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Research Article

The Preparation of Bioimprinted Whole-cell Biocatalyst and Its Application in Bioconversion of Biodiesel

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Abstract: Biodiesel has attracted considerable attention as an environmentally friendly alternative fuel. Lipase is the most popular enzyme for biodiesel production and immobilization has been deployed to improve enzyme stability and reusability. Exploitation of high activity lipase is the key point for biodiesel production. Whole-cell biocatalysts have been applied in the biosynthesis of biodiesel and bioimprinting is a promising approach for enzyme performances improvement. In this study, based on the *S. cerevisiae* cell-surface display system with α-agglutinin as anchor, a whole-cell biocatalyst of codon-optimized *Rhizopus oryzae* lipase was constructed and bioimprinted with oleic acid, gaining 5-fold increase on enzymatic activity in the alcoholysis of soybean oil to biodiesel. Moreover, the conversion of FAME was up to 95.45±2.73% after a 27-h reaction at 60°C. Our results indicated that combining bioimprinting with yeast display technique to prepare whole-cell biocatalyst could result in potential enzymes for bioconversion of biodiesel in organic solvents.

Keywords: Biodiesel, bioimprinting, *Rhizopus oryzae* Lipase (ROL), whole-cell biocatalyst, yeast surface display

INTRODUCTION

Nowadays, lipases (EC 3.1.1.3) have become the most popular enzymes for biodiesel biocatalysis. Transesterification reactions catalyzed by lipases can be performed under mild temperature and normal atmospheric pressure and small amounts of water and free fatty acids in raw substrates have no influence on the synthesis reaction. However, there are several technical difficulties for the application of lipase in the production of biodiesel, such as enzyme instability in organic solvent, time-consuming and high cost in processes like free enzyme purification, separation and production, etc. (Adamczak *et al*., 2009). Yeast surface display technique has been developed for whole-cell biocatalyst preparation (Kondo and Ueda, 2004). The enzymes are produced and immobilized on the cell wall of yeasts spontaneously during cultivation, which cuts down the cost of enzymes and saves immobilization materials as well as facilitates the process of purification and immobilization (Fukuda *et al*., 2008).

Bioimprinting has been developed to improve enzymatic activity and stability in non-aqueous medium (Gonzalez-Navarro and Braco, 1997). When lipase was bioimprinted with fatty acid, the three-dimensional structure of the enzyme remains "frozen" in a modified form in the organic phase, as if 'remembering' the structure of the ligand. This was caused by interfacial activation by the hydrophobic substrate. This interfacial activation was accompanied, for most lipases, with the opening of an α-helical "lid" covering the active site in aqueous surroundings. Fatty acids are suitable bioimprinted materials, which facilitate interfacial activation and "open" conformation exposure of lipase (Fishman and Cogan, 2003). However, the memory will be lost in aqueous systems (Yilmaz, 2002).

In the present work, based on the yeast whole-cell lipase biocatalyst successfully constructed by displaying codon-optimized *Rhizopus oryzae* Lipase (ROL), its activity was further improved via bioimprinting, aiming to get high conversion in biodiesel production.

MATERIALS AND METHODS

Strains, genes and plasmids: We have successfully constructed a yeast displaying lipase whole-cell biocatalyst, in which *S. cerevisiae* MS-1 (purchased from Meishan Mauri Yeast Co., Ltd., China) was used as the host strain for cell surface display system and expression plasmid pGMRAK (Fig. 1) was transformed into the *S. cerevisiae* MS-1 (Chen *et al*., 2011). Moreover, this whole-cell biocatalyst presented great thermo stability in bioconversion of biodiesel in our previous report (Chen *et al*., 2012).

