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Isolation, Purification and Characterization of a Novel Solvent Stable Lipase From Pseudomonas Reinekei

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Isolation, purification and characterization of a novel solvent stable lipase from *Pseudomonas reinekei*

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Abstract

The Pseudomonas sp. have been long recognized for their exogenous lipolytic activities yet the genus still contains a lot of unexplored strains. Due to the versatile metabolic machinery and their potential for adaptation to fluctuating environmental conditions Pseudomonas sp. are of great interest for biotechnological applications. In this study, a new extracellularly produced lipolytic enzyme from Pseudomonas sp. (P. reinekei) was purified and characterized. The production of lipase from P. reinekei (H1) was enhanced 10-fold by optimizing the nitrogen source. The 50 kDa H1 lipase was purified using negative and positive mode anion exchange chromatography. The purified lipase was active over a broad pH range (5.0-9.0) and was stable for 24h at 40°C. The lipase showed significant stability, and indeed activation, in the presence of organic solvents with log P≥ 2.0. These features render this lipase of interest as a biocatalyst for applications such as biodiesel production, detergent formulations and biodegradation of oil in the environment.

Keywords: *Pseudomonas,* lipase, chromatography, solvent stability, fermentation

Introduction

Lipases also known as serine hydrolases are ubiquitous enzymes that belong to the triacylglycerol ester hydrolase family (EC 3.1.1.3). They are also termed carboxylesterases, since they can catalyse the hydrolysis (and synthesis) of long-chain triglycerides. They were first identified in pancreatic juice by Bernard in 1856 [1]. Later in 1901, their presence was observed in the bacterial genus *Bacillus* [2], and this initiated an ongoing exploration of lipase-producing microbes of which *Candida*, *Geotrichum, Rhizopus, Bacillus, Pseudomonas, Burkholderi* and *Streptomyces* are the most studied [3]. They have been widely used for synthesis of novel compounds in so called biocatalytic processes.

Enzyme catalysed water-based transformations can result in unwanted side reactions such as hydrolysis, racemization, polymerization and decomposition; and may have lower yields due to solubility of substrates/products [4]. Hence, the biocatalytic environment, from a processing and economic viewpoint, has shifted researchers' interest from aqueous to a non-aqueous environment [5]. Organic solvents are the most commonly used non-aqueous media for bio-catalysis [6]. However, enzymes may be inactivated, or denatured, in organic solvents thereby limiting their use in some cases [4]. Despite this drawback many

industrial processes such as the production of biodiesel, biopolymers, cosmetics and pharmaceuticals still employ enzymes in non-aqueous environments. Solvent stable lipases are one of the leading biocatalysts in non-aqueous environment due to their unique property of catalysing a wide variety of useful transformations. The benefits of non-aqueous biocatalysis have encouraged researchers to discover, or engineer, enzymes that are stable in non-aqueous environment. In this study, the biodiscovery, purification and characterisation of a novel solvent stable lipase from *Pseudomonas reinekei* is described. This novel enzyme will be of interest for biocatalytic applications in non-aqueous media.

Materials and methods

Chemicals and materials

Q-Sepharose high performance (HP) resin was purchased from GE Healthcare. All other chemicals were analytical grade and were purchased from Sigma-Aldrich.

Enzyme assay

Plate assay

Rhodamine B agar plates were used for the detection of lipolytic activity from microbial strains. Rhodamine B agar plates were prepared by using the Kouker and Jaeger Method [7].

Spectrophotometric assay

p-NPP (*p*-Nitrophenyl palmitate) was used as the substrate for the estimation of lipase activity as per Glogauer and colleagues [8]. Lipase activity was measured after 30mins of incubation at 28°C.

Zymogram assay

Lipolytic activity of proteins separated by Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE; non-reducing) was visualised before Coomassie Brilliant Blue staining. After non-reducing electrophoresis, gels were washed twice in 50mM Phosphate buffer saline with 1.5% v/v Triton X-100 at pH 7.0±0.2 for 30mins at room temperature and were then were treated with freshly prepared 100µM 4-Methylumbelliferyl butyrate (MUF-butyrate) for 10mins [9]. After incubation, activity bands resulting from 4-methylumbelliferyl (MUF) liberation were visualised under UV illumination.

Isolation and identification of solvent tolerant lipase producing strain:

Soil samples collected from the Wicklow mountains in Ireland from various locations were enriched in enrichment media [10] for 72hours at 28°C, 200rpm. The supernatant of the enriched samples were serially diluted (10^{-1} to 10^{-11}) with autoclaved double distilled water (ddH_2O). 100µl of each diluted sample was spread on Rhodamine B plates and incubated at 28°C for 48hours. Lipase producing colonies were aseptically picked and were sub-cultured on LB agar plates at 28°C to isolate pure colonies. The stability of lipase producing cultures in different solvents was determined by a plate-overlay method [11] against various solvents ranging from log P<0.2 to log P>2. Lipolytic strains stable in multiple solvents were 16S rRNA sequenced (Eurofins, Germany).

Lipase production

Fermentation time and inoculum percentage

1% to 15% (v/v) of an overnight grown culture in LB media was added to basal lipase production media containing 50g/L bacteriological peptone, 2 gm/L sodium chloride, 0.4gm/L magnesium sulfate, 0.5gm/L ammonium sulfate, 0.3gm/L dipotassium hydrogen phosphate, 0.03gm/L potassium hydrogen phosphate and 10g/L olive oil at pH 7.0±0.2. After every 24hrs of fermentation, cell free supernatant was analysed for lipolytic activity by the spectrophotometer assay.

Nitrogen source and percentage

1% w/v of different nitrogen sources (bacteriological peptone, tryptone, yeast extract, ammonium sulfate, *L*-Lysine and *L*-Arginine individually) were used as a substitute to 50g/L peptone in the basal lipase production media. After screening the best nitrogen source responsible for maximum lipase production was further explored a different concentration (0.25-5% w/v) to supplement the basal production media.

pH of production media

The pH of the production media, with the optimised nitrogen source and concentration, was adjusted between 5.0 (±0.2) to 9.0 (±0.2) to identify the optimum production pH.

Purification

With the optimized fermentation conditions, cell free supernatant was harvested by centrifugation at 4°C, 5000x*g* for 20mins. The supernatant was filtered through a 1.2µm pre-filter, followed by 0.45µm filter. The filtered supernatant was dialysed at 1:20 ratio in 10mM Tris-HCl buffer at pH 9.0 (±0.2) in 12kDa cut off dialysis membrane. Lipase was purified from the dialysate using two step anion exchange chromatography with Q-Sepharose High Performance resin (6 cm x 1.5 cm). The first purification step

was carried out at flow through mode; pre-equilibrated with 10mM Tris-HCl at pH 9.0 (±0.2); the second purification with bind and elute mode; pre-equilibrated with 10mM Tris-HCl pH 9.0 (±0.2) containing 250mM NaCl. The flow though from first column was collected and before using it for lipase purification from the second anion exchange chromatography 250mM of NaCl was added to it. Purified lipase was collected from second anion exchange chromatography when a step elution of 500mM NaCl was performed.

