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Characterization of a novel alkaline-stable lipase from *Acinetobacter haemolyticus* KV1 isolated from an oil palm mill effluent

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ABSTRACT

Aims: Bioprospecting for lipases remains limited despite its great deal of industrial application. This study reports on the purification and characterization of a novel lipase KV1 from *Acinetobacter haemolyticus* strain KV1.
Methodology and results: Strain KV1 was identified as *A. haemolyticus* using the 16S rDNA sequencing, phylogenetic and BIOLOG assessments. The intracellular lipase was purified to homogeneity using consecutive treatments of ammonium sulfate precipitation, dialysis and DEAE-cellulose ion exchange chromatography, affording ~3.5-fold of the purified lipase with an estimated relative molecular mass of 37 kDa. The PCR product of lipase KV1 revealed that the retrieved sequence contained the proposed complete lipase gene sequence at nucleic acid positions 1-954. The purified lipase exhibited its maximum relative activity at 40 °C and pH 8.0, respectively. Interestingly, the novel alkalophilic lipase KV1 retained its relative activities (> 50%) even up to 24 h between pH 7-11.
Conclusion, significance and impact of study: The findings revealed that relative activities of the intracellular lipase KV1 were the highest at 40 °C and pH 8.0, respectively. Pertinently, the remarkable stability of the lipase KV1 over a broad range of pH values (pH 7-11), as well as an optimum activity at 40 °C indicated it was an excellent enzyme for producing a wide range of industrial detergents, cleaning up enviro-agro-industrial wastes as well as catalysts in synthetic manufacturing processes. Therefore, its full characterization reported here deserves scientific and economic considerations.

Keywords: Acinetobacter haemolyticus; lipase; alkaline-stable; intracellular.

INTRODUCTION

Lipase-producing microorganisms identified so far, include bacteria, fungi and yeasts with lipases isolated from *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas* receiving greater attention in the body of literature (Sangeetha *et al*., 2011; Casas *et al*., 2012). In recent times, lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) have emerged as key enzymes in biotechnology, owing to their multifaceted properties covering a wide array of industrial applications such as food technology, detergent, chemical industry and biomedical sciences (Gupta *et al*., 2004; Batumalaie *et al*., 2017). Lipases catalyze hydrolysis of long chain triglycerides at the interface between the insoluble substrate and water, forming diacylglyceride, monoglyceride, glycerol and free fatty acids as products. Aside from catalyzing their natural substrates, lipases catalyze the enantio- and regioselective hydrolysis as well as synthesize a broad range of natural and non-natural esters (Bancerz *et al*., 2016; Batumalaie *et al*., 2017).

Acinetobacter sp. is a coccobacilliary, aerobic and Gram-negative bacterium that belong to the wider class of Gammaproteobacteria (Park *et al*., 2009; Anbu *et al*., 2011). The bacterial species is oxidase-negative, exhibiting twitching motility (Bancerz *et al*., 2016) and occur in pairs. *Acinetobacter* sp. grows optimally at 33-45 °C and within a pH range of pH 5-9 and the species is widely distributed in nature and commonly isolated from various sources which include soil (Bompensieri *et al*., 1996) and water (Blaise and Armstrong, 1973), for instance, from oil contaminated soil samples (Margesin *et al*., 2003), polluted fresh water (Blaise and Armstrong, 1973), sea water (Kaplan and Rosenberg, 1982), raw milk (Pratuangdejkul and Dharmsthiti, 2000) and clinical

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samples (Hostacka, 2000). Initial designations of the bacteria were founded on at least 15 different names, sporadically cited in the medical literature, with the most frequently used names viz. *Bacterium anitratum*, *Mima polymorpha Herella* (or *Herellea*) *vaginicola*, *Achromobacter*, *Diplococcus* B5W, *Micrococcus calcoaceticus* and *Cytophaga*. French group from the Pasteur Institute proposed a slightly clearer taxonomy based on the biological tests on morphology, nutrition, and *in vitro* growth characteristics; that was later confirmed by the Subcommittee on the taxonomy of Moraxella (Towner *et al*., 2013).

Likewise, lipases produced by the *Acinetobacter* sp. have been found useful for the bioremediation of alkanes and aromatic hydrocarbons. However, the use of the lipase remains limited due to confusion in the taxonomic identification and were designated by a variety of names (Snellman *et al*., 2002; Saffarian *et al*., 2015). *Acinetobacter* sp. lipases have been used to synthesize high molecular weight heteropolysaccharides, used as powerful emulsifiers. A recombinant *Acinetobacter* lipase (isolated from the intestinal sample of *Cyprinus carpio*) was added as a component in aqua feed (Ran *et al*., 2015). So far, the most commercially important applications for hydrolytic lipases are as additives in detergents, food ingredients and flavor development for dairy products. Therefore, their practical applications require unique features that include high substrate specificity as well as temperature and pH stability. In fact, lipases are often incorporated as components of detergent and dishwasher formulations for effective removal of fatty residues, useful in cleaning clogged drains as well as domestic usages (Bisht *et al*., 2013). These enzymes are also used in their purified form for drug targeting purposes, hence, the complete identification of the amino acid sequences and three-dimensional protein structures as well as certain degree of purity is imperative (Bisht *et al*., 2013). As nature offers an amazing diversity of enzymes, comprehensive biochemical characterization of lipases may prove necessary for fully maximizing their catalytic performance suitable for industrial applications, (Sangeetha *et al*., 2011).

Despite several reports on the beneficial usages of bacterial lipases, the uses of lipases from *Acinetobacter* sp. remain scarce in comparison to the more well-known lipases from *Pseudomonas*/*Burkholderia* species. Nevertheless, the use of *Acinetobacter* lipases has been gaining popularity following the growth of enzyme-related industries along with the widening search for novel enzymes for specific applications (Saffarian *et al*., 2015; Batumalaie *et al*., 2018). Herein, we report on the isolation and biochemical characterization of an *Acinetobacter* sp. Subsequently, the study also examined the lipase produced by the *Acinetobacter* sp. bacterium. The enzyme was purified and the optimal working temperature and pH for lipase activity as well as stability of the enzyme was also assessed.

MATERIALS AND METHODS

Chemicals

Acetic acid, copper (II) acetate-1-hydrate, isooctane, ammonium sulphate, boron, copper, manganese, molydenum, zinc, sodium chloride and methanol were acquired from Merck, Germany. Bovine serum albumin, Bradford reagent, agarose, triolein, tributyrin, Victoria blue, rhodamine B, nutrient broth and nutrient agar were purchased from Sigma-Aldrich, USA. Arabic gum, magnesium sulfate heptahydrate, magnesium chloride hexahydrate, calcium chloride dihydrate, potassium phosphate monobasic were acquired from Fisher Scientific, UK.

Sample collection and screening of lipase-producing bacteria

A sample of palm oil effluent was taken from a palm oil mill in Kulai, Malaysia and stored at 4 °C in a sterile glass vial prior to analysis. The sample was enriched in olive oil as the sole carbon source and shaken (150 rpm, at 40 °C, pH 7.0) overnight. Composition of the enrichment media is as follows: olive oil (2%); NaCl (0.2%); $MgSO_4 \cdot 7H_2O$ (0.04%); $MgCl_2 \cdot 6H_2O$ (0.07%); $CaCl_2 \cdot 2H_2O$ (0.05%); KH_2PO_4 (0.03%); K_2HPO_4 (0.03 %); $(NH_4)_2SO_4$ (0.05%) (Leow *et al*., 2004), to which trace element salt solutions containing boron (0.026%), copper (0.05%), manganese (0.05%), molydenum (0.006%) and zinc (0.07%) were then added (Leow *et al*., 2004; Wahab *et al*., 2012). The enriched culture was further screened for any possible growth of lipase-producing bacteria using tributyrin agar plate. Isolates with positive results (halo zones) on the tributyrin agar were subsequently grown in basal media.

Quanlitative determination of lipase activity

Qualitative determination of true lipase producers was carried out using triolein and rhodamine agar plates (Zottig *et al*., 2016) and the overall method is described below.

Triolein agar

Triolein agar comprising of triolein (0.25%), bacteriological agar (1%), nutrient broth (0.8%) and Victoria Blue (0.01%) was adjusted to pH 7.0 and homogenized for 5 mins prior to sterilization. The sterilized triolein agar was poured into petri dishes and allowed to set. A loop full of a pure colony of the bacteria was streaked onto the triolein agar and incubated. A positive result for lipase activity was represented by an intense blue color around a colony following 24-30 h of incubation (Zottig *et al*., 2016).

