Characterization of an extracellular lipase and its chaperone from Ralstonia eutropha H16

Applied Microbiology and Biotechnology

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Running title: Identification and analysis of an extracellular lipase from *Ralstonia eutropha* H16

Jingnan Lu[1], Christopher J. Brigham[2], ChoKyun Rha[3], Anthony J. Sinskey[2, 4, 5]*

Department of Chemistry[1], Department of Biology[2], Biomaterials Science & Engineering Laboratory[3], Division of Health Sciences and Technology[4], Engineering Systems Division[5], Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

* Corresponding author. Mailing address: Bldg. 68-370, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, USA. Phone: (617) 253-6721. Fax: (617) 253-8550. E-mail: asinskey@mit.edu

Keywords: *Ralstonia eutropha*, lipase, chaperone, triacylglycerol, palm oil, emulsification

ABSTRACT

Lipase enzymes catalyze the reversible hydrolysis of triacylglycerol to fatty acids and glycerol at the lipid-water interface. The metabolically versatile *Ralstonia eutropha* strain H16 is capable of utilizing various molecules containing long carbon chains such as plant oil, organic acids, or Tween as its sole carbon source for growth. Global gene expression analysis revealed an upregulation of two putative lipase genes during growth on trioleate. Through analysis of growth and activity using strains with gene deletions and complementations, the extracellular lipase (encoded by the *lipA* gene, locus tag H16_A1322) and lipase-specific chaperone (encoded by the *lipB* gene, locus tag H16_A1323) produced by *R. eutropha* H16 was identified. Increase in gene dosage of *lipA* not only resulted in an increase of the extracellular lipase activity, but also reduced the lag phase during growth on palm oil. LipA is a non-specific lipase that can completely hydrolyze triacylglycerol into its corresponding free fatty acids and glycerol. Although LipA is active over a temperature range from 10 to 70°C, it exhibited optimal activity at 50°C. While *R. eutropha* H16 prefers a growth pH of 6.8, its extracellular lipase LipA is most active between pH 7 and 8. Cofactors are not required for lipase activity, however EDTA and EGTA inhibited LipA activity by 83%. Metal ions Mg^{2+}, Ca^{2+}, and Mn^{2+} were found to stimulate LipA activity and relieve chelator inhibition. Certain detergents are found to improve solubility of the lipid substrate or increase lipase-lipid aggregation, as a result SDS and Triton X-100 were able to increase lipase activity by 20 to 500%. *R. eutropha* extracellular LipA activity can be hyper-increased, making the overexpression strain a potential candidate for commercial lipase production or in fermentations using plant oils as the sole carbon source.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases) are ubiquitous enzymes in nature. They play a crucial role in fat metabolism by catalyzing the hydrolysis of triacylglycerol to free fatty acids and glycerol at the interface of lipid and water (Gupta *et al.*, 2004; Jaeger & Reetz, 1998). Although lipases are highly chemo-, regio-, and chiral- selective enzymes, they also process esterolytic activities for the carboxyl ester bond cleavage of water-insoluble esters (Jaeger & Reetz, 1998; Reis *et al.*, 2009; Treichel *et al.*, 2010). Lipases can also catalyze the reverse reaction in the presence of a low water concentration (Franken *et al.*, 2010; Park *et al.*, 2005; Severac *et al.*, 2011).

Lipases are serine hydrolases with a conserved catalytic triad: serine, aspartate or glutamate, and histidine. These three amino acid residues always appear in this order, but are distant from each other in the lipase primary sequence. In the lipase tertiary structure, the catalytic triads are positioned within a close distance to each other in order to catalyze the hydrolysis reaction (Arpigny & Jaeger, 1999; Gupta *et al.*, 2004; Jaeger & Reetz, 1998; Jaeger *et al.*, 1999). The mechanism of cleavage involves the deprotonated serine hydroxyl group nucleophilically attacking the carbonyl carbon of the lipid ester bond. A proton is then transferred from the triad residues to the substrate hydroxyl group resulting in the cleavage of the ester bond between the fatty acid and glycerol backbone. The intermediate fatty ester is then attacked by water to regenerate the catalytic triad and fatty acid (Reis *et al.*, 2009). A characteristic α/β hydrolase fold was found in all lipase crystallographic structures solved to date, including bacterial *Psudomonas* and *Bacillus* lipases, fungal *Rhizomucor* lipases, and horse and human pancreatic lipases (Arpigny & Jaeger, 1999; Bourne *et al.*, 1994; Derewenda *et al.*, 1992; Jaeger & Reetz, 1998; Noble *et al.*, 1993; Roussel *et al.*, 1999; Schrag & Cygler, 1997; van Pouderoyen *et al.*, 2001). Since lipase enzymes catalyze reactions at the interface of water and neutral water-insoluble ester substrates, the catalytic triad that is buried in the structure must surface in order to access the substrate (Cherukuvada *et al.*, 2005; Reis *et al.*,

2009; Wang *et al.*, 2007). This major conformational change results in lipase activity being highly inducible by lipids, hydrolysable esters, Tween detergents, glycerol, or bile salts (Boekema *et al.*, 2007; Franken *et al.*, 2010; Gupta *et al.*, 2004; Kim *et al.*, 1996; Lotti *et al.*, 1998; Mahler *et al.*, 2000).

Due to the high stability, selectivity, and specificity of lipases, they have been used extensively in food, detergent, cosmetic, synthesis, and pharmaceutical industries (Jaeger & Reetz, 1998; Park *et al.*, 2005; Treichel *et al.*, 2010). Bacterial lipases, especially enzymes from *Bacillus*, *Pseudomonas*, *Burkholderia*, and *Straphylococcus* species, have been extensively studied and used commercially (Gupta *et al.*, 2004; Jaeger & Eggert, 2002; Pandey *et al.*, 1999; Rosenstein & Gotz, 2000; Sanchez *et al.*, 2002). These lipases are mostly extracellular which makes bulk production straightforward. Although many efforts have been made to increase lipase production in heterologous hosts like *Escherichia coli*, only a few heterologously-produced lipases are enzymatically active, because lipase gene expression and secretion are strictly regulated in the host organism (Rosenau & Jaeger, 2000).

