2020-10-01

Isolation and Characterization of a Novel Thermo-solvent-stable Lipase from Pseudomonas Brenneri and its Application in Biodiesel Synthesis

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Recommended Citation

Priyanka P., Kinsella G.K., Henehan G.T., Ryan B.J. (2020). Isolation, purification and characterization of a novel solvent stable lipase from Pseudomonas reinekei.*Biocatalysis and Agricultural Biotechnology*,101806. Doi: 10.1016/j.bcab.2020.101806

Isolation and characterization of a novel thermo-solvent-stable lipase from *Pseudomonas brenneri* and its application in biodiesel synthesis

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Short Running Title: Biofuel from novel lipases

Abstract

Pseudomonads are one of the most studied species of bacteria as they display remarkable metabolic and physiological versatility. This enables them to colonize a wide variety of terrestrial and aquatic habitats, generating biotechnologically interesting enzymes. Here, the partial purification and characterization of a novel, extracellularly-produced, lipase from *Pseudomonas brenneri* is described. The partially purified lipase was active over a broad pH range (5.0–9.0) and was stable at 70 °C for 45 min. The lipase displayed significant stability, and in some cases activation, in the presence of organic solvents with log P≥2.0. Such stability characteristics indicated that this lipase could potentially be useful as a biocatalyst for biodiesel production. This was subsequently demonstrated through the facile production of Fatty Acid Methyl Esters in the presence of olive oil and methanol. Possible applications for this novel, stable lipase include the bioremediation of oil in the environment.

Keywords: biodiesel production, lipase, *Pseudomonas*, solvent stability, thermal stability.

Highlights:

1. A novel lipase, from an under explored *Pseudomonas* genus has been isolated and characterized.
2. The novel lipase displays excellent thermo-organo-stability.
3. The novel lipase readily catalyzed Fatty Acid Methyl Ester synthesis in the presence of olive oil and methanol.

1. Introduction:

Enzyme biotechnology has progressed from catalysis in an aqueous medium to synthesis in non-aqueous media. Since non-aqueous biocatalysis is preferred for many processes, a significant research effort in recent decades has been devoted to establishing platforms for its enhancement of non-aqueous biocatalysis (Illanes, 2016). However, many enzymes are either inactivated or denatured in organic solvents which limits their usefulness (Kumar *et al.*, 2016). Physical (immobilisation), chemical (protein modification) and genetic methods (protein engineering) have been employed to tailor enzymes in order to increase their activity, stability and selectivity in non-aqueous environments (Villeneuve *et al.*, 2000). However, nature provides a vast natural microbial pool of novel enzymes which can be screened by exploring the diversity of the microbiome (Adrio and Demain, 2014). Solvent stable lipases are key biocatalysts in non-aqueous solutions and can catalyse various reactions (such as esterification, alcoholysis, acidolysis and interesterification; (Sharma and Kanwar, 2014)). Due to their wide ranging applications, lipases have long been subjected to intensive study. *Pseudomonas* sp. are a known source for the isolation of lipases. For example, a lipase from *P. cepacia* has been used for the synthesis of *cinnamyl propionate* (Badgujar, Pai and Bhanage, 2016) while lipases from *P. fluorescens* and *P. stutzeri* have been used in the cosmetic, oral care and pharmaceutical industries (Vescovi *et al.*, 2017; Cao *et al.*, 2012). In this laboratory, we have previously identified lipases with potential in organic synthesis (Priyanka *et al.*, 2019).

In this study, a novel extracellular lipase was identified in a *Pseudomonas brenneri* culture isolated from soil sample. The lipase was partially purified and characterised following production optimisation. This thermo-organo stable novel lipase was employed in the production of Fatty Acid Methyl Esters (FAMEs) as a proof of application exercise. Based on initial characterisation and this application, the novel lipase will be of further interest for various biocatalytic and biotechnological uses in non-aqueous media, as well as bioremediation of oil in the environment (Kumar, 2020).

2. Materials and methods:

2.1. Chemicals and materials:

All chemicals used were of analytical grade and purchased from Sigma-Aldrich. GE Healthcare supplied the Q-Sepharose high performance (HP) resin. The GC column for the analysis of FAME(s) was purchased from Bruka™.

2.2. Enzyme assays

2.2.1. Plate assay

Lipolytic activity was monitored during screening using Rhodamine B agar plates; the Kouker and Jaeger Method was employed to prepare these plates (Kouker and Jaeger, 1987).

2.2.2. Standard enzyme spectrophotometric assay

p-nitrophenyl palmitate (*p*-NPP) was the substrate used to measure lipase activity at pH 7.5 (Winkler and Stuckmaan, 1979), based on the adapted method of Glogauer and colleagues (Glogauer *et al.*, 2011) with minor deviation: lipase activity was measured under initial velocity conditions, after 30 min of static incubation at 28 °C.

2.3. Isolation and identification of the solvent tolerant lipase producing strain

Soil samples, collected from various locations on the Wicklow mountains in Ireland, were incubated in enrichment medium containing olive oil as a carbon source (Mo *et al.*, 2016) for 72 h at 28 °C, 200 rpm. Solvent tolerant lipase producing strains were isolated as described previously (Priyanka *et al.*, 2019).

2.4. Lipase production

2.4.1. Fermentation time and inoculum percentage

A 1–8% (v/v) aliquot of LB medium culture grown in an Erlenmeyer flask at 28 °C, 200 rpm was added to basal lipase production medium. LB cultures with an $O.D_{600nm}$ of 0.85 (measured after diluting the inoculum by 1:3) were selected as the inoculum for lipase production medium. Basal lipase producing medium contained 50g/L bacteriological peptone, 2 g/L sodium chloride, 0.4 g/L magnesium sulfate, 0.5 g/L ammonium sulfate, 0.3 g/L dipotassium hydrogen phosphate, 0.03 g/L potassium hydrogen phosphate and 10 g/L olive oil at pH 7.0. The cell free supernatant was analysed for lipolytic activity after 24 h of fermentation by the standard spectrophotometric assay.