Rhodamine B agar

Rhodamine B agar plate that comprised of nutrient broth (0.8%), NaCl (0.4%), and bacteriological agar (1%) was

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adjusted to pH 7.0, autoclaved (121 °C, 15 mins) and maintained at 55 °C in an oven. A 2.5% (v/v) final concentration of an emulsion (10 mL) consisting of sterilized olive oil and Rhodamine B (1 mg/mL) was prepared by dissolving in suitable amount of distilled water, sterilized by filtration, mixed well with molten agar and subsequently poured into petri dishes. Positive lipase activity was detected as orange fluorescent halo under UV (350 nm) after 24-30 h of incubation time at 40 °C (Rahman *et al*., 2007).

Morphological screening and biochemical tests

To ascertain the identity of isolate, detection of the morphological properties through various biochemical tests was carried out. Gram staining as well as biochemical test that include lactose fermentation (MacConkey's), catalase, oxidase, citrate, nitrate, lactose and motility (Wang *et al*., 2011).

Genomic DNA extraction, amplification, sequencing and analysis of 16S rDNA gene

Following manufacturer's protocol, DNA purification Kit (Promega, USA) was used to extract the genomic DNA from bacterial strain KV1. The concentration of isolated genetic material that determined using Nanodrop analysis was approximately 300 ng/µL and the extracted DNA was subjected to gel electrophoresis (1%) (Mobarak *et al*., 2011). The 16S rDNA gene of the isolate was amplified using polymerase chain reaction (PCR). The total volume of the amplification reaction mixture was 50 µL, which contained 5 µL of forward (fD1) and reverse (rP1) primers (20 pmol/µL), 5 µL template DNA (approximately 10 ng of DNA), 24 µL of Fermentas PCR MasterMix (200 µM dNTPs; 2 mM $MgCl_2$; 1.25 U *Taq* polymerase), and nuclease free water made up to the final volume. The chromosomal DNA was prepared from late exponential phase culture using Wizard Genomic DNA Purification Kit (Promega, USA).

The universal primers used in this present research were suggested by previous researchers (Weisburg *et al*., 1991) viz. fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') (forward) and rP1 (5'-ACGGTCACCTTGTTACGACTT-3') (reverse). The 16S rDNA gene amplification was performed for 30 cycles with temperature program set as follows: initial denaturation (94 °C for 5 min), denaturation (94 °C for 1 min), annealing (55 °C for 1 min) and final extension (72 °C for 10 min) (Weisburg *et al*., 1991). The PCR product was electrophoresed on agarose gel (1%). For sequencing, the PCR product was purified with QIAquick PCR purification kit (Qiagen, USA) and the sequencing of nucleotide was performed by the 1st Base Laboratory (Malaysia).

Phylogenetic analysis of 16S rDNA gene and BIOLOGTM GEN III microplate identification

The discrepancies between forward and reverse sequences were resolved and the resultant multiple sequences were aligned using Biology Workbench 3.2 (CLUSTAL-W) (http://workbench.sdsc.edu/). For identifying the bacterial species, the sequence of nucleotide was compared with the sequences maintained by the National Center for Biotechnology Information (NCBI) using the BLAST tool (http://www.ncbi.nlm.nih.gov). The phylogenetic analysis was conducted using MEGA6 software. Construction of the neighbor-joining phylogenetic tree was done using the Tamura and Nei (2004) model of nucleotide substitution (Tamura *et al*., 2004). Maximum Composite Likelihood analysis was also conducted to calculate the tree based on the least number of evolutionary steps. Five hundred bootstrap replications were performed. Bootstrap values were calculated by repeated random sampling of the data to provide an indication of the confidence limits of a particular group. A total of 15 sequences of *Acinetobacter* species (AY586400.2; KF185109.1; FJ263930.1; AB859671.1; HE651915.1; AY639376.1; HM566065.1; HM566042.1; JX8673691; EU016146.1; GU124484.1; CP010350.1; KT445980.1; JF742664.1; HQ424441.1) as well as an outgroup i.e. *Pseudomonas aeruginosa* strain ATCC 10145 (NR114471.1) were used in the construction of the phylogenetic tree. In addition, BIOLOG analysis that encompassed of 94 phenotypic tests i.e. 71 carbon source utilization and 23 chemical sensitivity assays (Wragg *et al*., 2014) was outsourced to Focus Biotech (Malaysia).

Quantitative determination of lipase activity

Lipase activity assay and determination of protein content

Based on the standard curve of oleic acid (Wragg *et al*., 2014), lipase activity was estimated by measuring the amount of free fatty acid released. The activity assay was carried out at 40 °C for 30 min at 200 rpm using olive oil as the substrate. The released free fatty acid was extracted with isooctane, colored with copper reagent and read at 715 nm using a spectrophotometer (HITACHI U-3210), with isooctane as the blank. One unit (U) of lipase activity is defined as the amount of enzyme releasing 1 µmole of fatty acid/min. The lipase activity is represented as percentage relative activity when examining the stability of the lipase KV1. The protein content was established using a standard curve prepared from standard solutions of bovine serum albumin (BSA: Sigma, USA) and Bradford reagent (Sigma, USA), monitored at 595 nm in a spectrophotometer (HITACHI U-3210) using solution without bovine serum albumin as blank (Kwon *et al*., 1986). All determinations were carried out in triplicates.

Purification of crude lipase KV1

The bacterial pellets were re-suspended in 15 mL of lysis buffer (50 mM Tris-Cl, pH 8.0) and disrupted by sonication (10 mins). The cell lysate was cleared by centrifugation at 10,000× g for 30 mins at 4 °C and filtered through a 0.45 µm membrane filter (Ran *et al*., 2015).

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Ammonium sulfate precipitation

Purification of the crude KV1 lipase was carried out by ammonium sulfate precipitation executed at 4 °C. The crude lipase extract was precipitated by treating with ammonium sulfate at varying saturation levels (20-80%) (w/v) and left to stand for 4 h prior to precipitation by centrifugation (10,000× g, 15 min at 4 °C). All the collected precipitates were re-suspended in a minimum amount of deionized water and dialyzed against it by routinely replacing the water, and the process continued overnight. All the concentrated fractions were subjected to protein and enzyme activity assays for establishing the fraction containing maximum activity (Bradford *et al*., 1976).

Diethylaminoethyl (DEAE)-cellulose ion exchange chromatography

The concentrated crude lipase was subjected to DEAE-cellulose ion exchange chromatography (GE Healthcare Life Sciences, USA) that was previously equilibrated with 10 mM sodium phosphate buffer at pH 7.2. The protein was eluted using a linear gradient of 0 to 1 M NaCl in the same buffer at a flow rate of 1 mL/min. Finally, the purified lipase KV1 proteins were dialyzed using Amicon Ultra centrifugal filter unit (Sigma-Aldrich, St. Louis, MO) with 50 mM phosphate buffer and was electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Ekinci *et al*., 2016).

SDS-PAGE

A pre-stained low molecular weight calibration kit for SDS Electrophoresis (Fermentas, USA) was used as a standard. The separated proteins were visualized through staining with Coomassie brilliant blue R-250. The single protein band obtained after purification was confirmed by the peptide mass fingerprinting (Ekinci *et al*., 2016).

Amplification of full length lipase KV1 gene

By comparing the amino acid sequences of lipases from different species of *Acinetobacter* and *Pseudomanas*, two highly conserved regions (HGGG and GDSAG) were identified (Figure. 5) and the design of the degenerate primers was based on these regions. The amplified partial fragment of *LipKV1* gene was then compared with other *Acinetobacter* lipases using a multiple sequence alignment (Multalin) software. The *LipKV1* showed 96% similarity to the previously reported *LipA2* gene (Accession number: GQ227702.1). Hence, for amplifying the full-length sequence of lipase *KV1* gene, a set of primers was again designed based on the lipase gene sequence (*LipA2*) of *Acinetobacter* sp. XMZ-26 (Zheng et al.,2011) using Primer3 (v. 0.4.0) online application, and the sequences of the primers are as follows: LipA2-F (5'-ATGACACAACAATCGAGCATGCAC-3') and LipA2-R (5'-TCAATTGAACATTGGCTTTAACGA-3'). The primers were synthesized at 1st BASE ® Laboratories, Malaysia. Polymerase Chain Reaction (PCR) was carried out in a reaction mixture (100 µL) containing DNA template (10-100 ng), 10 mM deoxynucleotide triphosphates (dNTPs), 10× PCR buffer (10.0 µL), 25 mM $MgCl_2$, oligonucleotide primers: AX-F (30 pmol) and AX-R (30 pmol), and *Taq* DNA polymerase (2 U). The gene was amplified with a thermocycler (Gene Amp PCR system 2400, Perkin Elmer, Foster, CA) with the temperature program of pre-denaturation at 94 °C for 5 min, 30 cycles PCR of 30 s denaturation at 94 °C, 45 s annealing at 50 °C and 50 s extension at 72 °C. The final elongation step at 72 °C was for 10 min and preservation was at 4 °C. The amplified PCR products were electrophoresed on 1.0 % agarose gel (w/v) at 70 mA for 30 min and were stained with ethidium bromide (1 µg/µL) for 10 min (Leow *et al*., 2004).

