophenyl-α-D-galactopyranoside (PNPG) was used as a substrate. One unit of enzyme activity was defined as the amount of enzyme that releases 1 nmol of *p*-nitrophenol from the substrate per minute.

2.6 Cultivation and induction conditions for cell-surface display expression

Transformants were preincubated in 5 ml of YPD medium overnight at 30 °C with shaking at 180 r/min, and the 5-ml culture was used as a starter to inoculate 50 ml of YPGL medium in a 250-ml baffle flask. At optical density at 600 nm (OD_{600}) close to 2.0, galactose was added to 2% (w/v) final concentration.

2.7 Identification of the expression of β-1,3-1,4-glucanase in plate assay

A two-layer detection plate was used to identify the expression of the β-1,3-1,4-glucanase gene. The transformants were inoculated on YPG plates (1% (w/v) bacto-peptone, 2% (w/v) yeast extract, and 3% (v/v) glycerol). After 24 h, the YPG plates were covered with 5 ml of 0.5% (w/v) agar solution containing 0.1% (w/v) lichenan and 2% (w/v) galactose to induce the expression of the β-1,3-1,4-glucanase gene. After an induction at 30 °C for 20 h, the plates were stained with 0.1% (w/v) Congo-red solution until halos appeared. Colonies that formed clear halos were the recombinant yeasts expressing the β-1,3-1,4-glucanase gene (Zhang Q. *et al*., 2008).

2.8 Preparation of β-1,3-1,4-glucanase-displaying yeast cells

To prepare the β-1,3-1,4-glucanase-displaying yeast cells, 2 ml of culture broth was collected at various time points after induction and centrifuged at 10 000×*g* at 4 °C for 3 min. The pellet was washed with distilled water twice and resuspended in 400 μl of 0.05 mol/L sodium acetate buffer (pH 6.0) to give an OD_{600} of 2 for further use.

2.9 Assay of surface-displayed β-1,3-1,4-glucanase activity

The activity of β-1,3-1,4-glucanase was determined by the Congo-red method (van Rensburg *et al*., 1997). Barley β-glucan, used as the substrate in this assay, was dissolved in 0.05 mol/L sodium acetate (pH 6.0) to obtain a final concentration of 100 μg/ml. The reaction mixture, with a total volume of 1 ml, contained 400 μl of the yeast cell suspension and 600 μl of the substrate solution. The mixture was incubated at 50 °C at pH 6.0 for 1 h, heated at 100 °C for 10 min, and then cooled to room temperature. The supernatant was separated by centrifugation at 10 000×*g* at 4 °C for 3 min. One millilitre of Congo-red (100 μg/ml) was added to the supernatant. Absorbance of the supernatant was measured at OD_{540} (Wood *et al*., 1988). One unit of β-1,3-1,4-glucanase activity was defined as the amount of enzyme required to hydrolyze 1 μg β-glucan per hour at 50 °C and pH 6.0.

2.10 High performance liquid chromatography (HPLC) analysis of galactose concentration

The concentration of galactose was determined by HPLC. One millilitre of sample was taken out of the culture broth and immediately filtered through a cellulose acetate filter with 0.45-mm pores. The filtrate was frozen and kept at −20 °C until analyzed. HPLC analysis of galactose was performed using a Waters 2410 RI detector. The HPLC apparatus was operated at 90 °C with 0.1 mmol/L Ca-ethylenediamine tetraacetic acid (EDTA) solution at a flow rate of 0.25 ml/min as the mobile phase. Waters Sugar PAK-I columns, 6.5 mm×300 mm, were used for the separation.

2.11 Measurement of thermal stability of β-1,3-1,4-glucanase in yeast cells

Yeast cells harvested from culture broth were washed with distilled water twice and resuspended in 0.05 mol/L sodium acetate (pH 6.0). The suspension was then incubated at 50, 60 and 70 °C for 1–3 h. Periodically, aliquots of cell suspensions were taken and immediately cooled on ice. The residual β-1,3-1,4-glucanase activity was measured as described above. The percentage of residual β-1,3-1,4-glucanase activity was calculated by dividing the enzyme activity after incubation by the initial enzyme activity and then multiplying by 100. The thermal stability of free β-1,3-1,4-glucanase was also measured.

