Expression and characterization of thermotolerant lipase with broad pH profiles isolated from an Antarctic *Pseudomonas* sp strain AMS3

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A gene encoding a thermotolerant lipase with broad pH was isolated from an Antarctic *Pseudomonas* strain AMS3. The recombinant lipase AMS3 was purified by single-step purification using affinity chromatography, yielding a purification fold of approximately 1.52 and a recovery of 50 %. The molecular weight was approximately ~60 kDa including the strep and affinity tags. Interestingly, the purified Antarctic AMS3 lipase exhibited broad temperature profile from 10-70 °C and stable over a broad pH range from 5.0 to pH 10.0. Various mono and divalent metal ions increased the activity of the AMS3 lipase, but Ni^{2+} decreased its activity. The purified lipase exhibited the highest activity in the presence of sunflower oil. In addition, the enzyme activity in 25 % v/v solvents at 50 °C particularly to n-hexane, DMSO and methanol could be useful for catalysis reaction in organic solvent and at broad temperature

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30   **Abstract**

31   A gene encoding a thermotolerant lipase with broad pH was isolated from an Antarctic

32   *Pseudomonas* strain AMS3. The recombinant lipase AMS3 was purified by single-step purification

33   using affinity chromatography, yielding a purification fold of approximately 1.52 and a recovery of

34   50 %. The molecular weight was approximately ~60 kDa including the strep and affinity tags.

35   Interestingly, the purified Antarctic AMS3 lipase exhibited broad temperature profile from 10-70 °C

36   and stable over a broad pH range from 5.0 to pH 10.0. Various mono and divalent metal ions

37   increased the activity of the AMS3 lipase, but Ni^{2+} decreased its activity. The purified lipase

38   exhibited the highest activity in the presence of sunflower oil. In addition, the enzyme activity in 25

39   % v/v solvents at 50 °C particularly to n-hexane, DMSO and methanol could be useful for catalysis

40   reaction in organic solvent and at broad temperature

42   **1.0 Introduction**

43   Psychrophiles produce cold active enzymes, which are more active at low and moderate

44   temperatures compared to homologous mesophilic enzymes. These enzymes are capable of

45   maintaining the flexibility and dynamics of their active site at low temperatures. The enzymes are

46   highly useful in biotechnological application as they save energy costs, prevent undesired chemical

47   transformation, prevent the loss of volatile compounds and do not require expensive heating or

48   cooling systems [Margesin *et al.,* 2003].

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50   Lipases or triacylglycerol acylhydrolases (EC 3.1.1.3) are long chain fatty acid ester hydrolases and

51   are currently attracting enormous attention because of their biotechnological potential. Lipases

52   occur widely in nature and are largely produced by animals, plants and microbes; microbial lipases

53   are the most studied and commercialized. Microbial lipases have gained huge attention from

54   industry because of their flexibility in temperature, pH and organic solvent [Verma et al., 2012].

55   Lipases have been widely used for biotechnological and industrial applications such as in the food

56   and leather industries, oil processing, environmental management and the production of

57   surfactants, detergents, and pesticides. The temperature stability of lipases has been regarded as

58   the most important characteristic for use in industry. Cold-adapted lipases are largely used in the

59   detergent industry, where cold washing reduces both energy consumption and the wear and tear of

60   textile fibers. These lipases are also preferred in the food industry, as these enzymes can be

61   inactivated at reasonably low temperatures, thereby conserving the nutritional quality of the food.

62   Lipase specificity to catalyze triglycerides in particular has attracted application in nutraceutical

63   industry. Specific structured triacylglycerols (SSTs) [Xuebing *et al.,* 2000] is a product from mainly

64   produced via lipase reactions in which the chemical catalysts are unable to do. They are also used in

65   environmental applications such as wastewater treatment and the bioremediation of fat-

66   contaminated cold environments [Lu *et al.,* 2000].

67   Chemical processes, including biomass conversion, bioremediation and fermentation, are highly

68   desired to be completed at lower temperatures because this approach reduces the risk of

69   contamination by mesophiles and saves on energy consumption [Cotarlet *et al.,* 2011]. In

70   conjunction with these needs, studies focused on enzymes with unique properties (i.e. broad

71   temperature and pH profiles) have gained an increasing interest. To the best of our knowledge,

72   there is no report pertaining to Antarctic lipases exhibiting activities at broad temperature and pH

73   ranges. This current work describes the gene isolation, expression, recombinant lipase purification

74   and characterization of a lipase from Antarctic *Pseudomonas* with broad temperature and pH

75   profiles.

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79   **2.0 Materials and methods**

80   **2.1 Sources of bacteria**

81

82   The bacterium was isolated from Antarctic soil. The isolated denoted as AMS3. Identification was

83   performed using 16SrDNA and homology to *Pseudomonas* spp. The 16SrDNA sequence was sent to

84   NCBI under accession no (**KR821141).** The isolate was screened to produce lipase via qualitative

85   approach using selective media tributyrin, Rhodamine B and Victoria Blue agar plates containing

86   tributyrin, triolen and olive oil respectively as substrate.[Samad *et al.,* 1989]. Lipase producer will

87   hydrolyze the lipid to free fatty acid. Lipolysis is observed directly by changes in the appearance of

88   the substrate such as forming a clearing zone and change in colour of indicator dye used. (Scholze et

89   al., 1999).

90

91   **2.2 Quantitative assay for lipase activity**

92   The lipase assay was performed with a colorimetric method using olive oil as the substrate as

93   previously described by Kwon and Rhee (1986) [Kwon *et al.,* 1986]. A reaction mixture of 1.0 ml of

94   enzyme, 2.5 ml olive oil emulsion (50 % olive oil + 50 % phosphate buffer, the emulsion was mix

95   using homogenizer at 2500 rpm) and 0.02 ml $CaCl_{2.}2H_2O$ was used. The reaction mixture was

96   incubated for 30 min with shaking (250 rpm) at 37 °C. The reaction was stopped by the addition of

97   5.0 ml isooctane. The upper layer (4.0 ml) was moved to a test tube, and 1.0 ml of cupric acetate

98   pyridine pH 6.1 was added. The concentration of free fatty acid dissolved in isooctane was

99   determined by measuring the absorbence at 715 nm. One unit of lipase activity is defined as the

100   rate of release one micromole of free fatty acid in one min.

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105   **2.3 Cloning of the lipase gene**

106   **2.3.1 Genomic library construction**

107   *Pseudomonas* sp. strain AMS3 genomic DNA was extracted using the Qiagen DNA Extraction kit

108   (Qiagen, Germany). Partial digestion of *Pseudomonas* AMS3 genomic DNA was performed using

109   *Sau*3A1 (Fermentas, Germany) to prepare a clonable size of genomic DNA. The plasmid was

110   digested with *Bam*H1 to generate compatible ends for ligation with *Sau*3A1 partially digested

111   genomic DNA. The plasmid DNA (50 μl) was digested with *Bam*H1 for 1 h in a 100 μl reaction

112   mixture that included the following components: (1) distilled water (37 μl), (2) 10X buffer E (10

113   mM Tris-HCl, pH 7.4, 300 mM KCl, and 5 mM $MgCl_2$) (3) 0.1 mM Dithiothreitol (DTT) (10 μl), (40

114   BSA (0.1μg;1 μl), (5) DNA (50 μg), and (6) enzyme (10U; 2 μl). The mixture was incubated at 37 °C

115   for 1 h. The cleaved products were dephosphorylated by the direct addition of calf intestinal

116   alkaline phosphatase (1 U, 1 μl) for 1 h at 37 °C. The enzyme was deactivated by heating at 65 °C for

117   20 min in the presence of EDTA (5 mM). The *Sau*3A1 partially digested genomic DNAs with

118   maximum sizes in the range of 2-9 kb were ligated with the pET 51b vector (*Bam*H1 digested and

119   dephosphorylated).

120

121   **2.3.2 Screening for positive recombinant clones**

122   Putative lipase activity was indicated by the formation of halos around the colonies on tributyrin-

123   ampicillin agar. Cold active lipase activity was confirmed by incubating the plates at 37 °C for 16 h

124   followed by another 72 h of incubation at 4 °C. Colonies that produced clearing zones on tributyrin-

125   ampicillin agar were isolated and restreaked on Victoria Blue agar and Rhodamine B agar plates to

126   confirm lipase activity.

127

128

129 **2.3.3 Gene sequencing**

130 Plasmid from positive transformant was extracted and digested with the BamH1. The insert was

131 predicted around 9 kb. The T7 promoter and terminator was use to sequence the gene via primer

132 walking. Result from the starting primer was sent to 1st BASE Laboratories Sdn Bhd (Shah Alam,

133 Selangor, Malaysia) for primer walking. The primer was designed base on each sequencing result.

134

135 **2.3.4 Analysis of the lipase gene**

136 Lipase sequence analysis was performed using the National Centre of Biotechnology (NCBI)

137 database. Analysis of the lipase gene was performed using Biology Workbench

138 (http://www.biology.ncsa.sdsc.edu) and the Expasy Molecular Biology Server

139 (http://www.expacy.org/tools). The nucleotide sequence of the lipase gene has been submitted to

140 the Genbank database with the accession number **KP744536** and **KT215165.**

141

142 **2.3.5 Expression of recombinant lipase in *E. coli***

143 A cultured derived from a colony harboring the putative lipase gene (*E. coli BL21*/pETlipAMS3) was

144 cultured in LB media containing 50 mg/L ampicillin. A total of 200 ml of LB media was inoculated

145 with transformed cells or empty vector and incubated at 37 °C with shaking at 150 rpm. A total of

146 0.5 mM of IPTG was added to cultures with an A_{600} of 0.5-0.7 and the cells were grown at 20 °C for 8

147 h. Cell growth was measured spectrophotometrically at A_{600}.

148

149 **2.4 Recombinant AMS3 lipase purification**

150 The His-tagged recombinant AMS3 lipase was purified using Nickel Sepharose affinity

151 chromatography. The cell pellet was resuspended using 100 mM Tris-HCl (pH 7.4) binding buffer

152 containing 20 mM imidazole and 0.5 M NaCl. The sonicated cells were centrifuged at 12,000 x g for

153 30 min to separate the crude extract. The crude enzyme was filtered and subjected to affinity

154 columns that were pre-equilibrated with binding buffer. The recombinant enzyme was eluted using

155 elution buffer containing 100 mM Tris-HCl with 0.5 M imidazole and 0.5 M NaCl (pH 7.4). The active

156 fractions were pooled together and the protein homogeneity was determined using SDS-PAGE.