Characterization of the purified lipase KV1

Characterization of the relative activity of the purified KV1 lipase was executed using a standardized protein concentration of 1.58 mg/mL.

Effect of temperature on lipase activity and stability

The effect of temperature on the relative activity of KV1 lipase was determined at temperatures ranging from 5-80 °C for 30 mins, prior to the activity assay. Using the same temperature range (5-80 °C), the enzyme stability test was conducted by pre-incubating the lipase for 30 mins and the stability was monitored for 25 h (Zheng *et al*., 2011).

Effect of pH on lipase activity and stability

The effect of pH was evaluated using various buffer systems: 50 mM acetate buffer (pH 4-6), potassium phosphate buffer (pH 6-8), Tris-Cl buffer (pH 8-9), glycine-NaOH (pH 9-11), and Na_2HPO_3 buffer (pH 11-12), under agitation rate of 200 rpm for 30 mins. The pH stability test was performed by pre-incubating the lipase in various buffers (pH 4-12) for 30 mins with agitation at 200 rpm and the stability was monitored for 25 h (Zheng *et al*., 2011).

RESULTS

Bacterial isolation, screening, morphological and biochemical analyses

Identification of the bacterial strain (KV1) from water sample obtained from effluent of an oil palm mill was performed. Analysis of the basic cellular morphology of the bacteria using a light microscope (Figure 1A) and SEM analysis (Figure 1B) revealed that the isolates were generally of coccobacillary in shape and occurred in pairs (Figure 1). On the tributyrin agar, the colonies appeared pale and of mucoid with smooth to pitted surface. The KV1 bacterium was Gram-negative, appearing pink to red during post-staining and grew optimally at 40 °C. The

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results for the morphological and biochemical tests for bacterial strain KV1 are summarized in Table 1.

The 16S rDNA nucleotide sequence of the *A. haemolyticus* KV1 studied in this present research was deposited in the GenBank database with an accession number KU363981.1. The 16S rDNA partial sequence of the KV1 strain analyzed here (GenBank accession number: KU363981.1) was 99 % similar with the partial sequences of *A. haemolyticus* maintained by the GenBank database (accession numbers: AY586400.2; HE651915.1; AB859671.1; AY639376.1; JX867369.1), suggesting that the KV1 bacterium was possibly *A. haemolyticus*. The neighbour-joining tree (Figure 2) constructed from the sequence data was identical to the tree obtained using Maximum Composite Likelihood method. Two distinct congeneric clusters with high bootstrap supports (95-99%) were formed based on the sequence data. The bootstrap support values indicated the percentage support for a grouping by randomly resampling the data (Harvey *et al*., 2003). In the neighbour-joining phylogenetic tree, the KV1 bacterium partial 16S rDNA sequence was grouped together with other *A. haemolyticus* sequences maintained by the GenBank database with high bootstrap support of 99% (Figure 2), indicating that the KV1 bacterium was *A. haemolyticus*. The series of the BIOLOG® biochemical tests confirmed that bacterium KV1 was indeed *A. haemolyticus* (Table 2). Scientific classification of the isolate KV1 was obtained as follows: Kingdom - Bacteria; Phylum - Proteobacteria; Class - Gammaproteobacteria; Order - Pseudomonadales; Family - Moraxellaceae; Genus - *Acinetobacter*.

Table 1: Morphological and biochemical characteristics of bacterium KV1.

Parameters	Properties
Size	Small, 0.5~1 mm
Color	Non-pigmented
Shape	Domed, mucoid with smooth to pitted surface and coccobacillary
Gram	– (pink/red)
Catalase test	+
Nitrate test	–
Oxidase test	–
Lactose utilization test (MacConkey Agar)	+
Citrate test	+
Motility test	–

+, positive result; –, negative result.

Table 2: Morphological and biochemical characteristics of bacterium KV1.

Properties	Results	Properties	Results
D-Trehalose	–	D-Cellobiose	–
D-Maltose	–	Guanidine HCl	+
Surcrose	–	Niaproof 4	+
Stachyose	–	L-Lactic acid	–
pH 6	+	Tetrazolium violet	+
pH 5	–	Glucuronamide	+
D-Raffinose	–	D-Gluconic acid	–
α-D-Lactose	–	Muric acid	–
D-Melibiose	–	Quinic acid	+
β-Methyl-D-glucoside	–	α-Keto-glutaric acid	+
D-Salicin	–	Vancomycin	+
D-Fucose	+	Tetrazolium blue	+
N-Acetyl-D-glactosamine	–	p-Hydroxy-phenylacetic acid	–
N-Acetyl-neuraminic acid	–	D-Lactic acid methyl ester	–
1% NaCl	+	Citric acid	+
4% NaCl	+	D-Saccharic acid	–
D-Galactose	+	Nalidixic acid	–
N-Acetyl-β-D-mannosamine	–	Potassium tellurite	–
Inosine	–	Tween 40	+
1% sodium lactate	+	γ-Amino-butryric acid	+
Fusidic acid	–	Sodium butyrate	+
D-Serine	+	Gelatin	–
D-Sorbitol	–	Acetic acid	+
D-Mannitol	–	Formic acid	–
D-Arabitol	–	Propionic acid	+
myo-Inositol	–	Sodium bromate	–
Glycerol	–	Lincomycin	+
D-serine	–	Troleandomycin	+
L-Glutamic acid	+	Rifamycin SV	+
Glycyl-L-proline	–	Minocycline	–
L-Pyroglutamic acid	–	β-Hydroxy-D,L butyric acid	+
		L-Alanine	+

+, positive result; -, negative results.

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Table 3: Purification of the crude lipase from *A. haemolyticus*.

Samples	Volume (mL)	Lipase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude lysate	100	35.3	3.8	9.2	1	100
Ammonium sulfate precipitation	30	29.1	3	9.7	1.1	82
Dialysis	18	26.4	2.1	12.6	1.4	75
DEAE-cellulose	10	23.3	1.2	19.4	2.1	66
Dialysis	3	20.5	0.61	33.5	3.5	58

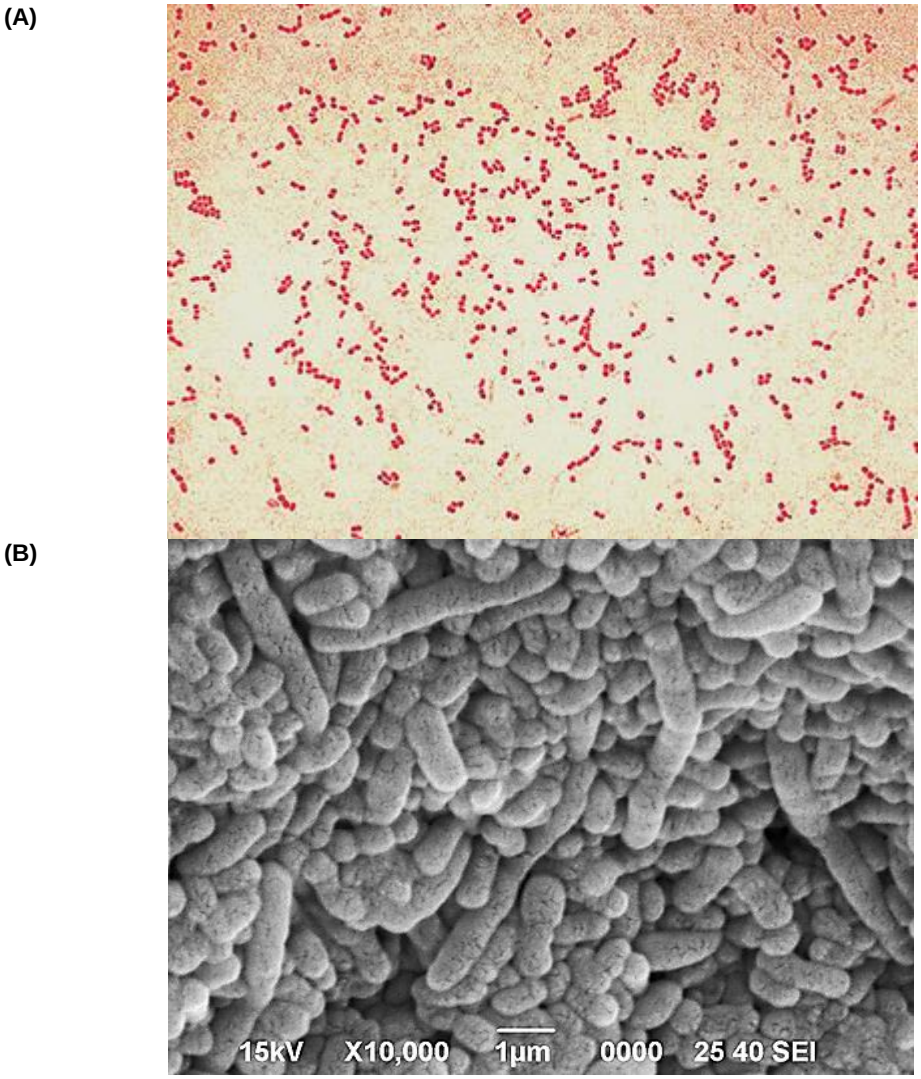

Figure 1: (A) Gram stain and (B) SEM analysis of bacterial strain KV1.