2.4.2. Nitrogen source

1% (w/v) of different nitrogen sources (bacteriological peptone, yeast extract, tryptone, ammonium sulfate, Urea, L-Lysine, L-Arginine, Glutamic acid, Glutamine and Asparagine respectively) were used as a substitute for 50 g/L peptone in the basal lipase production medium, as described in previous work (Priyanka *et al.*, 2019).

2.5. Lipase partial purification

Following fermentation using the optimized conditions (pH 6.8), cell free supernatant was harvested by centrifugation at 4 °C, 5000*g* for 20 min. This was then initially filtered through a 1.2 µm pre-filter and, subsequently, a 0.45 µm filter. The filtered cell free supernatant was dialyzed in a 14 kDa cut-off dialysis membrane (Sigma-Aldrich) at 1:20 ratio in 10 mM Tris HCl, pH 8.5, for 4 h at room temperature under continuous stirring using a magnetic stirrer. After 4 h, the buffer was replaced with fresh buffer and dialysis was continued overnight (15 h) at 4 °C. After dialysis, the crude extract was alkaline precipitated for 30 min at pH 9.0 at room temperature. After centrifugation at 5000*g* for 20 min at 4 °C and filtration through 0.45 µm filter; the alkaline precipitated filtrate was adjusted to pH 8.5 using HCl. This filtrate was loaded onto an anion exchange Q-Sepharose HP resin pre-equilibrated with 10 mM Tris-HCl pH 8.5. Partially purified lipase was collected from the anion exchange chromatography resin when a step elution with 750 mM NaCl in 10mM Tris-HCl pH 8.5 was performed.

The partially purified lipase was dialysed (1:20) in Tris-HCl buffer pH 8.5 overnight at 4 °C. Following dialysis, the protein sample was transferred to an air-tight container and was stored at -80 °C for 12 h. The frozen sample was later freeze dried at -54 °C, 0.002 milli bar. The freeze-dried protein sample was reconstituted based on requirements for characterization and application.

2.6. Stability studies/characterisation

All stability studies were carried out using 30IU/ml of lipase with a specific activity of 3.18 IU/mg. The stability of partially purified lipase was determined over a wide pH range (3.0–10.0) at 28 °C using the following buffers: 50 mM Glycine-HCl (pH 3.0, 4.0), 50 mM Tris-Acetate (pH 5.0, 6.0), 50 mM Tris-HCl (pH 7.0, 8.0, 9.0) and 50 mM Borate Buffer (pH 10.0). The corresponding percent relative activity was expressed relative to the highest activity after 24h of incubation at 28°C. Following enzyme incubation at 60 °C for 4 h, the lipase thermal half-life ($T_{1/2}$) was estimated. The partially purified lipase was analysed for stability in a variety of organic solvents by gently mixing the selected solvent and the enzyme at both 28 °C and 40 °C in screw cap glass vials. The

effect of modifiers such as metal ions, enzyme inhibitors and surfactants on the lipase, at 28 °C and 40 °C, were explored in a similar way. For organic solvent and modifier stability studies, a percentage residual activity value was measured relative to control (i.e. the enzyme solution without any solvents/additives). A Lineweaver–Burk plot was employed to estimate Michaelis–Menten steady state kinetic constants of K_m and $V_{max.}$ In all cases, with the exception of substrate specificity, lipolytic activity was measured using the standard spectrophotometric assay employing *p*-NPP as substrate.

2.7. FAME synthesis using olive oil

500 IU of lipase (specific activity of 3.18IU/mg) was used for the transesterification of 1gm olive oil using methanol (molar ratio 9:1 for methanol: oil). The reaction mixture was maintained at 40 °C and 120 rpm for 72 h in a shaker water bath. After transesterification, the sample was centrifuged at 5,000*g* for 10 min and the solvent layer (top layer) containing FAME was carefully pipetted into a clean sealed glass container. FAMEs generated were subsequently analysed by TLC and GC. TLC detection of FAME post transesterification was carried out as per Kim and colleagues (2014). In brief, a 90:10 (v/v) *n*-hexane:diethyl ether solvent mix was used as the mobile phase and after full development of TLC plate, the FAME spots were visualized using a 10 % (v/v) ethanoic phosphomolybdic acid spray, followed by drying at 105 °C for 5 min.

A Scion-436GC (comprising a GC column BR-SWax, 0.25 mm x 30 m, with FID detector) was used for FAME GC analysis. FAMEs generated by transesterification of olive oil were identified by comparing their RT (Retention Time) with the RT of a standard FAME mix (Sigma-Aldrich; Religia and Wijanarko, 2015). The GC method published by Agilent (David, Sandra and Vickers, 2005) was used for all the FAME analysis.

3. Results and Discussion

3.1. Isolation and identification of solvent tolerant lipase producing strain

Two lipolytic cultures isolated from soil samples (GPS location 53°00'12.4"N, 6°20'47.9"W; a woodland in a national park) were stable in cyclohexane, ethanol, *n*-hexane, heptane and methanol by plate overlay method (Patel, Nambiar and Madamwar, 2014). 16S rRNA sequencing of these strains identified one of the lipolytic cultures as *Pseudomonas reinekei* (see Priyanka *et al.*, 2019) while the other isolate was identified as *Pseudomonas brenneri*. The stability of the extracellular

lipase produced by *Pseudomonas brenneri* in the presence of *n*-hexane, as observed by plate overlay method, is shown in Figure 1.