157

158 **2.5 Protein determination**

159 The protein content was measured by the Bradford method [Bradford *et al.,* 1976]; BSA was used as

160 a standard. During chromatography, the protein concentrations in the fractions were monitored by

161 measuring the absorbance at 280 nm.

162

163 **2.6 SDS-PAGE analysis**

164 The purified fractions that showed lipase activity were pooled and subjected to SDS-PAGE

165 according to the method described by Laemmli (1970) [Laemmli *et al.,* 1970]. The sample was

166 electrophoresed on a 12 % resolving gel (2.5 ml 1.5 M Tris-HCl (pH 8.8), 100 μl 10 % SDS, 4.4 ml

167 distilled water, 3 ml Bis-acrylamide (12 %), 50 μl APS and 10 μl TEMED) and a 6 % stacking gel (2.5

168 ml 0.5 M Tris-HCl (pH 6.8), 100 μl 10 % SDS, 5.9 ml distilled water, 1.5 ml 12 % Bis-acrylamide and

169 50 μl APS). Electrophoresis was performed at a constant voltage (210 V) at room temperature.

170 Coomassie Brilliant Blue R 250 was used to stain the gel for 10 min, and then the gel was destained

171 with destaining solution. The molecular mass of the protein was estimated using a protein standard

172 marker (Unstained protein marker from Thermo scientific U.S.A).

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175 **2.7 Characterization of AMS3 lipase**

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177 **2.7.1 Effect of temperature**

178   The effect of temperature on the recombinant lipase was studied by measuring the enzyme activity

179   at varying temperatures (5 °C, 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C) for 30 min at pH 7.

180   The activity was assayed following the method of Kwon and Rhee (1986) and the assay was done

181   using olive oil as substrate. To assess the effect of temperature on the thermostability of the lipase,

182   the enzyme solution was pre-incubated in 50 mM Phosphate buffer (pH 7) at different

183   temperatures (5 °C, 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C) for 30 min and assayed at the

184   optimum temperature.

185

186   **2.7.2 Effect of pH.**

187   The optimum pH of the recombinant lipase was studied by emulsifying the substrate (olive oil) in

188   buffers of different pH values (4.0-12.0). The following buffers (50 mM) were used: (1) sodium

189   acetate (pH 4.0-6.0), (2) potassium phosphate (pH 6.0-8.0), (3) Tris-HCl (pH 8.0-9.0) and (4)

190   glycine-NaOH (pH 9.0-12.0). To assess the pH stability of the lipase, the enzyme solution was pre-

191   incubated in different pH buffers (pH 4.0-12.0) at 50 °C for 30 min, followed by the lipase assay.

192

193   **2.7.3 Effect of metal ions**

194   The effect of metal ions on lipase activity was studied using the method described by Kwon and

195   Rhee (1986) after the enzyme was pre-incubated at 50 °C for 30 min in 50 mM Phosphate buffer

196   (pH 8.0) and either 1 mM or 5 mM of various metal ions in different reaction vessels (Li^+, Rb^+, Na^+,

197   Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, K^+, Zn^{2+}, Ni^{2+} or Co^{2+}).

198

199   **2.7.4 Natural oil specificity**

200   Natural oils (olive oil, corn oil, sun flower oil, canola oil, sesame oil, coconut oil and palm oil) were

201   tested for AMS3 lipase activity. The oils were assayed at 50 °C for 30 min colorimetrically.

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205 **2.7.5 Effect of organic solvents**

206 The effects of organic solvents on lipase activity were determined by measuring the residual

207 activity after pre-incubation of the enzyme with different solvents at a concentration of 25 % (v/v)

208 in 50 mM Phosphate buffer (pH 8.0) at 50 °C for 30 min. The residual activity was measured

209 following the method of Kwon and Rhee (1986) and using olive oil as a substrate. The solvents used

210 had varying *log P* values as shown in parenthesis. The following solvents were used: (1) DMSO (-

211 1.45), (2) methanol (-0.76), (3) acetonitrile (-0.33), (4) ethanol (-0.24), (5) acetone (-0.24),

212 propanol (0.28), (6) chloroform (2.0), (7) benzene (2.0), (8) toluene (2.5), (9) xylene (3.1) and (10)

213 n-hexane (3.5).

214

215 **2.7.6 Statistical analysis**

216 The standard deviations of the triplicate data were performed using deviation (SD) in Microsoft

217 office excel 2010 (Microsoft Corporation USA). The data are mean ± standard deviation of three

218 determinations and indicated as error bars. When the error bar cannot see seen, they are less than

219 the size of symbol.

220

221

222 **2.8 Secondary structure and thermal denaturation measurement of AMS3 lipase using**

223 **Circular dichroism (CD) spectropolarimeter**

224 Circular dichroism (CD) spectra were recorded using JASCO J-810 spectropolarimeter at 25 °C. The

225 purified AMS3 lipase was dialysed over night with 10 mM phosphate buffer pH 7 prior to CD

226 spectral analysis. The secondary content measurement was conducted from wavelength of 190 to

227 260 nm on a 1mm path length. A few temperatures have been set to measure the changes of

228 secondary structure from 10 °C to 90 °C. The protein concentration was 0.1 mg/ml and the cell

229 pathlength 0.1 cm. The data been collected every 1nm (band with) and the data pitch every 0.5 nm.

230 Protein secondary structures content were estimated from the far-UV CD spectra based on

231 webserver, perry.freeshell.org [Raussens *et al.,* 2003]

232

233 The thermal denaturation of AMS3 lipase was measured at 222 nm from 10 °C to 90 °C at a 1

234 °C/min heating rate. Wavelengths 222 nm measures α-helical content of the protein. Data collected

235 from higher wavelengths usually have lower absorption. T_m is defined as a midpoint of sigmoidal

236 melting curves using 0.5 mg/ml protein. The data was collected every 1 degree per min. Data pitch,

237 bandwidth, response, scanning speed, and accumulation were set to be 0.1 degree, 1 nm, 1 seconds,

238 1 degree per min and 3 times, respectively.

239

240

241 **3.0 Results and discussion**

242 **3.1 Lipase gene isolation and expression in *E. coli***

243 To isolate the lipase gene, a genomic library approach was completed. To achieve this, a library was

244 constructed via fragmentation or partial digestion of genomic DNA using *Sau*3A1. The plasmid

245 (pET51b) was linearized using *Bam*HI. Among thousands of transformants from the shotgun

246 cloning, three of them produced a clearing zone. The positive recombinants harboring the putative

247 lipase gene were assumed to have cold active lipolytic activity when the recombinant clone was

248 initially grown at 37 °C for 24 h and transferred at 4 °C. The clearing zone only appeared after 3

249 days of incubation at 4 °C. The colonies were transferred to two selective media plates, triolein and

250 Rhodamine B plates, to confirm lipase gene expression. Colonies 1 and 2 gave a positive result by

251 the intense blue and orange coloration around the colonies. Colony 3, however, did not exhibit such

252 coloration. These results show that these two colonies produced a putative true lipase compared to

253   colony 3 that was a putative esterase producer (data shown at supporting document). To identify a

254   true lipase producer the transformant must able to hydrolyse triglyceride that contain long chain

255   fatty acid (Scholze et al., 1999). Colony 1 and 2 were sent for sequencing and the partial sequence

256   from both colonies shown to contain putative gene from α/β hydrolase family. Colony 1 (denoted as

257   AMS3 lipase) was selected due to the high catalytic activity at 20 °C and was used for the remainder

258   of the study.

259   Generally, protein functional and structural studies often require a large amount of pure, correctly

260   folded protein, which is commonly produced in *Escherichia coli* expression systems. However, these

261   overexpressed proteins are sometimes not efficiently processed by the *E. coli* post-translational

262   machinery, resulting in protein misfolding. A dense insoluble aggregate of misfolded proteins is

263   generally known as inclusion bodies (IBs). Most of the lipases from *Pseudomonas* are expressed in

264   the form of IBs. Even under the control of pET-25b(+), a vector containing a signal sequence

265   responsible for the translocation of expressed protein to the periplasm, the lipases from

266   *Pseudomonas* sp. MIS38 and *Pseudomonas* sp. strain KB700A were still overexpressed in non-active,

267   insoluble forms [Claudia *et al.,* 2009 and Rashid *et al.,* 2001]

268
269   **3.2 Lipase sequence analysis**
270
271   Putative plasmid contained recombinant lipase AMS3 was sent for sequencing. A few sets of primer

272   were used to complete the sequence. The presence for open reading frame (ORFs) was analyzed

273   using the ORF finder software from the NCBI. One open reading frame (ORF) was found. With a

274   predicted size of 1353 bp encoding a 450 amino acid was determined**.** The protein sequence is

275   made up by two different domains namely the GST C (KT215165) domain and lipase (KP744536)

276   domain at the N and C terminal sections respectively. The lipase (AMS3 lipase) molecular mass

277   without the GST C sequence was predicted to be 43.6 kDa with pI value of 6.3. The BLASTp result

278   revealed that AMS3 lipase showed homology to α/β hydrolase family. Multiple sequence alignment

279   was done using Biology Workbench software to predict the conserve region of the lipase. Aligment

280   between known lipase from various microorganisms exhibited the conserved region of the lipase

281   **(Fig 1)**. A typical lipase conserved region contains the pentapeptide (GXSXG) sequence. The lipase

282   catalytic triad which made up by Ser, Asp and His were also conserved. No signal peptide sequence

283   was observed as predicted using SignalP software. Similarly, Group III lipases including lipases

284   from *Pseudomonas fluorescens* and *Serratia marcescens* has no signal peptide. The protein origin can

285   be reveal from G+C content. The G+C content was calculated using http: www.watson.nih.go.jp

286   (FramePlot 2.3.2). AMS3 lipase content 55 % of G+C almost similar to lipase from *Pseudomonas*

287   *fragi (*59.3 %) [Claudia *et al.,* 2009]. The N terminal section of AMS3 lipase contains a GST C

288   (glutathione-S-transferase C family) similar to *Pseudomonas* Ag1. Glutathione have been reported

289   as essential factor for chaperon to activate the lipase from *Pseudomonas*.[Rosenau *et al.,* 2000 and

290   Tanaka *et al.,* 2000]. According to Tanaka and co-workers, glutathione was found to be important

291   for 'lipase activator factor' or *Lif* protein from *Pseudomonas* sp. strain 109 to fold an active form

292   [Tanaka et al., 2000].