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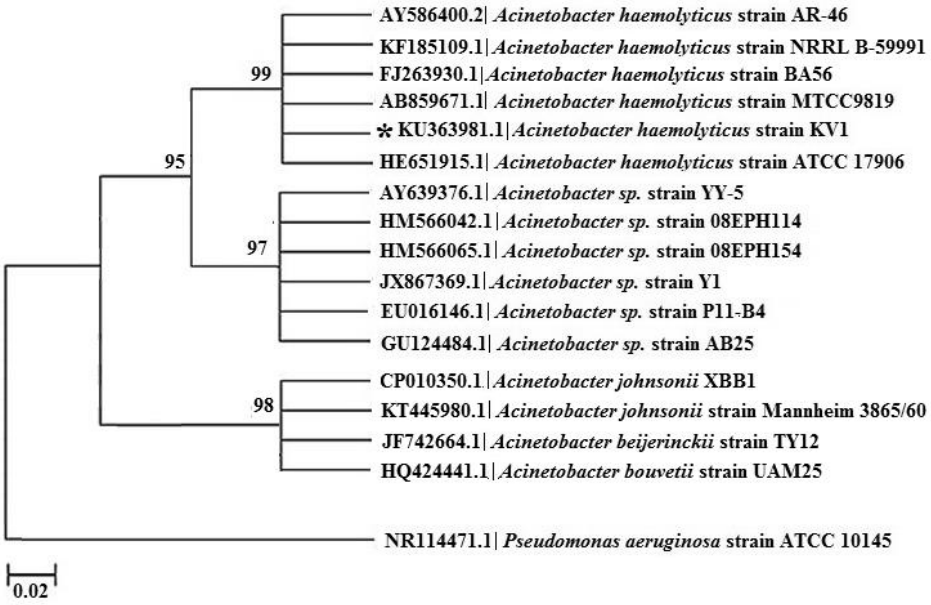

Figure 2: Phylogenetic tree of 16S rDNA sequence obtained for *A. haemolyticus* strain KV1 (KU363981.1) with sequences of other *Acinetobacter* species and an outgroup (*P. aeruginosa* strain ATCC 10145) from GenBank database. Scale bar represents 0.02 substitutions per site.

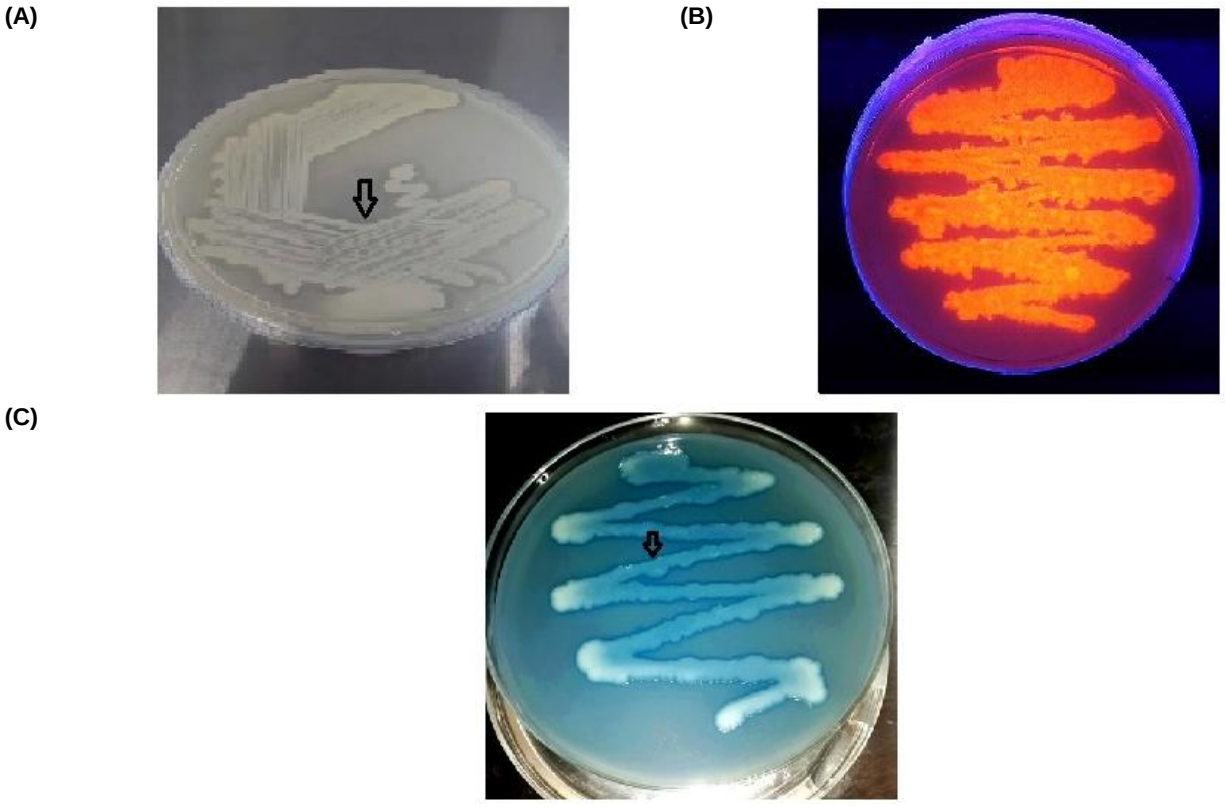

Figure 3: Qualitative determination of lipase activity. (A) Tributyrin agar plate showing clearing zone around positive colony. (B) Rhodamine agar plate showing orange fluorescence around positive colony under UV irradiation. (C) Triolein agar plate showing intense blue color around positive colony.

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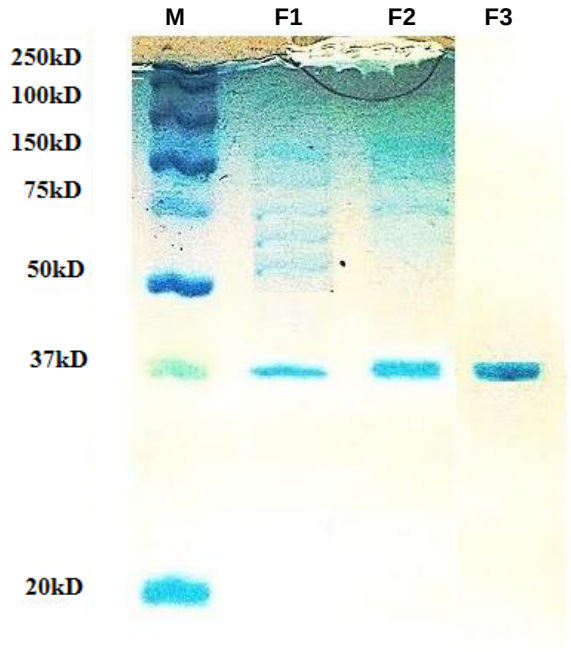

Figure 4: SDS-PAGE analysis of crude lipase from *Acinetobacter haemolyticus* strain KV1 at various stages of purification. Lane 1, molecular mass markers; lane 2, crude enzyme; lane 3, 60% ammonium sulphate; lane 4, DEAE cellulose purification.

Likewise, the primary screening for lipase activity using tributyrin, triolein and rhodamine B agar plates confirmed the *A. haemolyticus* KV1 lipase exhibited true lipase activity. The positive colony formed a clearing zone on the tributyrin agar plates (Figure 3A) but also conferred orange fluorescence and intense blue on rhodamine B agar plate (Figure. 3B) and triolein agar plate (Figure. 3C), respectively. Further confirmation test was performed quantitatively on the positive *A. haemolyticus* KV1 bacterial strain which showed lipase activity and specific activity corresponding to 5.87 µmol/mL and 1.25 U/mg, respectively.