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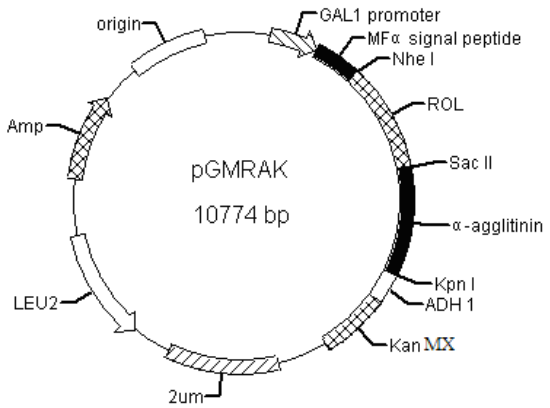

Fig. 1: Construction of the plasmid pGMRAK, for ROL displaying yeast

Screening by halo assay: Transformants were spread on YGCG plate medium containing 0.2% tributyrin as substrate (Kato *et al*., 2007). Colonies hydrolyzing tributyrin were identified by the formation of clear halos. *S. cerevisiae* MS-1 harboring pGMRAK (MS-1/pGMRAK) and *S. cerevisiae* MS-1 harboring pGMAK (MS-1/pGMAK) as the control were spread on the medium. The activities of the lipases were examined by the halo formed around the colony.

Preparation of bioimprinted and non-bioimptinted whole-cell biocatalyst: To prepare the whole-cell biocatalyst for the bioconversion of biodiesel, MS-1/pGMRAK was cultivated in YGCG medium at 30°C for 120 h (stationary phase). After cultivation, the cells were collected by centrifugation (3,000 g, 15 min), washed with distilled water and 50 mM phosphate buffer pH 7.0, mixed with and without oleic oil, lyophilized for 24 h by a freeze drying system (Mingarro *et al*., 1995), to prepare bioimprinted whole-cell biocatalyst and non-bioimptinted one. Then, the biocatalyst powder was washed with hexane to remove the imprint molecules and vacuum dried to remove the solvent. The dry biocatalyst powder was kept under refrigeration.

Measurement of the lipase activity: The hydrolytic activity of lipase displayed on yeast cell surface was measured with p-Nitrophenyl Palmitate (pNPP) as the substrate, according to Prim *et al*. (2000). Bioimprinted whole-cell biocatalyst and non-bioimptinted one were measured, respectively. One unit of hydrolytic activity was defined as the amount of enzyme that releases 1 μmol of pNP/min under the assay conditions described (n = 3).

Biodiesel biosynthesis and its gas chromatography analysis: Alcoholysis reaction was carried out in a water bath in 50 mL screw-capped vessel with reciprocal shaking at 150 rpm. A typical reaction mixture was consisted of soybean oil and methanol using hexane as the solvent. Lyophilized bioimprinted whole-cell biocatalyst or non-bioimprinted one was added for the conversion of triglycerides to FAME (fatty acid methyl ester). After termination of the reaction, the FAME formation in the reaction mixture were detected by Gas Chromatography (GC) (Agilent Technologies, series HP7890) (Wilmington, Del., USA) equipped with an automatic injector, a Flame Ionization Detector (FID) and fitted with a HP-88 capillary column (60 m× 0.25 mm i.d. ×0.20 μm film thickness). The amount of sample injected was 1 μL and the GC conditions were: Injector and Detector temperature were 250°C, The initial oven temperature 90C° (5 min) was increased to 180°C at 10°C/min (10 min), then increased to 220°C at a rate of 5°C/min (8 min). Hydrogen was used as the carrier gas. Heptadecanoic acid (C17:0) was used as internal standard. Conversion of FAME is defined as the FAME amount produced over the initial amount of oil (g/g).

