Innovative approaches for effective selection of lipase-producing microorganisms as whole cell catalysts for biodiesel production

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The high cost of commercial lipases limits their industrial application in the production of biodiesel or fatty acid methyl esters (FAME). This disadvantage has encouraged the search for lipase-producing microorganisms (LPMs) as potential whole cell catalysts for FAME production. The aim of this study, therefore, was to evaluate innovative procedures for easy selection and testing of LPMs as a low-cost whole cell catalyst, based on catalytic performance, methanol tolerance and physico-chemical cell surface properties. The latter (in particular the cell surface hydrophobicity and charge) were analyzed because of their crucial role in microbial adhesion to surfaces and the concomitant increase in cell immobilization and bioavailability of hydrophobic substrates. Biocatalysis experiments performed in the presence of nutrient, rapeseed oil and methanol were an effective tool for studying and identifying, in just two experiments, the capacity of different LPMs as biocatalysts in organic media, as well as the methanol tolerance of the cell and the lipase. This indicates the potential for using live microorganisms for FAME production. Another finding was that the inhibitory effect of methanol is more significant for lipase activity than LPM growth, indicating that the way in which alcohol is supplied to the reaction is a crucial step in FAME production by biocatalysts. According to these results, the application of these innovative assessments should simplify the search for new strains which are able to effectively catalyze the FAME production process.

Introduction

Increasing attention has been focused on microbial lipases (glycerol-ester hydrolases, E.C.3.1.1.3.) because of their diverse substrate specificity, stereo-specificity, tolerance against heat and various organic solvents [1] and their application as biocatalysts in the production of fatty acid methyl esters (FAME) or biodiesel.

Enzymatic FAME production, as compared to the conventional alkaline catalytic process, promotes triglycerides transesterification and free fatty acid (FFA) esterification to FAME [2,3]. This enables the use of raw materials with high FFA content such as waste frying oils, microalgae oil and Jatropha oil [3].

The industrial use of commercial lipases for FAME production, however, is still limited by the high cost of the enzymes [4]. This disadvantage has encouraged the search for lipase-producing microorganisms (LPMs) as potential whole cell catalysts for FAME production [4,5]. LPMs include bacteria, fungi and yeasts [6].

According to Jaeger et al. [7] biotechnological applications of lipases produced by LPM often prompt a demand for techniques to determine not only their activity, but also their substrate. Additionally, for effective performance as whole cell catalysts, LPMs must be used in an immobilized form, preventing them from washing off the reactor, while simultaneously allowing their reutilization, according to Fukuda et al. [4]. Easy fixation of LPM to a support may be promoted by effective microbial adhesion and
favorable physico-chemical cell surface properties, such as a high cell surface hydrophobicity and appropriate cell surface charge \([8,9]\), respectively. An additional challenge of whole cell biotransformation applied to FAME production is related to the lipase inhibition caused by methanol used in the transesterification reaction \([10]\). In this sense, the search for and isolation of LPM should be focused on microorganisms with specific catalytic characteristics for FAME production, such as methanol tolerance and high cell surface hydrophobicity. Therefore, this research aims at developing innovative and simple techniques for testing LPMs as whole cell catalysts in FAME production. These approaches use physico-chemical cell surface properties, methanol tolerance and the occurrence of hydrolysis, esterification and FAME conversion as selection criteria.