2.12 Plasmid stability

A single yeast colony was used to inoculate the

YPD medium. Yeast cells were diluted with distilled water and plated onto YPD and MSD plates every 24 h. After incubation at 30 °C for 48 h, the numbers of colonies on the plates were counted. Plasmid stability (*X*) was determined by comparing the number of colonies on the YPD plate (*A*) with that on the MSD plate (*B*), i.e., X (%)=B×100/A.

3 Results

3.1 Construction and characterization of the food-grade vector YGMPNA-PM

The vector YGMPNA-PM was designed according to bi-directional vectors (Miller III *et al*., 1998; Li *et al*., 2008) which allowed for the expression of two genes under the control of $GAL1_P$ and $PGK1_P$ in different orientations. Three restriction sites, *Nhe*I, *Nco*I, and *Sac*II, were introduced into YEPlac181 for easy manipulation.

β-l,3-1,4-glucanases from barley and *B. subtilis* have similar substrate specificities for β-glucan and lichenan (Muller *et al*., 1998). In this study, the β-1,3-1,4-glucanase gene from *B. subtilis* mutant ZJF-1A5 was cloned and put under the control of $GAL1_P$ and *MFα1*. To display the enzyme, the C-terminal half of α-agglutinin was used as a surface anchor for target proteins. In comparison with the published α-agglutinin sequence, 7 nucleotide mutations were found in the open reading frame (ORF) of our α-agglutinin gene sequence, which led to the changes of 5 amino acid sequences (data not shown). The *MEL1* gene from *S. bayanus* var. *uvarum* was fused to the constitutive $PGK1_P$ to form the food-grade selection module in yeast. The final vector YGMPNA-PM was constructed as described in the Section 2.3 (Fig. 1).

3.2 Evaluation of *MEL1* as a food-grade selection marker in industrial yeasts

It is well known that the industrial *S. cerevisiae* strains are recalcitrant to the uptake of exogenous DNA (Navas *et al*., 1991). When α-galactosidase was adopted as a marker for positive selection of yeast transformants, the transformation efficiency was 10–20 transformants per μg of DNA, which was much lower than that of laboratory haploid yeast strains (50–70 transformants per μg of DNA). The expression of the *MEL1* gene gave the industrial yeast the ability to ferment melibiose. As a result, the transformants carrying vector YGMPNA-PM could grow in the minimum media containing melibiose as a sole carbon source. The transformants were also easily detected by the appearance of a blue colony on the X-α-gal-containing medium owing to the expression of *MEL1*. The growth of *S. cerevisiae*/YGMPNA-PM cells in MSD medium was monitored by determining the OD_{600} of the culture broth. After being grown in MSD for 160 h, the absorbance of *S. cerevisiae*/YGMPNA-PM at 600 nm was able to reach about 16 (Fig. 2). However, no growth was observed in the control strain harboring YEPlac181.

