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ISOLATION AND CHARACTERIZATION OF LIPASE PRODUCING BACTERIA FROM ANIMAL FATS

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ABSTRACT: Lipases are enzymes which have numerous applications in many industries like leather, soaps and detergents, pharmaceuticals, biofuel, food, textile *etc*. Many organisms like plants, fungi, bacteria are known to produce lipase. Lipolytic bacteria were isolated from Egyptian animal fats and grown on tributyrin media. Among the isolates from Egyptian animal fats three strains identified to *be Lactobacillus suebicus* DSM 5008 DSM, *Pseudomonas oryzihabitans* DSM 6835 THAM and *Staphylococcus epidermidis* 4b_r ESL. These strains were evaluated for potential probiotic properties. Absence of haemolytic activity and antibiotic resistance is considered as a safety prerequisite for the selection of a probiotic strains. One strain was γ-haemolytic (*i.e.* no haemolysis) and other were α-hemolysis. All of the strains were recorded highly lipolytic activity on Tributyrin agar base plates The highest lipase activity (651.9 µg/ml/min) was observed in *Pseudomonas oryzihabitans* DSM 6835 THAM but the lowest activity was recorded in *Staphylococcus epidermidis* 4b_r ESL (151.3 µg/ml/ min). On the other hand, *Lactobacillus suebicus* DSM 5008 DSM tested gave (337.9 µg/ml/min).

Key words: Lipase, isolation, characterization, lactic acid bacteria, hemolytic activity, antibiotic resistance.

INTRODUCTION

The term probiotic, literally meaning "for life", was first addressed by **Lilly and Stillwell (1965)** and was used to describe substances produced by protozoa to stimulate the growth of other organisms (**Argyri *et al.,* 2013**).

Microorganisms with potentials to produce lipases can be found in different habitats, including wastes of vegetable oils and dairy product industries, soils contaminated with oils, seeds, and deteriorated food (**Sharma *et al.,* 2001**). This indicates that nature offers a tremendous potential for identifying new sources of lipases with novel properties.

Taxonomically close strains may produce lipases of different types. There are many microorganisms known to produce different lipases (**Iizumi *et al.,* 1990**). Microbial lipases possessing either high alkalophilic or thermophilic properties have been reported from Alcaligenes (**Kakusho *et al.,* 1982**). *Pseudomonas fragi* (**Nishio *et al.,* 1987**) *P. nitroreducens* (**Watanabe *et al.,* 1977**) and other *Pseudomonas strains* were isolated from soil that produced lipase having different characteristics. **Choo *et al.* (1998)** isolated a psychrotrophic *Pseudomonas* strain from Alaskan soil, which produced a coldadapted lipase at low temperatures. The oily environment (sewage, rubbish dump sites and oil mill effluent) may provide a good environment for lipolytic microorganisms to flourish and for isolation of lipase producing microorganisms (**Graham *et al.,* 2007**). Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains (**Bhaskar *et al.,* 2013**).

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important industrial enzymes due to their versatile applications (M**ahadik *et al.,* 2002; Pandey, 2003**). Lipases catalyze a variety of reactions, such as partial or complete hydrolysis of triacylglycerols and reactions of esterification, transesterification and interesterification of lipids (**Colla *et al.,* 2010**).

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The recent interest in the production of lipases is associated with their applications as additives in food (flavour modification), fine chemicals (synthesis of esters), detergent (hydrolysis of fats), waste water treatment (decomposition and removal of oily substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather processing (removal of lipids from animal skins) and biomedical assays (blood triglycerides) (**Elibol and Ozer, 2000; Kamini *et al.,* 2000; Burkert *et al.,* 2004**). Additionally, lipases have an important application in the field of bioenergy, especially in biodiesel production, which is an expanding sector, as a result of the worldwide rising demand on the use of renewable energy (**Colla *et al.,* 2010**).