Materials and methods

**Strains and culture conditions**

**Isolation and enrichment of LPM from soil**

The LPM strains *Serratia* sp., *Myroides* sp., *Arthrobacter* sp. and *Bacillus* sp. were isolated from contaminated soils obtained from salmon (S1) and rapped oil (S2) produced in southern Chile. The samples were collected at a depth of 0–5 cm in December 2007 and January 2008 and transported in sterile glass bottles at 4°C to the laboratory. The LPMs were enriched using a procedure adapted from Ban et al. \([11]\). Shortly thereafter, 1.0 g of soil was placed in a 100 mL Erlenmeyer flasks containing 50 mL of liquid enriched selective medium (M1) (70 g/L polypeptone, 1 g/L NaNO\(_3\), 1 g/L KH\(_2\)PO\(_4\), 0.5 g/L MgSO\(_4\)·7H\(_2\)O and 30 g/L rapped seed oil) and shaken at 150 rpm and 25°C during three days. Then a selective medium with 1.5% agar (solid medium) was used to isolate LPM colonies at 20°C. Four axenic cultures were characterized through partial sequencing of the 16S rRNA genes. In addition to the four isolated strains, *Pseudomonas veronii* 547 and *P. veronii* 549, both isolated from aquifer sediments contaminated with halogenated hydrocarbons in Bitterfeld, Germany, as described by Vogt et al. \([12]\), as well as *P. fluorescens* 340 (ATCC 13525) were included in this study.

**Culture conditions**

Because lipase production is growth-associated \([13]\), microbial growth was checked on three different growth media for LPM: (a) M1: liquid enriched selective medium according to Ban et al. \([11]\) (70 g/L polypeptone, 1 g/L NaNO\(_3\), 1 g/L KH\(_2\)PO\(_4\), 0.5 g/L MgSO\(_4\)·7H\(_2\)O and 30 g/L rapped seed oil); (b) M2: basal medium following Haba et al. \([14]\) (7 g/L NaNO\(_3\), 2 g/L K\(_2\)HPO\(_4\), 1 g/L KH\(_2\)PO\(_4\), 0.1 g/L KCl, 0.5 g/L MgSO\(_4\)·7H\(_2\)O, 0.01 g/L CaCl\(_2\), 0.012 g/L FeSO\(_4\)·7H\(_2\)O, 1 g/L yeast extract and 2% of rapeseed oil); and (c) M3: basal medium according to Haba et al. \([14]\) using glucose instead of rapeseed oil as the carbon source. The growth of microorganisms was determined by measuring optical density (OD) at 578 nm using a UV-visible spectrophotometer (Varian Cary 400 Scan UV-Vis). A blank with the culture medium without the tested strain was used as control. Bacterial strains were maintained on tryptic soy agar (TSA) slants at 4°C \([14]\).

**Identification and phylogenetic characterization of bacterial strains**

The bacterial DNA was extracted from colonies grown on agar, suspended in 500 \(\mu\)L of DNA-free water, and mixed thoroughly. The 16S ribosomal RNA (rRNA) genes were subsequently amplified by a polymerase chain reaction (PCR) using the universal eubacterial primers \(\text{f}933\) (5′-GCA CAA GGC GTG GAG CAT GTG G-3′) and \(\text{r}1387\) (5′-GCC CGG GAA CTT ACCCG-3′) \([15]\). The PCR products were purified with an E.Z.N.A. \({^\text{TM}}\) Cycle-Pure Kit (Omega Bio-Tek, Doraville, GA, USA). Amplification was performed with GoTaq \({^\text{TM}}\) Flexi DNA polymerase (Promega, Madison, WI, USA). The polymerase chain reaction (PCR) mixture, containing 0.25 \(\mu\)L of GoTaq \({^\text{TM}}\) Flexi DNA Polymerase, 20 pmol of each primer, 6 \(\mu\)L of 25 mM MgCl\(_2\) solution, 5 \(\mu\)L of 2 mM of each deoxyribonucleotide triphosphate, and 10 \(\mu\)L of 5× Green GoTaq \({^\text{TM}}\) Flexi Buffer, was made up to 50 \(\mu\)L with DNA-free water. The 16S rDNA fragments were sequenced by Macrogen (Seoul, Korea). The closest related organisms were identified using BLAST software from National Centre for Biotechnology Information, NCBI (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). Partial sequences were deposited at GenBank (NCBI) under the accession numbers: GQ367572 and GQ367573 for *Bacillus* sp., GQ367570 and GQ367571 for *Serratia* sp., GQ367564 and GQ367565 for *Myroides* sp., GQ367566 and GQ367567 for *Arthrobacter* sp.