Purification of crude lipase and amplification of full length lipase KV1 gene

The purification of the crude lipase derived from *A. haemolyticus* KV1 was carried out using a consecutive two-step procedure by ammonium sulfate precipitation followed by DEAE-cellulose ion exchange chromatography (Table 3). Purification of the crude *A. haemolyticus* KV1 lipase by 60% ammonium sulfate precipitation resulted in a 1.1-fold purification with 82% recovery. Subsequent dialysis increased the purification fold to 1.4 at 75% recovery (Table 3). Purification by a single-step DEAE-cellulose ion exchange chromatography achieved near homogeneity to afford a 2.1-fold of the pure lipase, represented by a single band at the expected 37 kDa position on the SDS-PAGE gel (Figure 4). The purified lipase KV1 gave specific activity of 19.4 U/mg with a purification yield of 66%. Dialysis on the purified lipase further increased the purification fold to 3.5 at a 58% recovery (Table 3). It is pertinent to note that, the molecular mass of the *A. haemolyticus* KV1 lipase obtained in this study was well within the reported range of other *Acinetobacter* sp. lipases (23 to 62 kDa). Lipases having molecular mass of 32 kDa were the most frequently reported which included those from *A. calcoaceticus* BD413 (Kok *et al*., 1995), *Acinetobacter* sp. ES-1 (Ekinci *et al*., 2016), *A. venetianus* RAG-1 (Park *et al*., 2009) and *Acinetobacter* sp. RAG-1 (Snellman *et al*., 2002).

In this study, alignments of the partial *LipKV1* DNA sequence with other known lipase enzymes from *Acinetobacter* sp. (*LipA2*, *LipAYE*, *LipAB900*) revealed that the *LipKV1* shared the highest homology (96%) with that of *LipA2*. The PCR product was sent to 1st BASE Laboratories (Malaysia) for sequencing and the results revealed that the retrieved sequence of *LipKV1* contained the proposed complete lipase gene sequence at nucleic acid positions 1~954. The full sequence of KV1 lipase was obtained after removal of the invariant positions in the nucleotide sequence and was deposited in NCBI GenBank under the accession number of KX459517. Using online tool “ExPASy Translate tool” (http://web.expasy.org/translate/), the amino acid

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sequence (305 aa) of *LipKV1* was deduced and its open reading frame (ORF) was determined (Figure 5). The ORF of the putative *LipKV1* encoding a 305-deduced amino acid sequence was aligned with multiple amino acid sequences derived from lipases, LipA2 (GQ227702.1), LipAYE (B0V8H7), LipPf-5 (Q4KD54), LipAB900 (ABO11284.2) (> 95 % sequence similarity) (Table 4).

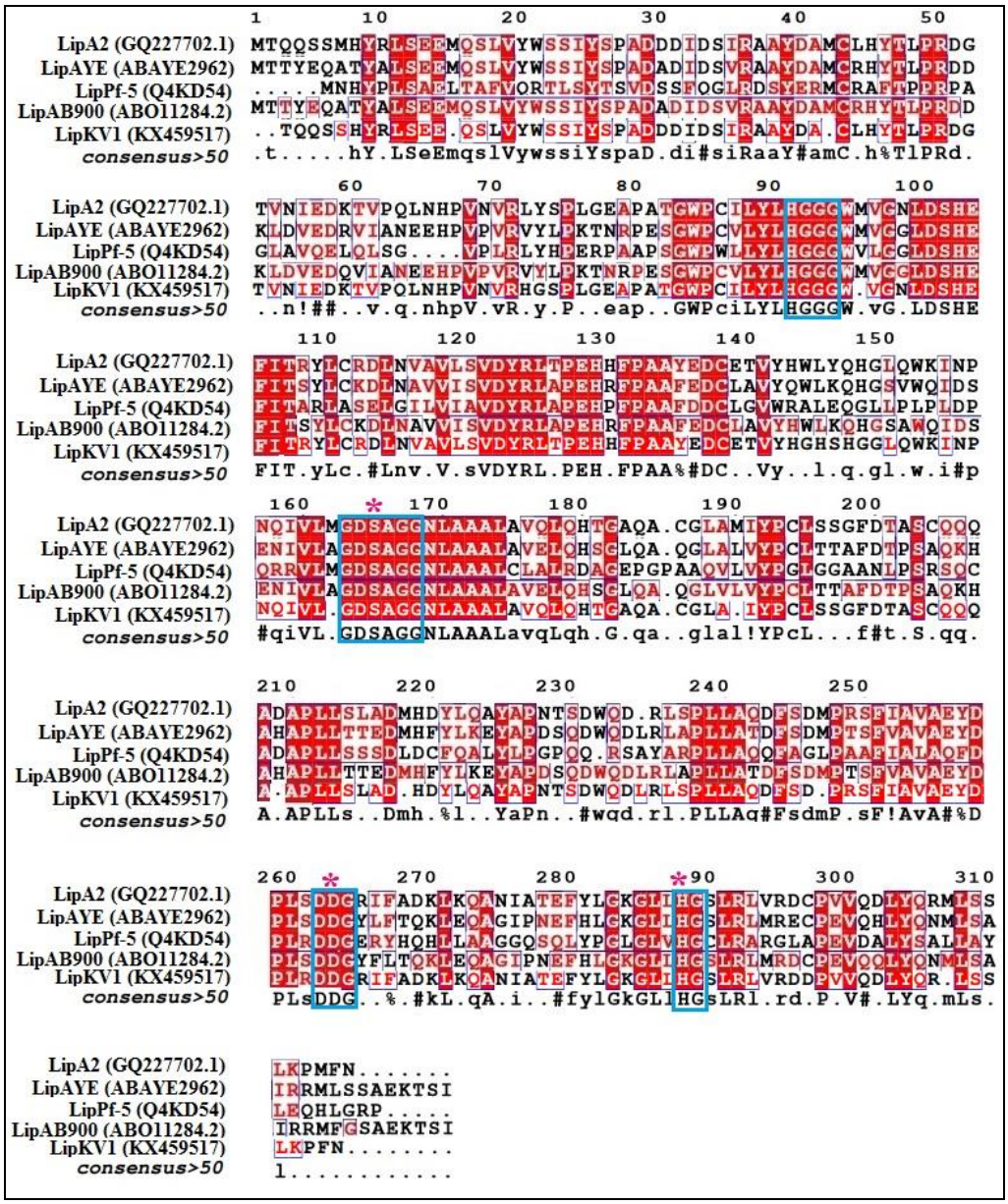

Figure 5: Alignment of the amino acid sequence of LipKV1 with four other lipases [LipA2 (GQ227702.1), LipAYE (ABAYE2962), LipPf-5 (Q4KD54), LipAB900 (ABO11284.2)] with high sequence identity. Residues involved in the catalytic triad are identified by asterisk (*). Conserved regions are enclosed in blue boxes.

The alignments indicated that a consensus showing that the LipKV1 catalytic triad was comprised of Ser 165 (in the motif GDSAGG), Asp 264, and His 289, respectively (Figure 5). A HGGG motif, characteristic of the *Acinetobacter* sp. lipase which forms an oxyanion hole which role is for stabilizing the tetrahedral intermediates (Martinez *et al*., 1994; Ito *et al*., 1998) was also identified. Small amino acids (Ala, Cys, Gly, Ser, and Thr)

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amounting to 28.7% in total amino acids were found mainly distributed around the active site of LipKV1.

Table 4: Multiple amino acid sequence alignment of different *Acinetobacter sp*. And *Pseudomonas* sp. lipase enzyme.

Gene	Identity (%)	Source	Accession number
LipA2	98	*Acinetobacter* sp. XMZ-26	GQ227702.1
LipAYE	97	*A. baumannii* AYE	B0V8H7
LipAB900	97	*A. baumannii* AB900	ABO11284.2
LipPf-5	96	*Pseudomonas fluorescens*	Q4KD54

Characterization of *A. haemolyticus* KV1 Lipase

Effect of temperature on lipase activity and stability

The effect of temperature was evaluated in this study and the relevant data are tabulated in Figure 6. The activity profile for the *A. haemolyticus* KV1 lipase was seen to increase when the reaction temperature was elevated. The maximum relative activity was attained at 40 °C (100%), beyond which the activity began to deteriorate. Relative activity was particularly low when the reaction temperatures were at the lowest (5 °C, 40%) and highest (80 °C, 20%). Activity of the lipase KV1 significantly dipped at 70 °C and beyond (Figure 6A). Time course profile for the stability of the lipase KV1 evaluated here (Figure 6B) showed that the lipase showed remarkable structural stability, retaining its catalytically competent form for extended durations of incubation (up to 25 h) over a wide range of temperatures. Lipase KV1 retained over 75% of its relative activity between 20-40 °C after 12 h of incubation. The lipase also exhibited a remarkable half-life of 25 h when the reaction set was set to its optimum temperature (40 °C). The half-lives of lipase KV1 were relatively long attaining duration for up to 10 h and 8 h for incubation temperatures of 50 °C and 60 °C, respectively.