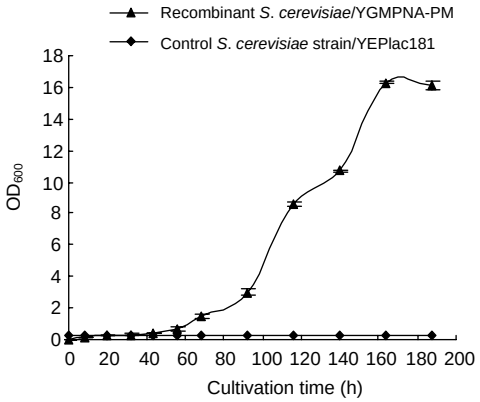

Fig. 2 Time courses of cell growth of *S. cerevisiae* in MSD medium

The data points represent the average of three independent experiments

Fig. 3 shows a comparison of α-galactosidase activity of *S. cerevisiae*/YGMPNA-PM between different cultivation modes (MSD and YPD). The α-galactosidase activity in both cultivation modes increased with the cultivation time. The enzyme activity reached its maximum at 44 h in YPD medium (104.38 U/ml) and at 116 h in MSD medium (41.72 U/ml). The peak enzyme activity in YPD medium was 2.5 times higher than that in MSD medium, which is likely to have resulted from the differences in nutrients between these two media. The α-galactosidase activity in YPD medium then decreased but almost remained constant in MSD medium. The plasmid stability in the cells cultivated in YPD and MSD media at 116 h was about 60% and 95%, respectively (data not shown).

After the transformants were obtained on MSD plates using α-galactosidase as the selection marker,

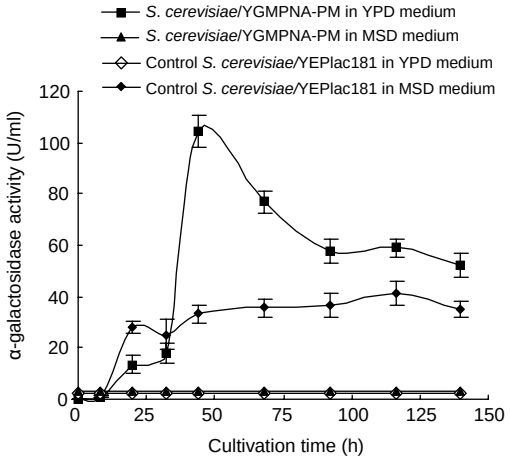

Fig. 3 Time courses of α-galactosidase activity of *S. cerevisiae* in different cultivation media
The data points represent the average of three independent experiments

we performed a plate assay to determine whether the transformants gained β-1,3-1,4-glucanase activity. A two-layer detection plate was used to identify the expression of the β-1,3-1,4-glucanase gene as described above. The desired *S. cerevisiae*/YGMPNA-PM transformants were preliminarily identified by the formation of halos strictly around colonies grown on the YP plates. Clear halos reflected the degradation of lichenan owing to the expression of the β-1,3-1,4-glucanase gene (Fig. 4). No halos were observed around the control strain/YEPlac181. Thus, the use of α-galactosidase as the selection marker was proved to be effective, and the resultant yeast strains/YGMPNA-PM could be called food-grade yeast.

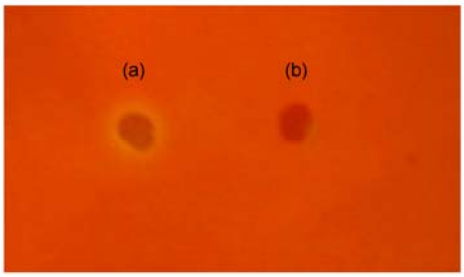

Fig. 4 Plate assay for detection of β-l,3-1,4-glucanase activity
Cells were grown on YPG plate at 30 °C for 24 h. The YPG plate was then covered with 5 ml of 0.5% (w/v) agar solution containing 0.1% (w/v) lichenan and 2% (w/v) galactose to induce the expression of β-1,3-1,4-glucanase. β-l,3-1,4-glucanase activity was indicated by the formation of a halo around the colony. (a) *S. cerevisiae*/YGMPNA-PM; (b) Control strain/YEPlac181

3.3 Localization of β-1,3-1,4-glucanase

To determined whether β-1,3-1,4-glucanase was secreted into the culture medium or retained by the cells, the culture broth and cell pellets were separated by centrifugation and β-1,3-1,4-glucanase activity was measured in both fractions. Table 2 shows that the activity of β-1,3-1,4-glucanase in *S. cerevisiae*/YGMPNA-PM cell pellets was 45.1 U/ml, but was undetectable in the supernatant, indicating that the β-1,3-1,4-glucanase activity was associated with *S. cerevisiae*/YGMPNA-PM cells, not secreted into the culture.