Crude and purified lipase fractions were analysed on 12% (v/v) reducing and non-reducing, SDS-PAGE. Protein bands were checked for lipolytic activity by zymogram assay and were also visualised by Coomassie Brilliant Blue staining. The relative molecular mass was calculated by comparing with the molecular weight marker (14.4kDa- 116kDa, Pierce™ Unstained Protein marker).

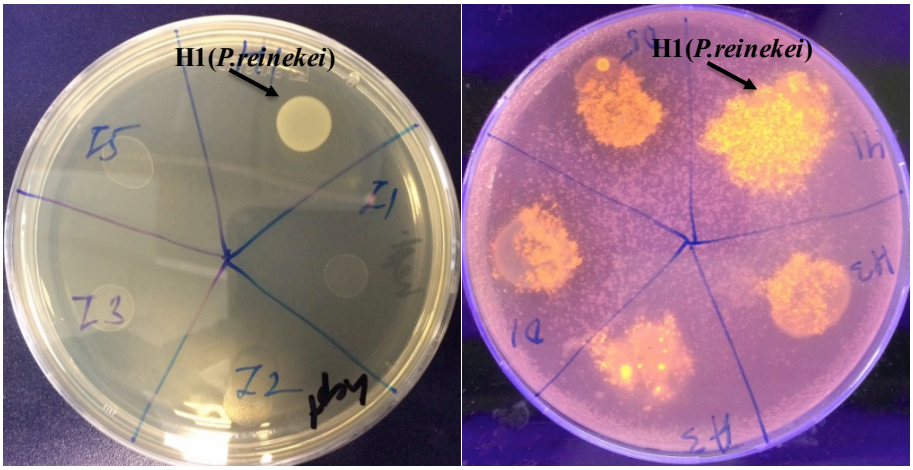
Stability Studies/Characterisation

The stability of purified lipase was monitored over pH 3.0-10.0 at 28°C using the following buffers: 50mM of Glycine-HCl (pH 3.0, 4.0), 50mM of Tris-Acetate (pH 5.0, 6.0), 50mM of Tris-HCl (pH 7.0, 8.0, 9.0) and 50mM of Borate Buffer (pH 10.0) and subsequent relative activity was expressed as percentage to highest activity. The effect of temperature at 20, 30, 40, 50, 60, 70 and 80°C was determined by pre-incubating the purified lipase solution at the relevant temperature for 1.0 hour and 24 hours and subsequent residual activity was expressed as a percentage of the activity at 4°C. The thermal half-life ($T_{1/2}$) of lipase was determined by incubating the enzyme solution at 45°C for 1.5hours and analysing for lipase activity by withdrawing samples at different intervals. Lipase stability in organic solvents was investigated by gently mixing purified lipase solution and the selected solvent in screw cap glass vials under continuous mixing at both 28°C and 40°C. Similarly, the effect of additives (metal ions, enzyme inhibitors and surfactants) on purified lipase was estimated at 28°C and 40°C. Enzyme activity is represented as a % residual activity and was measured relative to control (enzyme solution without any solvents/additives at same condition). The steady state Michaelis–Menten kinetic constants of K_m and V_{max} were determined by Lineweaver–Burk plot using the reaction rate at varying substrate concentrations (*p*NP-Palmitate) under standard assay conditions. The catalytic constant (K_{cat}) was calculated by using V_{max}, molecular weight and concentration of the enzyme. Lipolytic activity for all the characterisation trials (except substrate specificity) was estimated using spectrophotometric assay with *p*-nitrophenyl palmitate (*p*-NPP) as substrate.

Results and discussion

Isolation and identification of solvent tolerant lipase producing strain:

Two lipolytic cultures isolated from soil sample from 53°00'12.4"N 6°20'47.9"W 53.003435, -6.346639 were found to be stable in methanol, ethanol, n-hexane, heptane and cyclohexane by plate overlay method [11]. 16S rRNA sequencing of these strains identified one of the lipolytic cultures as *Pseudomonas reinekei (P. reinekei),* designated H1. The *Pseudomonas* genus demonstrates a great deal of metabolic diversity and attracts attention for industrial and environmental biocatalysis [12]. Figure 1 illustrates stability of *Pseudomonas reinekei* and its lipase respectively towards *n*-heptane by plate overlay method.



(A) (B)

Figure 1: Plate over lay method: (A) LB agar plates treated with *n*-heptane. The presence of growth indicated stability of the *P. reinekei* (H1) towards the organic solvent (*n*-heptane in the figure). (B) Stability of extracellular crude lipase towards *n*-heptane visualized by UV-illumination of Rhodamine B agar plates treated with *n*-heptane.

Lipase production

Production of lipases is known to be affected by media composition as well as factors like fermentation time, initial inoculum level, nitrogen source, pH, temperature etc. [13] and these were investigated following a one-factor at a time optimisation approach [14].

Fermentation time and Inoculum percentage

The percentage of inoculum (i.e. the initial cell count) during the fermentation process plays an important role in lipase production. The finite volume of a culture medium results in limited nutrients and the rate of nutrient consumption is dependent on bacteria cell population/growth stage [15]. Maximum lipase activity for *P. reinekei* (H1) was obtained with 15% (v/v) inoculum after 6 days of fermentation (Figure 2). The onset of lipase production is organism-specific but, in general, lipases are released during late logarithmic or stationary phase of growth [16]. Cultivation periods from 5.0 hours to 168 hours have been reported as optimal for different lipase producing organisms. Lipases from *Serratia marcesecens* [17] and

Pseudoalteromonas sp. WP27 [18] were shown to be produced to the highest level after 6 and 14 days of fermentation respectively.

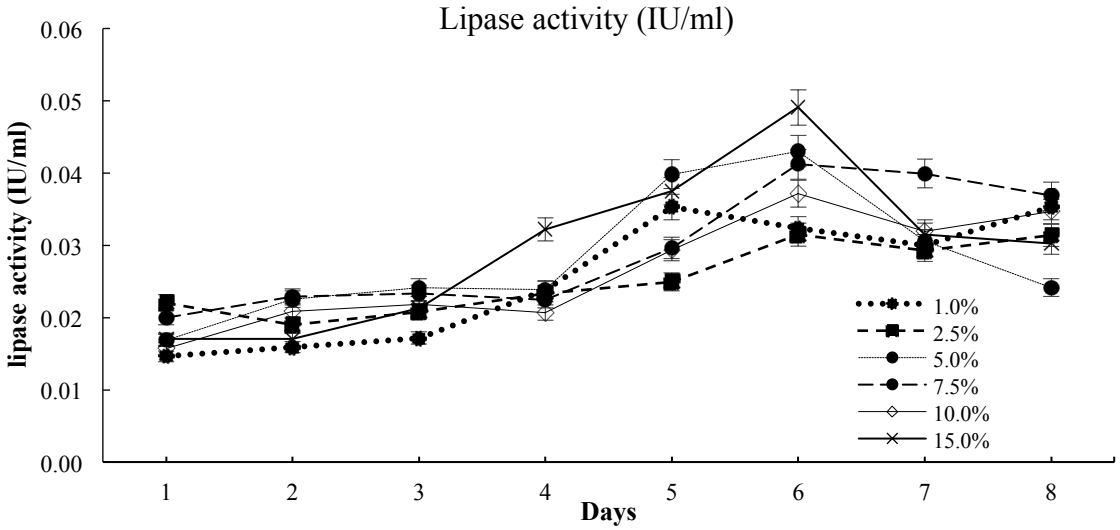


Figure 2: The effect of percentage inoculums (1-15% v/v) on lipase production as estimated via spectrophotometric activity assay. This shake flask experiment was performed in basal lipase production media at 28°C with 200rpm continuous shaking over a period of 8 days. Data represented here are the mean of three independent determinants with error bars as standard deviation.