3.2. Lipase production

Extracellular microbial lipases are useful for biotechnological applications as they can be easily recovered from the fermentation broth. However, optimization of media and growth conditions are vital for the successful development of a productive fermentation process (Padhiar, Das and Bhattacharya, 2011). Media composition, as well as factors like initial inoculum and fermentation temperature and time, are known to effect the extracellular production of lipases (Andualema and Gessesse, 2012). In this study, a one-factor at a time approach was employed to optimise these influencing variables for *P. brenneri production* (Ayinla, Ademakinwa and Agboola, 2017).

3.2.1. *Fermentation time and inoculum percentage*

At a suitable inoculum size, the nutrient and oxygen levels are appropriate for bacteria growth and therefore, maximum lipase production. Conversely, if the inoculum size is too small, insufficient biomass will lead to reduced levels of lipase secreted over the culture period. For example, for *P. stutzeri* MTCC 5618, a 1 % (v/v) inoculum resulted in 83.33 % lipase activity yield but an increased inoculum percentage (from 3 to 6 %; v/v) resulted in a decrease in enzyme activity (Thakur, Tewari and Sharma, 2014).

In the current study, maximum lipase activity for *P. brenneri* lipase was obtained with 2.5 % (v/v) inoculum after 2 days of fermentation in basal lipase producing medium (see Figure 2). The rate of lipase production is related to the organism, however, generally, extracellular lipases are produced in the late logarithmic or stationary phases of microbial culture (Gupta *et al.*, 2016). Various cultivation periods, ranging from 5 h to 168 h, have been described as optimal for different lipase producing organisms. Lipases from *P. reinekei* (Priyanka *et al.*, 2019) and *Pseudoalteromonas* sp. WP27 (Joseph, Upadhyaya and Ramteke, 2011) were maximally produced after six and fourteen hours of fermentation respectively. While in some studies, a fermentation time of 48 h for *Pseudomonas putida* 922 (Fatima and Khan, 2015) and four days for *P. aeruginosa* JCM5962 (Sachan and Singh, 2017) produced highest lipase activity.

3.2.2. Nitrogen Source

Tryptone and peptone are common organic sources of nitrogen used for microbial culture and enzyme production. For the lipase from *P. brenneri*, a 1 % (w/v) bacteriological peptone supplement resulted in a significant ($p \leq 0.05$, t-test; see Figure 3) increase in lipase production to 0.91 IU/mL and was the best nitrogen source of those explored. It has been reported that peptone is an inducer of lipase production as it provides NH_4^+ ions, which stimulate bacterial growth and increases enzyme production (Kumar *et al.*, 2012). A 1% (w/v) peptone supplement was found to be the best nitrogen source for lipase production by *Pseudomonas gessardii* (Ayinla, Ademakinwa and Agboola, 2017) and a 0.5 % (w/v) peptone level was optimal for lipase production in *Alkalibacillus salilacus* (Samaei-Nouroozi *et al.*, 2015). Media supplementation with specific amino acids has been shown to enhance lipase production in microbial sources (Alanine, Glycine, Lysine and Serine for *Streptococcus faecalis* lipase, Chander and Ranganathan, 1975; Arginine, Lysine, Aspartic acid and Glutamic acid for *Pseudomonas fragi* lipase, Alford and Pierce, 1963; and L-Lysine for *P. reinekei* lipase, Priyanka *et al.*, 2019)). Therefore, it was important to explore different amino acids supplements to examine their effect on lipase production.

3.3. Partial purification

Partial lipase purification was accomplished by a two-step process (see Table 1). The initial, and little used, purification step of alkaline precipitation (pH 9.0) removed some contaminant proteins from the lipase preparation. A second purification step of anion exchange chromatography (pH 8.5) eluted partially purified lipase, in an elution buffer containing 750 mM NaCl at pH 8.5. Increasing the pH to pH 9.0 triggered precipitation of the dialysate, which could not be used for purification on chromatographic resins. Hence, to avoid precipitation and to permit the use of the dialysate after alkaline precipitation for chromatography, the pH of dialysate was lowered to 8.5. Following the purification procedure; a lipase with specific activity of 3.18 IU/mg was achieved, with an overall yield of 56.89%.

3.4. Characterisation of P. brenneri lipase

3.4.1. Effect of pH

The lipase from *P. brenneri* showed maximum stability (>90% relative activity) between pH 5.0 to 8.0 (see Figure 4). However, at pH 3.0, 4.0 and pH 10.0; a significant loss of activity was seen ($p \leq 0.05$, t-test). The optimal pH of this lipase was 6.0-8.0, as is the case for many other

Pseudomonas lipases (Sugiura *et al.*, 1977; Lee and Rhee, 1993; Sharma, Tiwari and Hoondal, 2001). An optimal pH of 6.0-8.0 makes this lipase ideal for detergent formulations, as well as for flavour synthesis and bioremediation (Salihu and Alam, 2015).

3.4.2. *Thermostability*

Lipases from many *Pseudomonas* species are known to have optimum temperatures from 4°C to 90°C (11, 26, 28, 29). *P. brenneri* lipase showed a broad temperature range for optimum lipolytic activity. The lipase showed >80 % residual activity at 70 °C after 45 min of incubation (Figure 5) and by first order thermal deactivation, the half-life of the lipase at 60 °C was calculated as 190 min (Figure 6). The half-life of the lipase from *P. brenneri* was not examined at other temperatures. Thermostable lipases from *Pseudomonas* sp. have already been isolated and studied (Sugihara *et al.*, 1992; Rathi *et al.*, 2000; Sharma, Tiwari and Hoondal, 2001; Kulkarni and Gadre, 2002; Badgujar, Pai and Bhanage, 2016; Latip *et al.*, 2016); with the current lipase being of comparable thermal stability.