Lipase production is depends upon a number of factors including carbon, nitrogen sources, pH, temperature, aeration and inoculums size (**Heravi *et al.,* 2008**). *Marine vibrio* sp. VB-5 produces lipase that hydrolyzes n-3 polyunsaturated fatty acid (PUF A)-containing fish oil (**Ramji *et al.,* 2002**). As lipase producing microbes are widely distributed in nature, there is an immense need to explore natural habitats to isolate stable enzyme producing microbes. The aim of the study is to identify and characterize lipase producing bacteria isolated from Egyptian animal fats.

MATERIALS AND METHODS

Sample Collection

Egyptian animal fat samples were collected from various locations in Sharkia Governorate, Egypt. The samples were collected in sterilized polythene bags and transported to the laboratory.

Isolation of Lactic Acid Bacteria

Three grams of each sample (animal fats) were homogenized in 90 ml of Man Rogosa and Sharpe (MRS) broth media and incubated at 37°C for 48 hr., as described by **De Man *et al.* (1960).** Then, 0.1 ml of the culture was spread on MRS agar media and incubated for 48 hr., at 37°C as described by **Terzaghi and Sandine (1975**). The obtained colony was selected for estimation lipolytic activity.

Screening of the Isolates for Lipase Activity

Lipolytic organisms were screened by qualitative plate assay. Isolates were grown on Tributyrin agar base plates and incubated at 37°C for 48 hr., as described by **Sarantinopoulos *et al.* (2001)**. Zone of clearance was observed due to hydrolysis of tributyrin. The potential probiotic strains were conserved at -20°C in MRS broth with 30% glycerol.

Identification of Strains

Preliminary identification

All isolates were tested by Gram staining and for catalase reaction. Preliminary identification and grouping was achieved on the basis of cell morphology and phenotypic properties, namely carbon dioxide production from glucose and growth at different NaCl concentrations (2.5, 5, 7.5 and 10%, *W/V*) and at pH 4, 5, 6 and 7 according to **Asteri *et al.* (2009)**.

Identification of the selected strains by MALDI-TOF-MS

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) is a new technology for routine identification of bacteria in clinical or microbiological laboratories (**Biswas and Rolainand, 2013**). Each mass spectrometer consists of three functional units: an ion source, to ionize and transfer analytic ions into the gas phase; a mass analyzer, to separate ions by their mass to charge ratio (M/Z) and a detection device, to monitor ions. MALDI-TOF-MS is known since 1996 but this is a new technology and it is a soft ionization method which allows desorption of peptides and proteins from both whole different cultured bacteria and crude bacterial extracts. Protein mass patterns can be used for identification of bacteria at the genus, the species and in some case, the subspecies level (**Sauer and Kliem, 2010**). Identification of the selected ten isolates were conducted by this technique at Academic Park, Faculty of Medicine, Alexandria, University, Egypt. Shortly, one large colony or multiple small colonies (enough to fill about one half of a 10-µl inoculating loop) of a bacterial isolate to be tested was suspended in 70% ethanol in a 1.5-ml micro centrifuge tube. Extraction of bacteria,

matrix preparation, spotting of the steel target plate, and calibration of the instrument were performed as previously described by **Stevenson *et al.* (2010)**. A Bruker MALDI-TOF MicroFlex LT mass spectrometer was used to generate spectra from the bacterial extracts, and the Biotyper software (Version 2.0.4) was used to analyze the results.

Tolerence to pH

Tolerance to low pH is often indicative to stomach pH and was tested as described by **Conway *et al.* (1987)**

Hemolytic activity

Pure culture of bacterial isolates were streaked on the freshly prepared blood agar media, containing 5% (*W/V*) human blood (Michopoulos SA, Athens, Greece), and incubated at 37°C for 48-72 hr. Results were recorded based on the type of clear zone observed. *i.e.* α-hemolysis when the colony was surrounded by greenish zone β-hemolysis when the colony was surrounded by a clear white zone and γ-hemolysis when there was no change in the medium surrounding the colony according to **Argyri *et al.* (2013)**.

