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Establishment and application of a modified membrane-blot assay for *Rhizomucor miehei* lipases aimed at improving their methanol tolerance and thermostability

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ABSTRACT

Directed evolution has been proved an effective way to improve the stability of proteins, but high throughput screening assays for directed evolution with simultaneous improvement of two or more properties are still rare. In this study, we aimed to establish a membrane-blot assay for use in the high-throughput screening of *Rhizomucor miehei* lipases (RMLs). With the assistance of the membrane-blot screening assay, a mutant E47K named G10 that showed improved thermal stability was detected in the first round of error-prone PCR. Using G10 as the parent, two variants G10-11 and G10-20 that showed improved thermal stability and methanol tolerance without loss of activity compared to the wild type RML were obtained. The T_{50}^{60}-value of G10-11 and G10-20 increased by 12 °C and 6.5 °C, respectively. After incubation for 1 h, the remaining residual activity of G10-11 and G10-20 was 63.45% and 74.33%, respectively, in 50% methanol, and 15.98% and 30.22%, respectively, in 80% methanol. Thus, we successfully developed a membrane-blot assay that could be used for the high-throughput screening of RMLs with improved thermostability and methanol tolerance. Based on our findings, we believe that our newly developed membrane-blot assay will have potential applications in directed evolution in the future.

1. Introduction

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3), a family of industrial enzymes that catalyze the hydrolysis of fats or lipids, have been widely applied in the areas of food, detergents, paper manufacturing, leather, textiles, organic synthesis, production of cosmetics, and biodiesel production [1]. However, lipases can undergo deactivation in synthetic reactions due to changes in temperature, shear stress, exposure to interfaces, and chemical denaturants [2], which reduces the life span of lipases and increases the cost of production. Therefore, the development of lipases with tolerance to organic solvents and high temperature is essential for their application in industries.

Directed evolution of proteins to mimic natural evolution is a powerful way to enhance the stability, activity, and selectivity of enzymes [3,4]. Directed evolution usually consists of three steps: construction of a mutant library, expression of mutant proteins, and high-throughput screening for mutants with the desired property. Several methods such as the use of degenerated oligonucleotides, chemical mutagenesis, saturation mutagenesis, error-prone PCR, and DNA shuffling have been developed to obtain mutant libraries [2,5–7]. The constructed mutant libraries are usually very large, making high throughput screening the key step in directed evolution. Efforts have been made to use directed evolution for improving the properties of lipases, such as tolerance to high temperatures and organic solvents, for a long time, but very few studies have been focused on the simultaneous improvement of two or more properties [8]. One reason for this may be the lack of a fast and efficient assay for reducing the heavy workload of throughput screening for two or more evolved properties.

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Escherichia coli has become the first choice for the creation, expression, and screening of mutant libraries due to its advantages such as ease of genetic manipulation, availability of efficient genetic tools, high transformation efficiency, and rapid growth rates. There have been many reports on developing effective high throughput screening methods for the *Escherichia coli* expression system [9]. However, prokaryotic systems like the *Escherichia coli* expression system also present various disadvantages for heterologous protein expression due to misfolding (especially for eukaryotic proteins) and the absence of post-translational modifications, and their intracellular production often presents a risk of inclusion body formation [3]. *Pichia pastoris*, an effective host for the production of both secreted and intracellular heterologous proteins, can help overcome the disadvantages of the *Escherichia coli* system, since it is capable of disulfide bond formation, proteolytic maturation, N-and O-linked glycosylations, and other post-translational modifications [10]. At present, high-throughput screening of the *Pichia pastoris* expression system is mainly accomplished using phenotypic selection in microtiter well plates [11–15]. However, screening using microtiter well plates not only results in a heavy workload and requires expensive instruments for colony operation, but also takes a relatively long time.

To simplify the high-throughput screen steps, the plate assay was previously applied for lipases and other enzymes [16–18]. Plate assay is a simple and rapid technique, but the original colonies can be easily damaged, and only a few characteristics of the enzyme, such as temperature thermostability, can be determined by this assay. Membrane-blot assay has also been applied for rapid screening of enzymes [2,19,20]. In this assay, colonies are replicated on a membrane (nitrocellulose membrane or filter paper) and cultured in the induced solid media. After enzyme expression, the membranes that had been absorbed with enzymes are treated with the required conditions such as high temperature or organic solvent. Positive enzymes with the required characteristics are finally obtained, as revealed by fluorescence or development of color on the membrane. However, only one membrane is insufficient to balance colony growth and enzyme absorption. Colony growth requires a membrane with large pore size and good permeability, such as filter paper, but the enzyme adsorption capacity of membranes like filter paper is low and the enzymes are easily detached, especially in the washing step. Membranes with good adsorption performance are not conducive to the growth and introduction of strains, and the existence of strains would lower the clarity of coloration. Therefore, in the current study, we aimed to establish a membrane-blot screening assay for the high-throughput screening of lipases. Our membrane-blot screening assay had two membranes, one for colony growth and the other for enzyme absorption. Mutant pools of *Rhizomucor miehei* lipases (RMLs) in *Pichia pastoris* were constructed using error-prone PCR and were screened using the developed membrane-blot screening assay for improvement in the properties of thermal stability and methanol tolerance.