Nitrogen source

For the lipase from *P. reinekei* (H1), a 1% (w/v, or 68mM) *L*-Lysine supplement resulted in a significant ($P \leq 0.05$, t-test) increase in lipase production to 0.46±0.023 IU/mL and was the best nitrogen source of all the nitrogen sources examined (Figure 3). Both organic and inorganic nitrogen sources have traditionally been used for lipase production. Media supplementation with specific amino acids; such as alanine, glycine, lysine and serine, have previously been shown to stimulate lipase production in *Streptococcus faecalis* [19]. For example, tryptone, combined with Lysine, was the most effective inducer for lipase production in *Pseudomonas fluorescens* [20]. Similarly, lipase production was enhanced by the presence of arginine, lysine, aspartic acid and glutamic acid for *Pseudomonas fragi* [21].

Increased *L*-Lysine concentrations (above 1%, w/v) resulted in a decrease in lipase production. There was no significant difference (log $P>0.05$, t-test) in lipase concentration for 0.25%, 0.5% and 1% (w/v) *L*-Lysine (Figure 4).

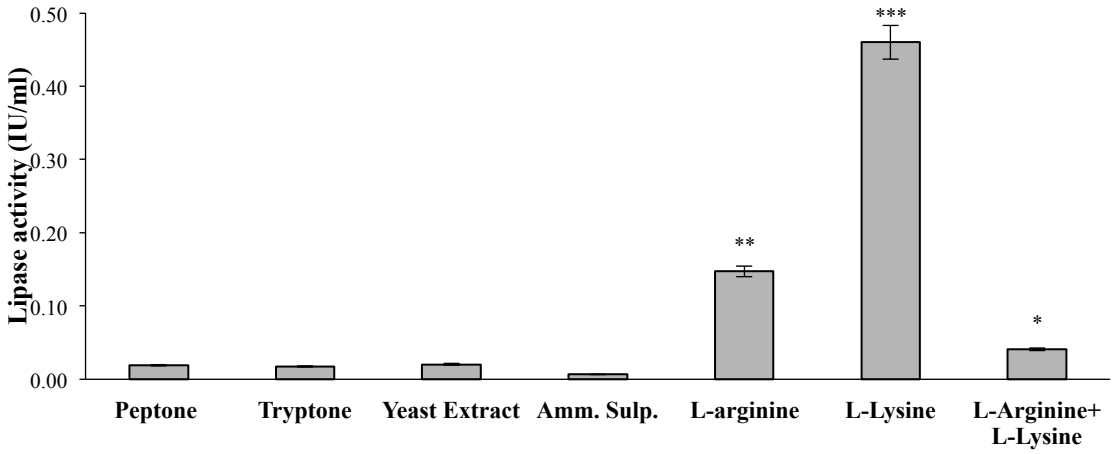


Figure 3: Influence of Nitrogen supplementation on Lipase production. Media supplementation optimization was performed by replacing the nitrogen source in basal lipase production media (without peptone) with 1% (w/v) of different nitrogen sources. A 15% (v/v) of inoculum was used for lipase production (28°C, for 6 days under continuous shaking at 200rpm). Amm sulp. represents 1% (w/v) of ammonium sulfate; while *L*-Arginine & *L*-Lysine represent lipase producing media containing 1% (w/v) of both *L*-Arginine and *L*-Lysine The data represented here are the mean of three independent experiments with standard deviations shown as error bars (* P≤0.05, **P≤0.01, ***P≤0.001 represents significant, very significant and extremely significant difference based on t-test)

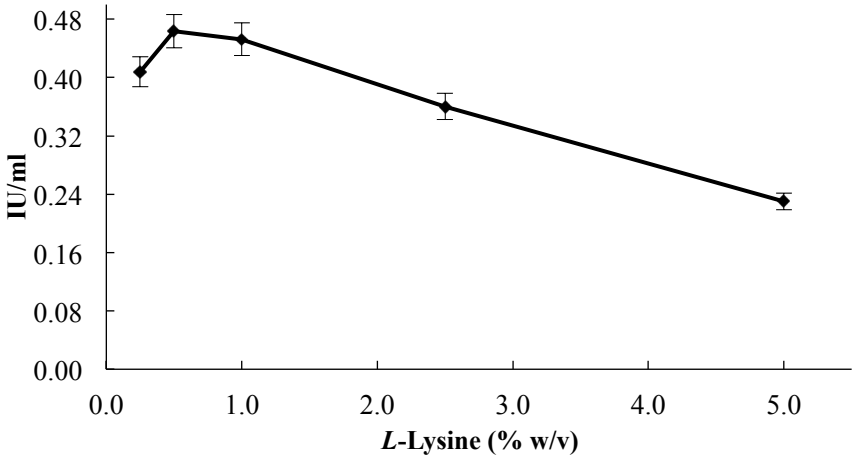
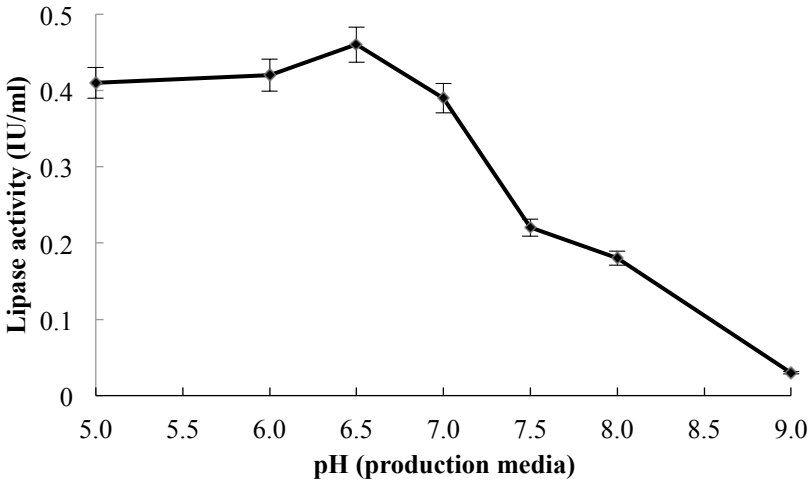


Figure 4: Influence of level of Lysine supplementation on Lipase production. Media supplementation optimization experiment was performed with different % (w/v) of *L*-lysine in the production media (basal media without peptone). 15% (v/v) of inoculum was used for lipase production (28°C, for 6 days under continuous shaking at 200rpm). No statistically significant difference was observed in lipase activity at 0.5% (w/v) and 1% (w/v) of L-Lysine. The data represented here are the mean of three independent experiments with standard deviations noted as error bars.