293

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295

296   **Fig 1**: Alignment of the AMS3 lipase sequence with *Pseudomonas* lipases of known three-
297   dimensional structure. The AMS3 was aligned with *P. aeruginosa* lipase (Q9L6C7),*P. fragi* lipase
298   (AY787823), *G. bacillus* T1 (AY166603) and *Proteus* sp. K107. - =Serine Pentapeptide, @= Aspartic
299   acid and ^=Amino acid involve in calcium binding. (*) blue: Conserved residue; (:) green:
300   conservation of strong groups; (.) blue: conservation of weak groups.
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304   **3.3 Purification of AMS3 lipase**

305   *E. coli* BL21 cells (DE3) harboring the pET51b/lipAMS3 plasmid was used to overexpress the

306   recombinant AMS3 lipase under the control of a T7 promoter. The crude AMS3 lipase containing

307   10x histidine and Strep tags was produced in a soluble form allowing for rapid purification of the

308   recombinant protein. The addition of fusion affinity tags and GST C *Pseudomonas* A3 increased the

309   size of AMS3 lipase to ~60 kDa. Purification was accomplished using the XK16 affinity

310   chromatography column (GE Healthcare, USA) packed with Nickel Sepharose resin. After the

311   column was equilibrated with binding buffer, the crude sample was loaded onto the column. Elution

312   was accomplished using increasing concentrations of imidazole. Pooled fractions resulted in a 50 %

313   yield. Some protein was retained in the low-yield fractions and not included in the pooled fraction,

314   resulting in some loss. Pooled fractions had an overall purification factor of approximately 1.52 fold.

315   In 2007, Yan et al. reported that their organic solvent-stable and thermostable lipase from

316   *Galactomyces geotrichum* Y05 had up to a 32 % recovery and a 3.2-fold purification factor using

317   ammonium sulfate, an ion exchange column and gel filtration purification [Yan *et al.,* 2007]. SDS-

318   PAGE analysis indicated that the molecular weight of the AMS3 lipase was approximately ~60 kDa

319   **(Fig. 2)**. Generally, lipases have molecular weights in the range of 16 to 69 kDa. According to

320   Schmidt-Dannert (1999), the *Staphylococcal* lipase family, which includes lipase BTL-2 from *B.*

321   *thermocatenulatus*, possesses molecular weights of 40 to 45 kDa [Schmidt-Dannert 1999].

322

323

324   **Fig 2**: SDS and native polyacrylamide gel electrophoresis (PAGE) profile of recombinant AMS3
325   lipase. Lane 1 : Crude lipase; Lane 2: Protein molecular weight marker(Fermentas, Germany); Lane
326   3: Purified AMS3 lipase; Homogeneity of AMS3 lipase was determined via native PAGE (Lane 4).The
327   mobility of the purified lipase was estimated around ~60kDa (indicated by arrow). The protein
328   loaded was approximately around 13μg.
329
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331

332   **3.4 Biochemical Characteristics of AMS3 lipase**

333   **3.4.1 Effect of temperature.**

334   Lipase activity was determined from 5 to 70 °C. The optimum temperature for the lipase was

335   activity was at 50 °C, and the activity dropped above 60 °C (**Fig. 3A**). Interestingly, the purified

336   lipase exhibited a broad temperature profile, whereby the lipase was still active at temperatures

337   ranging from 10-60 °C. This finding is unlike the typical characteristics of cold active lipase. Most

338   cold active lipases have been found to be active at temperature ranges from 15-35 °C [Gerday *et al.,*

339   2000]. Few "however" have reported the broad temperature observation from

340   psychrophilic/psychrotolerant bacteria. A *Pseudomonas* lipase from Greenland exhibited an almost

341   similar activity at wide temperatures [Schmidt *et al.,* 2010]. In contrast, AMS3 lipase exhibited

342   higher optimum activity compared to other reported lipases isolated from psychrophilic bacteria. r-

343   *LipA* from *Sorangium cellulosum* [Cheng *et al.,* 2011], *lipA1* from *Psychrobacter* sp. 7195 [Zhang *et*

344   *al.,* 2009], lipase from *Pseudomonas fragi* strain IFO 3458 (PFL) [Alquati *et al.,* 2002] and *lipX* from

345   *Psychrobacter* sp. C18 [Alquati *et al.,* 2011] were all reported to show maximum activity at 30 °C.

346   AMS3 lipase was mostly stable at 30 °C and below (**Fig. 3B**). The organic solvent-stable and

347   thermostable lipase from *Galactomyces geotrichum* Y05 was stable below 60 °C at a pH of 8.0 for at

348   least 12 h; however, above 60 °C, rapid inactivation occurred with only 36 % residual activity.

349   These characteristics differ from those of the AMS3 lipase and may be due to the effects of the

350   nature of the enzyme itself, which originated from Antarctica.

351

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353

354   **Fig 3**: Effect of temperature on purified AMS3 lipase activity. The lipase assay was done using olive
355   oil as a substrate. (A). Enzyme samples were assayed at various temperatures as mentioned in the
356   Methods section. The temperature that showed the highest lipase activity was considered to be the
357   optimum temperature for the AMS3 lipase. The effect of temperature on purified AMS3 lipase
358   stability (B). Enzyme stability was tested at different temperature. The enzyme was incubated at
359   various temperatures in 30 min. The assay was performed at optimum temperature. The relative
360   activity was calculated using 30 °C as 100%.
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364

3.4.2 Effect of pH

The broad temperature range of AMS3 lipase showed activity in the pH range from 5.0 to 10.0; the optimum activity was seen at pH 8.0 (**Fig. 4A)**. Lipase activity was reduced above a pH of 8.0, with a relative activity of 34 % at pH 9.0. Most of the studied *Pseudomonas* lipases have been known to be active around pH 7 to 8, stable at pH ranging from 6 to 9. To date, only a few lipases reported an optimum activity at pH 9 to 10. The cold-adapted lipase from *Psychrobacter* sp. 7195 [Zhang *et al.,* 2007] and a mesophilic lipase from *Pseudomonas fluorescens* JCM5963 [Zhang *et al.,* 2009] reported an optimum activity at pH 9, "whereas" lipases from *Acinetobacter* sp. XMZ-26 (cold-adapted) [Zheng *et al.,* 2011] and *Pseudomonas fluorescens* AK102 [Kojima *et al.,* 2003] reported an optimum activity at pH 10. The AMS3 lipase was stable from pH 5.0 to 10.0 after incubation for 30 min at 50 °C (**Fig. 4B**). Extracellular lipase from *Thermomyces lanuginosus* was also reported to be stable at in broad range alkaline pH from pH 8-12 [Zheng et al., 2011]. In contrast with AMS3 lipase the enzyme was stable from acidic to alkaline condition. These properties make the enzyme more valuable in detergent and oleochemical industries. Enzymes are proteinaceous, meaning that they have amino acids whose ionization can be affected by pH. The primary and secondary structures of the enzymes can be affected, thereby affecting the activity. The primary and secondary structures of the AMS3 lipase may be influencing these pH and temperature stabilities, leading to the variation in the ionization of the native AMS3 lipase enzyme at different pH.

383

384

Fig 4: Effect of pH on purified AMS3 lipase activity (A)**.** The lipase activity was measured at different pH using olive oil emulsion as substrate and assay at 50 °C for 30 min**.** Effect of pH on purified AMS3 lipase stability is shown in figure (B)**.** The enzyme was pre-incubated at different buffer for 30 min. The lipase assay was performed at 50 °C**.** Sodium acetate buffer was used for the

389   pH range of 4.0 to 6.0 (♦); potassium phosphate buffer for the pH range of 6.0 to 8.0 (■); Tris-HCl
390   buffer for the pH range of 8.0 to 9.0 (▲); and glycine-NaOH buffer for the pH range of 9.0 to 12.0.
391   (x). The relative activity was measure using pH 7 as 100%.
392

393

394   **3.4.3 Effect of metal ions**

395   The effect of metal ions was studied to determine which metal ions could enhance or reduce the

396   activity of AMS3 lipase. All of the metal ions studied enhanced lipase activity at 1 and 5 mM **(Fig. 5)**.

397   For Li^+ and Rb^+ lipase activity was heavily enhanced, with relative activities over then 160 %. Li^+

398   have been reported to improve the enantioselectivity of the lipase (Okamoto et al., 2006). Na^+ and

399   K^+ were also found to enhance AMS3 lipase activity. Metal ion activation of enzymes is important in

400   industrial applications for obtaining maximal catalytic efficiency. However, at 5 mM concentration,

401   Li^+, Ni^{2+} and Zn^{2+} reduced lipase activity to less than 50 %. In contrast, Sharma et al. (2002)

402   reported that Ca^{2+} ions increased the lipase activity of *Bacillus* sp. RSJ-1 by 16 %, similar to the

403   lipase activity of *B. coagulans* MTCC-6375 [Sharma *et al.,* 2002].

404

405

406

407

408   **Fig 5:** Effect of metal ions on AMS3 lipase stability**.** All assay was done using olive oil as substrate**.**
409   The enzyme was pre-incubated at 50 °C with various metal ions at concentrations of 1 mM (blue)
410   and 5 mM (red). The treated enzyme was assay at 50 °C for 30 min. The untreated enzyme was
411   considered as a control or 100 %.
412

413

414   **3.4.4 Substrate specificity.**

415   Assays addressing the specificity of the AMS3 lipase towards natural oils were conducted. The

416   results showed that the AMS3 lipase hydrolyzed all of the natural oils tested, and the highest

417   activity was seen with sunflower oil (**Fig. 6**). Olive oil is composed of mixed triglycerides

418 (monounsaturated C18:1). Most lipases show a preference for long-chain fatty acids. The

419 extracellular lipase Lip2 from *Yarrowia lipolytica* showed a much higher lipolytic activity toward

420 triglycerides in olive oil and safflower oil than toward hydrophilic esters such as methyl fatty acid

421 asters [Jaeger *et al.,* 1999].

422

423 The AMS3 lipase displayed the second highest activity with coconut oil, with a relative activity of

424 110 %, followed by canola oil, olive oil and sesame oil with relative activities of 106 %, 100 % and

425 90 %, respectively. Similarly, AMS3 lipase exhibited the same substrate specificity with an

426 extracellular lipase from *Pseudomonas sp* S5 [Rahman et al., 2005]. In contrast, a *Pseudomonas*

427 *aeruginosa* EF2 lipase reported olive oil as its preferred substrate [Gilbert et al., 1991]. AMS3 lipase

428 has a broader substrate specificity ranging from C12 to C18. The ability of AMS3 lipase to hydrolyze

429 long carbon chain triglycerides (C12 above) showed that the enzyme is a true lipase (Jaeger, et al.,

430 1994). According to Jaeger et al. (1994), surface pressure and lipid distribution are physical factors

431 that can impact the determinants of lipase activity [Hiol *et al.,* 1999]. Therefore, as reported by Hiol

432 et al. (1999), the differences in substrate specificity can arise not only from specific substrate

433 binding but also from differential activation of the enzyme at the interface [Lanser *et al.,* 2001].