The metabolically versatile betaproteobacterium *Ralstonia eutropha* strain H16 is able to grow on various carbon sources including lipids and some detergents (Tween) (Budde *et al.*, 2011a; Riedel *et al.*, 2012; Yang *et al.*,2010; Budde *et al.*, 2011b; Kahar *et al.*, 2004; Ng *et al.*, 2010). Since bacteria can only transport free fatty acids into the cytoplasm and utilize them via the β-oxidation pathway for the generation of the cellular building block acetyl-CoA and energy, lipids such as triacylglycerol and Tween compounds must be processed first by a secreted lipase enzyme (Budde *et al.*, 2011a; Budde *et al.*, 2011b; Gupta *et al.*, 2004; Treichel *et al.*, 2010). Previous microarray analysis by Brigham *et al*. on global gene expression of *R. eutropha* H16 revealed two putative lipase genes (locus tags H16_A1322 and H16_A3742) that were upregulated during trioleate growth. Deletion of lipase H16_A1322 (GeneID, 4249488) in *R. eutropha* H16 resulted in strain Re2313, which, unlike the wild type strain, was unable to emulsify palm oil in flask cultures. This suggested that lipase H16_A1322

played a role in the breakdown of triacylglycerol molecules in palm oil, and this breakdown of oil provided diacylglyerol, monoacylglycerol, and free fatty acids for emulsification of palm oil remaining in the culture (Brigham *et al.*, 2010). In this study, we have characterized the *R. eutropha* H16 extracellular lipase (encoded by H16_A1322; henceforth known as LipA) and identified its concomitant chaperone (henceforth known as LipB). The properties of this lipase including relevant physicochemical characteristics and substrate specificities were examined and reported here.

MATERIALS AND METHODS

Bacterial strains and plasmids

Experiments were performed with the strains and plasmids listed in Table 1. Mutants were derived from wild-type *Ralstonia eutropha* H16 (ATCC 17699).

Growth media and cultivation conditions

All *R. eutropha* strains were cultivated aerobically in rich and minimal media with an initial pH of 6.8 at 30°C. Rich medium consisted of 2.75% (w/v) dextrose-free tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD). Minimal medium was produced with the following salts: 4.0 g/L NaH_2PO_4, 4.6 g/L Na_2HPO_4, 0.45 g/L K_2SO_4, 0.39 g/L $MgSO_4$, 0.062 g/L $CaCl_2$, 0.05% (w/v) NH_4Cl, and 1 ml/L of a trace metal solution. The trace metal solution was prepared with 15 g/L $FeSO_4 \cdot 7H_2O$, 2.4 g/L $MnSO_4 \cdot H_2O$, 2.4 g/L $ZnSO_4 \cdot 7H_2O$, and 0.48 g/L $CuSO_4 \cdot 5H_2O$ in 0.1 M HCl. Carbon sources used were 1% palm oil (Wilderness Family Naturals, Silver Bay, MN) or 0.5% Tween-60 (Sigma-Aldrich). For all *R. eutropha* cultures, 10 µg/mL final concentration gentamicin was added. Kanamycin at 300 µg/mL concentration was added to *R. eutropha* with plasmid.

A single colony of *R. eutropha* from a TSB agar plate was used to inoculate 5 mL of TSB medium. The culture was then incubated on a roller drum for 24 h before being used to inoculate a 100 mL minimal medium flask culture, containing carbon sources mentioned

above, to an initial OD_{600} of 0.05. The 100 mL minimal medium culture was continuously shaken in a 30°C incubator at 200 rpm. Aliquots were removed from the flask culture at intermittent time points for analysis. OD_{600} of cultures of each strain were measured throughout the cultivation period.

Plasmid and strain construction

Gene deletions from *R. eutropha* H16 genome were carried out by a standard procedure described previously (Quandt & Hynes, 1993; York *et al.*, 2001). Standard molecular biology techniques were performed for all DNA manipulations (Chong, 2001)

The plasmid for markerless deletion was constructed by first amplifying approximately 500 base pairs of DNA sequence upstream and downstream of the target deletion gene using primers with identical sequence overlap at the end (Online Resource 1). Overlap PCR using these primers resulted in a DNA fragment that contained both the upstream and downstream region of the target deletion gene. The resulting DNA fragment and parent plasmid, pJV7 (Table 1), were digested with the restriction enzymes XbaI and SacI (New England Biolabs, Ipswich, MA) and then ligated together to create the gene deletion plasmid. The gene deletion plasmid was transformed into *Escherichia coli* S17-1 (Simon *et al.*, 1983), which was used as a donor for the conjugative transfer of mobilizable plasmids. A standard mating-procedure was performed to introduce the gene deletion plasmid into *R. eutropha* via conjugation (Slater *et al.*, 1998). Deletion strains were screened via diagnostic PCR with pairs of internal and external primer sets (Online Resource 1).

Lipids extraction and thin layer chromatography analysis

Lipids from palm oil culture supernatants were qualitatively analyzed by thin layer chromatography (TLC). A 10 mL aliquot of culture was taken at different time points during the growth of *R. eutropha* H16 and mutant strains on palm oil as the sole carbon source. Samples were spun down via centrifugation at 4,000 × *g*, room temperature to separate supernatant from cell pellets. The lipids in the supernatant were extracted with 5 mL of

chloroform/methanol (2:1, v/v) for 1 min with continuous mixing by vortex. The chloroform layer was removed, allowed to dry, and re-dissolved in fresh chloroform to a final concentration of 5 mg/mL. Aliquots of 10 µL (50 µg lipids) were spotted on silica gel TLC plate (EMD Chemicals, Gibbstown, NJ; 250 µm thickness). A mixture with 10 µg each of triacylglycerol (TAG: 1,2-distearoyl-3-oleoyl-rac-glycerol), diacylglycerol (DAG: 1,2-dipalmitoyl-rac-glycerol), monoacylglycerol (MAG: 1-palmitoyl-rac-glycerol) and free fatty acid (FFA: palmitate) (Nu-check Prep, Inc., Elysian MN) was also spotted as a standard. The TLC plate was developed first with chloroform/methanol/water (60:35:5, v/v) to 5 cm above the origin and then with hexane/diethyl ether/acetic acid (69.5:29.5:1, v/v). To visualize TAGs and lipase products, a 3% (w/v) cupric acetate solution in 8% (v/v) phosphoric acid was sprayed lightly and evenly onto the plate. The plate was placed in an oven (~200°C) for 10 to 30 min to char and then imaged with a camera (Canon, PowerShot Digital SD1200 IS).

Lipase Activity assay

Extracellular lipase activity was estimated using a modified protocol from Ng *et. al.* with *p*-nitrophenyl palmitate (*p*NP) as a substrate (Ng *et al.*, 2010). The assay mixture contains 100 mM glycine-HCl buffer at pH 7.0 and 0.1% (w/v) polyvinyl alcohol. Cell-free supernatant was added to the assay mixture to a final volume of 900 µL and incubated at room temperature for 5 min. The reaction was initiated by the addition of 0.19 mg *p*NP in 100 µL dimethyl sulphoxide. The absorbance was recorded at OD_{405} by a spectrophotometer (Agilent 8453 UV-visible). Control assay mixtures do not contain substrate or cell-free supernatant. One enzyme unit (U) was defined as one µmol *p*-nitrophenol liberated per min using extinction coefficient of 1.78×10^4 $M^{-1}cm^{-1}$.