Influence of media pH

pH plays a significant role in enzyme stability through maintaining an enzyme's three-dimensional structure required for its biological activity [22]. Enzymes remain metabolically active at a favourable pH range during fermentation. The maximum lipase production (0.46±0.02 IU/mL) was achieved when the initial pH of the production media was 6.5 (±0.2). Above pH 7.0 (±0.2), there was a significant reduction

188 (P≤0.05, t-test) in lipase production (Figure 5). A pH 7.0 (±0.2) was found to be optimum for lipase
189 production in *Pseudomonas gessardii* [23], *P. fluorescens* [24] and *P. aeruginosa* [25]. In comparison,
190 *Pseudomonas putida* 922 produced maximum lipase after 48 hours of incubation in a production media at
191 pH 10 [26].



192

193 Figure 5: Influence of initial media pH on lipase production. Optimization was achieved by adjusting the pH of
194 lipase producing media containing 1% (w/v) of *L*-Lysine. A 15% (v/v) inoculum was used for lipase production
195 (28°C, for 6 days under continuous shaking at 200rpm). The data represented here are the mean of three independent
196 experiments, with standard deviations noted as error bars.

197

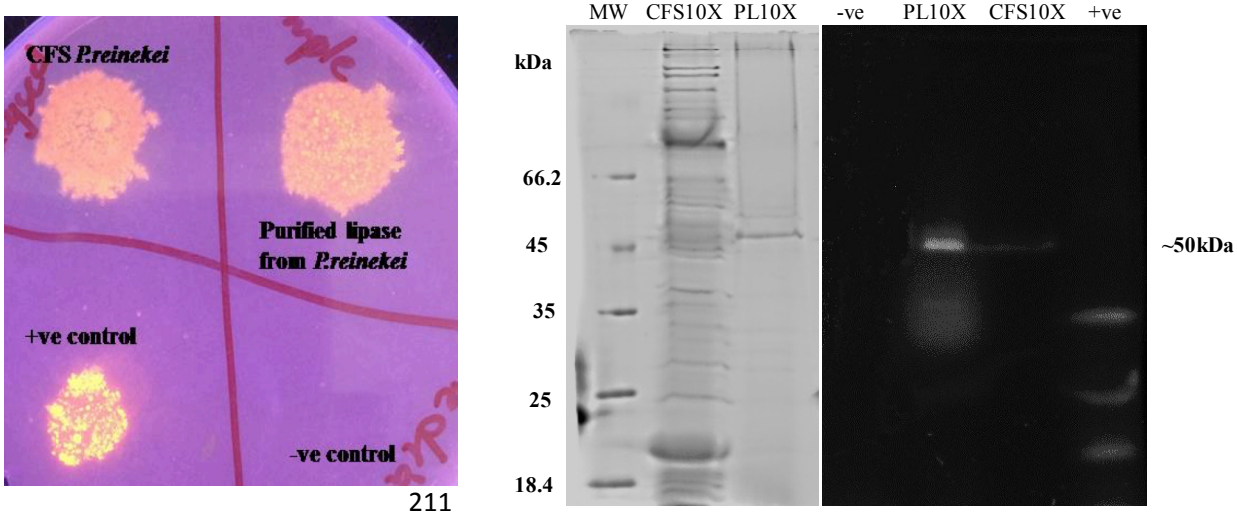
198 *Purification of P. reinekei Lipase*

199 The isolation of the lipase from the optimized fermentation parameters was achieved by a two-step procedure
200 (Table 1). The first purification column; an anion exchange Q-Sepharose HP (negative mode chromatography)
201 removed contaminant proteins from the lipase preparation. The second chromatography step (positive mode
202 chromatography; bind and elute), an anion exchange on Q-Sepharose HP resulted in isolation of 50 kDa
203 lipase (Figure 6).

204

Table 1: The purification of lipase from *P. reinekei* (H1) by two-step purification. Anion exchanger Q-Sepharose HP was used as negative (chromatography 1) and positive (chromatography 2) mode of purification to achieve an overall yield of 13.72%, 4.23IU/mg specific activity and a 4.65fold purification.

Purification step	Total activity (IU)	Total protein (mgs)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Cell free supernatant	75.1	82.48	0.91	1	100
Dialysate	64.2	82.06	0.79	0.98	97.13
Chromatography 1	49.4	47.60	1.04	1.14	67.66
Chromatography 2	6.8	1.60	4.23	4.65	13.72



(A) (B) (C)

Figure 6: (A) Rhodamine B agar plate representing the presence of lipolytic activity in purified lipase. CFS: Cell free supernatant from *P.reinekei*, +ve control: *M.meihei* lipase, -ve control: Bovine serum albumin. (B) 12% (v/v) non-reducing SDS-PAGE gel Stained with Coomassie Brilliant Blue. Lane 1: molecular weight marker, lane 2: Cell Free Supernatant; lane 3: Purified Lipase (C) Zymogram of 12% (v/v) non-reducing SDS-PAGE. Lane 1: Negative control (Bovine Serum Albumin); Lane 2: Cell Free Supernatant; Lane 3: Purified Lipase; Lane 4: lipase from *M.meihei* as positive control. The estimated size of purified lipase was ~50kDa.

Previous studies have shown sorbitol and other polyols to effectively reduce or inhibit aggregation of IgG solutions [27]. Therefore, to enhance the stability of lipase and to avoid precipitation due to aggregation 5% (w/v) sorbitol was added to purified lipase sample with no effect on the characteristics of the purified lipase.

Characterisation of P. reinekei lipase

pH stability

Significant loss of activity was seen at pH 3.0 (±0.2), 4.0 (±0.2) and pH 10 (±0.2); conversely >90% relative activity was observed between pH 5.0 (±0.2) to 8.0 (±0.2) (Figure 7). Generally, *Pseudomonas* lipases have neutral or alkaline pH optima [28], however *P. gessardii* lipase had an acidic optimum at pH 5.0 and was found to be active even at pH 2.0 [29]. Given that the purified from *P. reinekei* (H1) was stable from pH 5.0-9.0 it could prove advantageous in application areas such as detergents, leather tanning and fine chemicals manufacture [30]. Furthermore, an optimal pH of 5.0 makes this lipase ideal for oleochemical and food industries, as well as for the hydrolysis or modification of triacylglycerols to improve nutritional properties of food [31].

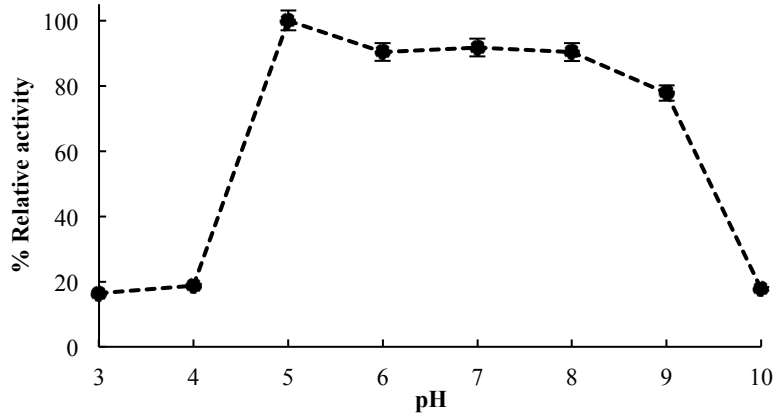


Figure 7: The relative activity of purified *P. reinekei* (H1) lipase was measured after 24hours incubation at 28°C in the presence of different pH buffers (pH 3.0-10.0). The relative lipase activity was measured by spectrophotometric assay. The data represent the mean of three independent experiments and the standard deviations are noted as error bars.