**Detection of lipase activity through diffusion agar methods**

To evaluate the occurrence of lipase activity in the strains, three different agar media were used: (a) *Soil extract agar medium*. A minimal medium was prepared according to Ko et al. \([16]\), using 10% soil extract, 0.02% urea, 1.5% agar and 0.1% (v/v) rapeseed oil previously emulsified with 10% (v/v) Tween 80. For a positive test, a visible clear zone occurred on the agar media after 4–8 days of incubation at 25°C. (b) *Tween 80 agar medium*. Nutrient agar medium BBL\({^\text{TM}}\) was supplemented with 0.01% CaCl\(_2\)-H\(_2\)O and 1% (v/v) Tween 80. For a positive test, an opaque halo occurred on the agar \([14]\). A blank containing the medium without bacteria was used as control. (c) *Rhodamine agar medium*. A test for extracellular lipase activity detection was performed according to Haba et al. \([14]\), using 0.8% of BBL\({^\text{TM}}\) nutrient broth supplemented with 0.4% NaCl, 1% of agar–agar, 2.5% (v/v) rapeseed oil and 10 mL of Rhodamine B (1 mg/mL). Lipase activity from the culture supernatant was observed in the dish as an orange fluorescent halo under UV light at 350 nm after 24–26 hours of incubation at 20°C.

**Procedures for selecting LPMs as biocatalysts for FAME production**

**Characterization of bacterial cell surface properties**

The electrophoretic mobility of bacterial suspensions in 10 mM KNO\(_3\) at pH 6.2 was determined by using a Doppler electrophoretic light scattering analyzer (Zetamaster, Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom) at 100 V following the procedure described earlier \([9]\). The zeta potential \(\zeta\) as an indirect measure of cell surface charge was calculated from the electrophoretic mobility according to the method of Helmholtz–Smoluchowski \([9]\).

Water contact angle \((\theta_w)\) measurements were used to assess the surface hydrophobicity of the cells. The strains were harvested in the late exponential phase by centrifugation for 10 min at 10,000 rpm. Then the cells were washed ten times by resuspension in 10 mM KNO\(_3\) and centrifugation, respectively. Bacterial lawns needed for \(\theta_w\) measurements were prepared by collecting cell
suspensions in 10 mM KNO₃ on 0.45-μm pore-size Micropore filters (Schleicher & Schuell, Dassel, Germany), mounting the filters on glass slides and drying them for 2 hour at room temperature. Cell surface hydrophobicity was determined from the contact angles of water drops on the bacterial lawns using a DSA 100 drop-shape analysis system (Krüss GmbH, Hamburg, Germany) [9]. All experiments were performed using two different growth media: M2 and M3, respectively.

**Column adhesion experiments**

Column percolation experiments using *Arthrobacter* sp. and *P. fluorescens* grown cells on LB medium were performed at 25°C following the method of Rijnaarts et al. [8]. Glass columns with a length of 9.2 cm and internal diameter of 1 cm were wet-packed with 1-mm-diameter glass beads (Roth AG, Reinach, Switzerland). The columns were connected to a peristaltic pump and a bacteria suspension in 0.1 M PBS (phosphate buffer saline) and 0.7 < OD 578 < 0.8 was pumped through the columns at a constant flow rate in down-flow mode. The use of identical empty bed flow rates led to different hydraulic flow velocities depending on the respective porosities. The hydraulic flow rate, porosity and pore volume for each column were 1 ± 0.04 mL/min, 0.49 ± 0.04 and 3.15 ± 0.07 mL, respectively. The adhesion efficiency (%) of bacteria was calculated as follows:

\[
\text{adhesion efficiency} = \frac{C_0 - C}{C_0} \times 100
\]

where, ‘C’ is the OD in the influent and ‘C’ is the OD in the effluent. Experiments were performed in duplicate. The omission of nutrients in the suspensions prevented significant bacterial proliferation during the short-term experiments.