Table 2 Distribution of β-1,3-1,4-glucanase activity

Strain	Activity of the expressed enzyme[a]		
	Culture supernatant	Cell pellet (U/ml)	Relative activity (%)
Strain (YGMPNA-PM)	ND	45.1±3.2	100
Strain (YGMNA-PM)	ND	ND	0

[a] Values are averages of three independent experiments. ND: not detected

3.4 Evaluation of the β-glucan-utilizing ability of *S. cerevisiae*/YGMPNA-PM

The expression of the β-1,3-1,4-glucanase gene in *S. cerevisiae*/YGMPNA-PM was induced by galactose in YPGL medium. Although the growth rate of yeast cells in YPGL medium was lower than that in YPD medium, this induction mode was used for lower repression of the galactose promoter ($GAL1_P$) than glucose-containing medium (Choi *et al*., 1994).

The culture course shows that β-1,3-1,4-glucanase activity in *S. cerevisiae*/YGMPNA-PM cells increased quickly after galactose addition and reached 45.1 U/ml after 32-h induction (Fig. 5). Subsequently, the enzyme activity decreased and then reached a plateau after 48 h induction. The reduction of β-1,3-1,4-glucanase activity was likely to have resulted from the loss of plasmid under non-selective pressure and the depletion of galactose. Fig. 5 shows that galactose was exhausted rapidly after 20 h. The metabolism of galactose by yeast was the expression-limiting factor in this work. Currently, we are constructing a *GAL1*-disrupted industrial *S. cerevisiae* strain to improve further the β-1,3-1,4-glucanase activity, because $GAL1_P$ is responsible for the first step of galactose catabolism (Štagoj and Komel, 2008).

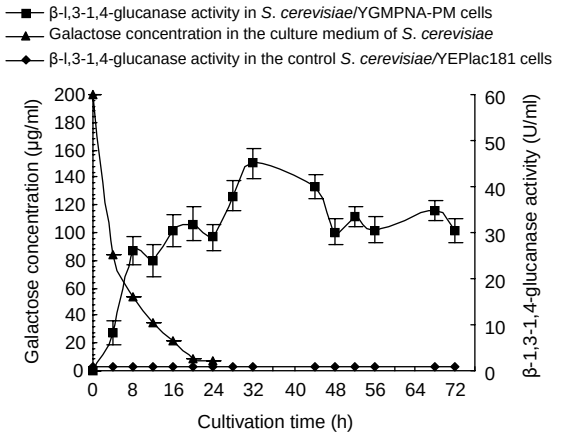

Fig. 5 Time courses of β-l,3-1,4-glucanase activity and consumption of galactose in YPGL medium after galactose addition

The data points represent the average of three independent experiments

3.5 Thermal stability of the β-1,3-1,4-glucanase in yeast cells

To investigate the thermostability of the enzyme in *S. cerevisiae*/YGMPNA-PM cells, the displayed enzymes were incubated at 50, 60 and 70 °C. Periodically, aliquots of cell suspension were taken and used for the residual activity measurement (Fig. 6). The residual activity of the free β-1,3-1,4-glucanase immediately decreased to 51.9% at 50 °C and to 20.1% at 60 °C, and was hardly detected at 70 °C after 2-h incubation. In contrast, the residual activity of β-1,3-1,4-glucanase in *S. cerevisiae*/YGMPNA-PM cells increased with incubation time and reached 129.2% at 60 °C and 109.2% at 70 °C after 1-h incubation, and then gradually decreased. After incubation for 3 h at 60 and 70 °C, the enzyme activity in *S. cerevisiae*/YGMPNA-PM cells remained at 64.6% and 35.8%, respectively, which indicated an improved thermostability.