Thermostability

Optimum temperature for lipolytic activity of purified lipase from *P.reinekei* using p-NPP spectrophotometer assay was 35°C (Figure 8). Purified lipase from *P. reinekei* lost 70% of its initial activity at temperatures greater than 50°C within one hour of incubation (Figure 9). However, the lipase retained 90% of activity at 40°C after 24hours. Lipases from *Pseudomonas* species have broad temperature optima from 4°C to 90°C. Lipase from *Pseudomonas* sp. PF 16 had an optimum temperature of 4°C [32]; while lipase from *Pseudomonas* sp. AG-8 showed optimum activity at 45°C [33]. The inter-connection between the habitat of

micro-organism isolated and the enzyme properties [34] could be a possible reason for explaining the lower thermostability of *P. reinekei* (H1) lipase. As the soil sample for H1 isolation was from temperate environmental conditions, lower thermostability of enzymes was expected. However, thermostability is a desirable characteristic for enzymes used in applications at high temperatures; the same can be achieved by protein engineering such as physical immobilization, chemical modification and crosslinking [35].

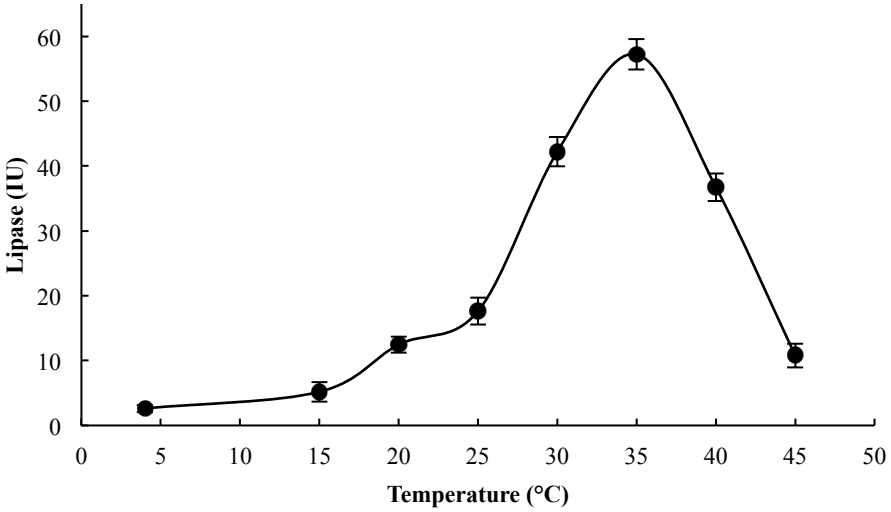


Figure 8: The optimum temperature for lipolytic activity of purified lipase was investigated by incubating the enzyme-substrate solution at various temperatures (4, 15, 20, 25, 30, 35, 40 and 45°C) for 30mins. Activity of lipase (IU) was calculated. The data represented are the mean of three independent experiments and the standard deviations are noted as error bars.

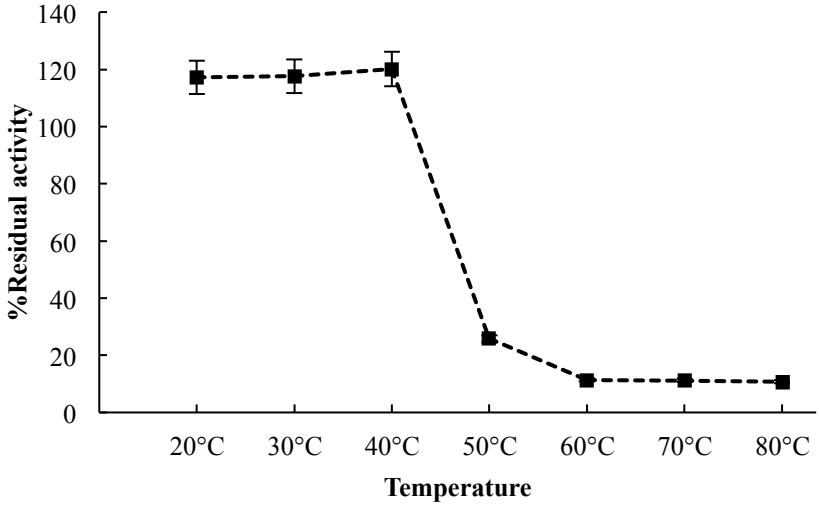


Figure 9: The thermal stability of purified lipase was investigated by incubating the enzyme solution at various temperatures (20, 30, 40, 50, 60, 70 and 80°C) for 1hour. Residual activity (%) at each temperature and time point was calculated relative to that at 0 hour, as 100%. The data represented are the mean of three independent experiments and the standard deviations are noted as error bars.

By first order thermal deactivation, half-life of purified lipase at 45°C (Figure 10) was calculated as 89mins according to eq. (1) and eq. (2) [36].

$$ln\, A_t = ln\, A_0 - k_d\, t \qquad (1)$$

$$T_{1/2} = \frac{\ln 0.5}{-k_d} \qquad (2)$$

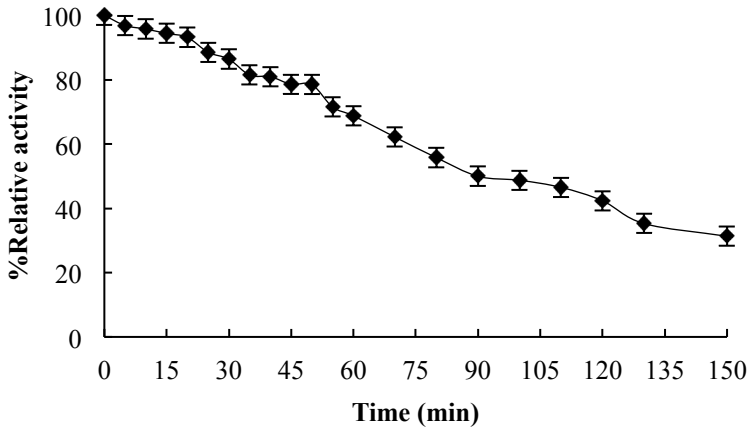


Figure 10: The thermal half-life of purified lipase was calculated by incubating the enzyme solution at 45°C. Relative activity (%) at each time point was calculated considering initial activity as 100% using the spectrophotometer assay. The data represented are the mean of three independent experiments and the standard deviations are noted as error bars.