**Methanol tolerance, hydrolysis, esterification and FAME production**

An innovative biocatalyst experiment was developed to evaluate the strains’ performance in an organic media (alcohol and oil).

The set-up consisted of petri dishes with mineral nutrient agar to support the strains’ growth, covered by a liquid layer of rapeseed oil mixed with the strain. In these experiments, two forms of supplying methanol for FAME production were used: in liquid phase and gaseous phase. For the liquid phase form, the methanol was added directly with a pipette over the agar, at two different methanol:oil molar ratios, 1:1 (35 g/L) and 3:1 (105 g/L), respectively [17]. For the gaseous phase form, a small vessel with 1 mL methanol was placed inside each petri dish to allow methanol supply for FAME production were used: in liquid phase and gaseous phase. For the liquid phase form, the methanol was added directly with a pipette over the agar, at two different methanol:oil molar ratios, 1:1 (35 g/L) and 3:1 (105 g/L), respectively. For the gaseous phase form, a small vessel with 1 mL methanol was added with a sterile syringe over the agar, at two different methanol:oil molar ratios, 1:1 (35 g/L) and 3:1 (105 g/L), respectively. For the gaseous phase form, a small vessel with 1 mL methanol was added with a sterile syringe over the agar, at two different methanol:oil molar ratios, 1:1 (35 g/L) and 3:1 (105 g/L), respectively. For the gaseous phase form, a small vessel with 1 mL methanol was added with a sterile syringe over the agar, at two different methanol:oil molar ratios, 1:1 (35 g/L) and 3:1 (105 g/L), respectively. For the gaseous phase form, a small vessel with 1 mL methanol was added with a sterile syringe over the agar, at two different methanol:oil molar ratios, 1:1 (35 g/L) and 3:1 (105 g/L), respectively. For the gaseous phase form, a small vessel with 1 mL methanol was added with a sterile syringe over the agar, at two different methanol:oil molar ratios, 1:1 (35 g/L) and 3:1 (105 g/L), respectively.

To evaluate the effect of nutrients on the LPM biocatalytic performance, two different nutrient agars were used (M1 and M2) in the reactions for 160 hour at 20°C, in duplicate. All media were prepared without oil and with 1.5% of agar added.

Before the inoculation, the bacteria were cultivated overnight in 150 mL of LB (Lysogeny broth) liquid medium at 20°C, centrifuged at 10,000 rpm during 15 min. The centrifuged bacteria obtained were mixed with 10 mL of sterile rapeseed oil in a vortex for 2 min. One milliliter of this preparation was mixed with 1 mL of sterile rapeseed oil and added to each petri dish. The emulsion was spread with a spatula over the agar.

To evaluate the product formation, samples were taken every 24 hour with a pipette. Hydrolysis was determined by measuring the acid value and FFA content in the oil, whereas the occurrence of esterification was evaluated in terms of the behavior of FFA concentration, considering that a decrease in FFA content indicates its esterification to FAME. Finally, FAME production was determined by the methyl ester content in the samples.

**Quantitation of FAME and FFA content**

FAME yield was calculated as the quantity of FAME content from the upper layer of the samples obtained after each reaction, which had previously been centrifuged.

FAME content was quantitated using an Agilent 6890N (Agilent Technologies, USA) gas chromatograph equipped with a split-splitless injector (240°C) and a flame ionization detector (at 270°C) GC-FID, connected to a CP-Sil 88 capillary column (50 m long, 0.25 mm internal diameter and 0.25 μm film thickness). The carrier gas flow rate was 1 mL/min. The column temperature was maintained at 40°C for 2 min, increased to 220°C at a rate of 8°C/min, and maintained at this temperature for 5 min. Methyl heptadecanoate was used as internal standard. FFA content was determined by titration with ethanol using phenolphthalein as an indicator. All samples had previously been centrifuged.

