Full Length Research Paper

Characterization and optimization of lipase activity produced by *Pseudomonas monteilli* 2403-KY120354 isolated from ground beef

Abdel-Hamied M. Rasmey*, Akram A. Aboseidah, Salha Gaber and Fatma Mahran

Department of Botany and Microbiology, Faculty of Science, Suez University, Suez, Egypt.

A total of 56 Gram negative bacterial isolates were recovered from twenty ground beef samples and were screened for their potentiality to produce lipase. Forty four bacterial isolates were recorded as positive producers for lipase on tween as carbon source in solid medium. Also, the highly producer isolates were screened for lipase activity in submerged culture using olive oil as carbon and the most active isolate was 2043 which gave an activity of 20.0 ± 0.29 U/ml. The bacterial isolate 2403 was identified phenotypically according to Bergey's Manual and genotypically using 16S rRNA genes analysis as *Pseudomonas monteilli*. Effect of some different factors on lipase activity were studied and the maximum lipase activity was achieved at reaction medium of pH 6 and incubated at 40°C for 60 min. Also, addition of Ba^{2+} in the reaction medium enhanced the lipase activity, while the other tested metals reduced the enzyme activity.

Key words: Food contamination, lipase activity, olive oil, cultural conditions, *Pseudomonas*.

INTRODUCTION

Recently, there has been a progressive increase in the number of publications related to properties and industrial applications of microbial metabolites. Microbial lipases are one of the most important extracellular enzymes which have been the main focus of scientific research due to its huge biotechnological usage over the years (Ullah et al., 2015). Lipase (EC 3.1.1.3) is an ester hydrolase, which catalyzes the hydrolysis of long-chain triacylglycerols to form glycerol and fatty acid in the presence of excess water (Sharma et al., 2001; Gupta et al., 2004; Pascale et al., 2008). Lipases are now applied in the modern food industry instead of traditional chemical processes and used in the production of a variety of products like fruit juices, baked foods, fermented vegetables, cheese, soups and sauces. Modification of lipids (oils and fats) is one of the important processes in food industry (Hasan et al., 2006). Lipases are also used to give special flavour and taste to food by

synthesis of fatty acids and alcohols, which are accepted as flavour and fragrance compounds (Gandhi, 1997). Improvement of flavour in cheeses, the acceleration of cheese ripening, the production of cheese-like products and the lipolysis of butter fat and cream is achieved by the help of lipase enzymes which primarily release short-chain (C4 and C6) fatty acids that form sharp flavor (Saxena et al., 1999). Also, lipases are used as additives to detergents in association with proteases and cellulases (Pandey et al., 1999).

Lipases are found throughout all kingdoms of life, which are prokaryotes including bacteria and archaea and eukaryotes including plants, animals and fungi (Cai-hong et al., 2008). Microbial lipases are more stable than their corresponding plant and animal origin and their production is more convenient, safer and can be obtained in bulk at low cost (Vakhlu and Kour, 2006). Generally, we could deduce that bacterial enzymes are more preferred over fungal enzymes because of their higher activities and neutral or alkaline pH optima. In order to increase the cell yields and the enzymatic activities of the cells or to produce altered enzymes, genetic and environmental manipulations can be performed more readily on bacterial cells due to their short generation times, their simple nutritional needs and easy screening procedures for desired properties (Hasan et al., 2006).

A variety of lipases are produced from both Gram-positive and Gram-negative bacteria, but greater part of bacterial lipases comes from Gram-negative bacteria. The most important Gram-negative bacterial genus is *Pseudomonas* which contains at least seven lipase producing species that are *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Pseudomonas fragi*, *Pseudomonas glumae*, *Pseudomonas cepacia*, *Pseudomonas fluorescens* and *Pseudomonas putida* (Jaeger et al., 1994; Kojima et al., 2003). Besides *Pseudomonas* species, *Achromobacter*, *Alcaligenes*, *Burkholderia* and *Chromobacterium* strains are the most common lipase producing gram-negatives (Gupta et al., 2004).

The production of lipases by a microbial cell depends on the presence of a lipid, such as olive oil or any other inducer, such as triacylglycerols, fatty acids and tweens in the culture medium (Treichel et al., 2010). Olive oil is considered the most suitable lipid substrate due to its advantage of including high concentration of oleic acid and being more economical (Jensen, 1983). The effects of some variables on the activity of crude and purified lipases have been studied by some researchers. So, some different factors such as pH, temperature, metallic ions, organic solvents, among others, can increase or decrease lipase activity (Smaniotto et al., 2014).

The main goal of this study was to isolate and identify a lipase-producing bacterial strain from food samples and the other purpose was the determination of the optimum conditions maximize the enzyme activity of the produced enzyme by the selected highly producer isolate.