3.4.3. Effect of metal ions and additives

Enhanced activity of lipase in the presence of Ca^{2+} and Mg^{2+} has been reported (Posner and Morales, 1972; Rukman and Henny, 2015). This enhanced activity is reportedly based on improved cross linking of the polypeptide chain in lipase with Ca^{2+}/Mg^{2+} bridges, making the enzyme-metal ion complex more rigid and stable (Kyu Kim *et al.*, 1997). However, the presence of Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, Co^{2+}, Mn^{3+} ions did not affect the activity of *P. brenneri* lipase. Conversely, a loss in activity was observed in the presence of Zn^{2+} and Fe^{3+} ions. The presence of EDTA also resulted in significant loss of lipolytic activity, suggesting the enzyme could be a metalloenzyme. Lipases from various *Pseudomonas* sp. have also been reported as metalloenzymes (Lee and Rhee, 1993; David, Sandra and Vickers, 2005; Parwata, Asyari and Hertadi, 2014; Priyanka *et al.*, 2019). No loss of activity in the presence of 4-mercaptaethanol indicated the absence of essential disulfide bonds in *P. brenneri* lipase. Non-ionic detergents are mild detergents which typically display a limited interaction with an enzyme surface; however, ionic detergents, and specifically SDS, cause protein unfolding due to non-specific binding to the enzyme surface (Mogensen, Sehgal and Otzen, 2005). These detergent differences could underpin the significant loss in activity in the presence of SDS, but minimal loss in the presence of Poly-80 and Triton X-100 (see Table 2).

3.4.4. Effect of solvents

Polar organic solvents, such as methanol or ethanol, are responsible for lipase inactivation by causing structural changes and by stripping essential water required for enzyme activity. Lipase from *Pseudomonas aeruginosa* AAU2 lost 90 % of its activity in the presence of 25 % (v/v) methanol after 24 h of incubation at 37 °C (Bose and Keharia, 2013). Similarly, *Pseudomonas* sp. DMVR46 lipase retained only 8.9 % and 30.5 % of its lipolytic activity in the presence of methanol and ethanol respectively, when a 3:1 enzyme:solvent mix was used at 37 °C within 4 h (David, Sandra and Vickers, 2005). In the current study, *P. brenneri* (H3) lipase retained >80 % of activity in the presence of 20 % (v/v) methanol after 24 h of incubation at both 28 °C and 40 °C (see Table 3). The lipase was found to be stable in 10 % (v/v) ethanol after 24h of incubation. However, increased concentrations of both methanol and ethanol generated a statistically significant loss in activity. Conversely, non-polar organic solvents with higher log P possess a reduced ability to strip the essential water from the enzyme structure. Hence, lipases tend to be more stable and active in high log P solvents (cyclohexane, *n*-hexane, *n*-heptane etc.). Enhanced lipolytic activity was observed for *P. brenneri* lipase in the presence of 50% (v/v) cyclohexane, *n*-hexane and *n*-heptane respectively. The interaction of the solvent in the region shielding the catalytic site may result in an increased catalytic activity in the presence of these solvents as the lipase is maintained in a flexible, open and active arrangement (Bose and Keharia, 2013). Similar observations have been noted for a lipase from various *Pseudomonas* sp. where increased lipolytic activity has been reported in presence of hydrophobic solvents (Parwata, Asyari and Hertadi, 2014; Priyanka *et al.*, 2019).

3.4.5. Substrate specificity

The substrate specificity of lipases provides useful information for the rapid selection of enzyme/substrate partnerships to catalyse desired reactions. A preference for long chain fatty esters is a desirable characteristic for lipases (Li *et al.*, 2014). *P. brenneri* lipase demonstrated a wide substrate specificity range (from medium length to long chain phenyl esters i.e. C8:0 to C16:0). No, or minimal, hydrolysis was seen with short esters such as C2:0; while maximum hydrolysis activity was seen towards C12:0 (*p*NP-dodecanoate; see Figure 7).

3.4.6. Enzyme Kinetics

K_m values as low as 0.037 mM, and a V_{max} as high as 188.6mmoL/L/min, using *p*-NP hydrolysis have been reported for lipases from *Pseudomonas aeruginosa* SRT 9 (Borkar *et al.*, 2009). For the *P. brenneri* lipase the V_{max} and K_m values were 5.17±0.12mmoL/min/mg and 0.37±0mM respectively. The kinetic parameters were studied using *p*NP-palmitate as the substrate at 28°C. Kinetic parameters were estimated from a Lineweaver Burk plot (see Figure 8).

3.5. Application of P. brenneri lipase in FAME generation using olive oil

As the lipase from *P. brenneri* (H3) was stable towards various organic solvents and showed specificity towards long-chain esters, it was considered suitable for biodiesel production. Methanol is widely used as the alcohol for transesterification reactions due to its widespread availability and solubility (Musa, 2016). Generally, for complete conversion of oil to methyl esters (also known as biodiesel or FAME), at least a 3-fold molar ratio of alcohol to oil is required in the reaction mixture (Bose and Keharia, 2013). A higher molar ratio of alcohol to oil is required to increase the contact between oil and alcohol and to drive the reaction equilibrium towards biodiesel synthesis. Increased alcohol to oil ratios result in enhanced biodiesel yield in a shorter time (Helwani *et al.*, 2009). Therefore, in this study, a molar ratio of 1:12 for olive oil:methanol was used for transesterification reaction. After 72h of incubation at 40°C, *P. brenneri* lipase generated FAME(s) as detected by TLC (see Figure 9), with a five-batch average yield of 85 % (±5 %). This yield is comparable to commercially available immobilised enzymes commonly used in biodiesel production; Novozym435 (>90%) and *Candida sp.* 99–125 (87%) (Tan *et al.*, 2010). The composition of the crude FAMEs synthesized was compared to the standard FAME mix (Sigma-Aldrich) using GC (Figure 10; Eder, 1995).