Effect of pH, temperature, metal ions, and detergents

The effect of pH on lipase activity was tested using 100 mM buffers at various pH values from 3 to 12. Glycine-HCl buffer was used for the pH range from 3 to 7; pH values

from 7.5 to 9 were achieved using Tris buffer; and glycine-NaOH buffer was utilized for pH values from 9.5 to 12. Cell-free supernatant was incubated with the various pH buffers at room temperature for 15 min prior to initiation of the reaction with the *p*NP substrate.

Temperature effect on lipase activity was assayed using a circulating-bath system (VWR) coupled to the spectrophotometer. Assay mixture of glycine-HCl (pH 7.0) and supernatant were incubated at various temperatures from 0 to 80°C for 15 min prior to the start of the reaction.

To test the effect of metals and chelating agents on lipase activity, metal ions ($ZnCl_2$, $MgCl_2$, $FeCl_2{\cdot}4H_2O$, $CaCl_2{\cdot}2H_2O$, $CuCl_2{\cdot}2H_2O$, $MnCl_2{\cdot}4H_2O$, or $NiCl_2{\cdot}6H_2O$) and chelators (EDTA, EGTA) at concentrations of 0.1 mM or 1 mM were added to the assay mixture with glycine-HCl (pH 7.0) buffer and assayed at room temperature. To determine the metal ion preference for the lipase, 1 mM chelators were first added to the assay mixture and supernatant to chelate metal ions from the solution. Following chelation, a 1 mM metal ion solution was added to help restore the activity of the lipase prior to activity assay.

To determine the effect of detergents on lipase activity, Tween 20 (polyoxyethylene (20) sorbitan monolaurate), Tween 40 (polyoxyethylene (20) sorbitan monopalmitate), Tween 60 (polyoxyethylene (20) sorbitan monostearate), Tween 80 (polyoxyethylene (20) sorbitan monooleate), sodium dodecyl sulfate (SDS), Triton X-100 (tetramethylbutyl phenyl-polyethylene glycol), or Triton X-305 (octylphenoxypolyethoxylethane) at final concentrations of 0.001, 0.01, or 0.05% (v/v) was added to the assay mixture with glycine-HCl (pH 7.0). The reaction was carried out at room temperature.

All experiments were carried out in triplicates and the values reported are averages of the three ± standard deviation.

RESULTS

Identification of LipA lipase and LipB chaperone pairs

Given the lack of evidence that *R. eutropha* H16 can directly uptake palm oil and utilize it for growth, TAGs in palm oil must first be hydrolyzed to free fatty acids (FFAs) by a secreted extracellular lipase and then taken up by the cells. TAG hydrolysis during cultivation of *R. eutropha* H16, Re2313, Re2314, and Re2315 (Table 1) in palm oil cultures were qualitatively analyzed by TLC (Fig. 1). At culture time points of 8, 24, 48, and 72 h, an increase in intensity of the spots corresponding to free fatty acids (FFA), diacyglycerol (DAG), and monoacyglycerol (MAG) with a concomitant decrease in the TAG spot intensity were detected from cultures of wild-type (H16) cells. These observations suggested the presence of an extracellular lipase produced by *R. eutropha* H16 cells for the hydrolysis of the TAGs in palm oil into DAGs, MAGs, and FFAs during growth. Strain Re2313, a Δ*lipA* derivative of H16, was unable to hydrolyze TAGs to the extent of wild-type cells, which resulted in unchanged TAG spot intensities on the TLC plate over the entire cultivation time. The same phenotype was observed for the mutant strain Re2315 with a deletion of both *lipA* and H16_A3742 (annotated as a putative lipase) genes. However a single deletion of the H16_A3742 gene (strain Re2314) had no defect in TAG cleavage and resulted in a similar phenotype as compared to the wild type. This suggested that only the *lipA* gene is responsible for the production of the extracellular lipase that is able to hydrolyze TAGs in palm oil and liberate FFAs for cellular usage in *R. eutropha* H16 cultures.

Introduction of the *lipA* gene into the mutant strain Re2313 via the overexpression plasmid pCJB201 was able to restore the TAG cleavage function of this strain (Fig. 2). Increasing the *lipA* gene dosage in both wild type and Re2313 resulted in more rapid TAG hydrolysis compared to just the wild type cell with vector alone (pBBR1MCS-2). The TAG spot intensity on the TLC plate decreased dramatically over the first 24 h and completely disappeared by 48 h when *lipA* was overexpressed. FFAs derived from TAGs built up in the media throughout cultivation time in the overexpression strains. This is likely the result of

rapid FFA production outpacing FFA uptake and incorporation into the cell or accumulation of excess FFAs not needed for cell growth.

Expression of *lipA* in heterologous hosts, such as *E. coli* and yeast, failed to produce an active lipase, which led us to the characterization of a lipase-specific foldase gene, *lipB* (H16_A1323), that is just downstream of *lipA*. Deletion of *lipB* (strain Re2318) showed the same growth phenotype in palm oil cultures as Re2313 (Online Resource 2). Introduction of the plasmid-borne *lipA* gene to Re2318 did not restore TAG hydrolysis (data not shown), suggesting that LipA is a Class I lipase that requires its specific chaperone (i.e. LipB) for folding and secretion (Arpigny & Jaeger, 1999; Frenken *et al.*, 1993a; Frenken *et al.*, 1993b; Hobson *et al.*, 1993). LipA from *R. eutropha* H16 shares 48% and 44% sequence identity to the *Ralstonia* sp. M1 and *Pseudomonas* sp. lipases, respectively, while the chaperone LipB only had 45% and 27% sequence identity to chaperones of the same class (Gilbert, 1993; Kim *et al.*, 2001; Quyen *et al.*, 2004; Quyen *et al.*, 2005).

Further analysis of the secreted protein in supernatants of *R. eutropha* H16, H16/pCJB201 (*lipA* overexpression), Re2313 (Δ*lipA*), and Re2318 (Δ*lipB*) were grown in cultures containing palm oil. After 24 h, culture supernatants were harvested and subjected to SDS-PAGE analysis. The presence of a ~40 kDa protein on the gel was observed, but only for strains containing intact *lipA* and/or *lipB* genes (Online Resource 3). The LipA enzyme H16_A1322 has a molecular weight of 38.6 kDa based on its primary amino acid sequence. H16/pCJB201 overexpressing *lipA* exhibited in an increase in the amount of protein at ~40 kDa as compared to wild type. This observation suggests that LipA and LipB work together as an extracellular lipase/chaperone pair.