**Results and discussion**

**Characterization of the strains**

**Strain isolation**

Oil-contaminated environments are known for their great diversity of LPM [18]. Thus, for this research two different oil-contaminated soils were chosen as possible sources of LPM. The genetic analyses of 16S rDNA sequences of the three isolated strains from soils contaminated with salmon grease (S1) revealed a high similarity with known bacteria belonging to genus *Serratia* sp., *Myroides* sp. and *Arthrobacter* sp. The strain isolated from contaminated soil with rapeseed oil (S2) was similar to *Bacillus* genus. All of the genera found have been reported to be sources of LPM [19].

**Culture conditions and maintenance of microorganisms**

Using M1, a medium containing rapeseed oil as its carbon source and polypeptone as the organic nitrogen source, all bacterial strains reached a high density at 6 hour, with *Serratia* sp. exhibiting the best growth performance. Using M2, a medium with rapeseed oil as carbon source and yeast extract as the organic nitrogen source, all strains, except *P. veronii* 547, reached the stationary phase after 6 hour. However, when using M3, a medium with glucose as carbon source and yeast extract as the organic nitrogen source, bacterial strains grew slowly except for *Pseudomonas*, which reached the stationary phase at 17 hour with a higher growth rate (Fig. 1). *Serratia* sp., by contrast, did not grow when glucose was used as a carbon source in M3 medium. Similar results were obtained by Szczesna-Antczak et al. [20] in cultures of *Myroides circinelloides* and *M. racemosus* when changing the carbon source of olive oil to glucose, and a decrease in growth and lipase activity was observed. Similarly, Ban et al. [11], using the fungus *Rhizopus oryzae*, showed that a large amount of glucose causes a reduction in lipase activity.
Figure 1

Bacteria strain growth in M3, a medium with glucose as the carbon source and yeast extract as the organic nitrogen source. Closed circle: Myroides sp.; closed square: Arthrobacter sp.; closed inverted triangle: Bacillus sp.; closed triangle: P. veronii 547; open square: P. fluorescens 340; open triangle: P. veronii 549; open circle: Serratia sp.

Substrate composition is an important factor in lipase activity expression, as has been shown by different authors [6,21]. Accordingly, the composition of the culture medium, in particular the incorporation of different lipids, can result in the production of different lipases [6]. Also, the nitrogen source is an important factor in lipase activity, such that organic nitrogen sources have been shown to increase the activity [13]. According to Shirazi et al. [13], lipids stimulate lipase production. However, Pseudomonas strains grew better in glucose compared to rapeseed oil. Therefore, different agar methods were applied to select only lipase-producing strains for the subsequent experiments.

### Table 1

<table>
<thead>
<tr>
<th>Microbial Strains</th>
<th>Physico-chemical cell surface properties</th>
<th>Whole cell characteristic</th>
<th>Biocatalyst performance in GM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contact angle [°]</td>
<td>Zeta potential [mV]</td>
<td>Methanol tolerance</td>
</tr>
<tr>
<td></td>
<td>Rapeseed oil</td>
<td>Glucose</td>
<td>Rapeseed oil</td>
</tr>
<tr>
<td>Serratia sp.</td>
<td>a</td>
<td>a</td>
<td>++</td>
</tr>
<tr>
<td>Myroides sp.</td>
<td>66</td>
<td>67 ± 4</td>
<td>–30 ± 1</td>
</tr>
<tr>
<td>Arthrobacter sp.</td>
<td>91 ± 3</td>
<td>99 ± 5</td>
<td>−28 ± 3</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>84 ± 5</td>
<td>77 ± 6</td>
<td>−44 ± 3</td>
</tr>
<tr>
<td>P. veronii 547</td>
<td>49 ± 4</td>
<td>22 ± 2</td>
<td>−8 ± 1</td>
</tr>
<tr>
<td>P. fluorescens 340</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>P. veronii 549</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

LM: liquid phase methanol experiments; GM: gaseous phase methanol experiment; N.A: not analyzed; +: growth or positive reaction (+: low; ++: medium; +++: high); –: no growth or negative reaction.

*Contact angle and zeta potential of 23 and −13, respectively (cell cultured in brain heart infusion (BHI)) [21].