4. Conclusion

In the current study, a novel lipase secreted by *Pseudomonas brenneri* was identified, isolated, characterised and applied to FAME synthesis. The results of this study indicate that this lipase is may be a metalloenzyme which is thermo- and organic solvent stable and is activated in the presence of organic solvents with log P>2.0. The lipase displayed excellent operational lipolytic activity between 10°C and 60°C towards *p*-NPP as substrate. The lipase was employed in the synthesis of crude FAMEs from olive oil, in the presence of methanol, as a proof of application.

This initial work indicates the potential application of this enzyme in biodiesel synthesis using diverse oil sources, as the enzyme displayed specificity towards a wide range of esters. Additional work will be required to extend the application of this novel enzyme in biodiesel synthesis, with the possibility of enhanced biocatalysis through novel strategies for immobilisation and stabilisation (Kumar, 2019).

Acknowledgement

This research was funded by a *Fiosraigh* Scholarship (PB04146) granted by Dublin Institute of Technology/Technological University Dublin to PP.

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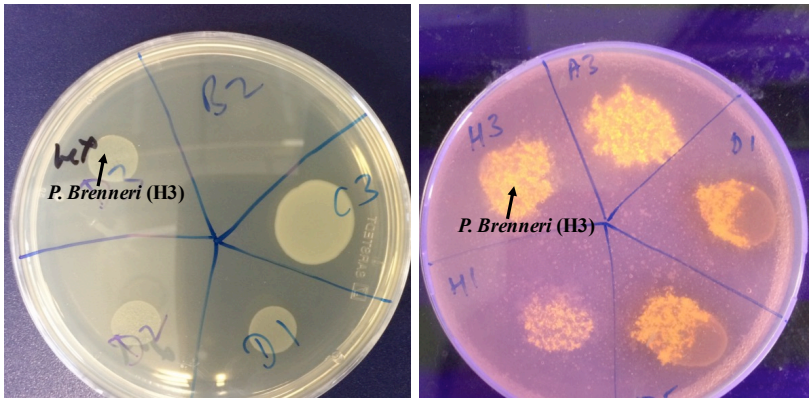

Figure 1: Plate over lay method: (a) LB agar plates treated with *n*-hexane. The presence of growth indicated stability of *P. brenneri* towards *n*-hexane. (b) Stability of extracellular crude lipase towards *n*-hexane as visualized by UV-illumination of Rhodamine B agar plates treated with *n*-hexane

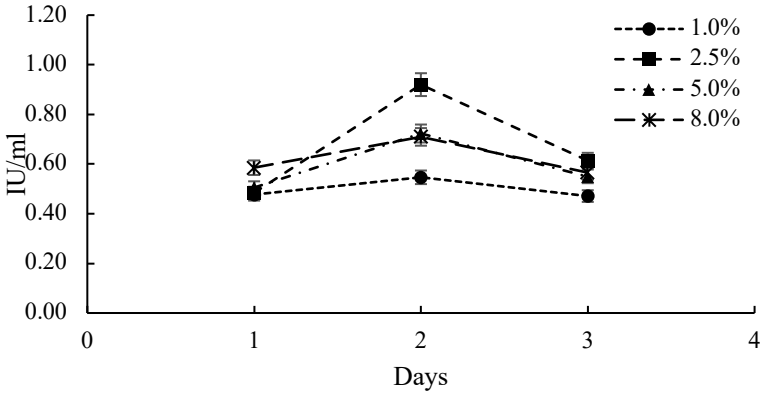

Figure 2: The effect of different percentage inoculums (1-8% v/v) on lipase production as estimated via a *p*-NPP spectrophotometry assay. This shake flask experiment was performed in basal lipase producing media at 28°C with 200rpm continuous shaking. Data represented here are the mean of three independent determinants with error bars as standard deviation.