MATERIALS AND METHODS

Collection of samples

A total of 20 ground beef samples were obtained from different markets located at Suez Governorate, Egypt. Each sample represents a mixture of three aliquots of the source collected randomly. The samples were collected aseptically in sterilized polyethylene bags, transferred to the laboratory immediately and processed for microbiological analysis within 1 h of collection.

Isolation of bacteria

Twenty five grams from each food sample were weighed out and homogenized into 225 ml of sterile buffered peptone water and incubated at 37°C for 4 h. One milliliter was transferred to tryptone soy broth (TSB) and incubated them at 37°C for 18 h. After incubation, a loopful of each tube was streaked aseptically on MacConkey (MAC) agar plates and incubated at 37°C for 24 h. The growing individual colonies were picked up, and subcultured on fresh agar medium. The purified cultures were transferred to TSA slants, incubated at 37°C for 24 h and stored at 4°C.

Detection of lipase enzyme

Screening for lipolytic activity of the isolated bacteria was performed on a modified tween agar base plates contained (g/L dist. H_2O): 0.3% beef extract, 0.5% peptone, 5% NaCl, 1% tween 40, 2% agar and 0.01 to 0.001% malachite green. The plates were inoculated by the tested bacteria and incubated at 37°C for 2 days. Lipolytic activity was observed as clear zone around the stab due to hydrolysis of tween 40.

Lipase screening fermentation medium

The secondary screening for highly producer isolates was carried out in liquid medium with olive oil instead of tween and without the dye. After 48 h of incubation at 37°C, the broth medium was centrifuged at 4000 for 20 min. The clear supernatant obtained was used as the extracellular enzyme source.

Lipase activity assay

Lipase activity was assayed by titrimetric method using olive oil as a substrate. One milliliter of crude enzyme was added to the assay substrate containing 10 ml of 10% homogenized olive oil in 10% gum acacia, 2 ml of 0.6% $CaCl_2$ solution and 5 ml of 0.2 mol/L phosphate buffer pH 7.0. The mixture was incubated on an orbital shaker with 100 rpm at 37°C for 1 h. The reaction was stopped by addition of 20 ml of acetone: ethanol mixture (1:1). The content of each flask was titrated against 0.1 M NaOH using a phenolphathelin indicator until a pink color appeared and the endpoint recorded. One unit of enzyme is defined as the amount of enzyme required to hydrolyze µmol of fatty acids from triglycerides. The quantity of fatty acids liberated in each sample was calculated based on the equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following equation:

µmol fatty acid/ml subsample = [(ml NaOH for sample − ml NaOH for blank) × N × 1000] / 5 ml

where N is the normality of the NaOH (0.1 in this case) and 5 ml is the volume of reaction mixture.

Characterization and identification of the highly lipase producer isolate

Morphological characterization

Colony characterization: Colony morphology was recorded with respect to colour, form, surface, elevation, margin and opacity.

Gram staining and microscopic characterization: The gram stain was carried out on 24 h cultures according to the Hucker method (Collins and Lyne, 1985). The shape of bacterial cells under microscope was recorded and the photo microscopy was taken using Nikon microscope.

Physiological and biochemical characterization

Physiological and biochemical characteristics of the selected isolate were determined by the standard methods described in Bergey's Manual of Determinative Bacteriology (Holt, 1993). Catalase production, urease production, starch hydrolysis, gelatine liquefaction, methyl red, Voges-Proskauer test, citrate test, indole test, motility test, triple sugar iron agar (TSI) test, oxidase test, glucose and lactose fermentation tests were detected. The bacterial culture was grown on nutrient agar medium and incubated at different temperatures range (5, 25, 37, 45 and 50°C) for 24 h to determine its temperature profile.

Identification of bacterial isolate using 16S rRNA

DNA isolation: The locally isolated bacteria strains were grown for 24 h at 37°C on nutrient agar medium. Two milliliters of spore suspension were inoculated into the tryptic soy broth medium and incubated for 24 h at 37°C to form pellets of vegetative cells. The preparation of DNA extraction was done using Bacterial DNA Preparation kit (Jena Bioscience). (1) The pellets of vegetative cells were collected by centrifugation, the samples at 5000 rpm for 20 min, 300 μl Lysis Buffer and 2 μl RNase A were added to cell pellet and vortex vigorously for 30 to 60 s; (2) 8 μl Proteinase K was added and mixed by pipetting, incubated at 60°C for 10 min and cooled down for 5 min; (3) 300 μl Binding Buffer was added and vortex briefly; the tube was placed on ice for 5 min and centrifuged for 5 min at 10,000 g; (4) A spin column was placed into a 2 ml collection tube; the lysate was pipetted directly into the spin column, centrifuged for 1 min at 10,000 g and the flow through was discarded; (5) 500 μl Washing Buffer was added into spin column, centrifuged for 30 s at 10,000 g and the flow through was discarded; (6) Another 500 μl Washing Buffer was added into the spin column, centrifuged for 30 s at 10,000 g, the flow-through was discarded, centrifuged again at 10,000 g for 1 min to remove residual Washing Buffer and the 2 ml wash tube was discarded and placed the column in the elution tube. (7) 40 to 50 μl Elution Buffer was added into the center of the column, incubated at room temperature for 1 min, centrifuged at 10,000 g for 2 min and DNA was stored at -20°C.