Antibiotic resistance

For testing antibiotic resistance, LB agar plate was overlaid with 100 ml of LAB culture containing 10^8 CFU/ml and antibiotic Aztreonam, Amoxicillin/Clavulanic acid, Rifampin, Cefaclor, Nalidixic acid, Doxycycline, Cefoxitin, Cephradine, Tetracycline and Nemomycin at various final concentrations (10, 20/10 (30), 5, 30, 30, 30, 30, 30, 30, 30 µg, respective) were placed on inoculated plates under sterile conditions. After incubation for 24 hr., at 30°C, the diameter (mm) of inhibition zone was measured **(Angmo *et al.,* 2016)**. The commercial antibiotics sensitivity test against bacterial isolates, measured by the inhibition zone diameter (mm) according to **(Han *et al.*, 2015)**.

Production of lipase

The production media was prepared in 250 ml Erlenmeyer flask containing 50ml of nutrient broth media and 1% olive oil as substrate. The medium was sterilized autoclaving at 121°C for 20 minutes and then it was incubated with 1 ml of bacterial suspension and incubated at 37°C for 24 hr., as described by **Sarika and Aradhana (2016)**.

Enzyme extraction

Enzyme extract from the production media, 5 ml of sample were taken and centrifuged at 10,000 rpm for 10 minutes. The supernatant and pellets were collected separately and the enzyme mixture was stored at 4°C for further studies as described by **Sarika and Aradhana (2016)**.

Lipase assay

The crude enzyme preparation was the culture broth after separation of cells and particles. The enzyme was normally stored at 4°C until use. Lipolytic activity was determined by colorimetric method based on the activity in cleavage of p-nitrophenylpalmitate (p-NPP) at pH 8.0. The reaction mixture contained 180 µl of solution A (0:062 g of p-NPP in 10 ml of 2-propanol, sonicated for 2 min before use), 1620 µl of solution B (0.4% triton X-100 and 0.1% gum Arabic in 50 mM Tris-HCl, pH 8.0) and 200 µl of properly diluted enzyme sample. The product was detected at 410 nm wavelength after incubation for 15 min at 37°C. Under this condition, the molar extinction coefficient (410 nm) of p-nitrophenol (p-NP) released from p-NPP was 15000 M^{-1}. The calibration equation for p-nitrophenol (p-NP) was $y = 0.0001x + 0.9441$ where y is absorbance and x is concentration of lipase in µg/ml/min as studied by (**Odeyemi *et al.,* 2013**).

RESULTS AND DISCUSSION

Biochemical Tests for Identifying the Selected Bacterial Isolates

Forty five bacteria isolates were obtained from animal fat. All isolates were screened for extracellular lipase using Tributyrin agar media. Three of the isolates (25, 26 and 37) produced larger clear zone than the others, indicating higher lipase activity. These three isolates were identified based on Gram staining and catalase activity. All the bacterial strains were positive for catalase activity (Table 1). Both strains (26 and 37) were Gram positive but the strain number 25 was Gram negative (Table 1).

Table 1. Gram staining and catalase activity of different strains isolated from animal fats

Strain No.	Gram staining	Catalase activity
37	+	+
25	-	+
26	+	+

(+) Positive. (-) Negative

The effect of Nacl at different concentrations (2.5, 5, 7.5 and 10%) on growth of isolated strains from animal fats are listed in Table 2. The results showed that the strain number 26 has the highest salt tolerance followed by 25. But, the strain number 37 was tolerate against salt concentration 2.5%, while it was sensitive to 5, 7.5 and 10% These results are in agreement with those obtained by **Maragkoudakis *et al*., (2006**) Effect of different pH (4, 5, 6 and 7) on growth of isolated strains from animal fats are listed in Table 3. Isolates 25 and 37 were highly tolerant to different pH (4, 5, 6 and 7) but strain 26 was sensitive for pH 4. These results are in agreement with those obtained by (**Adamberg *et al.,* 2003**)

Identification of lipolytic bacteria by MALDI -TOF MS

In the case of 45 isolates (12.0%), MALDI-TOF MS measurement did not give a reliable identification result, with a log (score) of 1.7 of these 45 isolates only three could be sequenced, due to an insufficient volume of ethanol extract. The sequencing data compared with the phenotypic identification of these strains can be seen in Table 3.