Growth and Lipase activity

Strains H16, Re2313, and Re2318 were individually cultivated in minimal media containing 1% palm oil as the sole carbon source (Fig. 3A). Wild type H16 with an intact *lipA* gene reached an OD_{600} of 15.0 by 48h. Strain Re2313 lacks *lipA* for the production of

extracellular lipase, and not only was unable to hydrolyze TAGs in palm oil, but also did not grow significantly in the presence of unemulsified palm oil (Fig. 1). Strain Re2318, which lacks lipase chaperone LipB, also was unable to grow on palm oil.

Activity of LipA was determined in the cell supernatants during palm oil cultivation of wild type, Re2313, and Re2318 strains (Fig. 3B). Both Re2313 and Re2318 lack the gene for the production or secretion of active lipase, respectively, thus no lipase activity was detected (Fig. 3B. In strain H16, lipase activity was determined to be ~47 mU/mL after 5 h of culture and remained active till after 24 h (Fig. 3B). Reintroduction *in trans* of the *lipA* gene or the *lipB* gene into strains Re2313 or Re2318, respectively, restored growth of these strains on palm oil and also their lipase activities (Fig. 4). Re2313/pCJB201 exhibited four times higher lipase activity than the wild-type strain, due to the increase of lipase gene dosage (Fig. 4B). Strains Re2313/pCJB201 and H16/pCJB201 exhibit a decreased lag in growth in palm oil cultures as compared to the wild type, due to the increase in lipase activity (Fig. 4A). Although the chaperone gene was overexpressed in the same way as the lipase gene in Re2318/pJL36, the growth rate and lipase activity was found to be similar to the wild-type strain (Fig. 4). Reintroduction of the chaperone gene was able to restore the secretion mechanism of the lipase, but did not result in higher than wild-type levels of lipase activity. Lipase activity reached 1 U/mL when *lipA* was overexpressed in strain H16, thus explaining the rapid TAGs cleavage, compared to H16 with vector alone, as also detected by TLC (Fig. 2).

LipA production by *R. eutropha* was induced by growth on various carbon sources (Table 2). Initial extracellular lipase activity was detected at 3 h after inoculation (data not shown). Lipase activity was dependant on the carbon source used; the activity at 10 h varied from 0.5 mU/mL in Tween 20 to 200 mU/mL in Tween 60. LipA was also produced and active with non-lipid carbon sources such as fructose. Of the carbon sources tested in this

study, the LipA activity was observed in the following order (from highest to lowest activities): Tween 60> Tween 80>Palm Oil>Tween 40>Fructose> Tween 20 (Table 2).

Decreased lag phase due to lipase overexpression

Growth of strain H16/pCJB201 in palm oil cultures not only resulted in quick TAG cleavage (Fig. 2), but also shortened the lag phase of growth (Fig. 4A, 5). Wild-type culture experienced a ~22 h lag phase when grown on palm oil as the sole carbon source. H16/pCJ201, on the other hand, reached exponential growth phase in less than 12 h. The increased amount of DAGs, MAGs, and FFAs liberated from TAG hydrolysis in the growth media acted as a natural surfactant and enhanced the oil emulsification during growth on palm oil (Online Resource 4). Thus, the amount of time needed for the cells to adapt to the two-phase heterogeneity of the palm oil growth medium decreased significantly.

Substrate specificity

Substrate specificities of lipase enzymes classify them into one of the following categories: non-specific, regio-specific, or fatty acid-specific. Non-specific hydrolysis will result in complete breakdown of the TAG molecule into FFAs and glycerol, while regio-specific lipase will catalyze hydrolysis only at C1 and C3 position of the glycerol backbone. Fatty acid-specific lipase will only cleave fatty acid esters of certain chain lengths such as C12 lipids or C16 lipids (Arpigny & Jaeger, 1999; Gupta *et al.*, 2004; Treichel *et al.*, 2010). The TLC analysis of H16/pCJB201 resulted in complete hydrolysis of TAGs, DAGs, and MAGs in the media into FFAs (Fig. 2), suggesting that LipA is a non-specific lipase able to act at random for the complete breakdown of TAGs.

Effect of temperature, pH, metal ions, and detergents

The effects of temperature, pH, metal ions, and detergents on lipase activity were studied using culture supernatants of *R. eutropha* grown in Tween 60 for 10 h, since Tween 60 as the carbon source showed the highest induction of LipA activity compared to other carbon sources tested (Table 2). Lipase activity was measured at various temperatures from 0

to 80°C. LipA was active from 10 to 70°C (Fig. 6A). The optimal activity was observed at a temperature of 50°C, in which the activity reached 27 U/mL at pH 7. Although *R. eutropha* prefers 30°C for growth, its extracellular lipase is quite thermostable and remained active even at high temperatures.

Buffers of pH 3 to 12 were used to determine the pH optimum for LipA activity. The pH for best growth and lipase production was shown to be pH 6.8 for *R. eutropha*. LipA showed maximum activity at pH 7.0 to 8.0, when the activity reached 3 U/mL at room temperature (Fig. 6B). LipA was active in buffers ranging from pH 5 to pH 10 with a slight preference towards the alkaline pH buffers.

Although a metal cofactor is typically not involved in the mechanism of catalysis of lipases, divalent cations were reported to stimulate or inhibit lipase activity (El Khattabi *et al.*, 2003; Gupta *et al.*, 2004; Rosenstein & Gotz, 2000). Divalent metal ions Zn^{2+}, Mg^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, and Ni^{2+}, along with their chelators (EDTA and EGTA), were added at different concentrations to the assay mixture to examine their effect on LipA activity (Table 3). At concentrations of 0.1 mM, transition metal chelator EDTA and Ca^{2+}-chelator EGTA imparted little inhibition on LipA activity. At the same concentration, Zn^{2+} and Cu^{2+} inhibited LipA activity by ~50% while all other metal ions had little to no effect. The activity of LipA decreased dramatically to 3 - 18% of total activity when 1 mM of either chelators, Zn^{2+}, Fe^{2+}, Cu^{2+}, or Ni^{2+} were added. A 1 mM concentration of Mg^{2+} only inhibited LipA by 28%, while Ca^{2+} and Mn^{2+} ions had no affect at all on lipase activity. This suggested that LipA activity could be inhibited by Zn^{2+}, Fe^{2+}, Cu^{2+}, or Ni^{2+} but only slightly inhibited by Mg^{2+}, while activity was not affected by Ca^{2+}, and Mn^{2+} ions.

To test if Mg^{2+}, Ca^{2+}, and Mn^{2+} can reverse the inhibition caused by chelators, 1 mM of each ion was added separately to assay mixtures after the addition of 1 mM of both chelators (Fig. 7). While chelators EDTA and EGTA inhibited the LipA activity by 83%, addition of Mg^{2+}, Ca^{2+}, or Mn^{2+} alleviated this inhibition and helped restore the LipA activity

back to 80 to 95% of its original levels. Other metals had no affect on the inhibition caused by the addition of chelators. These results demonstrate that Mg^{2+}, Ca^{2+}, and Mn^{2+} divalent metal ions can stimulate lipase activity and alleviate chelator caused inhibition.