PCR amplification: The 16S rRNA encoding gene was amplified by the polymerase chain reaction (PCR) from purified genomic DNA primers using specific primers 16SF: 5′-GAGTTTGATCCTGGCTTAG-3′ and 16SR: 5′-GGTTACCTTGTTACGACTT-3′. The PCR amplification was performed as the following: PCR amplification was performed by using Qiagen Proof-start Tag Polymerase kit (Qiagen, Hilden, Germany). The following substrates were combined in a total volume of 25 μl including about 2 μl of template DNA (20 ng/μl), 12.5 μl PCR Master Mix, 20 pmol (2 μl) each of forward and reverse primers and the total reaction volume was completed by 8.5 μl of water DNAase free water and this was done on the ice. The complete reaction mixture was incubated at automated thermocycler TC-3000 (Biotechnology Research Center, Suez Canal University, Ismalia). The reaction conditions were: an initial denaturation at 94°C for 5 min, 37 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s. A final extension was conducted at 72°C for 5 min. PCR product was analyzed by electrophoresis on 1.5% (w/v) agarose in 1X TAE buffer and gels photos were captured using gel documentation system then analyzed by Gel Docu advanced ver.2 software. PCR products of about (1500 bp) were purified from gel with QIA quick gel extraction kit (Qiagen, Hilden, Germany).

DNA sequencing: Purified PCR product was sequenced by cycle sequencing with didesoxy mediated chain-termination (Sanger et al., 1977). The obtained sequence of 16S rRNA of the bacterial isolate was first analyzed using the advanced BLAST search program at the NCBI website: http:// www.ncbi.nlm.nih.gov/BLAST/ in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny was evaluated using CLUSTALW program (http://clustalw.ddbj.nig.ac.jp/top-ehtml). The phylogenetic tree was displayed using the TREE VIEW program. Phylogenetic tree derived from 16S rRNA gene sequence was generated in comparison to 16S rRNA gene sequence from different standard bacteria strains obtained from GenBank.

Effect of some different factors on lipase activity

According to the previous titration method, the higher producer isolate for lipase was selected for determining the effect of different factors on lipase activity. The effect of pH of the reaction mixture on lipase activity by the tested isolate was studied adjusted at different values using different buffers (phosphate pH 7 to 11; KCl-NaOH pH 12 to 13; and acetate pH 3 to 6). Different incubation temperatures (20, 30, 40, 50 and 70°C) were studied on lipase activity by the tested isolate. The effect of different incubation periods (15, 30, 60, 90, 120 and 150 min) were studied on lipase activity by the selected isolate. Effect of several metal ions (2 ppm) included Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+} and Ba^{2+} were studied on lipase enzyme activity, while using the optimum of other parameters (pH, temperature and incubation period).

RESULTS AND DISCUSSION

Isolation and detection of lipolytic bacteria

Lipase producing microbes have been found in diverse habitats such as industrial wastes, vegetables, dairies, soil contaminated with oil, oilseeds, and decaying food (Rohit et al., 2001). The shelf life of any food material can be affected by large numbers of microbial somatic cells. Increased somatic cell numbers are positively correlated with activities of extracellular protease and lipase in freshly produced food. Activities of the two enzymes can supplement those of bacterial hydrolases, hence shortening the time to spoilage. A total of 56 distinct morphological Gram negative bacterial isolates were isolated on MAC agar medium from 20 ground beef samples collected from different markets located at Suez Governorate, Egypt. In a similar study by Ullah et al. (2015), out of 52 bacterial isolates recovered from thirty soil samples only 21 isolates were lipase producers.

Table 1. Detection of lipase activity of isolated bacteria.