**Detection of lipase activity by agar diffusion methods**

To detect lipase activity in different cultures in a minimal medium, soil extract agar with rapeseed oil as the sole carbon source was used. All strains grew and a clear halo was evident on the agar after four days of incubation except for P. fluorescens 340 and P. veronii 549. The same results were found using Tween 80 agar method after 48 hour of incubation, where P. fluorescens 340 and P. veronii 549 did not show any lipase activity. The rhodamine test was used to detect extracellular lipase activity in the supernatant of the different cultures. All strains showed fluorescent halos on the agar when exposed to UV light after 24 hour of incubation, indicating the production of extracellular lipases, with the exception of P. fluorescens 340 and P. veronii 549.

As had been expected based on the growth experiment (Fig. 1), P. fluorescens and P. veronii 549 did not show any lipase activity in the agar diffusion tests and showed better growth in glucose as the carbon source. However, Tan and Gill [22] have found that when using other lipids such as olive oil as the carbon source, P. fluorescens shows high lipase activity. This indicates that in these experiments, rapeseed oil did not stimulate lipase production from P. fluorescens and P. veronii 549.

The results obtained allowed for selection of five LPM from the initial seven strains: Serratia sp., Myroides sp., Arthrobacter sp., Bacillus sp. and P. veronii 547.

**Procedure for selecting LPMs as biocatalysts for FAME production**

A special procedure was developed to select microorganisms with specific catalytic characteristics for FAME production, high adhesion performance, methanol tolerance and both microbial and lipase performance in an organic medium (rapeseed oil and methanol). This procedure was based on physico-chemical cell surface properties and biocatalysis performance in an organic medium.

**Physico-chemical cell surface properties**

To determine if any of the studied LPMs present the potential to be easily immobilized by their hydrophobicity and surface charge, LPMs were evaluated by the measuring contact angle and performing a zeta potential analysis, respectively [8] (Table 1). According
to Rijnaarts et al. [8], cells with contact angles of less than 20° are considered hydrophilic; those with contact angles between 20° and 50° are intermediate hydrophilic; and those with contact angles greater than 50° are hydrophobic. Using this classification, the strains were ordered from highest to lowest hydrophobicity as follows: *Arthrobacter* sp. > *Bacillus* sp. > *Myroides* sp. > *P. veronii* 547 (Table 1). In the case of *Serratia* sp. the reported literature indicates that it is an intermediate hydrophilic bacteria [23].

The hydrophobicity calculations indicate that *Arthrobacter* sp. may be more easily immobilized, in comparison to *P. veronii* 547. According to Rijnaarts et al. [8], greater hydrophobicity of cells and substrata results in greater attractive forces and higher levels of adhesion due to van der Waals force. This result was confirmed by adhesion experiments in packed columns (Fig. 2), where at a flow rate of 1 mL/min *Arthrobacter* sp. was retained in the column upper 80% of adhesion (Fig. 2). *P. veronii* was poorly retained in the column under the same conditions as *Arthrobacter* sp., and was washed from the column (Fig. 2). This indicates that weak adhesive bonds allowed shear forces to reduce adhesion [8]. As reported by Devanesan et al. [5], intermediate hydrophilic microorganisms such as *P. fluorescens* could be immobilized in alginate beads to avoid the washing problem. Hydrophilic microorganisms such as *R. oryzae* (contact angles between 60° and 76°) have been easily immobilized on reticulated polyurethane in airlift reactors and used successfully as whole catalysts for FAME production [11,24].

All strains presented negative values for zeta potential, with the least negative being *P. veronii* 457. According to Rijnaarts et al. [8] smaller (more negative) electrokinetic potentials of cells and solids and lower levels of ionic strength (I) result in greater repulsive electrostatic interactions and lower levels of adhesion.

The carbon source used did not significantly affect the zeta potential and contact angles found, except for *P. veronii* 547, where hydrophobicity increased when triglycerides were used instead of glucose as the carbon source (Table 1).

