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Short communication

# Combinatorial library strategy for strong overexpression of the lipase from *Geobacillus thermocatenulatus* on the cell surface of yeast *Pichia pastoris*

Ryosuke Yamada, Yusuke Kimoto, Hiroyasu Ogino *

*Department of Chemical Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan*

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ABSTRACT

The yeast cell surface display technique allows for the expression of a target protein on the yeast cell surface and has many applications such as the immobilization of enzymes and the development of biosensors. To increase the expression of the BTL2, a lipase from *Geobacillus thermocatenulatus*, on the cell surface of yeast *Pichia pastoris*, we developed a combinatorial library strategy for selecting appropriate expression cassette comprising sequences encoding a promoter, secretion signal, mature BTL2, anchoring protein, and terminator. The transformant GS115/D90, which comprised *P. pastoris ENO1* promoter sequence, *Hansenula polymorpha GAS1* secretion signal sequence, and *Saccharomyces cerevisiae GAS1* anchoring protein gene, exhibited 5-fold higher lipase activity compared to the control strain harboring a conventional expression cassette. Using the developed strategy, an appropriate expression cassette for the strong overexpression of target proteins on the cell surface of yeast could be rapidly and easily obtained.


## 1. Introduction

The yeast cell surface display technique allows for the expression of a target protein on the cell surface of yeast tethered by the anchoring protein that is genetically fused with the target protein. Yeast cell surface display is used for the immobilization of enzymes; the development of biosensors, vaccines, and antibodies; library screening; and biosorption; etc. [1,2]. To express the target protein on cell surface, the expression cassette composed of sequences encoding the promoter, secretion signal, target protein, anchoring protein, and terminator is required. Expression of the target protein on cell surface is controlled by many factors such as transcription efficiency of the promoter, the secretion efficiency imparted by the secretion signal, and the efficiency of immobilization provided by the anchoring protein [3–6]. Thus, selection of the appropriate promoter, secretion signal, and anchoring protein is important to overexpress target proteins on cell surface [6–8].

A variety of promoter sequences have been tested for their efficiency in overexpressing proteins in yeast, not only on the cell surface but also intra- and extracellularly. Use of the constitutive promoters *TDH3* and *PGK1* in yeast *Saccharomyces cerevisiae* [9] and *Pichia pastoris* [10,11] resulted in strong overexpression of target proteins. However, Da Silva and Srikrishnan reported that the strength of promoters in *S. cerevisiae* differed drastically depending on the target protein being expressed [9]. In addition, in a study of 26 types of promoter sequences combined with 3 types of target proteins in the yeast *P. pastoris*, Stadlmayr et al. reported that promoter strength was largely dependent on the compatibility between promoter and target protein sequences [12]. Besides promoter sequences derived from other species of microorganisms have been used for the overexpression of target proteins [13–15].

Multiple secretion signal sequences have also been tested to improve target protein secretion from yeast [4,16,17]. Many types of target proteins were successfully overexpressed and secreted using the α-factor secretion signal sequence from *S. cerevisiae* in *S. cerevisiae*, *P. pastoris*, and *Kluyveromyces lactis* [18–20]. Because the secretion signal sequence greatly influences the amount of protein secreted [17,21], selection of a secretion signal sequence appropriate for the target protein is very important.

Many anchoring proteins have been developed for use in yeast and bacteria [8,22]. The *S. cerevisiae* cell wall-anchoring domain of α-agglutinin, the glycosylphosphatidylinositol (GPI)-anchored cell wall protein involved in sexual adhesion of mating-type a and α cells, is most frequently used because it binds tightly to the cell surface through covalent bonds [23]. Use of the 3′-terminal region encoding 320 amino acids of this α-agglutinin cell wall-

* Corresponding author.
*E-mail address:* ogino@chemeng.osakafu-u.ac.jp (H. Ogino).

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anchoring protein has resulted in successful target protein display on the surface of *S. cerevisiae*, *Yarrowia lipolytica*, and *Kluyveromyces marxianus* [8,15,23]. However, anchoring protein selection had a significant effect on the efficiency of target protein immobilization. Van der Vaart et al. compared 8 different anchoring proteins fused to α-galactosidase in *S. cerevisiae* and observed that the amount of α-galactosidase produced varied up to 8-fold depending on the anchoring protein [6]. While several anchoring proteins from *P. pastoris* have been used [8], most were derived from *S. cerevisiae* and have not been optimized for cell surface expression in *P. pastoris*. The identification of anchoring proteins that promote strong overexpression of target proteins on the cell surface of *P. pastoris* is desired, as *P. pastoris* is a promising host for heterologous protein production [24].

Lipases are versatile biocatalysts that perform a wide range of bioconversions, including hydrolysis, esterification, interesterification, alcoholysis, acidolysis, and aminolysis [25]. Thus, lipases have potential applications in industries producing detergents, food, leather, textiles, oils and fats, cosmetics, paper, and pharmaceuticals. Lipases from thermophiles often exhibit extreme stability at elevated temperatures and in organic solvents [26]. The BTL2, a lipase from *Geobacillus thermocatenulatus*, exhibits such properties [27]. These desirable characteristics of BTL2 have led to its use in immobilized forms, for biodiesel fuel production [28], and for aliphatic ester synthesis [29]. Thus, an efficient method for the immobilization of BTL2 is needed.