434

435

436 **Fig 6**: Substrate specificity of purified AMS3 lipase toward natural oil. The assay was performed at
437 50 °C for 30 min. Substrate emulsion prepared with various natural oil with ratio 1:1 (buffer and
438 natural oil). Data shown are the means from triplicate measurements expressing % activity relative
439 to that with olive oil.
440

441

442 **3.4.5 Effect of organic solvents**

443 Persson et al., (2002) reported that the use of organic solvents in enzymatic catalysis is now well

444 recognized with several advantages in water-poor mediums including improved solubility of

445   hydrophobic substrates, a shift in thermodynamic equilibrium to synthesis from hydrolysis and

446   increased thermostability of the enzyme [Persson *et al.,* 2002]. Assays measuring the effects of

447   solvents were conducted to understand AMS3 lipase activity in organic solvents. The enzyme was

448   incubated in a 25 % v/v solvent at a temperature of 50 °C (**Fig. 7**). The results showed that AMS3

449   lipase exhibited some enhancements in stability expressed as relative activity in 25 % v/v solvent

450   compared to the control (without solvent). For instance, AMS3 lipase has a relative activity of 74 %,

451   89 % and 116 % in DMSO, methanol and n-hexane, respectively, compared with its original activity

452   at a temperature of 50 °C. The stability in the said organic solvents makes AMS3 lipase a good

453   candidate for synthesis of biodiesel and transesterification reactions. Lipases have a sheath of

454   water molecules tightly bound to them. This sheath protects the enzyme's hydrophilic surface and

455   possibly retains the native conformation even in non-polar solvents. Moreover, the changes in

456   enzyme stability in organic solvents are also greatly affected by the structure of the enzymes

457   (Gomez-puyou., 1992).

458

459   **Fig 7**: Effect of solvents on purified AMS3 lipase. All the assay was done using olive oil as a
460   substrate. Incubation time for treated enzyme with organic solvent was 30 min and assay at 50 °C.
461   Remaining lipase activity was expressed relative to that of the control (without solvent). Log P
462   (water/octanol coefficient) value of each solvents are represented in parenthesis.
463

464

465   **3.5 Secondary structure analysis of AMS3 lipase**
466

467   As illustrated in **Fig. 8A**, the resulting denaturation (sigmoidal graph shape) curve indicated a

468   structural transition of AMS3 lipase within the tested temperature range. The T_m value for AMS3

469   lipase for this transition was 62 °C **(Fig. 8A)**. The Tm measurement using circular dichroism is close

470   to the AMS3 lipase deactivation profile, which was at 55 °C. CD measurements have been widely

471   used to follow the equilibrium between helical structures and unordered conformations. The CD

472 spectra (molecular ellipticity) of AMS3 lipase was analyzed as a function of temperature between

473 190 and 260 nm. The wavelength 220 nm was set to monitor the transition of α-helical to

474 unordered structures as they exhibited characteristic signals at this wavelength. The high-tension

475 voltage (HT) value gradually increased until the T_m of AMS3 lipase and then decreased as the lipase

476 unfolded above the melting temperature point. This outcome might be due to loss of protein

477 secondary structure, followed by an increase in unordered conformations. This observation was

478 supported by the secondary structure content determination at different temperatures. As depicted

479 in **Fig. 8B**, the increased formation of loop structures or disordered regions were observed notably

480 at temperatures from 50-90 °C. In fact, AMS3 lipase exhibited a decreasing content of the alpha

481 helices. This behavior could turn out to be the deactivation factor for AMS3 lipase. The enzyme

482 gradually lost its activity beyond 50 °C. The random secondary structure conformation appears to

483 be increasing as temperature drops together with β-pleat contents. The increased loop

484 confirmation was found to be one of the factors for enzymes to work at low temperatures [Amico *et*

485 *al.,* 2002]. These findings could explain the AMS3 lipase broad temperature profile.

486

487 **Fig 8**: AMS3 lipase melting point and secondary content determination. Vertical line (arrow)
488 indicating the melting point value of AMS3 lipase when tested from temperature ranging from 10-
489 90 °C. (Figure 8A). Changes on the secondary structure content of AMS3 lipase were notably
490 observed as the enzyme was subjected at different temperature (Figure 8B)
491

492

493

494 **4.0 Conclusion**

495 The AMS3 lipase was successfully purified via single-step affinity chromatography. The purified

496 AMS3 lipase exhibited optimum lipolytic activity at pH 8 with a broad temperature profile from 10-

497 70 °C which is unique for an enzyme isolated from Antarctic microorganism. In addition, the

498 enzyme had shown to be stable when treated with different buffer condition ranging from pH 5-10.

499 This factor will make the enzyme very useful for detergent industry. As shown with other lipases,

500 the AMS3 lipase efficiently hydrolyzed unsaturated fatty acids and sunflower oil with the highest

501 activity. Various divalent and monovalent metal ions increased the catalytic activity of the AMS3

502 lipase, but Ni^{2+} completely decreased its activity. Additionally, the AMS3 lipase exhibited stability in

503 25 % v/v of n-hexane, methanol and DMSO at 50 °C. These unique properties are believed to

504 provide tremendous economic benefits in pharmaceuticals, cosmetics and synthesis of functional

505 lipids as well as other low-high temperature industrial applications.

506

507

508 **5.0 References**

509 1. Alquati, C., Gioia, L.D., Santarossa, G., Alberghina, L., Fantucci, P., Lotti, M., 2002. The cold-active
510 lipase of *Pseudomonas fragi*. Eur J Biochem. 269: 3321-3328
511
512 2. Amico, S.D., Claverie, P., Collins, T., Georlette, D., Gratia, E., Hoyoux, A., Meuwis, M.A., Feller,
513 Gerday, C., 2002. Molecular basis of cold adaptation. Philos T R Soc B. 357: 917-925
514
515 3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities
516 of protein using the principle of protein-dye binding. Anal Biochem. 72: 248-254
517
518 4. Chen, R., Guo, L., Dang, H., 2011. Gene cloning, expression, and characterization of a cold-adapted
519 lipase from a psychrophilic deep-sea bacterium *Psychrobacter* sp. C18. World J Micro Biot. 27: 431-
520 441
521
522 5. Cheng, Y.Y., Qian, Y., Li, Z.F., Wu, Z.H., Liu, H., Li, Y.Z., 2011. A novel cold-adapted lipase from
523 Sorangium   cellulosum   strain   So0157-2:   Gene   cloning,   expression   and   enzymatic
524 characterization.Int J Mol Sci. 12: 6765-6780
525
526 6. Claudia, A., Luca, D.G., Gianluca, S., Lilia, A., Piercarlo, F., Marina, L.,2009 The cold-active lipase of
527 *Pseudomonas fragi* Heterologous expression, biochemical characterization and molecular modeling.
528 Eur J Biochem. 269,13:3321–3328,
529
530 7. Cotarlet, M., and Bahrim, G.E., 2011. Optimization of cold-adapted amylases and protease
531 production by psychrotrophic *Streptomyces* 4 Alga using response surface methodology. Truk J
532 Biochem. 36(2): 83-92
533
534 8. Gilbert, E. J., Cornish, A., Jones, C. W. 1991. Purification and properties of extracellular lipase from
535 Pseudomonas aeruginosa EF2. J Gen Microbiol. (1991), 137, 2223-2229.
536

537 9. Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J.P., Claverie, P., Collins, T., Amico, D.S., Dumont, J.,
538 Garsoux, G., Georlette, D., 2000. Cold-adapted enzymes: From fundamentals to biotechnology.
539 Trends Biotechnol.18: 103-107

540

541 10. Gomez-puyou. A., 1992. Biomolecules in organic solvent. U. S. A. CRC Press.

542

543 11. Hiol, A., Jonzo, M.D., Druet, D., Comeau, L., 1999. Production, purification and characterization of
544 an extracellular lipase from *Mucor hiemalis f. hiemalis*. Enzyme Micr. 25: 80-87.

545

546 12. Jaeger, K.E., Dijkstra, B.W., Reetz, M.T., 1999. Bacterial biocatalysts: Molecular biology, three
547 dimensional structures, and biotechnological applications of lipases. Annu Rev Microbiol. 53. 315-
548 351

549

550 13. Kojima, Y., Kobayashi, M., Shimizu, S., 2003. A novel lipase from *Pseudomonas fluorescens*
551 HU380: gene cloning, overproduction, renaturation-activation, two-step purification, and
552 characterization. J Biosci Bioeng. 96(3): 242-249

553

554 14. Kwon, D. Y. and Rhee, J. S., 1986. A simple and rapid colorimetric method for determination of
555 free fatty acids for lipase assay. J Am Oil Chem Soc. 63(1): 89-92

556

557 15. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of
558 bacteriophage T4. Nature 227: 680-685

559

560 16.Lanser, A.C., Manthey, L.K., Hou, C.T., 2001. Regioselectivity of new bacterial lipases determined
561 by hydrolysis of triolein. Curr Microbiol. 44 (5) : 336–340

562

563 17. Lu, M., Wang, S., Fang, Y., Li, H., Liu, S., Liu, H., 2010. Cloning, expression, purification and
564 characterization of cold-adapted α-amylase from *Pseudoalteromonas arctica* GS230. Protein J. 29:
565 591-597

566

567 18. Leow, T. C., Rahman, R. N. Z. R. A., Basri, M., and Salleh, A. B., 2007. "A thermoalkaliphilic lipase
568 of *Geobacillus* sp. T1," Extremophiles, vol. 11, no. 3, pp. 527–535,

569

570 19. Margesin, R., Gander, S., Zacke, G., Gounot, A. M., Schinner, F., 2003. Hydrocarbon degradation
571 and enzyme activities of cold-adapted bacteria and yeasts. Extremophiles . 7:451-458

572

573 20. Persson, M., Mladeoska, I., Wehtje, E., Adlercreutz, P., 2002. Preparation of lipases for use in
574 organic solvents. Enzym. Microb. Techn. 31: 833-841