From the growth experiment and the physico-chemical cell surface properties, it was established that LPMs have an affinity for hydrophobic substrates such as triglycerides. Even the intermediate hydrophilic *P. veronii* 547 increased its hydrophobicity in the presence of rapeseed oils. These results indicate that if a methanol-tolerant strain is cultivated under conditions favorable to lipase production and simultaneously in the presence of methanol, triglycerides could be used to maintain the growth and lipase production and probably as raw material for FAME production. Additionally, because methanol is a hydrophilic alcohol, this property could be used to decrease its inhibitory effect on the whole cell catalyst. These hypotheses were considered in the development of the subsequent procedure.

**Table 1** shows the results of the biocatalyst experiment using liquid phase methanol at two different methanol:oil molar ratios and the use of gaseous phase methanol.

Several strains grew using liquid phase methanol, under two different methanol-to-oil ratios. However, using gas phase methanol, higher LPM growth was detected, indicating that despite continuous methanol refilling, LPMs were less affected by methanol. *Myroides* sp. grows neither in liquid nor in gaseous methanol, indicating a higher sensibility to methanol presence, and was discarded as a possible whole cell catalyst. *Serratia* sp., *Arthrobacter* sp., *Bacillus* sp. and *P. veronii* 547 showed methanol tolerance, reinforcing other research on organic solvent-tolerant microorganisms [25–27].

Despite the significant LPM growth, no conversion of triglycerides to FAME was detected in the liquid phase methanol experiment (Table 1). According to Shimada et al. [17], when using a methanol:oil molar ratio of 1:2, liquid methanol becomes immiscible with oil, negatively affecting lipase activity.

In the case of the gaseous phase methanol experiment, *Serratia* sp., *Arthrobacter* sp. and *P. veronii* 547 showed conversion of triglycerides to FAME. *Serratia* sp. and *P. veronii* 547, which are intermediate hydrophilic, showed the greatest FAME conversion yield, with 9.5 and 5%, respectively. Recent findings on *Serratia* sp.’s resistance to organic solvents might explain its better performance in an organic medium [28]. In addition, the agar medium composition influenced FAME conversion yield. In fact, FAME conversion was detected only when using the M2 medium. These results indicate the important role of substrate composition in lipase production and obviously in FAME conversion yield.

According to Heipieper et al. [25], the main target for the toxicity of alcohols or organic solvents is the membrane. They inhibit cell growth by partitioning preferentially in membranes, disturbing the integrity of this cellular barrier and leading to non-specific permeation. The toxic effects of aliphatic alcohols on growth depend on the value of log *P* (partition coefficient of the solvent for the standard octanol/water two-phase system), increasing its toxicity with higher log *P* values. Therefore, as methanol has a lower value of log *P* (–0.76) compared to other alcohols such as ethanol (–0.28), its toxic effect on microbial growth is lower compared to the effect on lipase activity [29].

By contrast, as the lipases produced by the studied bacterial strains are mainly extracellular, they diffuse into the oil, acting in...
the interfacial area between the oil and alcohol, whereas low quantities of water (provided by nutrient agar) facilitate the increase of interfacial area, thus promoting lipase activity. This explains the superior performance of intermediately hydrophilic bacteria. In addition, because the experiments were performed without agitation, lipase activity may have been inhibited by direct contact with immiscible methanol in the liquid phase experiment. However, in the gaseous phase experiment, the bacteria and lipases were probably protected by diffusion barriers between the gaseous methanol and the oil, decreasing the inhibitory effect of the methanol. According to this assessment, the inhibitory effect of methanol is most significant for lipase activity than for LPM growth, and the use of methanol in the gaseous phases enabled determination of its effect on strain growth and lipase activity. The use of nutrient agar suggests that it is possible to maintain the strain growth and FAME production where the vegetable oil can be used as both a carbon source and a raw material for producing biofuel. This information, coupled with the physico-chemical cell surface properties, provides an indication of the potential of several strains as whole cell catalysts for FAME production.