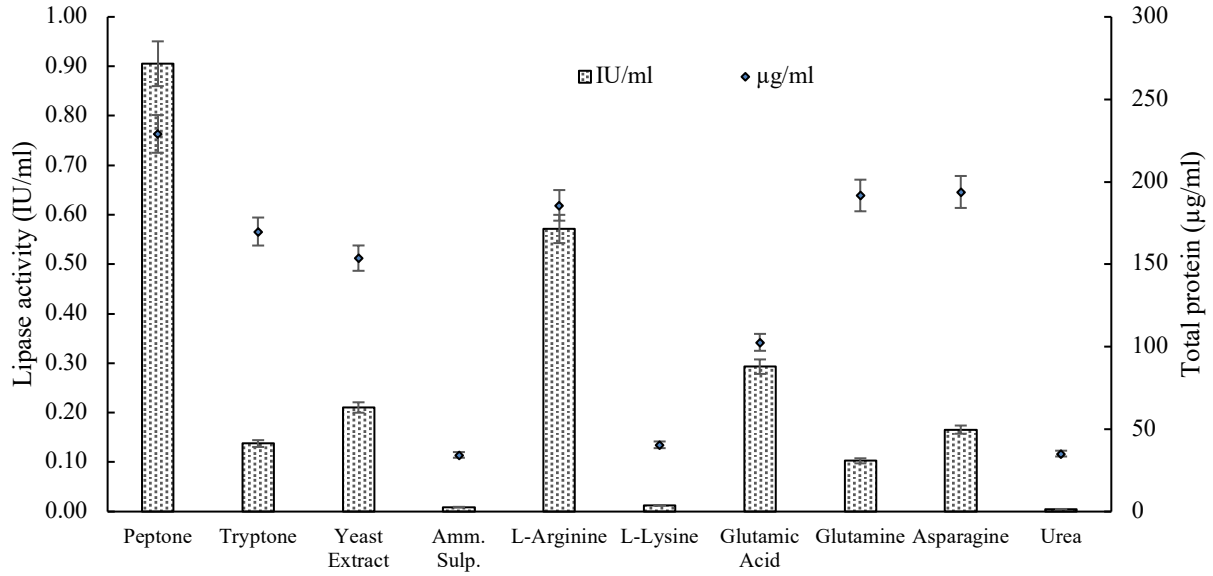

Figure 3: A small scale (250ml), shake flask, media supplementation optimization experiment was performed by replacing the nitrogen source in basal lipase producing media (without peptone) with 1% (w/v) of different nitrogen sources. 2.5% (v/v) of inoculum was used for lipase production at 28°C for 2 days under continuous stirring at 200rpm. Peptone represents the control (basal lipase producing media). Amm. sulp. represents 1% (w/v) of ammonium sulfate. Data represented here are the mean of three determinants with standard deviations as error bars.

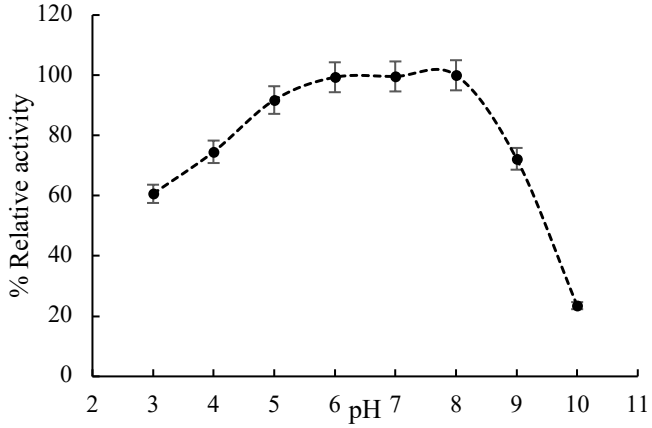

Figure 4: The relative activity of *P. brenneri* lipase was measured after 24h incubation at 28°C in the presence of different buffers (pH 3.0–10.0). The relative lipase activity was measured by the standard spectrophotometric assay. Data represented here is the mean of three independent experiments and the standard deviations are noted as error bars.

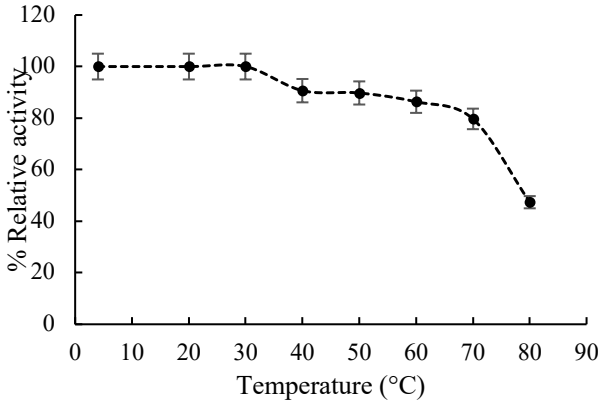

Figure 5: The thermal stability of lipase from *P. brenneri* was investigated by incubating the enzyme solution at various temperatures (4, 20, 30, 40, 50, 60, 70 and 80 °C) for 45mins. Residual activity (%) at each temperature after 45mins of incubation was calculated relative to that at 0h as 100%. Data represented here are the mean of three independent experiments and the standard deviations are noted as error bars.

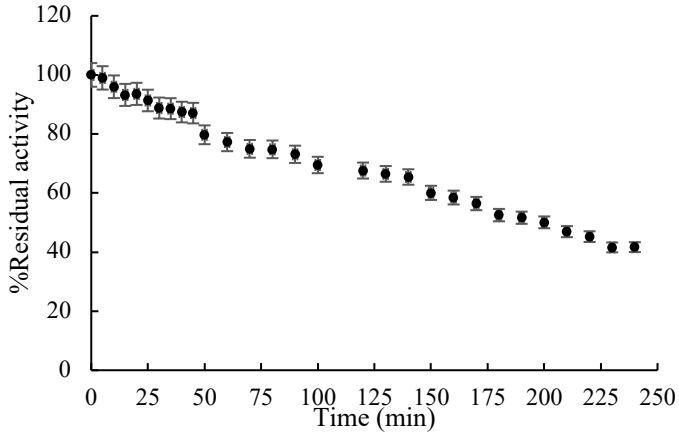

Figure 6: The thermal half-life of lipase from *P. brenneri* was estimated by incubating the enzyme solution at 60°C. Relative activity (%) at each time point was calculated considering initial activity as 100% using the spectrophotometer assay. Data represented here is the mean of three independent experiments and the standard deviations are noted as error bars.