575

576 21. Rashid, N., Shimada, Y., Ezaki, S., 2001. Atomi H. Imanaka T. Low-temperature lipase from
577 psychrotrophic *Pseudomonas* sp. Strain KB700A. Appl Environ Micro. 67(9): 4064-4069

578

579 23. Rahman, R.N.Z.R.A., Baharum, S. N., Basri. M., Salleh, A. B., 2005. High-yield puriWcation of an
580 organic solvent-tolerant lipase from Pseudomonassp. strain S5. Anal. Biochem. 341 (2005) 267–
581 274

582

583 24. Raussens, V., Ruysschaert, J.M., Goormaghtigh, E., 2003. Protein concentration is not an absolute
584 prerequisite for the determination of secondary structure from circular dichroism spectra: a new
585 scaling method. Anal Biochem. 114-21

586

587   25. Rosenau, F., Jaeger, K.E., 2000. Bacterial lipases from *Pseudomonas*: regulation of gene
588   expression and mechanisms of secretion. Biochimie. 82: 1023–1032.
589
590   26. Samad, M.Y.A., Razak, C.N.A., Salleh, A.B., Yunus, W.M.Z.W., Ampon, K. and Basri, M., 1989. A plate
591   assay for primary screening of lipase activity. J Microbiol Meth. 9: 51-56
592
593   27. Schmidt, M., Larsen, D.M., Stougaard, P., 2010. A lipase with broad temperature range from an
594   alkaliphilic gamma-proteobacterium isolated in Greenland. Environ Technol 31(10):1091-100
595
596   28. Schmidt-Dannert C., 1999. Recombinant microbial lipases for biotechnological applications.
597   Bioorgan Med Chem. 7,10: 2123-2130.
598
599   29. Scholze, H., Stutz, H., Paltauf, F., Hermetter, A., 1999. Fluorescent inhibitors for the qualitative
600   and quantitative analysis of lipolytic enzymes. Anal. Biochem. 276:72–80.
601
602   30. Sharma, R., Soni, S., Vohra, R., Gupta, L. And Gupta, J., 2002. Purification and characterization of a
603   thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ-1. Process Biochem.37: 1075-
604   1084.
605
606   31. Sugihara, A., Shimada, Y., Tominaga, T., 1991. A novel *Geotrichum candidum* lipase with some
607   preference for the 2-position on a triglyceride molecule. Appl Microbiol Biotechnol. 35(6):738-740.
608
609   32. Tanaka, J., Nihira, T., Yamada, Y., 2000. Glutathione as an essential factor for chaperon-mediated
610   activation of lactonizing lipase (LipL) from *Pseudomonas* sp. 109. J Biochem. 127(4):597-601.
611
612   33. Verma, N., Thakur, S., Bhatt, A.K., 2012. Microbial Lipases: Industrial Applications and
613   Properties. Int J Biol Sci. 1(8), 88-92
614
615   34. Xuebing, X., 2000. Production of specific-structured triacylglycerols by lipase-catalyzed
616   reactions: a review. Eur J Lipid Sci Tech. 2000; 287–303
617
618   35. Yan, J., Yang, J., Xu, L., Yan, Y., 2007. Gene cloning, overexpression and characterization of a novel
619   organic solvent tolerant and thermostable lipase from *Galactomyces geotrichum* Y05. J Mol Catal B-
620   Ezym. 49:28-35.
621
622   36. Zhang, A., Gao, R., Diao, N., Xie, G., Gao, G., Cao, G., 2009. Cloning, expression and characterization
623   of an organic solvent tolerant lipase from *Pseudomonas fluorescens* JCM5963. J Mol Catal B-Enzym.
624   56: 78-84
625
626   37. Zhang, A., Gao, R., Diao, N., Xie, G., Gao, G., Cao, G., 2009. Cloning, expression and characterization
627   of an organic solvent tolerant lipase from *Pseudomonas fluorescens* JCM5963. J Mol Catal B-Enzym.
628   56: 78-84
629
630   38. Zhang, A., Wei, J., Lin, S., Zeng, R.Y., 2007. Cloning, expression and characterization of a cold-
631   adapted lipase gene from an Antarctic deep-sea psychrotrophic bacterium, *Psychrobacter* sp. 7195. J
632   Microbiol Biotechn. 17(4): 604-610
633

634   39. Zheng, Y. Y., Guo, X. H., Song, N. N., Li. D. C., 2011. Thermophilic lipase from Thermomyces
635   lanuginosus: Gene cloning, expression and characterization. J. Mol. Catal. B: Enzym. 69 (2011)
636   127–132.
637
638   40. Zheng, X., Chu, X., Zhang, W., Wu, N., Fan, Y., 2011. A novel cold-adapted lipase from *Acinobacter*
639   sp. XMZ-26: gene cloning and characterization. Appl Microbiol Biot. 90: 971-980
640

Alignment of the AMS3 lipase sequence.

Fig 1: Alignment of the AMS3 lipase sequence with *Pseudomonas* lipases of known three-dimensional structure. The AMS3 was aligned with *P. aeruginosa* lipase (Q9L6C7),*P. fragi* lipase (AY787823), *G. bacillus* T1 (AY166603) and *Proteus* sp. K107. - =Serine Pentapeptide, @= Aspartic acid and ^=Amino acid involve in calcium binding. (*) blue: Conserved residue; (:) green: conservation of strong groups; (.) blue: conservation of weak groups

```
AMS_lipase            ----------------------------------------VPRGSASLRA
chain_A_t1            -------------------------------------------SLRA
pseudomonas_fragi     ------------------------------------------MDDSVN
pseudomonas_arg_Q9L6C7  -----------------MKKKSLLPLGLAIGLASLAASPLIQASTYTQ
proteus_sp_k107       -------------------------------------------MS
pseudomonas_flo       METGIFDYKNLGTEGSKTLFADAMETAITLYSYHNLDNGFAVGYQHNGLG

AMS_lipase            NDAPIVLLHGFTGWGREE--MFGFKYWGGVRGDIEQWLNDNGYRTYTLAV
chain_A_t1            NDAPIVLLHGFTGWGREE--MFGFKYWGGVRGDIEQWLNDNGYRTYTLAV
pseudomonas_fragi     TRYPILLVHGLFGFD--R--IGSHHYFHGIK----QALNECGASVFVPII
pseudomonas_arg_Q9L6C7  TKYPIVLAHGMLGFD--N--ILGVDYWFGIP----SALRRDGAQVYVTEV
proteus_sp_k107       TKYPIVLVHGLAGFN--E--IVGFPYFYGIA----DALRQDGHQVFTASL
pseudomonas_flo       LGLPATLVGALLGSTDSQGVIPGIPWNPDSEKAALEAVQKAGWTPISASA
                          *    *   .: *      .  :   .  .  . .:.   *

AMS_lipase            GPLSSNWDRACEAYAQLVGGTVDYGAAHAAKHGHARFGRTYPGLLPELKR
chain_A_t1            GPLSSNWDRACEAYAQLVGGTVDYGAAHAAKHGHARFGRTYPGLLPELKR
pseudomonas_fragi     SAANDNEARGDQLLKQIHN----------------------LRRQVG
pseudomonas_arg_Q9L6C7  SQLDTSEVRGEQLLQQVEE----------------------IVALSG
proteus_sp_k107       SAFNSNEVRGKQLWQFVQT----------------------LLQETQ
pseudomonas_flo       LGYAGKVDARGTFFGEKAGYTTAQVEVLGKYDDAGKLLEIGIG--FRGTS
                            .

AMS_lipase            GGRIHIIAHSQGGQTARMLVSLLENGSQEERE------YAKAHNVSLSPL
chain_A_t1            GGRIHIIAHSQGGQTARMLVSLLENGSQEERE------YAKAHNVSLSPL
pseudomonas_fragi     AQRVNLIGHSQGALTARYVAAIAP-------------------------
pseudomonas_arg_Q9L6C7  QPKVNLIGHSHGGPTIRYVAAVRP-------------------------
proteus_sp_k107       AKKVNFIGHSQGPLACRYVAANYP-------------------------
pseudomonas_flo       GPRETLISDSIGDLISDLLAALGPKDYAKNYAGEAFGGLLKNVADYAGAH
                        :   :*..* *        :.:

AMS_lipase            FEGGHHFVLSVTTIATPHDG---------------TTLVNMVDFTDRFFD
chain_A_t1            FEGGHHFVLSVTTIATPHDG---------------TTLVNMVDFTDRFFD
pseudomonas_fragi     -----ELIASVTSVSGPNHG---------------SELADRLRLAFVPGR
pseudomonas_arg_Q9L6C7  -----DLIASATSVGAPHKG---------------SDTADFLR-QIPPGS
proteus_sp_k107       -----DSVASVTSINGVNHG---------------SEIADLYRRIMRKDS
pseudomonas_flo       GLTGKDVVVSGHSLGGLAVNSMETADLSNYKWAGFYKDANYVAYASPTQS
                          . :  *  ::       .                     .:

AMS_lipase            LQKAVLEAAAVASNVPYTSQVYDFKLDQWGLRRQPGE-SFDHYFERLKRS
chain_A_t1            LQKAVLEAAAVASNVPYTSQVYDFKLDQWGLRRQPGE-SFDHYFERLKRS
pseudomonas_fragi     LGETVAAALTTSFSAFLSALSGHPRLPQNALNALNAL-TTDGVAAFNRQY
pseudomonas_arg_Q9L6C7  AGEAILSGLVNSLGALISFLSSGSTGTQNSLGSLESL-NSEGAARFNAKY
proteus_sp_k107       IPEYIVEKVLNAFGTIISTFSGHRGDPQDAIAALESL-TTEQVTEFNNKY
pseudomonas_flo       AGDKVLNIGYENDPVFRALDGSSFNLSSLGVHDKPHESTTDNIVSFNDHY
                         . :              . :          . .:       . :         :

AMS_lipase            PVWTSTDTARYDLSVSGAEKLNQWVQASPNTYYLSFSTERTYRGALTGNH
chain_A_t1            PVWTSTDTARYDLSVSGAEKLNQWVQASPNTYYLSFSTERTYRGALTGNH
pseudomonas_fragi     PQGLPDRWG-----GMGPAQVN-------AVHYYSWS------GIIKGSR
pseudomonas_arg_Q9L6C7  PQGIPTSAC-----GEGAYKVN-------GVSYYSWS----------GS-
proteus_sp_k107       PQALPKTPG-----GEGDEIVN-------GVHYYCFG------SYIQGLI
pseudomonas_flo       ASTLWNNVLP------FSIVNLPTWVSHLPTAYGDGMETTRILESGFYDQM
                         .            . :          .          .
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                                                          @
AMS_lipase            YPELGMNAFSAVVCAPFLGSYRNPTLGIDDRWLENDGIVNTVSMNGPKRG
chain_A_t1            YPELGMNAFSAVVCAPFLGSYRNPTLGIDDRWLENDGIVNTVSMNGPKRG
pseudomonas_fragi     LAESLN------LLDPLHNALRVFDSFFTRETRENDGMVGRFSSH-----
pseudomonas_arg_Q9L6C7 -SPLTN------FLDPSDAFLGASSLTFKNGT-ANDGLVGTCSSH-----
proteus_sp_k107       AGEKGN------LLDPTHAAMRVLNTFFTEKQ--NDGLVGRSSMR-----
pseudomonas_flo       ETTRDSTVIVANLSDPARANTWVQDLNRNAEPHKGNTFIIGSDGNDLIQG
                          .    *                          .: ::    .  .
                                                 ^
AMS_lipase            SSDRIVPYDGTLKKGVWNDMGTYNVDHLEIIG----VDPNPSFDIRAFYL
chain_A_t1            SSDRIVPYDGTLKKGVWNDMGTYNVDHLEIIG----VDPNPSFDIRAFYL
pseudomonas_fragi     -----------LGQVIRSDYPLDHLDTINHMA----RGSRRRINPVELYI
pseudomonas_arg_Q9L6C7 -----------LGMVIRDNYRMNHLDQVNQVFG---LTSLFETSPVSVYR
proteus_sp_k107       -----------LGKLIKDDYAQDHIDMVNQVAG---LVG-YNEDIVAIYT
pseudomonas_flo       GNGADFIEGGKGNDTIRDNSGHNTFLFSGHFGNDRVIGYQPTDKLVFKDV
                                   : .:    .      .       .
AMS_lipase            RLAEQLASLRP---------------------------------
chain_A_t1            RLAEQLASLQP---------------------------------
pseudomonas_fragi     EHAKRLKEAGL---------------------------------
pseudomonas_arg_Q9L6C7 QHANRLKNASL---------------------------------
proteus_sp_k107       QHAKYLASKQL---------------------------------
pseudomonas_flo       QGSTDLRDHAKVVGADTVLTFGADSVTLVGVGHGGLWTEGVVIG
                       .  :    *   .
```