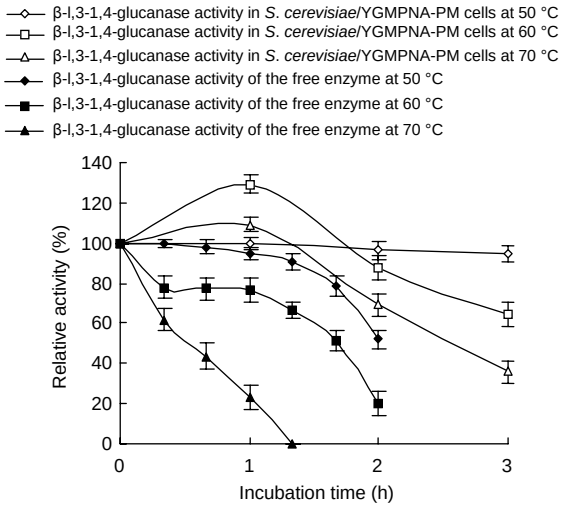

Fig. 6 Time courses of residual β-l,3-1,4-glucanase activity in whole cells of *S. cerevisiae*/YGMPNA-PM

The data points represent the average of three independent experiments

4 Discussion

β-1,3-1,4-glucanase can hydrolyze β-glucan and is considered to be helpful in beer production. To date, many studies have been carried out to improve its activity and performance by DNA recombinant techniques (Chen *et al*., 2001; Teng *et al*., 2007; Li *et al*., 2009). However, little attention has been paid to the practicability and safety of the application of β-1,3-1,4-glucanase in the food industry. Table 3 shows a comparison of different systems used in studies of the expression of β-1,3-1,4-glucanase genes. In this study, the construction of a novel food-grade industrial arming yeast displaying β-1,3-1,4-glucanase was described and evaluated.

Generally, auxotrophic markers such as *URA3*, *HIS3*, *LEU2*, and *TRP1* are the most commonly used selection markers in yeast transformation, and they are parts of food-grade markers. However, transformation by complementation of auxotrophic mutations cannot be employed for industrial polyploid yeasts. Estruch and Prieto (2003) tried to construct a *Trp*$^-$ industrial yeast using a module containing the *G418* resistance cassette, flanked by direct repeats from the *MEL1* gene of *S. cerevisiae*. They obtained the desired yeast strains after four gene disruption rounds, but a remaining functional wild-type *Trp* copy was still present in the genome of the strain, which caused a high reversion frequency of the *Trp*$^-$ phenotype. Food-grade industrial recombinant yeast can also be constructed by other selective methods, such as self-cloning and co-transformation. In a previous study, our lab constructed a food-grade industrial yeast secreting β-1,3-1,4-glucanase by homologous recombination using "*loxP-KanMX-loxP*" as the selection marker, which permitted antibiotic marker removal between the two repeats "*loxp*" after transformation using plasmid pSH47 carrying the

Table 3 Expression comparison of β-1,3-1,4-glucanase under different conditions

Expression system	Expression condition		Safety	Enzyme activity (U/ml)	Thermal stability	References
	Expression way	Selection marker				
E. coli BL21	Secretion	Kanamycin	No	24.1	Low	Qiao *et al*., 2009
L. lactis MB138	Display	Erythromycin	No	12.0	−	Li *et al*., 2009
Industrial *S. cerevisiae*	Secretion	G418	No	69.3	−	Zhang Q. *et al*., 2008
P. pastoris	Secretion	Kanamycin/His4	No	67.9	Low	Teng *et al*., 2007
Haploid *S. cerevisiae*	Secretion	*URA*	Yes	Weak	−	van Rensburg *et al*., 1997
Haploid *S. cerevisiae*	Secretion	*LEU*	Yes	Weak	Low	Hinchliffe and Box, 1984
Industrial *S. cerevisiae*	Display	α-galactosidase	Yes	45.1	High	This study