Using MALDI-TOF MS among the isolates from Egyptian animal fats three strains identified to be *Lactobacillus suebicus* DSM 5008DSM (37), *Pseudomonas oryzihabitans* DSM6835THAM (25) and *Staphylococcus epidermidis* 4b_r ESL (26). These strains were evaluated for potential probiotic properties (Table 4).

Lipolytic and haemolytic activities

Absence of haemolytic activity and antibiotic resistance is considered as a safety prerequisite for the selection of a probiotic strains. One strain was γ-haemolytic (*i.e.* no haemolysis) and other were α-hemolysis. All of the strains were recorded highly lipolytic activity on Tributyrin agar base plates (Table 5). None of the strains tested appeared to be haemolytic and, from this point of view, they can all be considered to be safe. Absence of haemolytic activity and antibiotic resistance is considered as a safety prerequisite for the selection of a probiotic strain (**FAO/WHO, 2002**). None of the examined strains exhibited haemolytic activity when grown in Columbia human blood agar. Most of the strains (37, 25 and 26 strains) were g-haemolytic (*i.e.* no haemolysis), isolated from dairy products which showed g-haemolysis except of few that showed α-haemolysis (**Maragkoudakis *et al.,* 2006**). Using the agar assay with tributyrin as substrate, only three isolates produced a halo round the colonies. These were 37, 25 and 26 isolates. This is in agreement with previous findings that characterize LAB as being only weakly lipolytic, in comparison with other groups of microorganisms (**El Soda *et al.,* 1995**).

The sensitivity test was conducted for the three potential probiotic strains (*Lactobacillus suebicus, Pseudomonas oryzihabitans* and *Staphylococcus epidermidis*) isolated from animal fat against 10 commercial antibiotics and the data were recorded in Table 6. Based on the average inhibition zone for each antibiotic with *L. suebicus, P. oryzihabitans* and *S. epidermidis* tested, there was an obvious variation in their sensitivity. Results in Table 6 show that *S. epidermidis* was resistant to Aztreonam, Amoxicillin/Clavulanic acid, Rifampin, Cefaclor, Nalidixic acid, Cefoxitin and Cephradine but it were intermediate to Tetracycline and Sensitive to Nemomycin. *L. suebicus* were resistant to each of Rifampin, Cephradine, Cefoxitin, Cefaclor, Amoxicillin/Clavulanic acid,

Table 2. Effect of NaCl at different concentration (2.5, 5, 7.5 and 10%) on growth of bacteria isolated from animal fats

Strain No.	NaCl Concentration (%)			
	2.5	5	7.5	10
37	+	-	-	-
25	+	+	+	-
26	+	+	+	+

(+): Resistant, (-): Sensitive

Table 3. Effect of different pH (4, 5, 6 and 7) on growth of bacteria isolated from animal fats

Strain No.	pH			
	4	5	6	7
37	++	++	++	++
25	++	++	++	++
26	-	++	++	++

(++): Resistant, (-): Sensitive

Table 4. Identification of bacteria isolated from animal fats by MALDI-TOF MS

Strain No.	Source	Strain
37	Animal fats	*Lactobacillus suebicus* DSM 5008DSM
25	Animal fats	*Pseudomonas oryzihabitans* DSM6835THAM
26	Animal fats	*Staphylococcus epidermidis* 4b_r ESL

Table 5. Lipolytic and haemolytic activities of selected strains with probiotic potential activity