Polyacrylamide gel

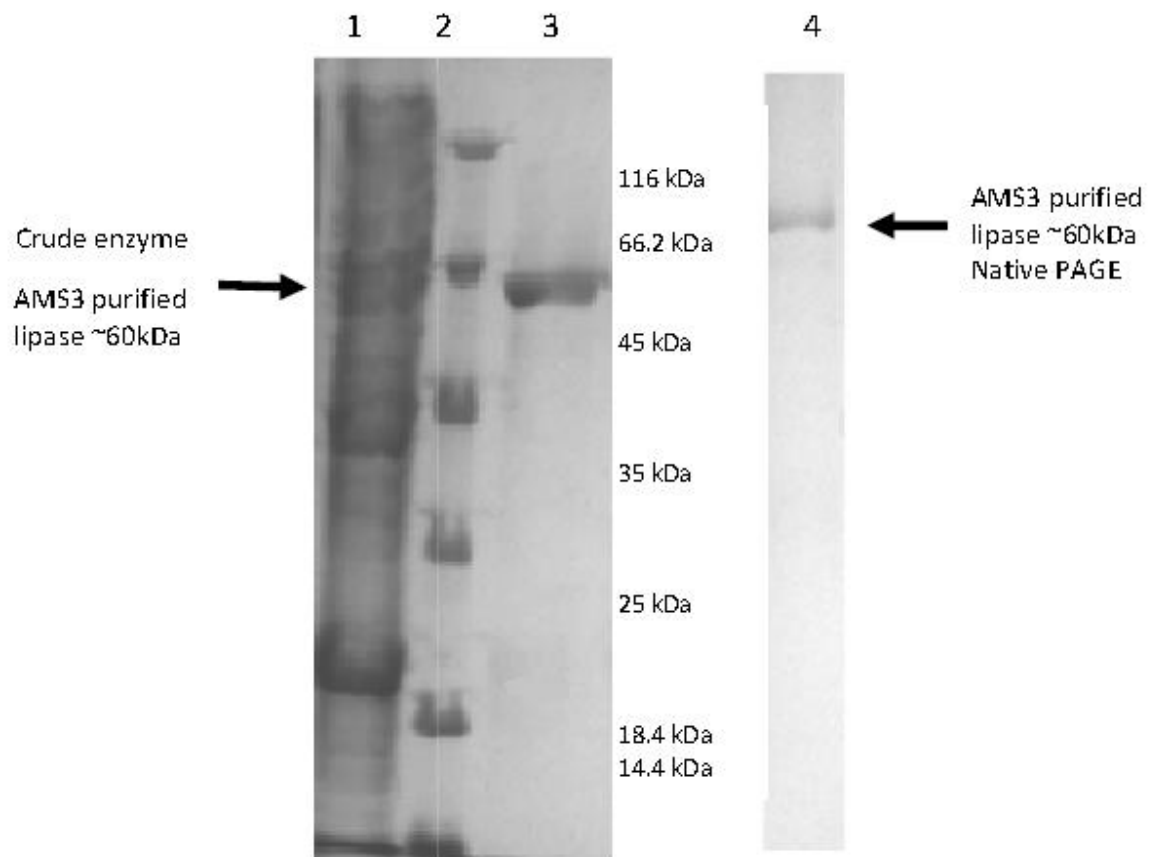
Fig 2: Polyacrylamide gel electrophoresis (PAGE) profile of recombinant AMS3 lipase. Lane; Lane 1 : Crude lipase; Lane 2: Protein molecular weight marker(Fermentas, Germany); Lane 3: Purified AMS3 lipase ; Homogeneity of AMS3 lipase was determined via native PAGE (Lane 4).The mobility of the purified lipase was estimated around ~60kDa (indicated by arrow). The protein loaded was approximately around 13μg .

Effect of temperature

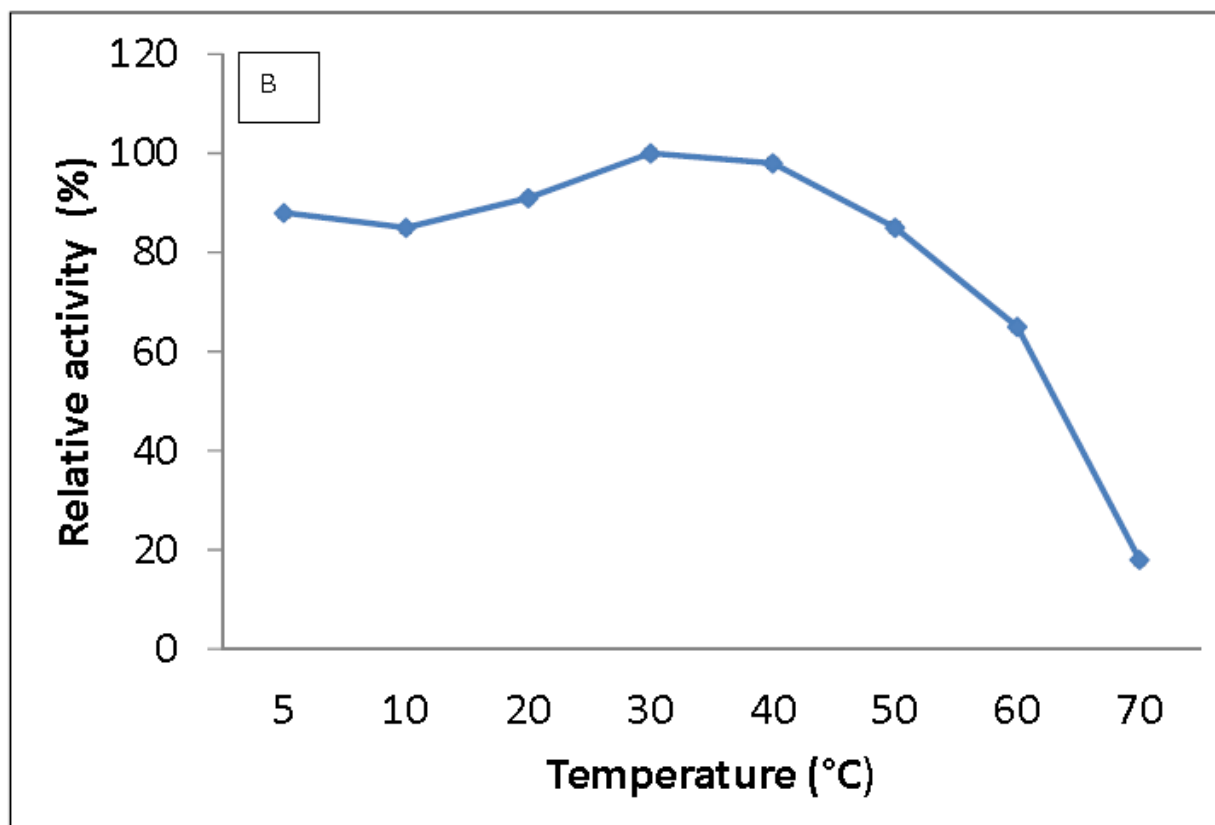
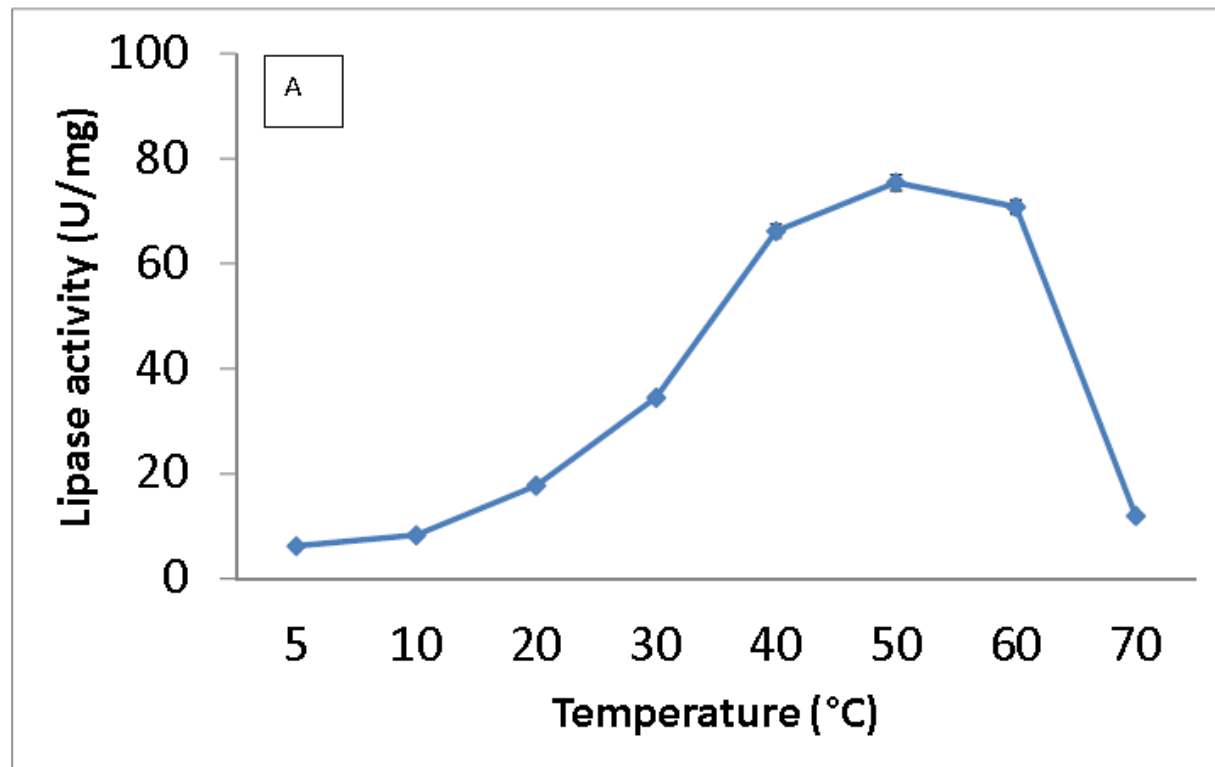
Fig 3: Effect of temperature on purified AMS3 lipase activity. The lipase assay was done using olive oil as a substrate. (A). Enzyme samples were assayed at various temperatures as mentioned in the Methods section. The temperature that showed the highest lipase activity was considered to be the optimum temperature for the AMS3 lipase. The effect of temperature on purified AMS3 lipase stability (B). Enzyme stability was tested at different temperature. The enzyme was incubated at various temperatures in 30 min. The assay was performed at optimum temperature. The relative activity was calculated using 30 °C as 100%.