Influence of Metal ions and chemical reagents

The presence of Ca^{2+} and Mg^{2+} ions has been reported to enhance the hydrolytic activity of lipases [37,38]. However, the activity of *P. reinekei* (H1) lipase was unaffected ($P \geq 0.05$, t-test) by the presence of Ca^{2+}, Mg^{2+}, K^{+}, Na^{+} ions irrespective of the incubation temperature. *P. reinekei* (H1) lipase lost activity in the presence of EDTA, suggesting this it may be a metalloenzyme and EDTA chelated metal ions required for its activity. Lipases from *Pseudomonas putida* 3SK, *Pseudomonas stutzeri,* and *Pseudomonas* sp. DMVR46 were also found to be metalloenzymes [12,39,40]. Significant loss in lipolytic activity ($P \leq 0.05$, t-test) for *P. reinekei* (H1) lipase was observed in presence of urea. Urea molecules interrupt the intra-chain hydrogen bonds in an enzyme and can cause direct denaturation [41]. However, lipase from *Pseudomonas* sp. *AG-8* [33] and *Pseudomonas* sp. 42A2 [42] have been shown to be stable in 6.0 M urea. Non-ionic detergents do not interact extensively with the protein surface and are therefore considered mild. Ionic detergents on the other hand, in-particular SDS, bind non-specifically to the enzyme surface, leading to protein unfolding [43]. Interestingly,

in the presence of 1mM (0.028% w/v) SDS, an enhanced activity of the *P. reineki* (H1) lipase was observed (Table 2). Enhanced activity at 28°C, coupled with no significant loss in activity at 40°C, could be explained by the concentration of SDS. Below the Critical Micelle Concentration CMC (8.2mM or 0.24% w/v at 25°C) SDS binds to the lid of lipase and activates it by conformational changes and the enzyme requires less interfacial activation [44]. Also, detergents may also alter the hydrophobicity of the enzyme and, therefore, the availability of substrate to the enzyme [45]**.** The absence of disulphide bonds in the novel *P. reinekei* lipase was confirmed as no activity loss was noted after incubation in β-Mercaptoethanol. A similar observation was seen with lipases from *Streptomyces bambergiensis* OC 25-4 [46] and *Pseudomonas aeruginosa* BN-1 [47]. Stability in surfactants like Tween, Triton X is desirable for lipases for their potential application in detergent formulations. The lipase from *P. reinekei* (H1) exhibited good stability towards surfactant and detergents, which enhances its' novel properties and extends its potential application range. The effect of detergents on this enzyme correlates with their hydrophilic/lipophilic balance (HLB), which is defined the detergent distribution between polar and non-polar phases [48]. Thus, non-ionic surfactants with low HLB value (Triton X-100: HLB 13.5; and Tween 80: HLB 15) are less detrimental on activity of lipase in comparison to SDS with a higher HLB of 40.

Table 2: The effect of various metal ions and effector molecules/chemicals (1mM) on the stability/activity of *P. reinekei* (H1) lipase was investigated and reported by the spectrophotometer assay. The residual activity (%) was calculated relative to that of enzyme solution at same temperature but in the absence of any additive, after 24 hours of incubation at 28°C and 40°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)

Substances	Residual activity ± SD (28°C)	Residual activity ± SD (40°C)
Control	100	100
Ca^{2+}	87.25±3.36**	98.88±4.94
Mg^{2+}	95.06±3.75	105.00±4.25
K^{+}	95.70±3.78	106.76±4.34
Na^{+}	100.48±4.02	90.87±4.54*
EDTA	26.36±1.32****	11.60±1.58****
β-Mercaptoethanol	122.73±5.14**	114.98±5.75*
Polysorbate 80	98.60±3.93	103.28±5.16
Triton X-100	100.48±4.02	90.87±4.54*
SDS	110.48±4.52*	88.24±5.41**

Effect of Organic solvents

The application of lipases for bioconversions in an organic solvent system is advantageous from a biotechnological viewpoint. Activity and stability in solvents are considered critical attributes in a lipase. *P.*

reinekei (H1) lipase showed significant stability in 20% (v/v) methanol and ethanol after 24hours of incubation (Table 3). Few lipases have been reported as stabilized/activated, in hydrophilic solvents; for example, Antarctic *Pseudomonas* lipase lost only 10% of its activity in presence of 25% (v/v) methanol, while showed 101.9% activity in 25% (v/v) ethanol [49]. The activation of lipase in the presence of some hydrophilic organic solvents can be explained by the interactions of certain amino acid residues with the organic solvent, changing the lipase conformation from the closed to the open form, thereby enhancing lipase activity [50]. Alternatively, some lipases are known to be able to maintain an essential hydration layer, due to the presence of surface polar/charged amino acid residues, which interact strongly with water molecules [40]. Hydrophobic organic solvents with higher log P (for example, cyclohexane, *n*-hexane, *n*-heptane) possess a reduced ability to strip essential water molecules from the enzyme surface than hydrophilic solvents (low log P). Enhanced lipolytic activity was observed for *P. reinekei* (H1) lipase in the presence of hydrophobic solvents (cyclohexane, *n*-hexane and *n*-heptane). The activation in lipolytic activity of *P. reinekei* lipase by hydrophobic solvents may be due to the interaction of solvent with hydrophobic amino acid residues present in the lid/flap covering the catalytic site of the enzyme, thereby keeping the enzyme in a flexible open conformation and consequently increasing its activity [3]. Similar observations have been noted for a lipase from *Pseudomonas stutzeri*, where the activity increased to 111% when it was incubated in 50% (v/v) *n*-hexane at 37°C for 30min [40].

Table 3: The effect of various organic solvents on the stability of *P. reinekei* (H1) lipase was investigated and reported by the spectrophotometer assay. Residual activity (%) was calculated relative to that of enzyme solution at same temperature but no additive after 1hour and 24hours of incubation at 28°C and 40°C. The data represented are the mean of three independent experiments and the standard deviations are noted (* P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 represents significant, very significant and extremely significant difference based on t-test).

Solvent	**%(v/v)**	**% Residual activity at 28°C after 1hour**	**% Residual activity at 40°C after 1hour**	**% Residual activity at 28°C after 24hours**
Methanol	10	96.24±4.81	97.19±4.81	92.71±4.63
	20	90.19±4.51	69.02±3.45***	97.86±4.80
Ethanol	10	97.57±4.81	82.86±4.12*	92.27±4.61
	20	87.90±4.39*	37.77±2.19****	92.07±4.60
Cyclohexane	50	243.64±5.81****	198.14±7.81****	193.28±6.88****
Hexane	50	138.72±7.81****	197.34±8.82****	275.95±7.56****
Heptane	50	181.14±9.05****	192.66±9.13****	324.37±4.81****

Enzyme Kinetics

The kinetics of the purified lipase from *P. reinekei* was studied using *p*NP-palmitate as the substrate of choice at 28°C. A Lineweaver Burk plot (Figure 11) was used to calculate the kinetic parameters V_{max}, K_m

and K_{cat} and were estimated to be 3.41±0.17mmol/min/mg, 0.48±0.02mM and 2601.66 respectively. Low K_m of *P. reinekei* lipase indicates a high affinity of this enzyme towards *p*NP-Palmitate. In this study the high K_{cat}, coupled with low K_m, values for *P. reinekei* (H1) lipase are beneficial both from economical and application perspective.

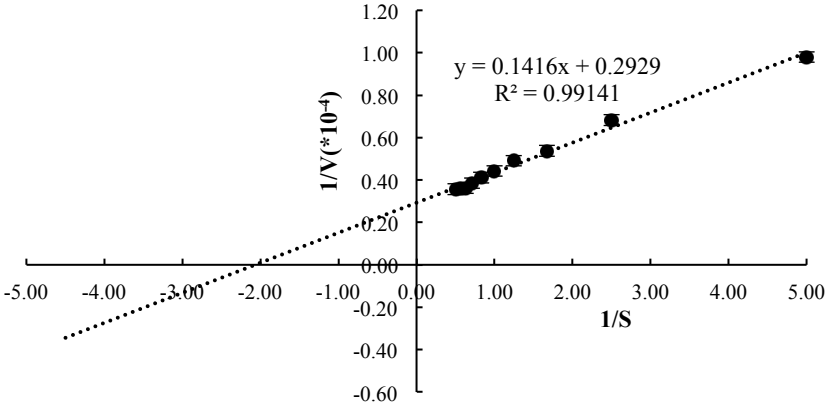


Figure 11: A Lineweaver Burk plot for the purified *P. reinekei* (H1) lipase using *p*NP-Palmitate as substrate over the substrate concentration range 0.2-2mM under standard assay conditions. The data represented are the mean of three independent experiments and the standard deviations are noted as error bars.