Strains	Lipase activity	Haemolytic activity
Lactobacillus suebicus	+	γ-haemolysis (no haemolysis)
Pseudomonas oryzihabitans	+	α-haemolysis
Staphylococcus epidermidis	+	α-haemolysis

Table 6.Antibiotics sensitity test against the potential probiotic strains *(Lactobacillus suebicus*, *Pseudomonas oryzihabitans* and *Staphylococcus epidermidis)* isolated from animal fats

Antibiotic	RA (5µg)		DO (30µg)		N (30µg)		CE (30µg)		TE (30µg)		FOX (30µg)		CEC (30µg)		NA (30µg)		AMC (30µg)		ATM (10µg)	
Strain	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L. suebicus	8	R	26	S	23	S	0	R	22	S	0	R	15	R	20	S	0	R	0	R
P.oryzihabitans	9.5	R	18	S	26	S	22	S	25	S	16	I	7	R	18	I	0	R	0	R
S. epidermidis	9	R	28	S	21	S	0	R	16	I	7.5	R	0	R	0	R	0	R	6.5	R

RA: Rifampin , DO : Doxycycline , N: Nemomycin ,CE : Cephradine, TE: Tetracycline , FOX: Cefoxitin, CEC: Cefaclor , NA: Nalidixic acid, AMC : Amoxicillin/Clavulanic acid , ATM : Aztreonam
(1) : Inhibition zone (mm) , (2) : S/R/I : S : sensitive , R : resistant , I : intermediate .

Aztreonam but it was sensitive to Nemomycin, Tetracycline, Doxycycline, Nalidixic acid, *P. oryzihabitans* was resistant to Rifampin, Cefaclor, Amoxicillin/ Clavulanic acid and Aztreonam but it was sensitive to Nemomycin, Doxycycline Cephradine, Tetracycline and was intermediate to Cefoxitin and Nalidixic acid.

Previous studies also confirm the generally lower resistance of the *Lactobacilli* species studied here towards tetracycline and chloramphenicol **(Charters *et al.,* 2001; Maragkoudakis *et al.,* 2006)**.

Various opinions exist as to whether it might be desirable that some probiotic strains show resistance to specific antibiotics that are, for instance, involved in antibiotic-induced diarrhea **(Charters *et al.,* 1998)**. On the other hand, the commercial introduction of probiotics containing antibiotic resistant strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens **(Curragh and Collins, 1992).** However, according to previous studies **(Charters *et al.,* 2001; Danielsen and Wind, 2003)** the antibiotic resistance observed for *Lactobacillus* strains in this work, are considered to be intrinsic or natural resistance because it is chromosomally encoded and, therefore, non-transmissible. Resistance to aminoglycoside antibiotics, such as gentamicin, streptomycin, kanamycin, is considered to be intrinsic in the *Lactobacillus* genus and is attributed to the absence of cytochrome-mediated electron transport, which mediates drug uptake. Also, the resistance to vancomycin by *Lactobacillus* strains has been attributed to the presence of D-Ala-Dlactate in their peptidoglycan instead of the normal dipeptide DAla-D-Ala, which is the target of the antibiotic **(Danielsen and Wind, 2003; Coppolaa *et al.,* 2005; Mera *et al.,* 2012)**. The vancomycin-resistant genes of *Lactobacill*i are also chromosomal and, therefore, not readily transferable to other species **(Morrow *et al.,* 2012)**.

Lipase Assay

L. suebicus, P. oryzihabitans and *S. epidermidis* have produced lipase and it was determined by colorimetric method. The highest lipase activity (651.9 µg/ml/min) was observed in *Pseudomonas oryzihabitans* but the lowest activity was recorded in *Staphylococcus epidermidis* (151.3 µg/ml/ min). On the other hand, *Lactobacillus suebicus* tested gave (337.9 µg/ml/min) (Table 7).