To improve the expression of BTL2 on the cell surface of *P. pastoris*, we used a combinatorial library strategy to select an appropriate expression cassette. We constructed a combinatorial plasmid library comprising sequences encoding 15 promoters, 15 secretion signals, and 15 anchoring proteins. *P. pastoris* cells with high lipase activity were then selected from transformants harboring the combinatorial plasmid library. Finally, the components of the expression cassettes harbored by *P. pastoris* transformants with high lipase activity were identified.

## 2. Materials and methods

### 2.1. Strains and media

The *Escherichia coli* strains HST08 (TaKaRa Bio Inc., Otsu, Japan) was used as a host for recombinant DNA manipulations. Recombinant *E. coli* cells were cultivated on LuriaBertani (LB) medium (10 g/L tryptone [Nacalai Tesque, Kyoto, Japan], 5 g/L yeast extract [Nacalai Tesque], and 5 g/L NaCl) supplemented with 100 μg/mL ampicillin sodium salt.

The yeast strains *P. pastoris* GS115 (Life Technologies, Carlsbad, CA, USA) was used as a host for cell surface display of BTL2 from *G. thermocatenulatus*. Recombinant *P. pastoris* cells were selected and cultivated using minimal dextrose (MD) medium (13.4 g/L Yeast Nitrogen Base without amino acids [Difco Laboratories, MI, USA], 0.4 mg/L biotin, and 20 g/L glucose) buffered with sodium phosphate (pH 7.0, 100 mM). Flask cultivation was performed using a 500 mL flask with baffles containing 150 mL of medium and a rotary shaker operated at 30 °C and 150 rpm. Cultivation was performed in 2-mL deep, 96-well plates containing 1.2 mL of medium/well, equipped with a gas-permeable seal on a rotary plate shaker operated at 30 °C and 1500 rpm.

### 2.2. Plasmid construction and yeast transformation

All primers used in this study are summarized in Supplementary Table 1. The construction methods of the plasmid pPPE_GPBTLAG and the plasmid library pPPE_LibBTLAnc (Fig. 1) are described in Fig. 2 and supplementary method.

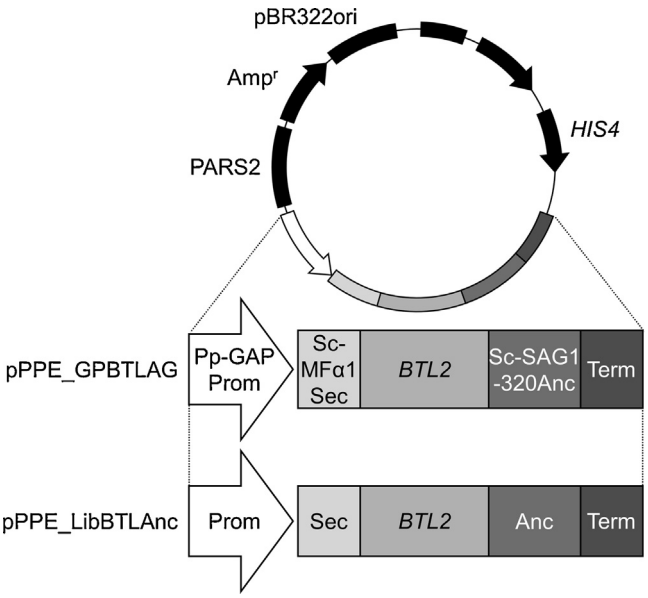


**Fig. 1.** Plasmids for *BTL2* gene expression in *P. pastoris*.
Pp-GAP Prom, GAP promoter sequence from *P. pastoris*; Sc-MFα1 Sec, secretion signal sequence of α-factor gene from *S. cerevisiae*; *BTL2* gene from *G. thermocatenulatus*; Sc-SAG1-320Anc, 3′-terminal region encoding 320 amino acids of α-agglutinin gene from *S. cerevisiae*; Term, *AOX1* terminator sequence from *P. pastoris*; Prom, 15 different promoter sequences from *S. cerevisiae*, *P, pastoris*, and *H. polymorpha*; Sec, 15 different secretion signal sequences from *S. cerevisiae*, *P, pastoris*, and *H. polymorpha*; Anc, 15 different anchoring protein genes from *S. cerevisiae*, *P. pastoris*, and *H. polymorpha*.

Constructed plasmids were transformed into *P. pastoris* GS115 using electroporation as previously described [30]. The *P. pastoris* strain harboring pPPE_GPBTLAG was named GS115/pPPE_GPBTLAG.

### 2.3. High-throughput screening of transformants with high lipase activity

*P. pastoris* GS115 cells transformed with the combinatorial plasmid library were cultured on MD agar media. High-throughput screening of transformants with high lipase activity was performed using a 96-well-plate based cultivation and enzyme assay. The transformants were cultivated for 48 h, then the 96-well-plates were incubated for 30 min at 60 °C for thermal activation. The resulting culture broths were used for measuring lipase activity.

### 2.4. Preparation of selected transformant cells for assay of lipase activity

Selected transformants were cultivated in 500-mL flasks for 48 h, collected by centrifugation at 3000*g* for 5 min at 4 °C, washed twice, and resuspended in sodium phosphate buffer (pH 7.0, 100 mM) at 5 $OD_{600}$. Cell suspensions were then incubated for 30 min at 60 °C for thermal activation and used for lipase activity assays.

### 2.5. Lipase activity assays

Lipase activity was assayed by the hydrolysis of 2,3-Dimercapto-1-propanol tributyrate coupled with 5,5′-dithiobis(2-nitr obenzoic acid) at 40 °C using the Lipase kit S (Dainippon Pharmaceutical Co., Osaka, Japan) according to the protocol specified by the supplier.