Effect of pH

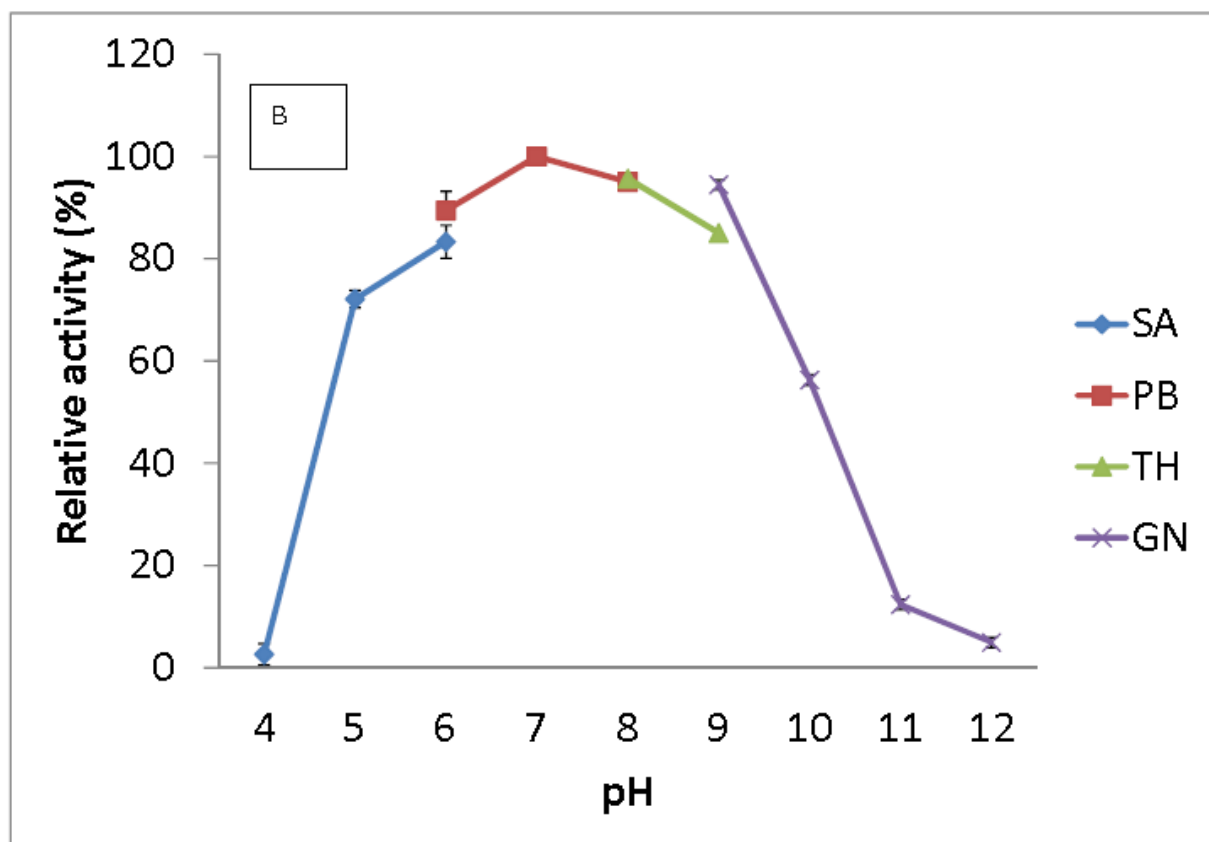
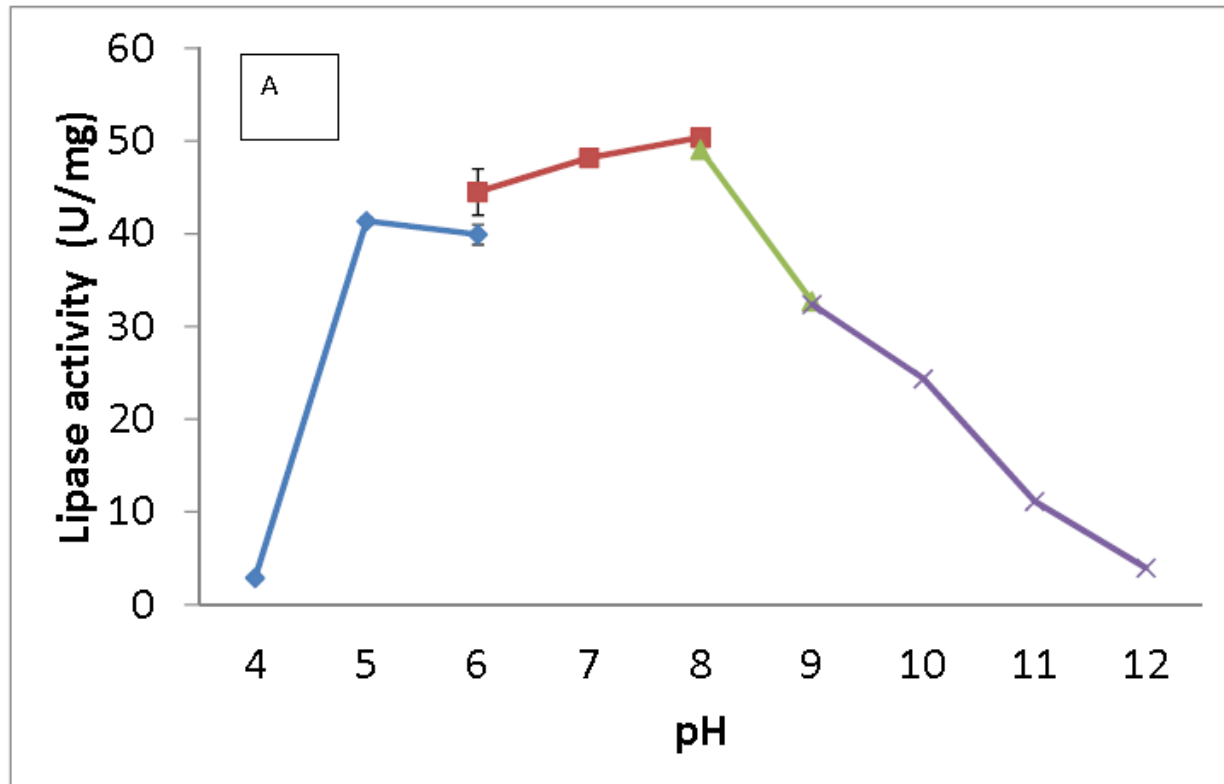
Fig 4: Effect of pH on purified AMS3 lipase activity (A). The lipase activity was measured at different pH using olive oil emulsion as substrate and assay at 50 °C for 30 min. Effect of pH on purified AMS3 lipase stability is shown in figure (B). The enzyme was pre-incubated at different buffer for 30 min. The lipase assay was performed at 50 °C. Sodium acetate buffer was used for the pH range of 4.0 to 6.0 (♦); potassium phosphate buffer for the pH range of 6.0 to 8.0 (■); Tris-HCl buffer for the pH range of 8.0 to 9.0 (▲); and glycine-NaOH buffer for the pH range of 9.0 to 12.0. (x). The relative activity was measure using pH 7 as 100%.