Substrate Specificity

Lipase from *P. reinekei* (H1) showed maximum catalytic efficiency for short chain ($C_{8:0}$) Phenyl ester (*p*NP-Octanoate). The catalytic efficiency reduced with increased chain length from $C_{10:0}$ to $C_{16:0}$, with no, or minimal, catalytic activity observed for short chain esters ($C_{2:0}$, $C_{4:0}$; Figure 12).

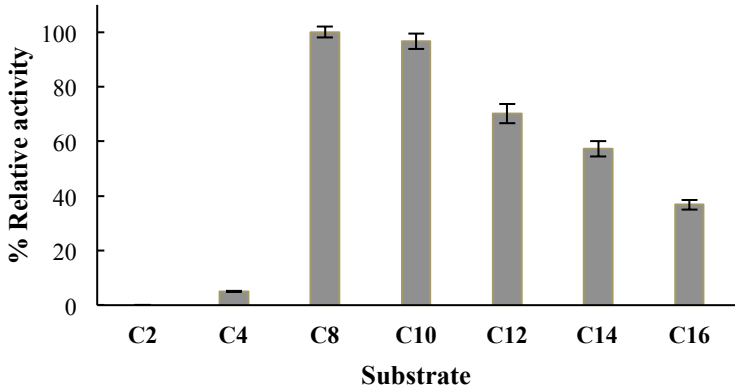


Figure 12: Substrate specificity of purified lipase from *P. reinekei* towards a range of *p*-NP esters. Specificity was checked using standard assay conditions reported via the spectrophotometer assay. The data represented are the mean of three independent experiments and the standard deviations are noted as error bars.

Amino acid sequence identification

Mass spectrophotometer analysis of the purified lipase from *P. reinekei* revealed four conserved peptide sequences: AGYTTAQVEVLGK; LLEIGIGFR; VLNIGYENDPVFR; ANTWVQDLNR. Internal sequencing primers were designed to bind where these *P. reinekei* lipases peptide sequence fragments most closely aligned the lipase from *P. fluorescens, Pseudomonas sp.* PMAC 25886, *P. yamanorum,* LipAMS8 from *Pseudomonas* sp. AMS8 and *Pseudomonas* sp. PAMC25886 (Figure 13). Examination of phylogenetic tree of the amino acid sequences by BlastP in NCBI revealed the location of lipase from *P. reinekei* to other lipases of *Pseudomonas* species (Figure 14).

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P. reinekei: H1     ------------------------------------------------------------      0
P. fluorescens      MGVFDYKNLGTEGSKALFADAMAITLYSYHNLDNGFAVGYQHNGFGLGLPATLVGALLGS      60
PAMC 25886          MGVFDYKNLGTEGSKALFADALAISLYSYHNLDNGFAVGYQHNGFGLGLPATLVGALLGS      60
P. yamanorum        MGVFDYKNLGTEGSKALFADAMAITLYSYHNLDNGFAVGYQHNGFGLGLPATLVGALLGS      60
LipAMS8             MGVFDYKNLGTEGSKALFADAMAITLYSYHNLDNGFAVGYQHNGFGLGLPATLVGALLGS      60
PTA-122608          MGVFDYKNLGTEGSKALFADAMAITLYSYHNLDNGFAVGYQHNGFGLGLPATLVGALLGS      60

P. reinekei: H1     ------------------------------------------------------AGYTTAQ      120
P. fluorescens      TDSQGVIPGIPWNPDSEKAALDAVHKAGWTPISASTLGYGGKVDARGTFFGEKAGYTTAQ      120
PAMC 25886          TDSQGVIPGIPWNPDSEKAALEAVNNAGWTPISASTLGYGGKVDARGTFFGEKAGYTTAQ      120
P. yamanorum        TDSQGVIPGIPWNPDSEKAALDAVNKAGWTPISASTLGYGGKVDARGTFFGEKAGYTTAQ      120
LipAMS8             TDSQGVIPGIPWNPDSEKAALEAVNKAGWTPISASTLGYGGKVDARGTFFGEKAGYTTAQ      120
PTA-122608          TDSQGVIPGIPWNPDSEKAALEAVNKAGWTPISASTLGYGGKVDARGTFFGEKAGYTTAQ      120
                                                                       *******

P. reinekei: H1     VEVLGK------LLEIGIGFR-----------------------------------------      180
P. fluorescens      VEVLGKYDGDGKLLEIGIGFRGTSGPRETLISDSIGDLVSDLLAALGPKDYAKNYAGEAF      180
PAMC 25886          VEVLGKYDGAGKLLEIGIGFRGTSGPRETLISDSIGDLVSDLLAALGPKDYAKNYAGEAF      180
P. yamanorum        VEVLGKYDGAGKLLEIGIGFRGTSGPRETLITDSIGDLVSDLLAALGPKDYAKNYAGEAF      180
LipAMS8             VEVLGKYDGDGKLLEIGIGFRGTSGPRETLITDSIGDLVSDLLAALGPKDYAKNYAGEAF      180
PTA-122608          VEVLGKYDGDGKLLEIGIGFRGTSGPRETLITDSIGDLVSDLLAALGPKDYAKNYAGEAF      180
                    ******      *********

P. reinekei: H1     ---------------------------------SMADLSGNKWSGFYKDSNYVAYASPT      240
P. fluorescens      GTLLKDVAAYAGSHGLTGKDVVVSGHSLGGLAVNSMADLSGNKWSGFYKDSNYVAYASPT      240
PAMC 25886          GTLLKDVAAYAGSHGLTGKDVVVSGHSLGGLAVNSMADLSGNKWSGFYKDSNYVAYASPT      240
P. yamanorum        GTLLKDVAAYAGSHGLTGKDVVVSGHSLGGLAVNSMADLSGNKWSGFYKDSNYVAYASPT      240
LipAMS8             GTLLKDVAAYAGSHGLTGKDVVVSGHSLGGLAVNSMADLSGNKWSGFYKDSNYVAYASPT      240
PTA-122608          GTLLKDVAAYAGSHGLTGKDVVVSGHSLGGLAVNSMADLSGNKWSGFYKDSNYVAYASPT      240
                                                    **************************

P. reinekei: H1     QSSAT-VLNIGYENDPVFR-------------------------------------WNVL      300
P. fluorescens      QSAGDKVLNIGYENDPVFRALDGSSFNFSSLGVHDKPHESTTDNIVSFNDHYASTLWNVL      300
PAMC 25886          QSSGDKVLNIGYENDPVFRALDGSSFNFSSLGVHDKPHESTTDNIVNFNDHYASTLWNVL      300
P. yamanorum        QSSGDKVLNIGYENDPVFRALDGSSFNFSSLGVHDKPHESTTDNIVNFNDHYASTLWNVL      300
LipAMS8             QSSGDKVLNIGYENDPVFRALDGSSFNFSSLGVHDKPHESTTDNIVSFNDHYASTLWNVL      300
PTA-122608          QSSGDKVLNIGYENDPVFRALDGSSFNFSSLGVHDKPHESTTDNIVSFNDHYASTLWNVL      300
                    **.   *************                                        ****