Detergents are known to either increase solubility of lipids (i.e. act as emulsifiers) or induce lipid aggregation, thus allowing lipase to better access the lipid substrate (Lin *et al.*, 1995; Reis *et al.*, 2009). To test the effect of detergents on LipA activity, 0.001, 0.01, and 0.1% (v/v) of Tween 20, Tween 40, Tween 60, or Tween 80, Triton X-100 or Triton 305, or SDS detergents was added to the enzymatic assay mixture at room temperature and pH 7.0 (Table 4). At low concentrations (0.001% v/v), Tween 40, Tween 60, Tween 80, and SDS all inhibited LipA activity by ~90%. However, an increase in the concentration to 0.01% (v/v) of Tween 40, Tween 60, Tween 80, and SDS helped restore the lipase activity. Interestingly Tween 40, which had restored LipA activity at 0.01% then again inhibited the activity at 0.1%. Addition of increasing concentration of Tween 20 resulted in LipA activity decrease by <90%. Triton X-100 was able to increase LipA activity by 586% at 0.001% and 333% at 0.1%. Increasing concentration of Triton X-305 reduced LipA activity by ~70%; however at 0.1% the LipA activity was restored to normal. This suggested that although some detergents could help stimulate LipA activity, the mechanism of detergent concentration to LipA activity is complex.

DISCUSSION

In this study, we have characterized the extracellular triacylglycerol hydrolase from *R. eutropha* H16. This lipase, LipA, contains the conserved lipase catalytic triad Ser, His, and Asp in its primary structure (Online Resource 5). Since LipA requires a lipase-specific foldase, LipB, to achieve its correct catalytic tertiary structure and be secreted outside of the cell, it belongs to the Class I lipase family (Arpigny & Jaeger, 1999; Frenken *et al.*, 1993b; Hobson *et al.*, 1993). Protein sequence homology revealed that LipA and LipB, the lipase and

chaperone pair of *R. eutropha* H16 were similar to those from *Ralstonia* sp. M1 (Quyen *et al.*, 2004; Quyen *et al.*, 2005). LipA primary sequence contains three cysteine residues (Online Resource 5) with the possibility of disulfide bond formation, and chaperone LipB could be required for this reduction *in vivo* (Kok *et al.*, 1996).

LipA from *R. eutropha* H16 was determined to be a non-specific lipase, because it was able to completely hydrolyze TAGs into FFAs and glycerol. Non-specific lipases hold great importance in the field of biodiesel fuel production from TAGs, since they can act at random and fully breakdown TAGs (Bajaj *et al.*, 2010; Fjerbaek *et al.*, 2009; Jegannathan *et al.*, 2010; Singh & Singh, 2010). Also, since LipA is a secreted lipase, its isolation process would be simplified, compared to intracellular lipases from other species, for lipase immobilization applications (Fjerbaek *et al.*, 2009; Gupta *et al.*, 2004; Jaeger & Reetz, 1998). LipA can also catalyze a diverse range of substrates such as Tween 20, Tween 40, Tween 60, and Tween 80, due to its broad range of selectivity. The reaction rate of such catalysis varies directly and extensively with the chemical properties of the substrate. LipA favored Tween 60 as a substrate compared to other Tween compounds, with the least favored being Tween 20. LipA could favor long chain length linear fatty esters, since the hydrolysis of Tween 60 can only liberate stearic acid (18 carbon linear chain).

Overexpression of the *lipA* gene on a plasmid not only increased the TAG hydrolysis rate in culture but also resulted in a shortened *R. eutropha* lag phase when grow on palm oil. Biotechnological usage of LipA would reduce the dependence on surfactants, since more DAGs, MAGs, and FFAs, that act as natural surfactants, can be liberated from the rapid hydrolysis of TAGs in the initial growth stage (Jaeger & Eggert, 2002; Skagerlind *et al.*, 1992). The industrial fermentation time could be shortened, using a strain of *R. eutropha* that overexpresses *lipA*, because the cells can reach the stationary phase in half of the normal growth time, due to the increased amount of FFAs present in the culture for cell growth and biosyntheses.

Lipase activity can be affected not only by the carbon sources used, but also by physico-chemical factors such as temperature, pH, metal ion, and detergent. Although the optimal temperature and pH for lipase production correspond with the growth temperature and pH of the host microorganisms, most secreted lipases are active in a wide range of temperatures and pHs (Gupta *et al.*, 2004). *R. eutropha* H16 LipA showed high activity at 50°C, which is similar to many other characterized bacterial lipases (Gupta *et al.*, 2004). Most of the previously studied lipases have temperature optima in the range of 30 to 60°C, although some extreme lipases were also found that exhibited high activity even at low or high temperatures (Jeon *et al.*, 2009; Joseph *et al.*, 2008; Kulkarni & Gadre, 1999; Nawani & Kaur, 2007). Adjustment of the temperatures for optimal lipase performance, depending on application, can be achieved through the use of stabilizers such as ethylene glycol, sorbitol, or glycerol (Franken *et al.*, 2010; Gupta *et al.*, 2004). LipA had neutral to slightly alkaline pH optimum that is similar to most known bacterial lipases (Gupta *et al.*, 2004; Kanwar *et al.*, 2002; Lesuisse *et al.*, 1993).

TAG hydrolysis by lipase does not require specific cofactors, although Ca^{2+} has been reported to stimulate the activity of lipases from *Bacillus, Pseudomonas, Chromobacterium*, and *Acinetobacter* species (Gupta *et al.*, 2004; Kanwar *et al.*, 2002; Rathi *et al.*, 2001). The function of Ca^{2+} in lipase is highly debatable. It has been hypothesized that the released fatty acids associate with Ca^{2+} to form calcium salts, and thus relieve product (FFA) inhibition (Godtfredsen, 1990; Macrae & Hammond, 1985). Also, the role of Ca^{2+} was thought to stabilize the lipase tertiary structure. It has also been theorized that there is a direct involvement of Ca^{2+} in the catalysis for the activation of water molecules; however, this theory is highly unlikely since the calcium-binding site was found to be far from the active site in the *Burkholderia glumae* lipase crystal structure (Jaeger *et al.*, 1999; Noble *et al.*, 1993; Rosenstein & Gotz, 2000; Verheij *et al.*, 1980); (Simons *et al.*, 1999). The metal chelators EDTA and EGTA acted as inhibitors to all lipases, including LipA. Although the role of Ca^{2+}

was not elucidated, it alleviated inhibition by chelation and stimulated LipA activity. Calcium ion could also be replaced by Mg^{2+} or Mn^{2+} without loss of activity in LipA. Reported in the literature, divalent cations Sr^{2+} or Ba^{2+} can also replace Ca^{2+} in lipase (Gupta *et al.*, 2004). The LipA primary sequence contains several aspartate residues that could be responsible for the binding of calcium (Online Resource 5). To test the residues involved in potential Ca^{2+} binding, site-directed mutagenesis could be employed. Furthermore, like many reported lipases, LipA activity was inhibited by metal chelators (Table 3, Fig. 7) and by various heavy metals (Gupta *et al.*, 2004).