Effect of metal

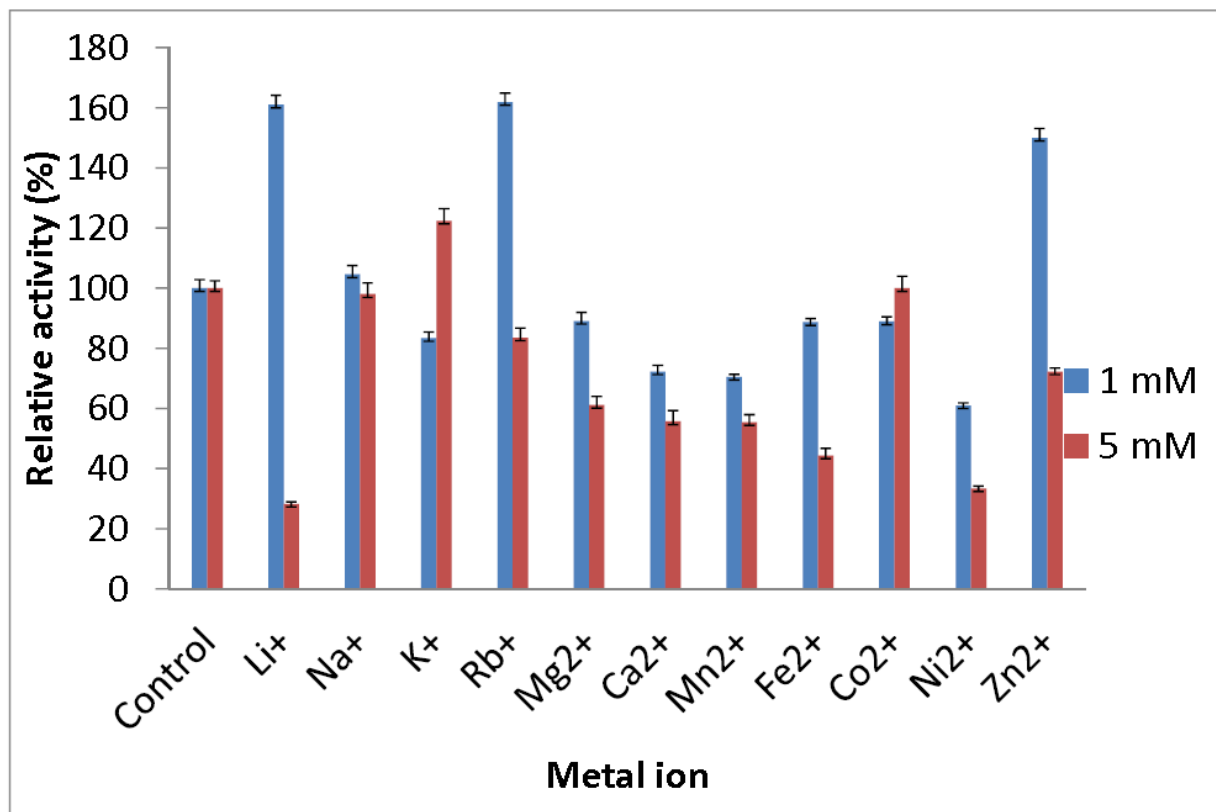
Fig 5: Effect of metal ions on AMS3 lipase stability. All assay was done using olive oil as substrate. The enzyme was pre-incubated at 50 °C with various metal ions at concentrations of 1 mM (blue) and 5 mM (red). The treated enzyme was assay at 50 °C for 30 min. The untreated enzyme was considered as a control or 100%.

Substrate specificity

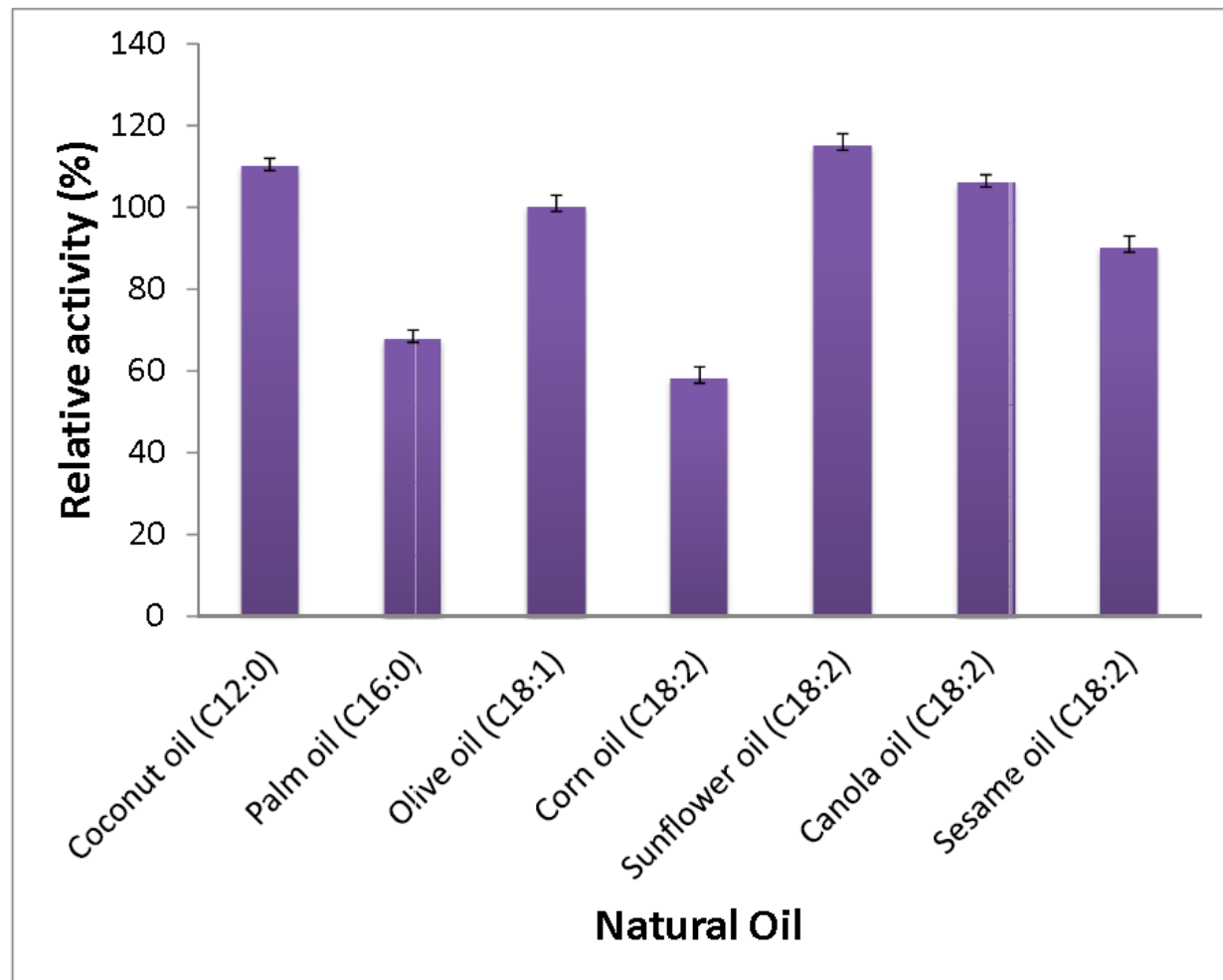
Fig 6: Substrate specificity of purified AMS3 lipase toward natural oil. The assay was performed at 50 °C for 30 min. Substrate emulsion prepared with various natural oil with ratio 1:1 (buffer and natural oil). Data shown are the means from triplicate measurements expressing % activity relative to that with olive oil .

Effect of solvent

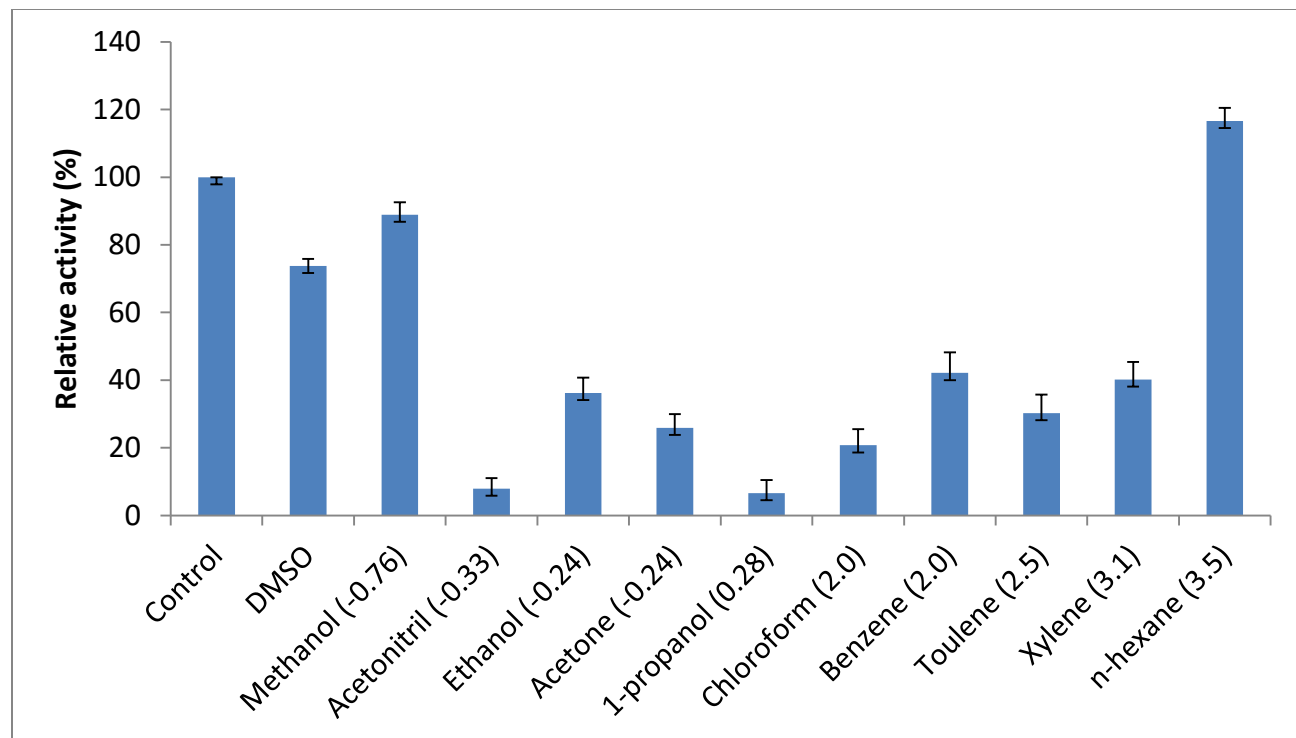
Fig 7: Effect of solvents on purified AMS3 lipase. All the assay was done using olive oil as a substrate. Incubation time for treated enzyme with organic solvent was 30 min and assay at 50 °C. Remaining lipase activity was expressed relative to that of the control (without solvent). Log P (water/octanol coefficient) value of each solvents are represented in parenthesis.

AMS3 lipase melting point

Fig 8: AMS3 lipase melting point and secondary content determination. Vertical line (arrow) indicating the melting point value of AMS3 lipase when tested from temperature ranging from 10-90 °C. (Figure 8A). Changes on the secondary structure content of AMS3 lipase were notably observed as the enzyme was subjected at different temperature (Figure 8B).