Isolate code	Lipolytic zone (mm)	Level
3071	22 ± 0.58	M
2105	19.3 ± 0.88	M
2055	21.6 ± 0.88	M
2004	19 ± 0.01	M
2001	18.6 ± 0.88	M
2016	21 ± 2.08	M
2052	20 ± 0.57	M
2054	18 ± 0.57	M
2103	14 ± 0.57	L
2113	15 ± 0.57	L
2116	24 ± 0.57	H
2117	14.3 ± 0.88	L
2123	19 ± 0.58	M
2130	17 ± 0.57	L
2403	32 ± 0.57	H
2406	21 ± 0.57	M
2435	21.6 ± 0.57	M
2444	24 ± 0.57	H
3001	27.3 ± 2.72	H
3034	21.6 ± 0.88	M
3046	16.6 ± 0.33	L
3049	20 ± 0.58	M
3055	20.6 ± 0.88	M
3070	25 ± 0.57	H

H: High level (> 24 mm), M: moderate (18 - 23 mm) and L: low (< 18 mm)

Several conditions must exist for lipolyzed flavor to develop from residual lipases in processed dairy foods, that is, large numbers (>10^6 CFU/ml) of lipase producers (Stead, 1986). The isolated bacteria were examined for their potentiality to produce lipase on tween agar base medium. The positive producer bacterial strains for lipase activity on agar medium were detected by appearance of lipolytic zones around the tested bacterial colonies. Many researchers studied the lipase activity by different microbial strains using tween as substrate and they reported that this substrate is more convenient and easy to use for lipase detection (Sierra, 1957; Anbu et al., 2011). Only 24 isolates out of the tested 56 isolates were lipase positive producers and the diameter of the lipolytic zones were recorded (Table 1). The obtained results revealed that a total of 14 isolates were moderate (18 to 23 mm), while five isolates were investigated as high (24 to 32 mm) producers for lipase activity and the remaining positive tested isolates were recorded as low (<18 mm) lipase producers. Evaluation of the lipase-producing efficiency, based on the clear zone around colony, indicated that all *Pseudomonas* isolates produced lipase enzyme (Benattouche and Abbouni, 2012). The highest producer isolates numbered 2116, 2403, 2444, 3001 and

3070 were selected for secondary screening to determine the most active isolate for lipase production in broth medium using olive oil as a substrate.

Assay of lipase activity by the highly producer isolates

Secondary screening was investigated on the selected highly producer bacterial isolates for lipase. Based on the obtained data in Figure 1, the highest lipase activity value (20.0 ± 0.29 U/mL) was recorded by the bacterial isolate 2403 on olive oil as carbon source in the used culture medium. Olive oil is considered the most suitable source for lipase production (Qamsari et al., 2011). Other researchers used olive oil and tween as carbon source and showed the lipase producing feasibility.

Identification of the bacterial isolate 2403

The selected bacterial isolate 2403 was characterized morphologically and physiologically according to standard methods. The bacterial colony appeared colorless, pinpoint with slimy surface and entire margin on agar plates. The microscopic examination of the tested isolate revealed that the isolate was gram negative, rod shaped, catalase positive, oxidase positive, etc., as recorded in Table 2. According to Bergey's manual of Determinative Bacteriology, the morphological and biochemical characterization of the bacterial isolate 2403 revealed that this isolate belongs to the *Pseudomonas* genus. *Pseudomonas* species have the ability to use various simple and complex organic compounds so they are involved in biodegradation of natural or man-made chemical compounds. Several species of bacteria principally *Pseudomonas* spp. have been reported to be the most important producer of extracellular enzymes like lipases (Stuer et al., 1986; Tan and Gill, 1987; Gowland et al., 1987; Harris et al., 1990; Fernandez et al., 1990; Hasanuzzaman et al., 2004).