RESULTS AND DISCUSSION

Expression of the ROL gene: A clear and distinguishable halo was observed around the colonies of ROL-displaying yeast on plate medium, but not for the control yeast (Fig. 2). The result indicated that the ROL-α-agglutinin fusion protein was successfully expressed and displayed in an active form. This is the first example of yeast displaying fully codon-optimized ROL as whole-cell biocatalyst with high enzymatic activity in the *S. cerevisiae* cell surface display systems with α-agglutinin as anchor. We could infer that the high activity of codon-optimized ROL was attributed to the increase of iso accepting tRNA molecules (Ikemura, 1985). Codon usage was considered as a key determinant of eventual expression of heterologous protein (Lithwick and Margalit, 2003). Additionally, compared with traditional immobilization methods, displaying of enzymes on the yeast cell surface as whole cell catalyst has at least two advantages. Firstly, the displayed enzymes can be readily produced by fermentation. No further work is required to purify or immobilize the enzymes. Secondly, enzymes displayed on the yeast cell surface can be modified directly by conventional genetic engineering, which enables error-prone PCR, DNA shuffling and gene codon optimization to be used quickly and efficiently to create strains with enhanced enzyme activity (Shiraga *et al*., 2004). Although it is also convenient to prepare whole-cell biocatalyst using BSPs to immobilize the cells, the immobilized materials cost high and the enzymes immobilize normally were intracellular ones, which means that the enzymatic process is slower than that of extracellular enzymes. Yeast displaying whole cell catalyst is either not extracellular, however, it can avoid the above problem, for it is located on the surface of cells.

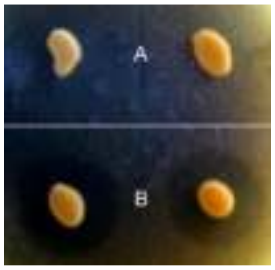

Fig. 2: Halo formation of ROL-displaying yeasts cultivated for 44 h

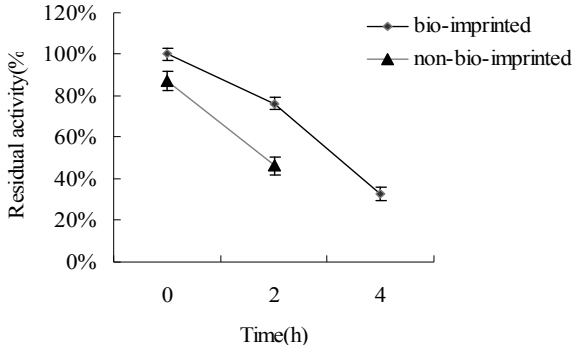

Fig. 3: Influence of bioimprinting on thermostability

Bioimprinting and activity, thermostability detection: It is confirmed that bioimprinting improve enzyme activity and catalytic efficiency. Bioimprinting of lipases with fatty acids has been applied in organic solvents to obtain highly active enzyme in many researches. Yan *et al*. (2009) reported a combined strategy including bioimprinting with dual imprint molecules and a cosolvent coupled to pH tuning, KCl salt activation, lecithin coating and immobilization on macroporous resin. This method effectively enhanced the activity and operational stability of *Geotrichum* sp., lipase. The modified lipase exhibited an 18.4-fold enhanced esterification activity towards methyl oleate synthesis and retained 90% activity following repeated use in 10 cycles. Yilmaz (2002) reported that bioimprinted lipase has yielded a 3.5- to 4.5-fold activity enhancement. Solvent free medium was equally effective as hexane medium. Still, a few research mentioned the influence of bioimprinting of lipase in the aqueous phase (Gonzalez-Navarro and Braco, 1998, 1997; Mingarro *et al*., 1995; Yilmaz, 2002). Here, we measured the bioconversion rate of biodiesel in organic phase and the hydrolytic activity in the aqueous phase for both bioimprinted whole-cell biocatalyst and non-bioimprinted one. The recombinant MS-1/pGMRAK was harvested at stationary phase (120 h) and imprinted with oleic acid. The hydrolytic activity of the bioimprinted whole-cell biocatalyst was 28.7±0.93 U/g dried cells, while that of the non-bioimptinted one was 25±0.89 U/g dried cells, which accounted for 87.1% of the bioimprinted one. However, in the biosynthesis of biodiesel in organic phase, bioimprinting with oleic acid resulted in a 5-fold increase of the conversion of triglycerides to biodiesel from 15.46 to 77.71%. By contrast, the effect of bioimprinting in the aqueous phase was not so significant. Bioimprinting only slightly improves hydrolytic activity for the bioimprinted catalyst because water may erase the bioimprinting effect due to the 'memory erase' as explained in previous studies (Gonzalez-Navarro and Braco, 1998, 1997; Mingarro *et al*., 1995). The enhanced activity in organic phase may be explained in that Oleic acid was used not only as the amphiphile for interfacial activation, but also as the substrate to induce conformational changes in the remaining cavity of the enzyme and facilitate matching of substrate functional groups after the imprint molecules were removed, leading to higher activity increase, which proved that substrate interfaces also affected the helical loop in the same way as amphiphile interfaces do.