Effect of pH on lipase activity and stability

Figure 7A depicts the profile for the effect of pH on the relative activity of the KV1 lipase. The activity of the lipase was seen to increase from pH 4.0 (35%) up to pH 8.0 (100%). The lipase activity was substantially higher at alkaline pH 8-12, retaining approximately 80% of its relative activity up to pH 12. In contrast, relative activity of lipase KV1 was the lowest at pH 4 and retained only 25% of the initial activity after 25 h. Examination on the effect of various pH showed that lipase KV1 was particularly stable in the alkaline pH range (pH 7-12), retaining relative activities > 70% and > 60% after 5 h and 25 h of incubation, respectively (Figure 7B). Again, lipase KV1 demonstrated the lowest relative activity at pH 4 followed by pH 5.

DISCUSSION

Identification of the bacterial strain (KV1) from water sample obtained from effluent of an oil palm mill was performed and it was evident that bacterium strain KV1 is *A. haemolyticus*. The results for the biochemical, 16S rDNA and phylogeny analyses in this study were confirmatory for *A. haemolyticus* as reported by previous researchers (Jagtap *et al*., 2010; Uttatree *et al*., 2010; Towner *et al*., 2013). However, comparison of the 16S rDNA gene sequences with that of other *Acinetobacter* sp. may not be sufficient to differentiate the strains within a species. Moreover, a single method of identification can be potentially misleading (Saffarian *et al*., 2015). In this perspective, the BIOLOG® result which confirmed that bacterium KV1 was indeed *A. haemolyticus*, hence complemented the aforementioned methods. This was exemplified in the bacterium showing true lipase activity on tributyrin, triolein and rhodamine B agar plates. Formation of halo zones surrounding each colonies was indicative of lipase production, in which the produced enzyme hydrolyzed the oily substrates into the alcohol and fatty components. Although there are several studies describing the characteristics of several species of *Acinetobacter*, available scientific data on the *A. haemolyticus* lipase is limited presumably due to the unclear taxonomic history of the *Acinetobacter* genus (Snellman *et al*., 2002; Anbu *et al*., 2011; Saffarian *et al*., 2015). It is believed that the mesophilic alkaline-stable *A. haemolyticus* lipase isolated in this study is exceptional and further investigation into such bacteria merits scientific consideration.

Ammonium sulfate precipitation involves a salting out process, in which the hydrophobic hydration interaction reduces the structural flexibility of water molecules in the relative proximity to apolar residues. This causes the decrease in entropy and thus becomes energetically unfavorable (Bompensieri *et al*., 1996), leading to some proteins to be precipitated out in solution of a certain salt concentration. Evidently, the use of 60% ammonium sulfate was sufficient to elevate the surface tension of the solution consequently raise the rate of hydrophobic interactions between the molecules of lipase KV1 and water. The stable interactions between lipase KV1 with the Tris-HCl buffer are reduced and the lipase KV1 protein is precipitated. Purification of lipase KV1 was successful based on the obtained 2.1-fold of the pure lipase showing a single protein band of 37 kDa. This was possible as the positively-charged DEAE-cellulose resin attracts the partially negatively-charged lipase KV1 protein thereby retaining the lipase proteins in the column. Increasing the NaCl concentration in the eluent subsequently changes the surface ionization of the bound lipase proteins and dislodges the lipase KV1 resin and is eluted from column. According to Gonzalez *et al*. (2010) similarity searching is considered effective and reliable only when sequences share similarities > 70%,as it would

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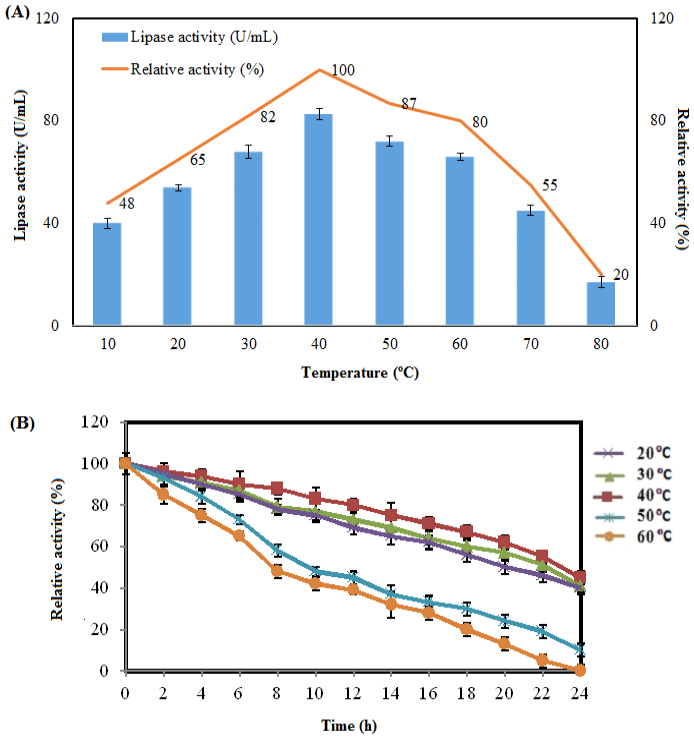

Figure 6: Effect of temperature on (A) lipase activity and (B) lipase stability on the purified KV1 lipase. Relative activities were the average values of triplicate measurements.

represent approximately a 90% probability or more that the enzymes have common biological processes and ancestry. In this perspective, the high similarity of *LipKV1* gene (96%) with that of *LipA2* suggests that the amplification of the full sequence of *LipKV1* from the genomic materials of *Acinetobacter* sp. XMZ-26 lipase (LipA2) was highly possible as well as reliable.

It has been shown that using thermally stable enzymes can be advantageous as well as useful for a wide range of industrial applications. This is because a higher reaction temperature could significantly influence the activity and stability of an enzyme (Isah *et al*., 2017) as well as increase reaction rate. Relative activities of lipase KV1 was particularly low at the lowest (5 °C, 40%) and highest (80 °C, 20%). This was largely associated with the structure of lipase KV1 being too rigid at such low temperature and has yet to unfold into its catalytically active form, and the excessive unravelling of the enzyme protein that caused the irreversible thermal inactivation of KV1 lipase, respectively (Kok *et al*., 1995). According to review of literature, the effect of temperature on enzyme activity is dictated by the active sites of enzymes. Since the active site is more flexible than the enzyme as a whole, losses in enzyme activity would usually precede denaturation (Kok *et al*., 1995). In this perspective, as the temperature of reaction was elevated, the active-site of the lipase KV1 becomes increasingly flexible its and its structure gradually distorted. As such the active form of lipase KV1 was converted into an inactive one along with the concomitant drop in its catalytic activity. Conversely, relative activity of the lipase was improved as the reaction temperature was increased. This was probably due to the enhanced effective collisions between the lipase and substrate molecules following the elevated overall kinetic energy within the system. Furthermore, increasing the reaction temperature would improve the reaction system as increasing the temperature in the system facilitates the diffusion process, promotes better integration and mutual solubility of the reactants as well as reduce viscosity of the reaction mixture (Wahab *et al*., 2014). Pertinently, the highest relative activity of KV1 lipase observed here at 40 ºC concurred with previous studies that reported on greater unfolding of the protein structure that rendered the lipases less rigid (Daniel *et al*., 2010; Wahab *et al*., 2014) and catalyzing more competently.