Molecular techniques utilizing amplification of target DNA provide alternate methods for diagnosis and identification (Kurtzman and Robnett, 1997). The identities of the bacteria were further confirmed by 16S rRNA sequencing. Approximately, 1200 bp sequence were obtained from DNA of bacterial isolate 2403 and then aligned with other 16S rRNA sequences available in the GenBank database. The results indicated 99% similarity with *Pseudomonas monteilli* (Figure 2). The nucleotide sequence of the isolate was deposited in the GenBank nucleotide sequence database under accession number KY120354. The ease of culture and availability of an increasing number of *Pseudomonas* strain genome sequences have made the genus a focus for scientific research (Zhang et al., 2009). Pseudomonads are characterized by their abilities to grow at broad range of temperatures and to hydrolyze and use large molecules of proteins and lipids for growth.
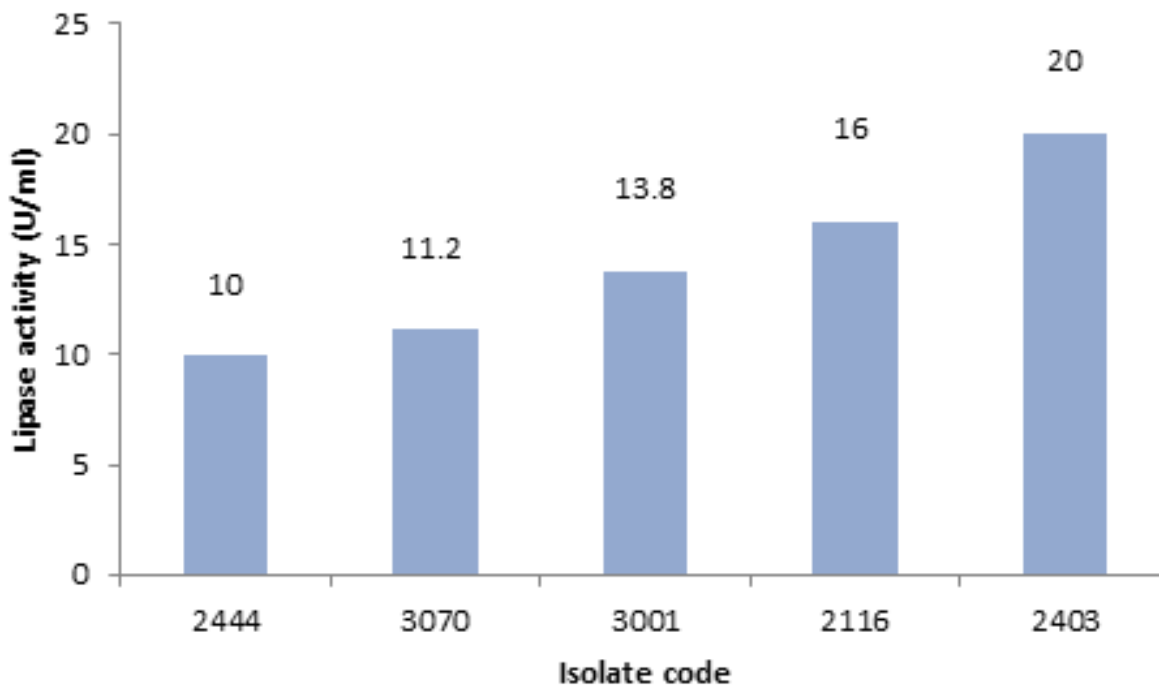
Figure 1. Lipase activity of the selected highly producer bacterial isolates on olive oil in broth medium.

Table 2. Phenotypic characterization of the bacterial isolate 2403.