As shown in Fig. 3, after 2 h and 4 h incubation at 60°C, the residual activity of bioimprinted whole-cell biocatalyst decreased to 76.28 and 32.76%, respectively. For the non-bioimprinted one, after 2 h incubation at 60°C, the residual activity decreased from 87.1% in the beginning to 46.45%. In addition, after 6 h incubation at 60°C, cell debris was observed in the bioimprinted sample implicating that yeast cells were fractured during the heating process, while it took only 4 h for the non-bioimprinted one to show the same state. These results indicated that the thermostability of bioimprinted whole-cell biocatalyst was improved in the aqueous phase compared with the non-bioimprinted one, which may be attributed to the protection of oleic acid in lyophilization process, resulting in less structural damage and more active enzyme structure maintaining.

Application in enzymatic bioconversion of biodiesel: Recently, many efforts have been endowed to the preparation of whole-cell biocatalyst by using BSPs to immobilize the cells. This method has been developed and applied in the field of biodiesel biosynthesis (Hama *et al*., 2006, 2007; Oda *et al*., 2005). Tamalampudi *et al*. (2008) reported that whole-cells of *Rhizopus oryzae* immobilized onto BSPs could catalyze the alcoholysis of Jatropha oil more effectively than Novozym 435. Matsumoto *et al*. (2001) firstly utilized yeast whole-cell biocatalyst of ROL to produce biodiesel from plant oil and methanol in a solvent-free and water-containing system. The FAME content in the reaction mixture was 71% after a 165-h reaction at 37°C with stepwise addition of methanol. Hama *et al*. (2010) compared the surfactant modified yeast whole-cell biocatalyst of ROL and the control one for