The data support that lipase KV1 is relatively thermostable as reflected in the exceptional retention of its activity. It also conveyed that the lipase is capable of retaining its active three-dimensional structure under elevated temperatures, thus suitable as biocatalysts for catalyzing commercial processes that require prolonged reaction times. At present, the use of robust and thermally stable enzymes is essential, particularly when lipases are used as additives in detergents. This is because higher temperatures are often required to remove difficult stains

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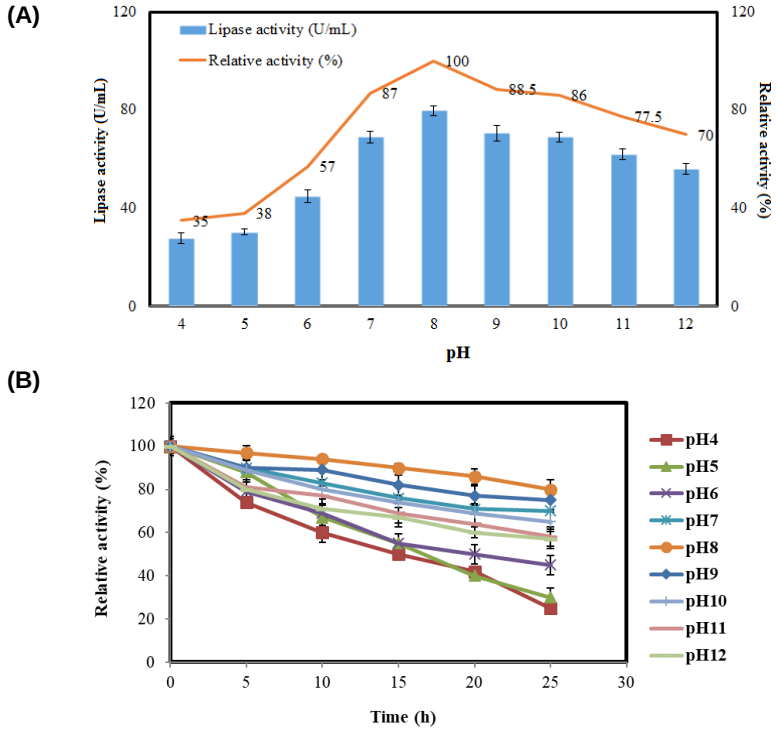

Figure 7: Effect of pH on (A) lipase activity and (B) lipase stability on the purified KV1 lipase. Relative activities were the average values of triplicate measurements.

such as food stains on synthetic materials or cotton (Bisht *et al*., 2013). Since the half-lives of lipase KV1 were relatively long, reaching up to 10 h and 8 h for incubation temperatures at 50 °C and 60 °C, respectively, thus indicate the lipase is satisfactorily stable for such purpose.

Due to the inherent poly-ionic nature of an enzyme, its three-dimensional structure is invariably susceptible to fluctuations in the distribution of charges on the surface of its protein as well as within its active site (Illanes, 2008). The fact that lipase KV1 could retain approximately 80% of its relative activity at pH up to 12, suggests that the enzyme is alkaline-stable. According to Wang *et al*., (2012), alkaline-stable is a class of lipase enzymes capable of retaining its activity in alkaline at pH 8-10.5 which resulted in remaining the activity from 80-90% and also indicating the alkalo-stable nature of the enzyme. Interestingly, the optimum pH for lipase KV1 is comparatively higher that those reported for *Staphylococcus aureus* (pH 6.5), *Fusarium oxyporum* (pH 7.0) and *Bacillus* sp. RSJ1 (pH 8.0) (Bora and Bora, 2012) but a little lower than *Geobacillus zalihae* (pH 9.0) (Wahab *et al*., 2014). The remarkably wide pH range of the *A. haemolyticus* KV1 lipase suggests its possible commercial applications. Conversely, lipase showed the lowest relative activity (25%) at pH 4 was presumably due to the extreme low pH that induced the partial loss of its catalytically active form. Such outcome seen here may be explained by (a) deprotonation at very low pH (producing more negatively charge enzymes) and (b) irreversible denaturation of its poly-ionic three-dimensional structures. The ionization state of the substrate would have also been affected too, which consequently altered binding of the substrate to the active site of lipase KV1 (Bisswanger, 2014).

It has been described that the catalytic behaviour of enzymes is highly dependent on the surrounding pH. Therefore, time-course profiling on tolerance of an enzyme with regards to the effect of various pH on its stability may prove necessary as well as useful. The data in this study thus convey that lipase KV1 is an alkaline-stable enzyme. A noteworthy point to highlight here, the half-lives of lipase KV1 that reached up to 25 h in buffers pH 8-12 were substantially lengthier than those reported by earlier studies (Borkar et al., 2009; Sharma et al., 2010). When compared to other lipases that showed high sequence identify (LipA2 (GQ227702.1), LipAYE (B0V8H7), LipPf-5 (Q4KD54), LipAB900 (ABO11284.2) to that of LipKV1, lipase KV1 is considerably more stable over a broad range of pH values (pH 7-12), such characteristic seen here was unique for lipases derived from the *Acinetobacter* species as previously reported (Martinez *et al*., 1994; Ito *et al*., 1998). This can be attributed to presence of higher number of surface acidic amino acids (negatively charge) (Bora and Bora, 2012) on the lipase KV1 protein. This aspect would have rendered the lipase more capable in accommodating changes associated with pH-induced local folding events. Hence,

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the active structure of lipase KV1 is retained and the lipase would remain active at high pH conditions for longer periods of time. To substantiate this aspect, further investigations using bioinformatic tools may therefore, be necessary. Crucially the alkaline-stable lipase i.e. lipase KV1 evaluated here may be useful in catalyzing processes viz. sewage treatment, leather processing and detergent formulations (Sharma *et al*., 2001). In fact, lipases used as additive in detergent formulations are those which are highly active in the alkaline region as well as having an optimum temperature of 40 °C (Sharma *et al*., 2001).

CONCLUSION

A novel lipase KV1 isolated from *A. haemolyticus* was purified and characterized. The findings revealed that relative activities of the intracellular lipase KV1 were the highest at 40 °C and pH 8.0, respectively. Pertinently, the remarkable stability of the lipase KV1 over a broad range of pH values (pH 7-11), as well as an optimum activity at 40 °C indicated it was an excellent enzyme for producing a wide range of industrial detergents, cleaning up enviro-agro-industrial wastes as well as catalysts in synthetic manufacturing processes.

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REFERENCES

Anbu, P., Noh, M. J., Kim, D. H., Seo, J. S., Hur, B. K. and Min, K. H. (2011). Screening and optimization of extracellular lipases by Acinetobacter species isolated from oil-contaminated soil in South Korea. *African Journal of Biotechnology* **10, 4147-4156.**

Bancerz, R., Osińska-Jaroszuk, M., Jaszek, M., Janusz, G., Stefaniuk, D., Sulej, J., Janczarek, M., Wilkołazka, A. J. and Rogalski, J. (2016). New alkaline lipase from *Rhizomucor variabilis*: Biochemical properties and stability in the presence of microbial EPS. *Biotechnology and Applied Biochemistry* **63, 67-76.**

Batumalaie, K., Khalili, E., Mahat, N. A., Huyop, F. Z. and Wahab, R. A. (2018). A statistical approach for optimizing the protocol for overexpressing lipase KV1 in *Escherichia coli*: Purification and characterization. *Biotechnology and Biotechnological Equipment*, **32, 69-87.**

Batumalaie, K., Edbeib, M. F., Mahat, N. A., Huyop, F. and Wahab, R. A. (2017). *In silico* and empirical approaches toward understanding the structural adaptation of the alkaline-stable lipase KV1 from *Acinetobacter haemolyticus*. *Journal of Biomolecular Structure and Dynamics* **28, 1-17.**

Bisht, D., Yadav, S. K. and Darmwal, N. S. (2013). An oxidant and organic solvent tolerant alkaline lipase by *P. aeruginosa* mutant: Downstream processing and biochemical characterization. *Brazilian Journal of Microbiology* **44, 1305-1314.**

Bisswanger, H. (2014). Enzyme assays. *Perspectives in Science* **1, 41-55.**

Blaise, C. R. and Armstrong, J. B. (1973). Lipolytic bacteria in the Ottawa River. *Applied Microbiology* **26, 733-740.**

Bompensieri, S., Gonzalez, R., Kok, R., Nutgeren-Roodzant, I., KJ, H. and Cascone, O. (1996). Purification of a lipase from *Acinetobacter calcoaceticus* AAC323-1 by hydrophobic-interaction methods. *Biotechnology and Applied Biochemistry* **23, 77-81.**

Bora, L. and Bora, M. (2012). Optimization of extracellular thermophilic highly alkaline lipase from thermophilic *Bacillus* sp. isolated from Hotspring of Arunachal Pradesh, India. *Brazilian Journal of Microbiology* **43, 30-42.**

Borkar, P. S., Bodade, R. G., Rao, S. R. and Khobragade, C. N. (2009). Purification and characterization of extracellular lipase from a new strain: *Pseudomonas aeruginosa* SRT 9. *Brazilian Journal of Microbiology* **40, 358-366.**