| Test	Result
Colony morphology	Slimy surface, pinpoint, entire margin, colorless
Gram stain	Negative
Cells shape	Rod shaped
TSI	Yellow color
Urease	Positive
Catalase	Positive
Indole	Positive
VP	Negative
MR	Positive
Citrate	Positive
H_2S test	Negative
Motility	Positive
Gelatin liquification	Negative
Protease	Positive
Oxidase	Positive
Glucose fermentation	Positive
Starch hydrolysis	Negative
Lactose fermentation	Negative
Temperature profile	
4°C	Negative (-)
25°C	Positive (++)
37°C	Positive (+++)
45°C	Positive (+)
50°C	Negative (-)
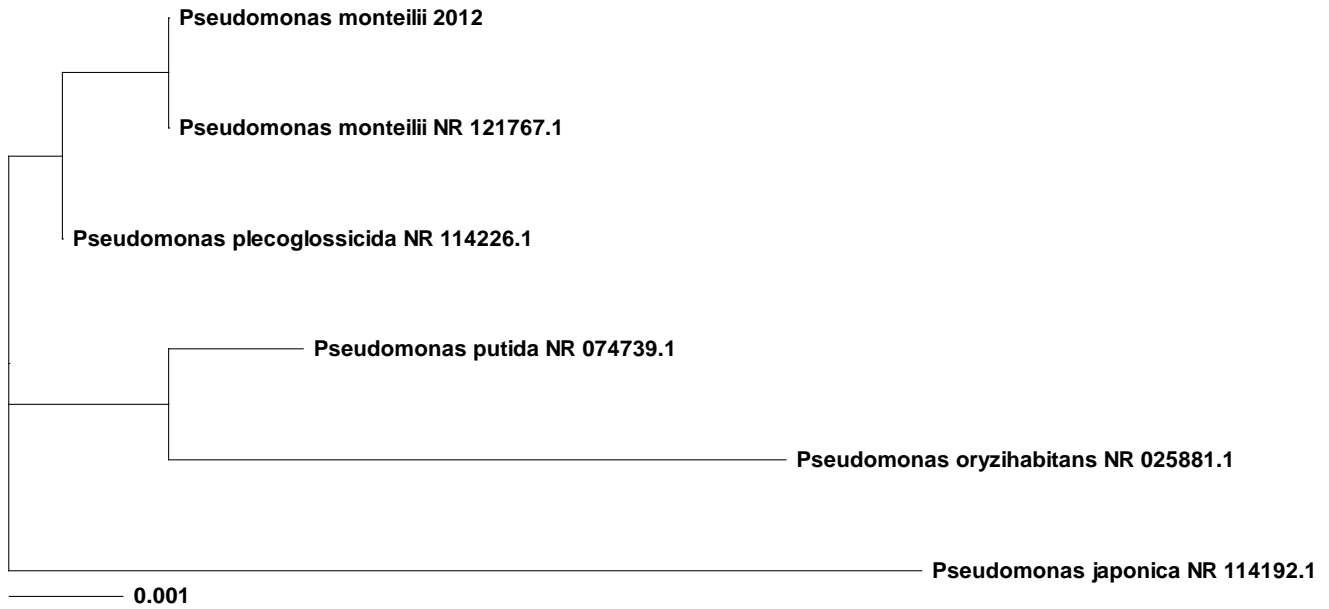
Figure 2. Phylogenetic tree of the bacterial isolate 2403 showing the most related strains in GenBank.
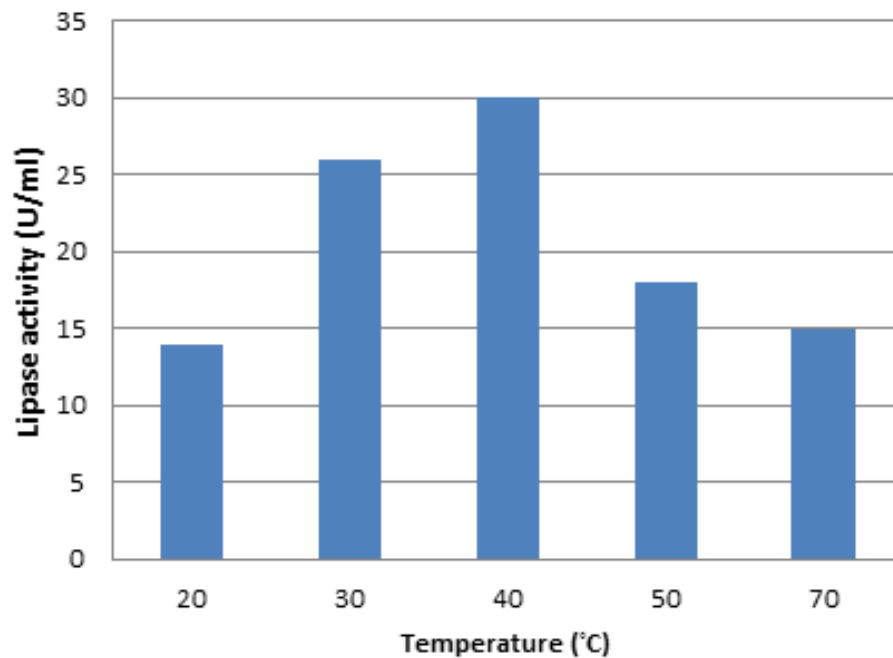

Figure 3. Effect of temperature on lipase activity of *Pseudomonas monteilli* 2403.

Optimization of lipase activity produced by *P. monteilli* 2403

Effect of temperature on lipase activity

The activity of lipase by *P. monteilli* was determined at broad range of temperatures varying between (20 and 70°C). As shown in Figure 3, lipase activity was recorded as 14 U/mL at 20°C and it was gradually increased until it reaches 30 U/mL at 40°C that was the optimum temperature, and then decreased by increasing temperature. Angkawidjaja and Kanaya (2006) reported that in general, the optimum growth and enzymes activity of *Pseudomonas* genus were obtained in the mesophilic
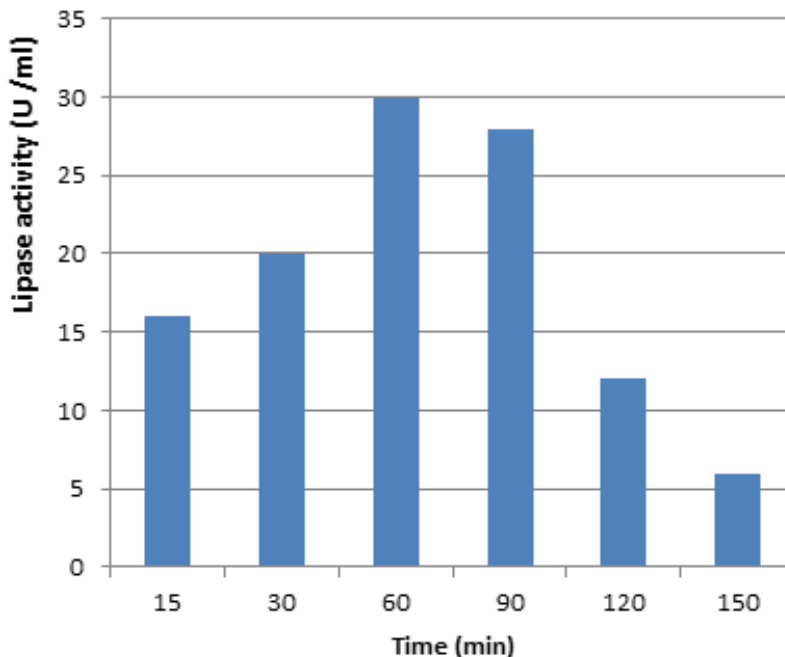
Figure 4. Effect of incubation time on lipase activity of *Pseudomonas monteilli* 2403.