Cre-recombinase expression module (Zhang Q. *et al*., 2008). However, the method was laborious because it involved several transformations and screening procedures to remove the selection marker. Co-transformation is also a technique that allows the construction of a food-grade yeast strain by using a vector carrying the gene of interest simultaneously with another plasmid carrying a selectable marker (Guerra *et al*., 2006). A drawback related to co-transformation is low transformation efficiency. As the criteria for better markers are high transformation efficiency and reliability of true transformant selection (Akada, 2002), we ruled out the co-transformation method. Recently, α-galactosidase was used as the food-grade selection marker in *L. lactis* and *S. thermophilus*. The characteristics of α-galactosidase make it a potential selective marker for yeast. It is convenient to screen and does not require the isolation of auxotrophic mutants of the industrial strain nor any antibiotic resistance genes. The *S. cerevisiae*/YGMPNA-PM using α-galactosidase as the selective marker in this work could be grown and selected on melibiose-containing medium, and the ability to express the β-1,3-1,4-glucanase gene of transformants was confirmed by the plate assay. The result proved the feasibility of using α-galactosidase as a selection marker in yeast.

Furthermore, the expression of the *MEL1* gene in yeast could be used to determine the number of pasteurization units of ale beer. The measurement of α-galactosidase activity has been proposed as a method of determining the number of pasteurization units (PU) of a lager beer (Enevoldsen, 1981; 1985). However, the α-galactosidase inactivation method is not generally applicable to ale beer at present, because the brewing ale strains (*S. cerevisiae*) do not produce α-galactosidase (Liljestrom-Suominen *et al*., 1988; Gasent-Ramirez *et al*., 1995). The *MEL1* selection module constructed in this study, when introduced into industrial ale strains, not only could be used as the selection marker for transformation, but also could extend the method to beers other than lagers.

The display of enzymes is one of the most attractive applications of cell-surface display in yeasts (Shibasaki *et al*., 2009). The enzymes displayed on the cell surface of the brewer's yeast offer an easy reproduction of biocatalysts and easy separation of products from catalysts (Kondo and Ueda, 2004). Here, we report that β-1,3-1,4-glucanase was successfully immobilized on the cell wall of *S. cerevisiae*/YGMPNA-PM by an α-agglutinin anchor system and could hydrolyze β-glucan efficiently. Also, the thermal stability of the displayed β-1,3-1,4-glucanase on the cell surface of yeast cells was much improved compared with the free form.

The thermal stability of β-1,3-1,4-glucanase plays an essential role in the degradation of barley β-glucan. During the malting and saccharification process, β-1,3-1,4-glucanase is heat-inactivated, leaving a high content of β-glucan in wort (Teng *et al*., 2006). Immobilization can increase the stability of enzymes (Sakai *et al*., 1991). Zhang W. *et al*. (2008) reported that the thermostability of *Rhizomucor miehei* lipase displayed on the cell surface of *S. cerevisiae* using α-agglutinin as an anchor was improved. Some studies pointed out that enzymes displayed in *P. pastoris* had better thermal stability than those displayed in *S. cerevisiae* because *P. pastoris* was more appropriate for protein folding and the formation of disulfide bonds (Tanino *et al*., 2006; Han *et al*., 2009).

However, *P. pastoris* is an unsuitable microorganism for use in the food industry. The mechanisms of the enhanced enzyme activity are still under investigation, but it has been postulated that the extent of glycosylation accounts for much of the stability difference between free and immobilized forms (Shusta *et al*., 1999; Tanino *et al*., 2006). Molecular rigidity of enzymes immobilized on the cell surface may be improved by attachment to a rigid support, which perhaps causes the enhanced thermostability in combination with the creation of a protected microenvironment (Mateo *et al*., 2007). Thus, the thermal stability of β-1,3-1,4-glucanase in *S. cerevisiae*/YGMPNA-PM cells was superior to that of the free form and was more feasible for high temperature industrial reactions in the beer industry.

In summary, we constructed a food-grade arming yeast strain that displayed β-1,3-1,4-glucanase with enhanced thermostability, thus meeting the requirements and market criteria for commercial use in the beer industry. Beer production with this yeast strain would represent a technical improvement and would also improve beer quality. Further studies on the fermentative properties and use of this recombinant arming yeast in beer brewing are under way.

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