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72, 248-254.**

Casas-Godoy, L., Duquesne, S., Bordes, F., Sandoval, G. and Marty, A. (2012). *Lipases*: an overview. Lipases and Phospholipases: *Methods and Protocols* **3-30.**

Daniel, R. M., Peterson, M. E., Danson, M. J., Price, N. C., Kelly, S. M., Monk, C. R., … Lee, C. K. (2010). The molecular basis of the effect of temperature on enzyme activity. *Biochemical Journal* **425, 353-360.**

Ekinci, A. P., Dinçer, B., Baltaş, N. and Adıgüzel, A. (2016). Partial purification and characterization of lipase from *Geobacillus stearothermophilus* AH22. *Journal of Enzyme Inhibition and Medicinal Chemistry* **31, 325-331.**

Gonzalez, M. W. and Pearson, W. R. (2010). Homologous over-extension: A challenge for iterative similarity searches. *Nucleic Acids Research* **38, 2177-2189.**

Gupta, R., Gupta, N. and Rathi, P. (2004). *Bacterial lipases*: An overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology* **64, 763-781.**

Harvey, M. L., Dadour, I. R. and Gaudieri, S. (2003). Mitochondrial DNA cytochrome oxidase I gene: Potential for distinction between immature stages of some forensically important fly species (Diptera) in Western Australia. *Forensic Science International* **131, 134-139.**

Hostacka, A. (2000). Influence of some antibiotics on lipase and hydrophobicity of *Acinetobacter baumannii*. *Central European Journal of Public Health* **8, 164-166.**

Illanes, A. (Ed.). (2008). Enzyme biocatalysis: Principles and applications. Springer, New York, NY, USA.

Isah, A. A., Mahat, N. A., Jamalis, J., Attan, N., Zakaria, I. I., Huyop, F. and Wahab, R. A. (2017). Synthesis of geranyl propionate in a solvent-free medium using *Rhizomucor miehei* lipase covalently immobilized on chitosan-graphene oxide beads. *Preparative Biochemistry and Biotechnology* **47, 199-210.**

Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S. and Hatada, Y. (1998). Alkaline detergent enzymes from alkaliphiles: Enzymatic properties, genetics, and structures. *Extremophiles* **2, 185-190.**

Jagtap, S., Gore, S., Yavankar, S., Pardesi, K. and Chopade, B. (2010). Optimization of medium for lipase production by *Acinetobacter haemolyticus* from healthy human skin. *Indian Journal of Experimental Biology* **48, 936-941.**

Kaplan, N. and Rosenberg, E. (1982). Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. *Applied and Environmental Microbiology* **44, 1335-1341.**

Kok, R. G., Thor, J. J., Nugteren-Roodzant, I. M., Brouwer, M. B., Egmond, M. R., Nudel, C. B., Vosman, B. and Hellingwerf, K. J. (1995). Characterization of the extracellular lipase, LipA, of *Acinetobacter calcoaceticus* BD413 and sequence analysis of the cloned structural gene. *Molecular Microbiology* **15, 803-818.**

Kwon, D. Y. and Rhee, J. S. (1986). A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *Journal of the American Oil Chemists' Society* **63, 89-92.**

Leow, T. C., Rahman, R. N. Z. R. A., Basri, M. and Salleh, A. B. (2004). High level expression of thermostable lipase from *Geobacillus sp*. strain T1. *Bioscience, Biotechnology, and Biochemistry* **68, 96-103.**

Margesin, R., Labbe, D., Schinner, F., Greer, C. W. and Whyte, L. G. (2003). Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Applied and Environmental Microbiology* **69, 3085-3092.**

Martinez, C., Nicolas, A., van Tilbeurgh, H., Egloff, M. P., Cudrey, C., Verger, R. and Cambillau, C. (1994). Cutinase, a lipolytic enzyme with a preformed oxyanion hole. *Biochemistry* **33, 83-89.**

Mobarak-Qamsari, E., Kasra-Kermanshahi, R. and Moosavi-Nejad, Z. (2011). Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110. *Iranian Journal of Microbiology* **3, 92-98.**

Park, I. H., Kim, S. H., Lee, Y. S., Lee, S. C., Zhou, Y., Kim, C. M., Ahn, S.C. and Choi, Y. L. (2009). Gene cloning, purification, and characterization of a cold-adapted lipase produced by *Acinetobacter baumannii* BD5. *Journal of Microbiology and Biotechnology* **19, 128-135.**

Pratuangdejkul, J. and Dharmsthiti, S. (2000). Purification and characterization of lipase from psychrophilic *Acinetobacter calcoaceticus* LP009. *Microbiological Research* **155, 95-100.**

Rahman, R. N. Z. R. A., Leow, T. C., Salleh, A. B. and Basri, M. (2007). *Geobacillus zalihae* sp. novel., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. *BMC Microbiology* **7, 77-86.**

Ran, C., He, S., Yang, Y., Huang, L. and Zhou, Z. (2015). A novel lipase as aquafeed additive for warm-water aquaculture. *PLoS One* **10, e0132049.**

Saffarian, A., Mulet, C., Naito, T., Bouchier, C., Tichit, M., Ma, L., Grompone, G., … Pédron, T. (2015). Draft genome sequences of *Acinetobacter parvus* CM11, *Acinetobacter radioresistens* CM38, and *Stenotrophomonas maltophilia* BR12, isolated from murine proximal colonic tissue. *Genome Announcements* **3, e01089-15.**

Sangeetha, R., Arulpandi, I. and Geetha, A. (2011). Bacterial lipases as potential industrial biocatalysts: An overview. *Research Journal of Microbiology* **6, 1-24.**

Sharma, M., Chadha, B. S. and Saini, H. S. (2010). Purification and characterization of two thermostable xylanases from *Malbranchea flava* active under alkaline conditions. *Bioresource Technology* **101, 8834-8842.**

Sharma, R., Chisti, Y. and Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology Advances* **19, 627-662.**

Snellman, E. A., Sullivan, E. R. and Colwell, R. R. (2002). Purification and properties of the extracellular lipase, LipA, of *Acinetobacter* sp. RAG-1. *The FEBS Journal* **269, 5771-5779.**

Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America* **101, 11030-11035.**

Towner, K. J., Bergogne-Bérézin, E. and Fewson, C. A. (Eds.). (2013). The biology of *Acinetobacter*: Taxonomy, clinical importance, molecular biology, physiology, industrial relevance (Vol. 57). Springer Science & Business Media, New York.

Uttatree, S., Winayanuwattikun, P. and Charoenpanich, J. (2010). Isolation and characterization of a novel thermophilic-organic solvent stable lipase from *Acinetobacter baylyi*. *Applied Biochemistry and Biotechnology* **162, 1362-1376.**

Wahab, R. A., Basri, M., Rahman, R. N. Z. R. A., Salleh, A. B., Rahman, M. B. A., Chaibakhsh, N. and Leow, T. C. (2014). Enzymatic production of a solvent-free menthyl butyrate via response surface methodology catalyzed by a novel thermostable lipase from *Geobacillus zalihae*. *Biotechnology and Biotechnological Equipment* **28, 1065-1072.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Wahab, R. A., Basri, M., Rahman, R. N. Z. R. A., Salleh, A. B., Rahman, M. B. A., Chaibakhsh, N. and Leow, T. C. (2012). Combination of oxyanion Gln114 mutation and medium engineering to influence the enantioselectivity of thermophilic lipase from *Geobacillus zalihae*. *International Journal of Molecular Sciences* **13, 11666-11680.**

Wang, H. K., Shao, J., Wei, Y. J., Zhang, J. and Qi, W. (2011). A novel low-temperature alkaline lipase from *Acinetobacter johnsonii* LP28 suitable for detergent formulation. *Food Technology and Biotechnology* **49, 96-102.**

Wang, H., Zhong, S., Ma, H., Zhang, J. and Qi, W. (2012). Screening and characterization of a novel alkaline lipase from *Acinetobacter calcoaceticus* 1-7 isolated from Bohai Bay in China for detergent formulation. *Brazilian Journal of Microbiology* **43, 148-156.**

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173, 697-703.**

Wragg, P., Randall, L. and Whatmore, A. M. (2014). Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest. *Journal of Microbiological Methods* **105, 16-21.**

Zheng, X., Chu, X., Zhang, W., Wu, N. and Fan, Y. (2011). A novel cold-adapted lipase from *Acinetobacter sp*. XMZ-26: Gene cloning and characterization. *Applied Microbiology and Biotechnology* **90, 971-980.**

Zottig, X., Meddeb-Mouelhi, F. and Beauregard, M. (2016). Development of a high-throughput liquid state assay for lipase activity using natural substrates and rhodamine B. *Analytical Biochemistry* **496, 25-29.**

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