Qualitative estimation of lipase activity of crude lipase (Table 7) showed the results of qualitative lipolytic activity of crude lipase from bacterial isolates. Lipase preparations from all the bacterial isolates possess significant lipolytic activity. As the level of purity increase improvement in lipolytic activity was also observed. Among the crude as well as partially purified preparations, lipase from *L. suebicus* showed maximum lipolytic activity followed by *P. oryzihabitans and S. epidermidis*. Lipolytic activity of crude lipase preparations from *L. suebicus, P. oryzihabitans and S. epidermidis* found to be almost similar to maintained positive

Table 7. Determination of crude lipase activity from different strains (*Lactobacillus suebicus, Pseudomonas oryzihabitans* and *Staphylococcus epidermidis)*

No. of sample	X(µg/ml/min)
Lactobacillus suebicus	337.9
Pseudomonas oryzihabitans	651.9
Staphylococcus epidermidis	151.3

controls. Whereas these preparations from bacterial isolates *L. suebicus, P. oryzihabitans* and *S. epidermidis* found to be possess, These results are in agreement with those obtained by (**Bhavani *et al.,* 2012**) lipolytic activity higher than maintained control shown in Table 7. After 72 hr. incubation of reaction mixture, maximum lipase activity was observed for *L. suebicus,* whereas least activity was observed for crude lipase of *L. suebicus, P. oryzihabitans and S. epidermidis*. Specific activity of crude lipase from *P. oryzihabitans and S. epidermidis* were observed to be greater than maintained controls whereas lipase activity of *L. suebicus, P. oryzihabitans and S. epidermidis* found to be comparable with maintained controls.

Lipase activity from *L. suebicus, P. oryzihabitans and S. epidermidis* was observed to be lesser than positive controls. These results are in agreement with those obtained by **Prasad (2014)**. Purified preparations from all the bacterial isolates was found to be higher than maintained controls.

Conclusion

In conclusion, the results of this study showed that 3 strains of *Lactobacillus suebicus*, *Pseudomonas oryzihabitans* and *Staphylococcus epidermidis* were found to possess desirable *in vitro* probiotic properties. These strains are good candidates for further investigation with *in vivo* studies to elucidate their potential health benefits as well as in fermentation studies to assess their technological characteristics for application as novel probiotic starters.

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عـــزل وتـوصيـف البكتيـريـــا المنتجـــة لإنـزيـــم الليبيـــز مـــن الـدهـــون الحيـــوانيـــة

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يدخل إنزيم الليبيز فى العديد من الصناعات مثل صناعة الجلود والصابون والنسيج والأغذية وغيرها، وكثير من الكائنات الحية مثل النباتات والطحالب والبكتيريا تنتج الليبيز، وتم عزل البكتيريا المحللة للدهون من دهون الحيوانات وتنميتها على بيئة الترايبوترين، وتم تعريف ٣ أنواع من البكتيريا المعزولة من دهون الحيوانات وهى *Lactobacillus suebicus* DSM 5008DSM, *Pseudomonas oryzihabitans* DSM6835THAM and *Staphylococcus epidermidis* 4b_r ES*L*، وهذه البكتيريا لها خصائص بروبيويك عالية، وتم إجراء اختبار تحلل الدم والمضادات الحيوية على البكتيريا وقد وجد أن بكتيريا اللاكتيك من النوع جاما (لا تحلل الدم) والأنواع الأخرى من النوع ألفا (تحلل الدم)، وأظهرت هذه العزلات تحلل عالى لإنزيم الليبيز على أطباق التربوترين أجار ويتم تقدير أنزيم الليبيز في هذه العزلات بطريقة لونية، وكانت بكتيريا *P. oryzihabitans* الأعلى في إنتاج الانزيم حيث بلغ نشاطها (651µg/ml/min) في حين كانت السلالة *S. epidermidis* أقلها نشاطا(151.3 µg/ml/min). وكانت السلالة *L. suebicus* متوسطة النشاط (337.9 µg/ml/min).

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