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

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Received 15 August 2017; Received in revised form 2 August 2018; Accepted 4 August 2018

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...
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 *Herella*) *viginicola*, *Achromobacter*, *Diplococcus BSW*, *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, isoctane, 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%); MgSO₄·7H₂O (0.04%); MgCl₂·6H₂O (0.07%); CaCl₂·2H₂O (0.05%); KH₂PO₄ (0.03%); K₂HPO₄ (0.03 %); (NH₄)₂SO₄ (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.

**Quantitative 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
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 mM dNTPs; 2 mM MgCl₂; 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’-AGAGTTTGATCCTGCGCTCAG-3’) (forward) and rP1 (5’-ACGGTCACCTTGTTACGACTT-3’) (reverse). The 16S rDNA gene amplification was performed for 30 cycles with temperature program as set 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; JX867369.1; 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).
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 Pseudomonas, 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'-ATGACACAAACATGGAGATGAC-3') and LipA2-R (5'-TCAATTGACATTGGGCTTAAACGA-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 MgCl2, 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 Na2HPO4 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
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.

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

+, positive result; –, negative results.

Table 2: Morphological and biochemical characteristics of bacterium KV1.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Results</th>
<th>Properties</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Trehalose</td>
<td>–</td>
<td>D-Celllobiose</td>
<td>–</td>
</tr>
<tr>
<td>D-Maltose</td>
<td>–</td>
<td>Guanidine HCl</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>Niaproof 4</td>
<td>+</td>
</tr>
<tr>
<td>Stachyose</td>
<td>–</td>
<td>L-Lactic acid</td>
<td>–</td>
</tr>
<tr>
<td>pH 6</td>
<td>+</td>
<td>Tetracilium violet</td>
<td>+</td>
</tr>
<tr>
<td>pH 5</td>
<td>–</td>
<td>Glucuronamide</td>
<td>+</td>
</tr>
<tr>
<td>D-Raffinose</td>
<td>–</td>
<td>D-Gluconic acid</td>
<td>–</td>
</tr>
<tr>
<td>α-D-Lactose</td>
<td>–</td>
<td>Muric acid</td>
<td>–</td>
</tr>
<tr>
<td>D-Melibiose</td>
<td>–</td>
<td>Quinic acid</td>
<td>+</td>
</tr>
<tr>
<td>β-Methyl-D-glucoside</td>
<td>–</td>
<td>α-Keto-glutaric acid</td>
<td>+</td>
</tr>
<tr>
<td>D-Salicin</td>
<td>–</td>
<td>Vancomycin</td>
<td>+</td>
</tr>
<tr>
<td>D-Fucose</td>
<td>+</td>
<td>Tetracilium blue</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>–</td>
<td>phenylacetic acid</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetyl neuraminic acid</td>
<td>–</td>
<td>D-Lactic acid methyl ester</td>
<td>–</td>
</tr>
<tr>
<td>1% NaCl</td>
<td>+</td>
<td>Citric acid</td>
<td>+</td>
</tr>
<tr>
<td>4% NaCl</td>
<td>+</td>
<td>D-Saccharic acid</td>
<td>–</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+</td>
<td>Nalidixic acid</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetyl-D-mannosamine</td>
<td>–</td>
<td>Potassium</td>
<td>–</td>
</tr>
<tr>
<td>Inosine</td>
<td>–</td>
<td>Tween 40</td>
<td>+</td>
</tr>
<tr>
<td>1% sodium</td>
<td>+</td>
<td>γ-Amino-butryic acid</td>
<td>+</td>
</tr>
<tr>
<td>lactate</td>
<td>–</td>
<td>Sodium butyrate</td>
<td>+</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>–</td>
<td>Gelatin</td>
<td>–</td>
</tr>
<tr>
<td>D-Serine</td>
<td>+</td>
<td>Acetic acid</td>
<td>+</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>–</td>
<td>Formic acid</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>–</td>
<td>Propionic acid</td>
<td>+</td>
</tr>
<tr>
<td>D-Arabitol</td>
<td>–</td>
<td>Sodium bromate</td>
<td>–</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>–</td>
<td>Lincomycin</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>–</td>
<td>Troleandomycin</td>
<td>+</td>
</tr>
<tr>
<td>D-serine</td>
<td>–</td>
<td>Rifampin SV</td>
<td>+</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>+</td>
<td>Minocycline</td>
<td>–</td>
</tr>
<tr>
<td>Glycyrl-L-proline</td>
<td>–</td>
<td>β-Hydroxy-D,L butyric acid</td>
<td>+</td>
</tr>
<tr>
<td>L-Pyroglutamic acid</td>
<td>–</td>
<td>L-Alanine</td>
<td>+</td>
</tr>
</tbody>
</table>

+, positive result; –, negative results.

Table 3: Purification of the crude lipase from \textit{A. haemolyticus}.

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

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

(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.

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.
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
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.](image)

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)
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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Identity (%)</th>
<th>Source</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>LipA2</td>
<td>98</td>
<td>Acinetobacter sp. XMZ-26</td>
<td>GQ227702.1</td>
</tr>
<tr>
<td>LipAYE</td>
<td>97</td>
<td>A. baumannii AYE</td>
<td>B0V8H7</td>
</tr>
<tr>
<td>LipAB90</td>
<td>97</td>
<td>A. baumannii AB900</td>
<td>AB011284.2</td>
</tr>
<tr>
<td>LipPH-5</td>
<td>96</td>
<td>Pseudomonas fluorescens</td>
<td>Q4KD54</td>
</tr>
</tbody>
</table>

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...
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.

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 °C 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.
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 than those reported for Staphylococcus aureus (pH 6.5), Fusarium oxysporum (pH 7.0) and Bacillus sp. RSJ1 (pH 8.0) (Bora and Bora, 2012) but a little lower than Geobacillus zailhae (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 identity (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,
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 environ-agro-industrial wastes as well as catalysts in synthetic manufacturing processes.

ACKNOWLEDGEMENTS

This work was supported by the Fundamental Research Grant Scheme (FRGS R.J130000.7826.4F649) and the Research University Grant Scheme (Q.J130000.2626.13H09) from the Universiti Teknologi Malaysia, Johor. We would also like to acknowledge valuable help and suggestions provided by our colleagues.

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