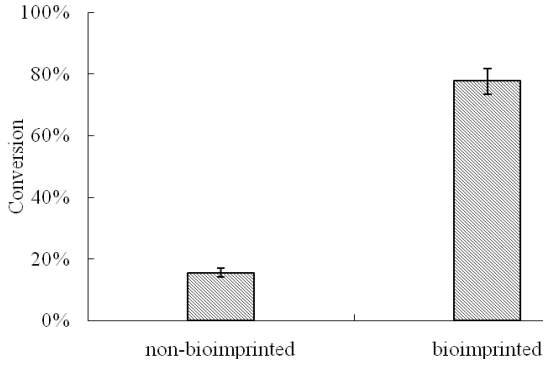

Fig. 4: Effect of bioimprinting on bioconversion of biodiesel

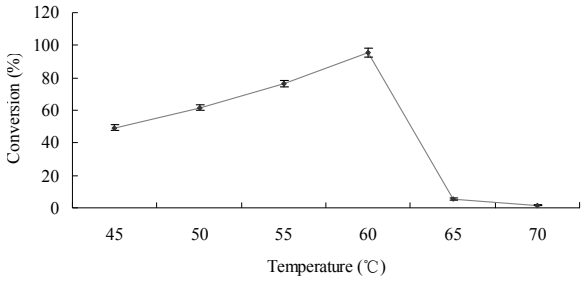

Fig. 5: Influence of the temperature in bioconversion of biodiesel

enzymatic synthesis of phospholipids and FAME in hexane. The result indicated that this modification could enhance the potential of the surface-displayed lipase for bioconversion. Thus, we combined cell surface display and bioimprinting strategies to construct a bioimprinted whole cell ROL biocatalyst for the biotransformation of biodiesel. In our study, the conversion of triglycerides to biodiesel by the bioimprinted whole-cell ROL biocatalyst was 77.71%, which was 5-fold of the non-bioimptinted one (15.46%) (Fig. 4). Figure 5 showed the main results of the alcoholysis reaction under standard reaction conditions at different temperatures. The conversion rate of biodiesel increased with the increase on temperature, reaching a maximum value of 95.45±2.73% at 60°C (Fig. 5), however, it decreased dramatically at both 65 and 70°C, which may be caused by the gradual denaturalization of the enzyme and cell lysis of yeast over 65°C. In general, to produce biodiesel from triglyceride by a sn 1, 3-Regioselective Lipase (including ROL), the maximum conversion of FAME was 67%, thus containing more than 30% of MAG and DAG in the reaction mixture (Arai *et al*., 2010; Caballero *et al*., 2009; Lee *et al*., 2006). In our research, the high conversion of FAME can be attributed to acyltransferase from the yeast cells (Zou *et al*., 1997), which catalyzed the acyl migration from the sn-2 position to the sn-1 or sn-3 position in partial glycerides (Kaieda *et al*., 1999). In addition, there were some free fatty acids in the soybean oil, which could be esterified to FAME by ROL resulting in higher conversion. Thus, a maximum FAME conversion 95.45±2.73% was achieved at 60°C. At last, GC data showed that the FAME contained methyl esters of C16:0, C16:1n7, C18:0, C18:1n9, C18:2n6 and C18:3n3 (Fig. 6).

CONCLUSION

In conclusion, for the first time we combined the strategies of codon-optimized display and bioimprinting to construct the yeast whole-cell biocatalyst with high activity (28.7±0.93 U/g dried cells for the bioimprinted whole-cell biocatalyst and 25±0.89 U/g dried cells for the non-bioimprinted one) and catalytic efficiency with FAME conversion up to 95.45±2.73%. This result provides an alternate method for the industrialization of biodiesel. In the further studies, unedible oils like crude *Jatropha curcas* seed oil, microbial oil and waste cooking oil etc. will be utilized as raw materials. Also, high density fermentation of the yeast whole cell biocatalyst and the production amplification of biodiesel catalyzed by the biocatalyst will be conducted.

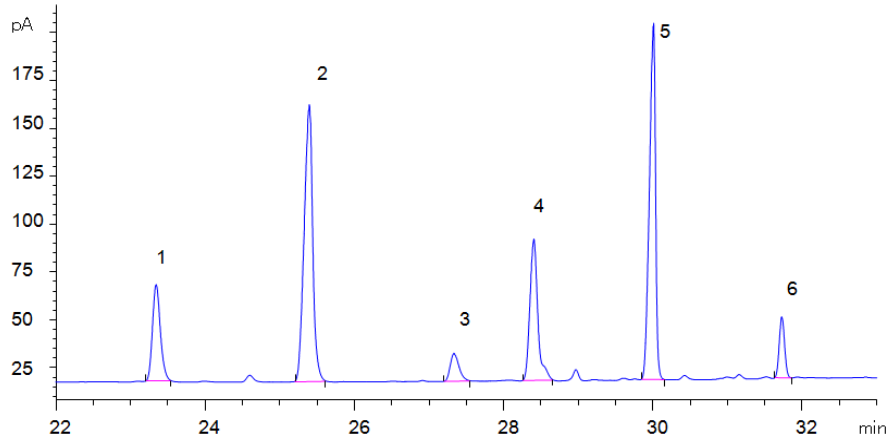

Fig. 6: GC chromatograms of FAME produced by optimized-ROL displaying yeast

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