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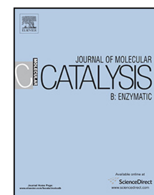

Improvement of *P. aeruginosa* 42A2 lipase preparations for FAMEs production, both in immobilized and soluble form

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ABSTRACT

Lipase application in industrial biocatalytic processes is a topic of great interest due to their versatility and mild conditions of use. Many lipases from different sources have been studied, improved and used in a variety of chemical reactions. One of the most recent applications is the transesterification of vegetal triglycerides with methanol to produce fatty acid methyl esters (FAMEs), aimed at the synthesis of enzymatic biodiesel. In general, this process has been performed using immobilized lipases as biocatalysts. However, last tendencies are promoting the use of soluble enzyme preparations to reduce the cost of the enzymatic preparations and to make the whole process more competitive with respect to the chemical systems. In this work, we evaluated both, the soluble and immobilized preparations of two cold-adapted lipases: LipC from *Pseudomonas aeruginosa* 42A2, and its thermo stable variant LipCmut, which were over expressed and produced using a low cost protocol. Several conditions of soybean oil transesterification with such lipase preparations were tested to evaluate the enzymatic FAMEs production process.

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1. Introduction

Biodiesel constitutes an important new alternative fuel. It is generally produced from transesterification of vegetable oils with short alcohols. It is a safe, nontoxic, biodegradable, renewable source, less contaminant than fossil fuels [1]. Nowadays enzymatic biodiesel is attracting the interest of industrial and policy-making partners because of the mild reaction conditions required, the high efficiency and selectivity of the catalysts available, the ease of glycerol removal, and the possibility to perform synthesis reactions in the presence of low water concentrations [2,3].

Lipases have been industrially very important enzymes in the last decades and are gaining interest nowadays, especially in the synthesis of biodiesel. A large variety of lipases have been used for transesterification or esterification [4], where they have been extensively used for biodiesel production in their immobilized form [1,5]. Success of immobilization was based on the possibility to reuse the enzymes and the increase of their stability, often reached with immobilization on expensive and sophisticated supports [6]. However, one of the main drawbacks for using immobilized lipases relies on the high costs of the enzyme, due to the use of expensive supports, and the high price of the immobilization process itself [3]. On the contrary, liquid lipases can be produced and sold at a price 30–50 times lower (P.M. Nielsen, personal communication) than immobilized enzymes, making the whole process more competitive and sustainable. Even though, there are still only a few works in the literature regarding the use of soluble lipases in transesterification reactions [3,7,8]. But such systems are gaining more and more interest due to the possibility to accept water in the biodiesel production process. Water is essential to maintain the specific tridimensional structure of some lipases, especially if used in soluble form [9]. Lipases from many sources have been tested in biodiesel production and among them, lipases from *Pseudomonas* strains have been found as good candidates for transesterification of vegetable oils [10,11]. At this respect, Shah and Gupta (2007) reported a yield of 98% production of FAMEs from *Jatropha* oil using *Pseudomonas cepacia* lipase immobilized on Celite [12], and Luo and coworkers screened a psychrophilic lipase from *Pseudomonas fluorescens* that resulted in a yield of 92% FAMEs production at 20 °C [13–15]. *Pseudomonas* species lipases have also been defined as enzymes with great resistance to methanol compared to other immobilized lipases tested [15], which can result in a higher FAMEs yield.

In a previous work, we described lipase C from *P. aeruginosa* 42A2, as a cold-adapted enzyme, with optimum temperature in the range 4–20 °C, but showing low stability at higher temperatures [16]. Therefore, a thermo resistant variant of LipC–LipCmut–, showing good stability until 60 °C while maintaining its cold-adapted properties, was obtained by rational design and saturation

Abbreviations: FAMEs, fatty acid methyl esters; FFAs, free fatty acids; GC, gas chromatography; IPTG, isopropil-β-D-1-tiogalattopiranoside; MeOH, methanol; RT, room temperature.

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Table 1
Bacterial strains and plasmid used.

Plasmid	Strain	Features	Reference
	P. aeruginosa PABST7.1	Δ*lip*A Δ*lip*H miniD-180 (*tet*A *tet*R *lac*Iq P*lac*UV5-T7 gene1)	[17]. Kindly provided by Dr. Rosenau
	P. putida KT2440	*P. putida* mt-2 without TOL plasmid, *hsdR*	[18] Kindly provided by Dr. Prieto
pBBLipC	*P. aeruginosa* PABST7.1 lipCHpBB	Contains *lip*C and *lip*H 42A2 genes at *Hind*III-*Xba*I sites of pBBRMCS1	[14]
pBBLipCmut	*P. aeruginosa* PABST7.1 lipCHmutpBB (variant D2_H8)	Contains mutated *lip*C and wild type *lip*H 42A2 at *Hind*III-*Xba*I sites of pBBRMCS1	[14]
pBBR1MCS		Shuttle vector for *E. coli* and *Pseudomonas*: Cm^r lacZ lacI	[22]
pMMB207		Shuttle vector for *E. coli* and *Pseudomonas*: Cm^r Ptac M13mp18 polylinker	[19] Kindly provided by Dr. García
pMMLipC	*P. aeruginosa* PABST7.1 lipCHpMM	Contains *lip*C and *lip*H 42A2 genes at *Kpn*I-*Xba*I sites of pMMB207	This work
pMMLipCmut	*P. aeruginosa* PABST7.1 lipCHmutpMM	Contains *lipCmut* and wild type *lip*H 42A2 at *Kpn*I-*Xba*I sites of pMMB207	This work

mutagenesis [14]. Both enzymes were cloned, characterized and immobilized onto several low cost supports for their evaluation as industrial biocatalysts in synthesis reactions (Cesarini et al., submitted for publication). In this work, we describe the evaluation of these two non-commercial lipases for FAMEs production, used both in their immobilized and soluble forms, for a possible application to the enzymatic biodiesel production. For this purpose, we first improved lipase expression and the protein concentration to get more powerful enzyme preparations. Next, we tested different transesterification conditions for FAMEs production using soybean oil as a feedstock.