Lipase activity is highly inducible and the activity is dependent on substrate concentrations at the interface of water and lipid. Detergents, at a specific concentration, can cause non-polar substrates to aggregate or solubilize in water thus dramatically increasing the activity of lipase (Boekema *et al.*, 2007; Reis *et al.*, 2009). The addition of small amounts of Triton X-100 could cause a shift in the solubility equilibrium and cause substrate aggregation because the substrate concentration exceeded the solubility limit. Such change at the interface strongly activated LipA activity. Detergents such as Tween 20 could compete with the LipA active site, thus inhibiting the activity of LipA in our assay system. Thus, dependant on the type and concentration of detergents, LipA activity was either enhanced or inhibited. LipA characterized here had similar activity response to detergents as LipA from *Ralsonia* sp. M1 (Quyen *et al.*, 2005). Since much is still unknown regarding lipase catalysis at the water-oil interface, the use of detergent-based inducers can only be determined experimentally at this time.

It has long been established that *R. eutropha* is the model organism for the production of polyhydroxyalkanoate (PHA), a polyester that can potentially replace current petroleum-based plastics in many applications. Palm oil is a promising carbon source because it is a readily available agricultural byproduct and has high density carbon content. Recently Riedel *et al.* were able to produce over 139 g/L of biomass using engineered *R. eutropha* with 74%

of cell dry weight as PHA, using palm oil as the sole carbon source (Riedel *et al.*, 2012). *R. eutropha* growth in palm oil, or any plant oil, presents challenges due to the heterogeneity between the oil feedstock and the aqueous media. Emulsifiers, such as the glycoprotein gum Arabic, were able to emulsify the palm oil in growth medium and make it more bioavailable without influencing *R. eutropha* growth (Budde *et al.*, 2011a). In this work, overexpression of extracellular LipA in *R. eutropha* allowed for rapid TAGs hydrolysis (Fig. 2) with liberated DAGs, MAGs, and FFAs acting as natural surfactants and ultimately resulted in a faster growth rate (Fig. 4A, 5). Such properties could potentially make LipA an enzyme with important biotechnological usage, especially in the production of PHA from palm oil.

ACKNOWLEDGEMENTS

The authors thank Dr. Charles F. Budde for his helpful ideas and discussions; Mr. John W. Quimby and Sebastian Riedel, Dipl.-Ing. (FH) for critical review of the manuscript. This work was funded by the Malaysia-MIT Biotechnology Partnership Program (MMBPP). We thank our MMBPP collaborators for their helpful discussions and support throughout the course of this study.

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FIGURE LEGENDS

FIG. 1

Thin layer chromatography (TLC) analysis of supernatants taken from the palm oil cultures of *R. eutropha* strains at 8, 24, 48, and 72 h. The TLC plate image shows the oil residue profile, including the presence of TAGs (triacylglycerols: 1,2-distearoyl-3-oleoyl-rac-glycerol), DAGs (diacylglycerols: 1,2-dipalmitoyl-rac-glycerol), MAGs (monoacylglycerols: 1-palmitoyl-rac-glycerol), and FFAs (free fatty acids: palmitate), during cultivation of *R. eutropha* strains H16 (WT), Re2313 (Δ*lipA*), Re2314 (ΔA3472), and Re2315 (Δ*lipA*ΔA3472) on palm oil (lanes 1 to 16). Standards were spotted and run on lane 17.

FIG. 2

Overexpression of *lipA* in palm oil cultures. TLC plate image of oil residue profile during cultivation of H16/pBBR1MCS-2, H16/pCJB201, Re2313/pBBR1MCS-2, and Re2313/pCJB201 using palm oil as the sole carbon source. Lane 1 was spotted with standards and lane 2 with palm oil in initial culture media. Lanes 3 through 18 were spotted with supernatants removed from the *R. eutropha* cultures at 8, 24, 48, and 72 h.

FIG. 3

Growth (A) and lipase activities (B) of *R. eutropha* strains H16 (WT), Re2313 (Δ*lipA*), and Re2318 (Δ*lipB*) in 1% palm oil cultures. Culture OD_{600} and lipase activity of H16 are indicated by the solid circles, Re2313 is shown with open circles, and Re2318 is represented by solid triangles.

FIG. 4

Growth (A) and lipase activities (B) of *R. eutropha* strains in 1% palm oil cultures. The culture OD_{600} and activity profiles of H16, Re2313, Re2318, Re2313/pCJB201, Re2318/pJL31, and H16/pCJB201 are

indicated by solid circles, open circles, solid triangles, open triangles, solid boxes, and open boxes, respectively.

FIG. 5

Viable colony counts of H16/pBBR1MCS-2 and H16/pCJB201 in palm oil cultures. Solid circles represent growth of strain H16 containing only empty vector (pBBR1MCS-2). Open circles indicate the colony-forming units of H16 with *lipA* overexpression (pCJB201). Data points represent the mean values of n = 3 ± standard deviation (error bars).

FIG. 6

Effect of temperature (A) and pH (B) on LipA lipase activity. Average values from both experiments from three replications were plotted in solid circles with standard deviation values represented as error bars.

FIG. 7

Metal ion alleviation of LipA lipase activity inhibition by chelators. Reactions were carried out at room temperature in pH 7.0 glycine-HCl buffer. Control was carried out without addition of chelators or metal ions. Metal ion solutions at a final concentration of 1 mM were added after the enzyme assay mixture was treated with 1 mM chelators. Average values from three experiments were plotted with error bars representing the standard deviation.

TABLES

Table 1

Strains and plasmids used in this work.

Strains or plasmid	Genotype	Reference
Strains		
R. eutropha		
H16	Wild-type, gentamicin resistant (Gen^r)	ATCC17699
Re2313	H16Δ*lipA* Gen^r	(Brigham *et al.*, 2010)
Re2314	H16Δ(H16_A3742) Gen^r	This work
Re2315	H16Δ*lipA* & A3742 Gen^r	This work
Re2318	H16Δ*lipB* Gen^r	This work
E. coli		
S17-1	Conjugation strain for transfer of plasmids into *R. eutropha*	(Simon *et al.*, 1983)
Plasmids		
pJV7	pJQ200Kan with Δ*phaC1* allele inserted into BamHI restriction site, confers kanamycin resistance (Kan^r)	(Budde *et al.*, 2011a)
pJL31	pJV7 with Δ*phaC1* allele removed by XbaI and SacI digestion and replace with Δ*lipB* allele (Kan^r)	This work
pBBR1MCS-2	Broad-host-range cloning vector (Kan^r)	(Simon *et al.*, 1983)
pCJB201	pBBR1MCS-2 with *R. eutropha lipA* gene inserted into the multiple cloning site (Kan^r)	(Brigham *et al.*, 2010)
pJL36	pBBR1MCS-2 with *R. eutroha lipB* gene inserted into the multiple cloning site (Kan^r)	This work

Table 2

Effect of carbon sources on lipase production[a].