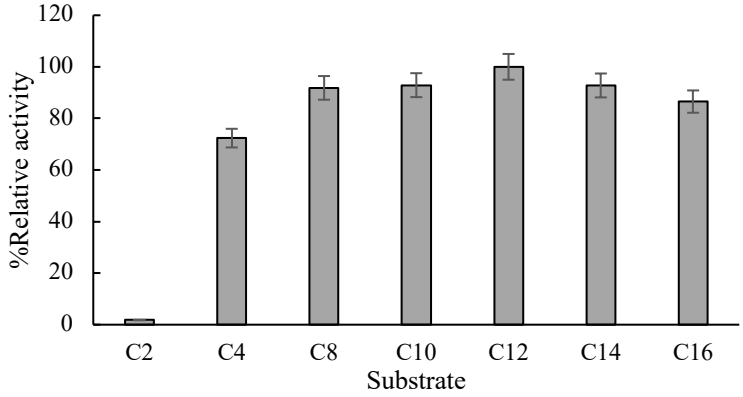

Figure 7: Substrate specificity of lipase from *P. brenneri* towards a range of *p*-NP esters of different alkyl chain length. Specificity was verified using the standard lipase activity assay. The data represented are the mean of three independent experiments and the standard deviations are noted as error bars.

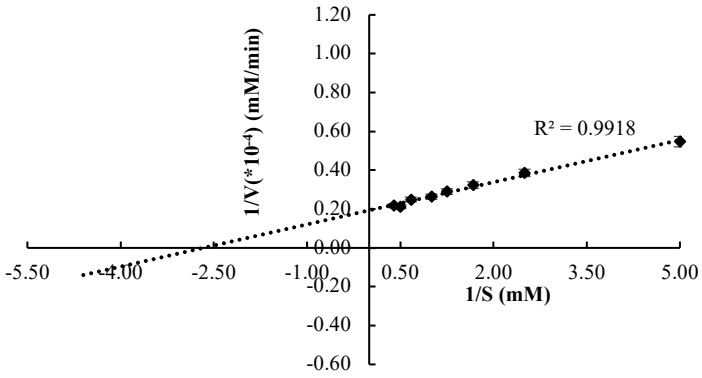

Figure 8: A Lineweaver Burk plot for *P. brenneri* lipase using *p*-NP-Palmitate as substrate, **S**, over the concentration range 0.2–2mM under standard assay conditions. The data represented are the mean of three independent experiments and the standard deviations are noted as error bars.

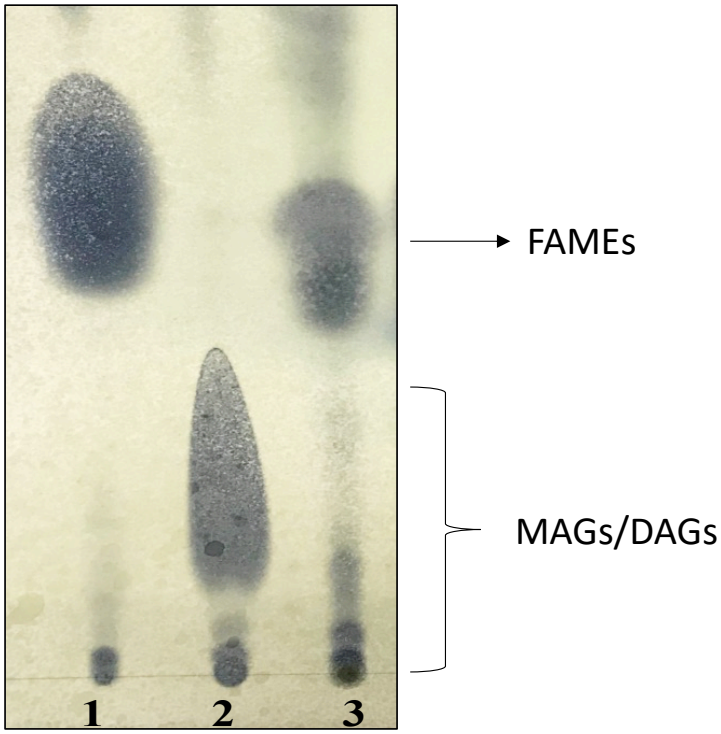

Figure 9: Thin Layer Chromatography of crude FAMEs (Fatty Acid Methyl Esters) on a silica gel plate developed using *n*-hexane:diethyl ether (90:10, v/v) and visualised using 10% (v/v) phosphomolybdic acid in ethanol. The annotated numbers are as follow; (1) FAME standard (ERMEF001), (2) olive oil (at transesterification time = 0 minutes), (3) crude FAMEs generated using lipase from *P. brenneri* (H3) after 72h of incubation at 40°C. MAGs and DAGs signify monoacylglycerols and diacylglycerols respectively.