2. Materials and methods

All chemicals and solvents were purchased from Sigma–Aldrich and Serviquimia (Spain) and, except when stated, were for analysis quality.

2.1. Bacterial strains and plasmids

Pseudomonas strains and the plasmids used in this work are listed in Table 1. *Escherichia coli* DH5α was used as recipient strain for recombinant plasmids and was grown in Luria–Bertani medium (LB, Panreac, Spain) at 37 °C (supplemented with 25 μg chloramphenicol mL^{-1} when necessary). *Pseudomonas* strains were routinely grown in LB medium (supplemented with 400 μg mL^{-1} chloramphenicol and 50 μg mL^{-1} tetracycline, when applied) at 30 °C on a reciprocal rotary shaker (180 rpm). *Pseudomonas* PABST7.1 is a PAO1 mutant deficient for *lip*A and *lip*H genes, where LipC is inactive due to the lack of the specific foldase LipH [17], and where plasmidic constructions of LipC and LipCmut were cloned. G.R.A.S. strain *Pseudomonas putida* KT2440, kindly provided by Dr. A. Prieto, was used here as homologous host for expression of *lip*C and *lip*Cmut [18]. Plasmid pMMB207, kindly donated by Dr. C. García, is a shuttle vector for *E. coli* and *Pseudomonas*, coding for Cm^r and containing the *Ptac* promoter upstream the M13mp18 polylinker [19]. It was used here to increase expression of the lipases in *Pseudomonas* strains.

2.2. DNA manipulation and cloning procedures

Standard molecular cloning techniques were employed [20]. Plasmid DNA was purified through chromatography kits (QIAprep Spin Miniprep, Qiagen and GeneJet Gel Extraction, and DNA Cleanup Micro Kit, ThermoScientific). Restriction nucleases and T4 ligase were obtained from Fermentas and Roche, respectively, and used according to the manufacturer's instructions. LipC and LipCmut genes were isolated by *Xba*I and *Kpn*I enzyme restriction from pBBLipC and pBBlipCmut constructions previously obtained [14,16]. Purified DNA fragments were ligated to pMMB207, previously digested with the same restriction enzymes, and cloned into *E. coli* DH5α. Recombinant vectors from the correct clones obtained were electroporated in *Pseudomonas putida* KT2440 and PABST7.1 strains.

2.3. Protein production and activity assays

Recombinant *Pseudomonas* clones bearing LipC and LipCmut genes were grown in LB medium, supplemented with the corresponding antibiotics, at 30 °C in a rotary shaker at 180 rpm. A pre-inocule of 20 mL was grown overnight and a further 1:50 dilution in 200 mL LB was set up for growth until OD = 0.8. At this point, promoter *Ptac* was induced with 0.5 mM IPTG and the cultures were let to grow overnight before use.

Supernatants, where LipC lipases are naturally secreted in *P. aeruginosa* strains, were recovered by centrifugation at 11,300 *g* for 20 min. In order to obtain the soluble enzyme form with high activity, supernatants were concentrated 1:20 by centrifugation, using Amicon Ultra 30 kDa filters (Millipore). When necessary, cell extracts were prepared by sonication with 2–4 cycles at 50 W for 2 min, using a Labsonic 1510 sonicator. Protein concentration was determined by using the Bradford reagent with bovine serum albumin as standard [21].

For plate detection of lipase activity, parental strains and mutant clones were grown on agar plates supplemented with 1% tributyrin. Lipase activity was detected by hydrolysis haloes visible around the colonies. Lipolytic activity of supernatants was checked by measuring the release of *para*-nitrophenol (*p*NP) from *p*NP-caprylate (optimum substrate for LipC and LipCmut), as a result of enzymatic hydrolysis, and was measured at λ = 405 nm. Hydrolytic activity for both, LipC and LipCmut, was measured at their optimum temperature of 4 °C, as previously described [16]. One unit of activity was defined as the amount of enzyme that released 1 mol of *p*NP per minute under the assay conditions used.

2.4. Immobilization on Accurel MP1000

Polypropylene matrix Accurel MP1000 (particle size under 1500 μm) was purchased from Membrana GmbH (Wuppertal, Germany). Adsorption immobilization was carried out by incubating the growth culture supernatants and the carrier for 24 h following the ratio of 2 mg protein per 1 g support. Immobilized preparations were recovered by vacuum filtration, dried and stored at 4 °C (Cesarini et al., submitted for publication).

2.5. Stability of LipC and LipCmut soluble forms

500 μL supernatants concentrated by AmiconUltra filters were characterized for thermal stability and methanol resistance during

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