or thermophilic range of temperatures. According to Adan et al. (2009), *Pseudomonas* spp. KE38 was grown at optimum at 25°C, but its lipase had maximum lipase activity at 45°C. In contrast to our results, Gilbert et al. (1991) indicated that maximum lipase activity by *Pseudomonas* spp. was detected at 30°C, while the results reported by Qamsari et al. (2011) showed that maximum lipase activity produced by *P. aeruginosa* KM110 was at temperature range (35 to 45°C). Also, Baharum et al. (2003) found that the maximum production of lipase by *Pseudomonas* spp. strain S5 was at 37°C. The highest lipase production (147.36 IU/ml) from *P. aeruginosa* SRT9 was also achieved at 37°C by Prita (2011). Optimization of temperature is vital for cell growth and enzyme production. Increase in temperature increases the rates of enzymatic reactions in cells till it reaches the optimum. Beyond the optimum temperature, the enzyme is inactivated due to denaturation of the protein causing slow metabolism of the cells and affect the cell growth and productivity (Sooch and Kauldhar, 2013).

Effect of incubation period on lipase activity

The incubation period effect was tested on lipase activity at different time intervals (15 to 150 mints) and the obtained results are as shown in Figure 4. The results showed that the enzyme activity increased by increasing the time of incubation and nearly remained constant in the period of 60 to 90 min. After that, it began to decrease by increasing the time of incubation until it reaches 5 U/ml at 150.0 min. Then, 60 min was the optimum time of incubation for enzyme activity and gave 30 U/ml. This decline in enzyme activity could be due to the denaturation and structure modification in the enzyme or to catabolite repression of enzyme (Sooch and Kauldhar, 2013).

Effect of pH on lipase activity

The effect of pH on lipase activity was studied at different pH values by using three different buffers covering the range of pH (4.0 to 12.0). Data in Figure 5 shows that lipase activity increased by increasing pH of the reaction medium until it reaches the optimum activity (35 U/ml) at pH 6. At pH above 6, the enzyme activity decreased until it lost at pH 12 (2 U/ml). Many microbial lipases have their optimum activity at a pH range of 7 to 9 (Zhang et al., 2005; Mahmoud et al., 2015). The optimal pH indicated that the lipase applicable at acidic pH conditions. According to Noman et al. (2010), the maximum lipase activity from *P. aeruginosa* BN-1 at 37°C was obtained by using medium of pH at 6.5. Also, the maximum activity of the lipase from *Pseudomonas* spp. BWS-5 (223.5 IU/ml) was obtained at pH 6.5 and temperature 37°C by Sooch and Kauldhar (2013) and thereafter, it decline. Also *P. fluorescens* SIK W1 has an acidic optimum pH 4.8 (Qamsari et al., 2011), while *P.*
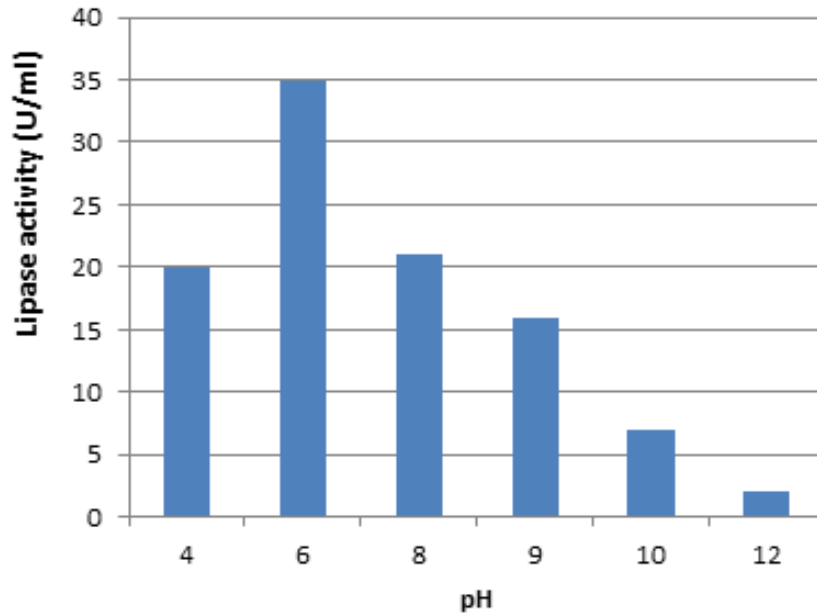
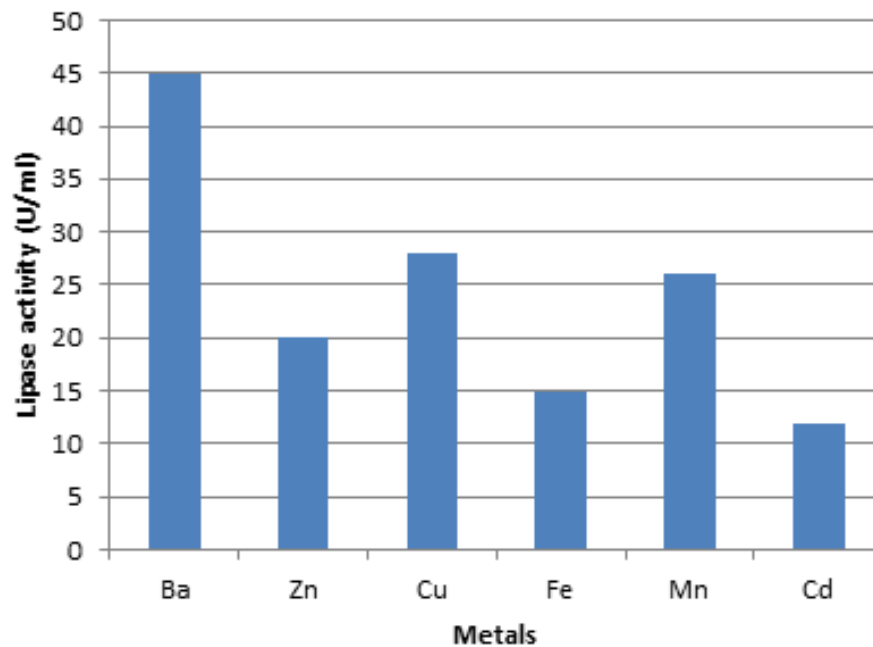**Figure 5.** Effect of pH on lipase activity of *Pseudomonas monteilli* 2403.