Carbon source	Lipase activity (mU/mL)
Fructose (0.5% w/v)	4.6±0.3
Palm Oil (0.5% v/v)	38.0±0.8
Tween 20 (0.5% v/v)	0.5±0.1
Tween 40 (0.5% v/v)	36.0±1.0
Tween 60 (0.5% v/v)	200.0±20.0
Tween 80 (0.5% v/v)	91.0±5.0

[a]Each value represents the mean ± standard error on n = 3. Supernatants of *R. eutropha* strain H16 grown in various carbon sources were collected at 10 h of culture time for activity assays. Lipase activity was determined at room temperature, pH 7.0 in glycine-HCl buffer.

Table 3

Effect of various metal ions or chelating agents on lipase activity[a].

Metal ion	Relative activity (%) (0.1 mM metal ion or chelator)	Relative activity (%) (1 mM metal ion or chelator)
None	100	100
EDTA & EGTA	91	15
$ZnCl_2$	43	3
$MgCl_2$	94	72
$FeCl_2$	86	18
$CaCl_2$	98	99
$CuCl_2$	68	11
$MnCl_2$	115	99
$NiCl_2$	90	7

[a]Enzymatic assay was carried out at room temperature with pH 7.0 glycine-HCl buffer.

Table 4

Effect of detergents on lipase activity[a].

Detergent	Relative activity (%) (0.001% v/v detergent)	Relative activity (%) (0.01% v/v detergent)	Relative activity (%) (0.1% v/v detergent)
None	100	100	100
Tween 20	120	66	2
Tween 40	5	117	8
Tween 60	7	10	27
Tween 80	5	9	83
Triton X-100	686	543	433
Triton X-305	77	27	112
SDS	11	38	126

[a]Lipase assay was conducted with pH7.0 glycine-HCl buffer at room temperature.