Finally, Table 1 shows an overview of the potential of the different LPMs studied as whole cell catalysts. Serratia sp., P. veronii 547 and Arthrobacter sp. show potential as whole cell catalysts, with methanol tolerance and the capacity to produce FAME through extracellular lipases. The lipase activity of the strains was strongly affected by the presence of methanol. The inhibitory effect of methanol was decreased by using a diffusional barrier, indicating that the way in which the alcohol is supplied to the reaction is a crucial step in FAME production by biocatalysts.

The lower hydrophobicity of Serratia sp. and P. veronii 547 indicates a lower adhesion efficiency, limiting their possible application as immobilized microorganisms.

**Conclusion**

This work introduces an innovative procedure for assessing the potential of new strains as whole cell catalysts for FAME production. Cell surface properties such as hydrophobicity and zeta potential allowed for the selection of LPMs with high adhesion performance, facilitating immobilization and reutilization. Biocatalysis experiments performed in the presence of nutrient, rapeseed oil and methanol were an effective tool for studying and identifying, in just two experiments, the overall capacity of different LPMs as biocatalysts in organic media, as well as the methanol tolerance of the cell and the lipase. These experiments also suggest that the inhibitory effect of methanol is more significant in lipase activity than in LPM growth.

The results indicate the possibility of using live microorganisms for FAME production which would decrease the cost of continued production of biocatalysts. According to these results, the application of these innovative procedures would provide a major boost to the search for new strains which would be able to catalyze the FAME production process.

**Acknowledgements**

This research was sponsored by CONICYT Project 79090009, PIA Project DI10-7001, Chilean FONDECYT Projects 3080021 and 1090382 and Chilean PBCT-CONICYT Project TPI-16.

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This innovative assessment showed itself to be an effective tool for studying and identifying, in just two experiments, the capacity of different LPMs as biocatalysts in organic media as well as the methanol tolerance of the cell and the lipase. The use of methanol in the liquid and gaseous phases enabled determination of its effect on strain growth and lipase activity. The use of nutrient agar suggests that it is possible to maintain the strain growth and FAME production where the vegetable oil can be used as both a carbon source and a raw material for producing biofuel. This information, coupled with the physico-chemical cell surface properties, provides an indication of the potential of several strains as whole cell catalysts for FAME production.

Finally, Table 1 shows an overview of the potential of the different LPMs studied as whole cell catalysts. Serratia sp., P. veronii 547 and Arthrobacter sp. show potential as whole cell catalysts, with methanol tolerance and the capacity to produce FAME through extracellular lipases. The lipase activity of the strains was strongly affected by the presence of methanol. The inhibitory effect of methanol was decreased by using a diffusional barrier, indicating that the way in which the alcohol is supplied to the reaction is a crucial step in FAME production by biocatalysts.

The lower hydrophobicity of Serratia sp. and P. veronii 547 indicates a lower adhesion efficiency, limiting their possible application as immobilized microorganisms.

**Conclusion**

This work introduces an innovative procedure for assessing the potential of new strains as whole cell catalysts for FAME production. Cell surface properties such as hydrophobicity and zeta potential allowed for the selection of LPMs with high adhesion performance, facilitating immobilization and reutilization. Biocatalysis experiments performed in the presence of nutrient, rapeseed oil and methanol were an effective tool for studying and identifying, in just two experiments, the overall capacity of different LPMs as biocatalysts in organic media, as well as the methanol tolerance of the cell and the lipase. These experiments also suggest that the inhibitory effect of methanol is more significant in lipase activity than in LPM growth.

The results indicate the possibility of using live microorganisms for FAME production which would decrease the cost of continued production of biocatalysts. According to these results, the application of these innovative procedures would provide a major boost to the search for new strains which would be able to catalyze the FAME production process.

**Acknowledgements**

This research was sponsored by CONICYT Project 79090009, PIA Project DI10-7001, Chilean FONDECYT Projects 3080021 and 1090382 and Chilean PBCT-CONICYT Project TPI-16.

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**References**