2. Materials and methods

2.1. Strains, vectors, and media

Restriction enzymes and DNA polymerase were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). *Escherichia coli* DH5α cells (TaKaRa, Dalian), *Pichia pastoris* GS115, and the pPIC9K plasmid were purchased from Invitrogen (Carlsbad, CA, USA). Olive oil, T4 DNA ligase, primers for RML cloning and polymerase chain reaction (PCR) were ordered from Sangon Biotechnology (Shanghai, China). pNPP was ordered from Aladdin Industrial Corporation (Shanghai, China). The *R. miehei* lipase sequence of the transformants were sequenced by Sangon Biotechnology (Shanghai, China).

2.2. Construction of the mutant library using error-prone PCR and its screening

The *R. miehei* lipase sequence (GenBank accession No. A02536) was used as the template for error-prone PCR with the primers RML-F (5′-CCGGAATTCGCCACCATGGTCCCTATTAAGCGTCAAA-3′) and RML-R (5′-ATAAGAATGCGGCCGCTTACGTGCACAACCCGGTATTTA-3′). Random mutagenesis with 1–2% error rates was obtained using the Genemorph II Kit (Stratagene) according to the manufacturer's instructions. The resulting PCR products were purified using a QIAquick spin column (Qiagen) and digested with *Eco*RI and *Not*I for 3 h. The digested products were then ligated into the pPIC9K plasmid using T4 DNA ligase for 12 h at 16 °C. The resulting libraries were transformed into *Escherichia coli* DH5α cells, which were then cultured in Luria–Bertani medium with 50 μg/mL ampicillin. The positive mutants were transformed into *Pichia pastoris* GS115 competent cells, which were grown in selective MD media (1.34% yeast nitrogen base, 400 μg/L biotin, 2% dextrose, and 2% agar).

2.3. Protocol for screening for enhanced thermal tolerance and improved methanol tolerance

Steps for the membrane-blot screening assay and staining protocol for enhanced thermal tolerance and methanol tolerance are shown in Fig. 1. Following incubation at 30 °C for 1 day, the transformants on MD plates (~400/plate) were lifted onto sterile filter circles and placed colony-up on BMMY plates that had been already placed by sterile nitrocellulose membranes in advance. The transformants were then again incubated for 48 h at 30 °C to induce protein expression. After induction of protein expression, the nitrocellulose membranes were immersed in phosphate buffered saline (PBS) solution (50 mM potassium phosphate, pH 8.0, 0.1% Triton X100) at 30 °C for 1 h. Next, the PBS solution was decanted and replaced by MeOH solution containing 0.1% Triton X100, and the nitrocellulose membranes were incubated at desired temperature for desired time. After incubation, the MeOH solution was decanted and the filters were developed by overlaying them with staining solution (1 mM 1-naphthyl palmitate, 3 mM Fast Blue RR, 0.5% Triton X100). After 10 min, the mutants displaying residual activity were identified based on the formation of purple colored dots. The colonies corresponding to the purple colored dots were then isolated from the MD plates for validation and further characterization. The *R. miehei* lipase sequence of the positive transformants were cloned by PCR and sequenced with the primers alpha-

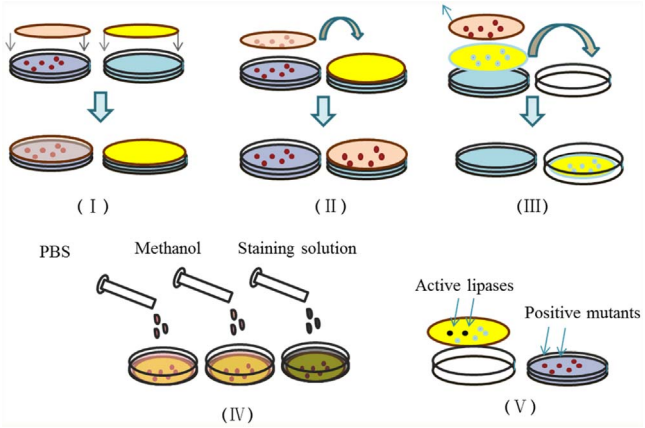

Fig. 1. Protocol for screening using the membrane-blot screening assay. i: sterile filter paper and nitrocellulose membrane were plated on the MD and BMMY plate, respectively; ii: the sterile filter paper was lifted onto the BMMY plate, the and colony was transferred from the MD plate to the BMMY plate; iii: After protein expression, the nitrocellulose membrane was removed for processing; iv: The nitrocellulose membrane was treated with PBS solution, MeOH solution, and staining solution in that order; v: Positive mutants were identified from the MD plate.