P. reinekei: H1     PFSIVNVPTWLSHLPTAYGDGLTRVLDSKFYDLTSRDS------------ANTWVQDLNR      360
P. fluorescens      PFSIVNVPTWISHLPTAYGDGLTRVLDSQFYDLTSRDSTIIVANLSDPARANTWVQDLNR      360
PAMC 25886          PFSIVNVPTWLSHLPTAYGDGLTRVLDSTFYDLTSRDSTIIVANLSDPARANTWVQDLNR      360
P. yamanorum        PFSIVNVPTWLSHLPTAYGDGLTRVLDSKFYDLTSRDSTIIVANLSDPARANTWVQDLNR      360
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AMS8                PFSIVNVPTWLSHLPTGYGDGLTRVLDSKFYDLTSRDSTIIVANLSDPARANTWVQDLNR      360
PTA-122608          PFSIVNVPTWLSHLPTGYGDGLTRVLDSKFYDLTSRDSTIIVANLSDPARANTWVQDLNR      360
                    **********.**********.*********           **********

P. reinekei: H1     ------------------------------------------------------------      420
P. fluorescens      NAEPHKGNTFIIGSDGNDLIQGGKGVDFIEGGKGNDTIRDNSGHNTFLFGGQFGQDRVIG      420
PAMC 25886          NAEPHKGNTFIIGSEGDDLIQGGKGVDFIEGGKGNDTIRDNSGHNTFLFGGQFGQDRVVG      420
P. yamanorum        NAEPHKGNTFIIGSDGDDLIQGGKGVDFIEGGKGNDTIRDNSGHNTFLFGGQFGQDRVIG      420
AMS8                NAEPHKGNTFIIGSDGNDLIQGGKGVDFIEGGKGNDTIRDNSGHNTFLFGGQFGQDRVIG      420
PTA-122608          NAEPHKGNTFIIGSDGNDLIQGGKGVDFIEGGKGNDTIRDNSGHNTFLFGGQFGQDRVIG      420

P. reinekei: H1     ------------------------------------------------------------ 476
P. fluorescens      YQPTDKLVFRDVEGSADWRDHAKVVGSDTVLSFGADSVTLVGVGLAGVWGDGISIS  476
PAMC 25886          YQPTDKLVFRDVEGSADWRDHAKVVGGDTVLSFGADSVTLVGVGLAGVWGDGISIS  476
P. yamanorum        YQPTDKLVFRDVEGSADWRDHAKVVGGDTVLSFGADSVTLVGVGLAGVWGDGISIS  476
LipAMS8             YQSTDKLVFKDVEGSADWRDHAKVVGGDTVLSFGADSVTLVGVGLAGVGGDGISIS  476
PTA-122608          YQSTDKLVFRDVEGSADWRDHAKVVGGDTVLSFGADSVTLVGVGLAGVGGDGISIS  476
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Figure 13: Alignment of *P. reinekei* (H1) lipase with lipase from *P. fluorescens, Pseudomonas sp.* PAMC 25886, *P. yamanorum,* LipAMS8 from *Pseudomonas* sp. AMS8 and *Pseudomonas* sp. PTA-122608.

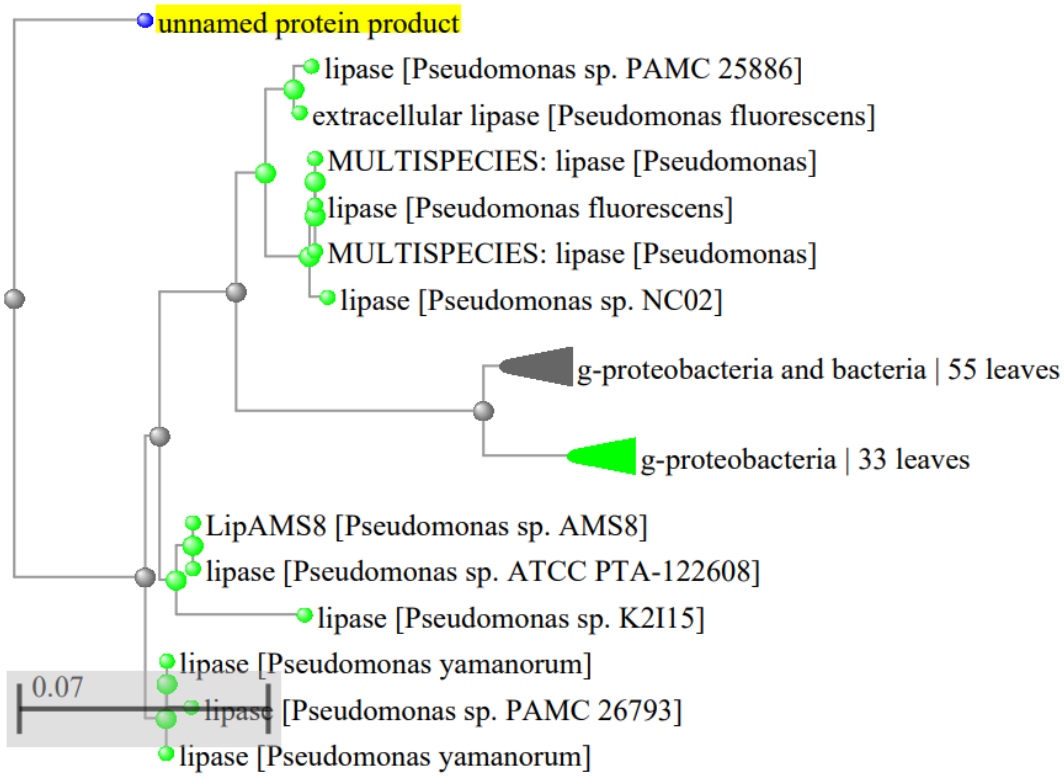

Figure 14: A distance tree based on conserved amino acid sequences in *P. reinekei* lipase generated via a BlastP alignment. The homology of *P. reinekei* lipase (highlighted in yellow and reported as 'unnamed protein product') to lipases from different *Pseudomonas* sp. is noted as within the same clade.

Conclusion

Pseudomonas sp. is one of the most studied bacterial species [51]; and lipases from these have been extensively explored. In this study, a novel lipase from *P. reinekei* was discovered and when fully characterised, displayed high stability in a variety of industrially relevant organic solvents. Furthermore, it was stable over wide pH range (5.0-9.0) and was moderately thermostable, suggesting that this enzyme may be a suitable candidate for bio-transformations in the food and pharmaceutical industries. Additionally, the novelty of *P. reinekei* strain, and the lipase explored here with its unique stability characteristics, makes this enzyme a potential catalyst for other biotechnological applications such as synthesis of biodiesel and biodegradable biopolymers. Further explorative work, including molecular cloning and lipase over expression, will assist in the application of this novel enzyme.

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