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

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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...
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, 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 biocatalytic 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 YE-Plac181 (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 inYP medium (1% (w/v) bacto-peptone and 2% (w/v) yeast extract) supplemented with either 2% (w/v) glucose (YPD) or 3% (w/v) glycerol and 2% (w/v) lactic acid (YPGL) (Tuan, 1997). Using α-galactosidase as a selection marker, yeasts harboring 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.