Figure

FIGURES

FIG. 1

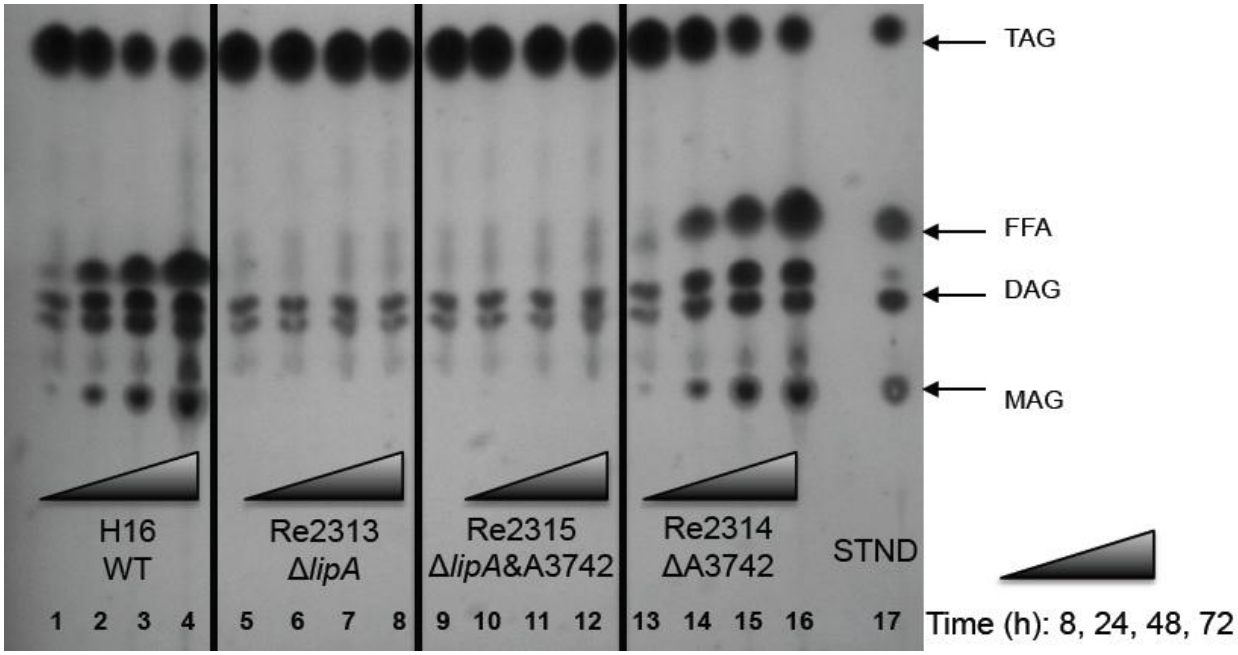


FIG. 2

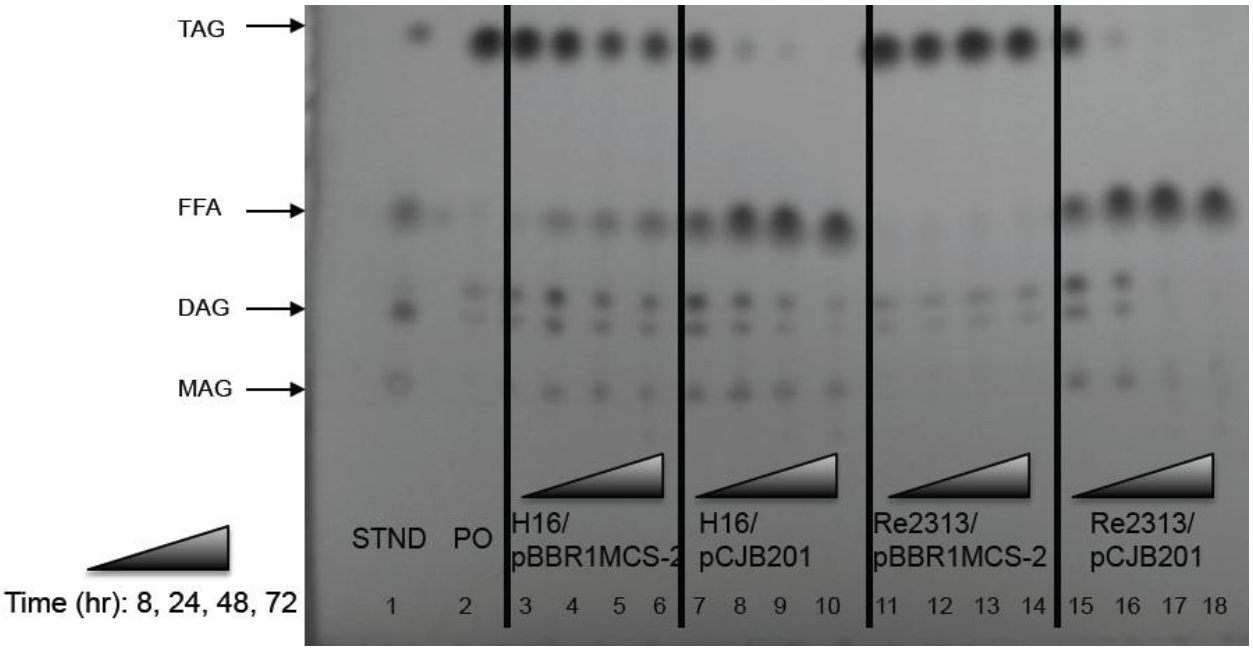

TAG
FFA
DAG
MAG
Time (hr): 8, 24, 48, 72
STND
PO
H16/ pBBR1MCS-2
H16/ pCJB201
Re2313/ pBBR1MCS-2
Re2313/ pCJB201
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

FIG. 3

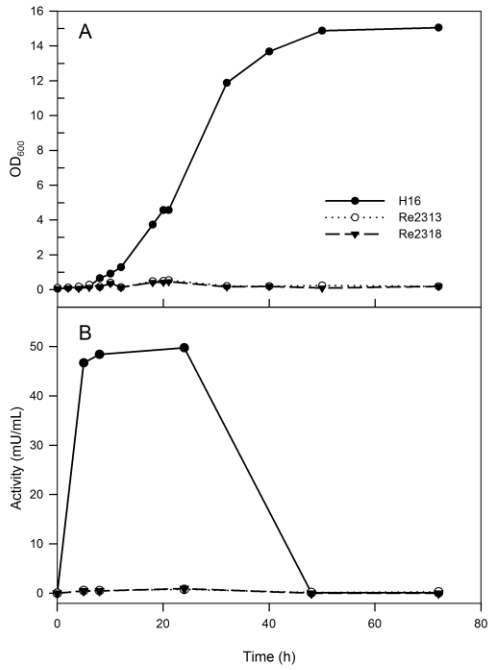

A
B
H16
Re2313
Re2318
OD600
Activity (mU/mL)
Time (h)
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
0
20
40
60
80

FIG. 4

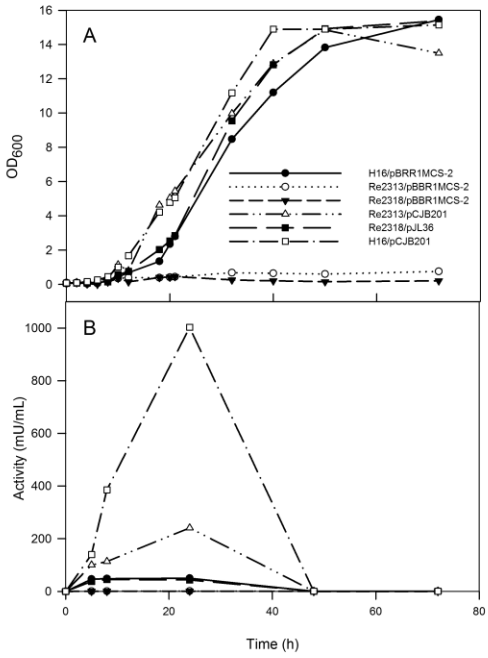

A
B
OD600
Activity (mU/mL)
Time (h)
H16/pBRR1MCS-2
Re2313/pBBR1MCS-2
Re2318/pBBR1MCS-2
Re2313/pCJB201
Re2318/pJL36
H16/pCJB201

FIG. 5

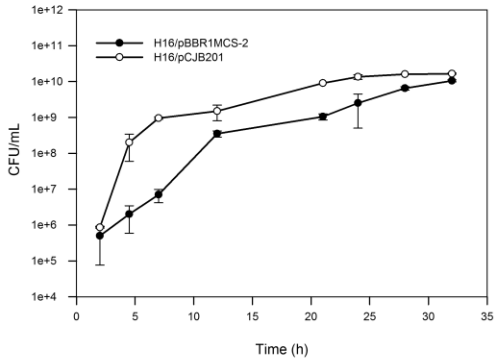

H16/pBBR1MCS-2
H16/pCJB201
CFU/mL
Time (h)
1e+12
1e+11
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
0
5
10
15
20
25
30
35

FIG. 6

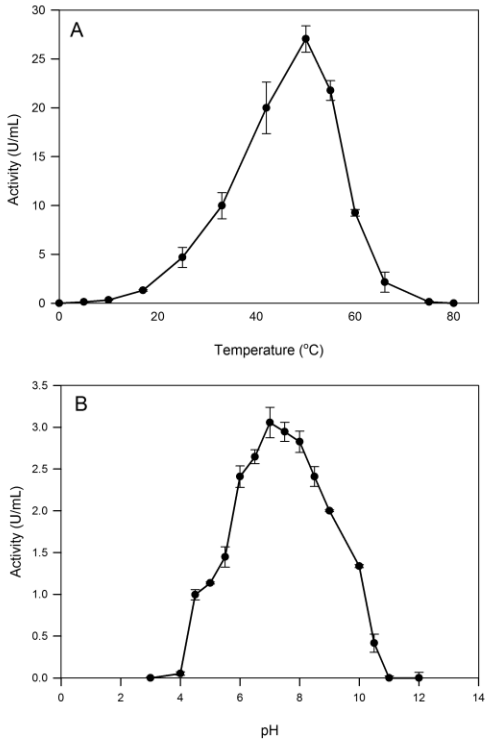
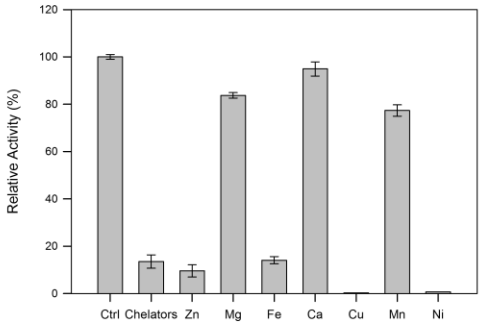


FIG. 7


Relative Activity (%)
120
100
80
60
40
20
0
Ctrl
Chelators
Zn
Mg
Fe
Ca
Cu
Mn
Ni

Supplementary Material

Click here to download Supplementary Material: JLu_lipase_Supplementary Material_Revised_Final.docx