Figure 6. Effect of some metals on lipase activity of *Pseudomonas monteilli* 2403.

fluorescens 2D (Makhzoum et al., 1996) and *P. cepacia* (Svendsen et al., 1995) have pH optima of 8.5 and 9.0, respectively. The similar finding to our results, Benattouche and Abbouni (2012) showed that *P. aeruginosa* was able to grow in the pH range from 6 to 8 and reached the maximum lipase activity of 38.5 U/ml at pH 7.

Effect of metal ions on lipase activity

Finally, different metal ions (Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+} and Ba^{2+}) were examined for their effect on lipase activity. As represented by Figure 6, it was revealed that Zn^{2+} lowered lipase activity to 20% and Cd^{2+} dramatically lowered lipase activity to 12%, while the activity nearly

remained constant (26 to 28%) using Cu^{2+} and Mn^{2+} and increased 28% using Ba^{2+}. This metal ions effect may be due to a change in the solubility and the behavior of the ionized fatty acids at interfaces, or the change in the catalytic properties of the enzyme itself (Matsumae and Shibatani, 1994). According to Qamsari et al. (2011), Zn^{2+} and Cu^{2+} salts also decreased activity by 32 and 27%, respectively. Also, Zouaoui and Bouziane (2011) had found that the hydrolytic activity of the enzyme was inhibited by heavy metals Zn^{2+}, Mn^{2+} and Cu^{2+} with 32% relative activities. Studies of Benattouche and Abbouni (2012) showed that Zn^{2+}, Mn^{2+} and Cu^{2+} reduced enzyme activity to less than 43% of its relative activity.

Salt ions like Ca^{2+}, Cd^{2+}, and Fe^{2+} enhanced the activity of immobilized biocatalyst, while a few ions like Co^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Al^{3+}, and Na^+ had mild inhibitory effect (Kumar and Kanwar, 2011).

Conclusion

P. monteilli strain 2403 was isolated from ground beef and recorded as highly producer isolate for lipase activity in the current investigation. The results of the present study provide useful information for the optimization of lipase activity by studying the effect of different factors such as pH, incubation period, temperature and metal ions to provide the best lipase activity produced by *P. monteilli*. The maximum activity of the enzyme was obtained when the reaction medium contained Ba^{2+} (2 ppm), adjusted at pH 6 and incubated at 40°C for 1 h. More studies will be conducted to purify and characterize the lipase produced by the selected strain under the current investigation and to detect and clone the responsible genes.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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