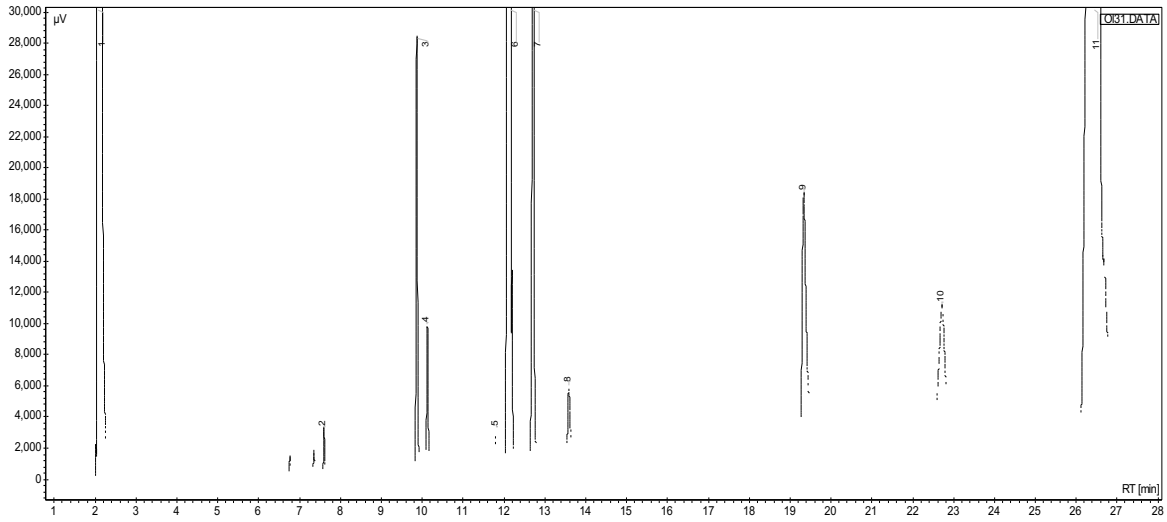

Figure 10: GC chromatogram of crude FAMEs mixture synthesized from the transesterification reaction of olive oil using lipase from *P. brenneri*. Peak 1: Solvent and Peak 6: Oleic Acid. Peak identification was carried out by comparing the Retention Time of the FAME with Retention of FAME 37-component standard FAME mix.

Table 1: Partial purification of lipase from *P. brenneri*. Anion exchanger (AEX) Q-Sepharose HP was used for purification to get an overall yield of 56.89% and 3.18IU/mg specific activity.

Purification step	**Total activity (IU)**	**Total protein (mgs)**	**Specific activity (IU/mg)**	**Purification (fold)**	**Yield (%)**
Cell free supernatant	120.9	86.1	1.40	1	100
Dialysate	114.7	58.5	1.96	1.40	94.86
Alkaline precipitation	102.8	51.2	2.01	1.43	89.57
AEX Chromatography	58.5	18.4	3.18	2.26	56.89

Table 2: The effect of various metal ions and effector molecules/chemicals (10mM) on the stability/activity of *P. brenneri* lipase was investigated and reported by the spectrophotometer assay. The residual activity (%) was calculated relative to that of enzyme solution in the absence of any additive, after 24h of incubation at 28°C. The data represented are the mean of three independent experiments and the standard deviations are noted (*P ≤ 0.05, **P ≤ 0.01, ****P < 0.0001 represents significant, very significant and extremely significant difference based on t-test)

Additive	% Residual activity
Control (no additive)	100.00 ± 0.00
$CaCl_2$	106.03 ± 2.76
$MgCl_2$	93.52 ± 2.32
KCl	103.12 ± 2.24
NaCl	99.84 ± 2.18
$CoCl_2$	91.14 ± 2.92
$MnCl_3$	94.55 ± 2.13
$FeCl_3$	6.68 ± 1.86****
$ZnSO_4$	0.00****
EDTA	0.00****
4-mercaptaethanol	103.10 ± 2.13
Poly-80	98.29 ± 2.11
Triton X-100	89.94 ± 2.75
SDS	59.70 ± 1.46**

Table 3: The effect of various organic solvents on the stability of *P. brenneri* lipase was investigated and reported by the standard lipase activity assay. Residual activity (%) was calculated relative to that of the buffered enzyme at same temperature, but with no additive after 1 h and 24 h of incubation at 28 °C and 40 °C respectively. The data represented are the mean of three independent experiments and the standard deviations are noted. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 represents significant, very significant and extremely significant difference based on t-test.

Temperature	Solvent (%v/v)	% Residual activity (1h)	% Residual activity (24h)
28°C	Control (no additive)	100.00 ± 0.00	100.00 ± 0.00
	10% Methanol	97.94 ± 2.59	98.64 ± 2.12
	20% Methanol	92.49 ± 2.45	80.02 ± 2.59*
	10% Ethanol	116.06 ± 2.38*	84.45 ± 2.33*
	20% Ethanol	93.63 ± 2.07	16.60 ± 1.99****
	50% *n*-hexane	138.38 ± 2.21***	131.02 ± 2.99**
	50% Cyclohexane	133.18 ± 2.04***	122.48 ± 2.74 *
	50% n-heptane	138.25 ± 2.66***	128.80 ± 2.71**
40°C	Control (no additive)	100.00± 0.00	100.00± 0.00
	10% Methanol	97.62 ± 2.62	102.50 ± 2.02
	20% Methanol	78.90 ± 2.65**	91.55 ± 2.40
	30% Methanol	55.35 ± 2.21***	6.10 ± 0.12****
	10% Ethanol	87.33 ± 2.89*	103.02 ± 2.78
	20% Ethanol	35.00 ± 1.40****	53.15 ± 2.22***
	50% *n*-hexane	114.81 ± 2.01*	111.78 ± 2.58*
	50% Cyclohexane	118.83 ± 2.44*	114.94 ± 2.57*
	50% *n*-Heptane	122.46 ± 2.98*	124.91 ± 2.04**

Table 4: Fatty Acid Methyl Ester composition of transesterified olive oil using lipase from *P. brenneri*. Peaks were labelled and identified with respect to the retention time of 37-component standard FAME mix.

Peak Label	Peak Identified as	Composition	Area %
2	Pentadecanoic acid	C15:0	0.23
3	*Cis*-10-heptadecenoic acid	C17:1	3.31
4	Stearic acid	C18:0	1.08
5	Linolelaidic acid	C18:2n6t	0.36
6	Linoleic acid	C18:2n6c	40.41
7	γ-linolenic acid	C18:3n6	7.83
8	Arachidic acid	C20:0	0.72
9	*Cis*-13,16-docosadienoic acid	C22:2	3.62
10	Lignoceric acid	C24:0	3.47
11	Nervonic acid	C24:1n9	38.97