J Genet Resour2021;7(2):188-195 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2021.20826.1237*

J|G|R

Production of Lipase by Isolated Halophile, *Halobacillus* sp. Strain AR11 from International Miankaleh Wetland

Sedigheh Rajaei-Maleki[1], Bagher Seyedalipour[*2], Salman Ahmady-Asbchin[1] and Gholamhossein Riazi[3]

[1] Department of Microbiology, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran
[2] Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran
[3] Biochemistry Department, Institute of Biochemistry and Biophysics (IBB), University of Tehran, Tehran, Iran

ARTICLE INFO

Article history:
Received 01 January 2021
Accepted 25 May 2021
Available online 02 June 2021

Keywords:
Halophilic bacteria
Lipase activity
Halobacillus truperi
Miankaleh wetland

Corresponding authors:
✉ B. Seyedalipour
b.seyedalipour@umz.ac.ir

p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

Lipases are particularly important because they specifically hydrolyze acyl glycerol, oils, which are of great interest for different industrial applications. Some halophilic microorganisms produced some lipases. Halophilic bacteria have greater ability to produce salt and thermo tolerant enzymes like amylases, proteases and lipases. Most industrial enzymatic functions may be stopped by concentrated salt solutions and high temperatures; therefore, halophilic enzymes that have optimal activity at a vast range of temperature, pH and ionic strength, would be considered as suitable biocatalysts in industrial processes. The aim of this study was to isolate and study the halophilic lipase producing bacteria from the Miankaleh Wetland. The lipase activity was measured using titrimetric methods. Three halophilic strains (AR11, AR18 and AR28) were isolated from the Miankaleh wetland and were screened for the production of hydrolytic enzymes and lipolytic activity. Among three isolates, one strain was selected for identification using the molecular methods and some morphological characteristics. The bacterium *Halobacillus truperi* AR11 with 1.82 ± 0.1 U/mL lipase activity was selected as the highest lipase producing isolate. Lipase enzyme produced by this potential isolate was also characterized for determining its optimal activity. Effect of different pH, NaCl concentration and temperature on lipase activity was determined. The optimum pH for AR11 was found to be 9, while the optimum temperature and NaCl concentration for lipase activity was found to be 45ºC and 5 % NaCl, respectively. This is the first report of isolation and molecular identification of lipase producing halophilic bacteria from the Miankaleh wetland.

Please cite this paper as: Rajaei-Maleki S, Seyedalipour B, Ahmady Asb-chin S, Riazi Gh. 2021. Production of lipase by isolated halophile, *Halobacillus* sp. strain AR11 from international Miankaleh wetland. *J Genet Resour* 7(2): 188-195. doi: 10.22080/jgr.2021.20826.1237

Introduction

Halophiles are a group of "salt-loving" microorganisms that live in saline environments. Since the early 1980s halophilic microorganisms as a group of extremophiles were considered with high potential for biotechnological applications (Paul *et al*., 2008). According to the NaCl concentrations need for growth, halophilic bacteria can be classified as non-halophiles (<1%), slight halophiles (from 1 to 3%), moderately halophiles (3-15% NaCl), and extreme halophiles (between 15 and30%) (Gutiérrez-Arnillas *et al*., 2016; Kushner, 1985; Oren, 2006; Sahay *et al*., 2011). Halophilic bacteria have a greater ability to produce salt and thermotolerant enzymes like amylases, proteases, and lipases (Govender, 2009; Rohban *et al*., 2009). Most industrial enzymatic functions may be stopped by concentrated salt solutions and high temperatures; therefore, halophilic

enzymes that have optimal activity at a vast range of temperature, pH, and ionic strength, would be considered as suitable biocatalysts in industrial processes (Ghasemi *et al*., 2010). Lipases (EC 3.1.1.3) and carboxylesterases (E.C. 3.1.1.1) are enzymes that hydrolyze the carboxyl ester bonds in acyl-glycerides (Niyonzima and More, 2014). They differentiate between the length of their substrate, while lipases hydrolyze long-chain acylglycerols, esterase prefers short-chain glycerol esters.

Despite this difference, they are collectively referred to as lipases or "lipolytic enzymes. Lipases have alkaline or neutral optimum pH and display stability over a much pH range of 4 to 11. The thermal stability of lipases ranges from 30 to 60 °C, and a larger number are stable in organic solvents (José Murillo *et al*., 2012). Applications of lipase are being considered in a range of industries such as food processing, washing, biosynthetic processes, and environmental bioremediation (Oren, 2002). Bacterial lipases are important enzyme applications in different industries because they are suitable for the environment, non-toxic and no dangerous residues (Hasan *et al*., 2005; Schreck *et al.*, 2014) and they are the third-largest groups of industrial biocatalysts after bacterial amylolytic enzymes and proteases. This paper reports on the production of an extracellular lipase and optimization of its activity by an isolated halophilic bacterium, phylogenetically related to genus *Halobacillus*, under various chemical and physical conditions.

Materials and Methods

Sample collection and isolation

Samples were collected in September 2015 from the soil, water, and sediment of the International Miankaleh Wetland located in the north of Iran (Fig. 1). To observe lipase production, bacteria were isolated from a direct method using the special medium containing (% w/v): agar 1.5, tributyrin (glycerol tributyrate) 1, tween 80 1, peptone 0.5, yeast extract 0.3, NaCl 5, pH 7.5 at 37°C. Lipase-producing bacteria made a clear zone on tributyrin agar plates (Castro-Ochoa *et al*., 2005). Growth was investigated qualitatively at various concentration of NaCl ranging from 1-15% (w/v) on nutrient agar. All purified isolates were preserved in the 15% glycerol solution for long storage. The pH optimum for bacteria growth was determined at various pH ranging from (5-11), the effect of salt was determined by varying salt concentrations (0-30% NaCl). For determining the temperature optima, the growth was carried out at various temperatures (15-55 ºC). The biochemical test such as Sugar utilization test, IMViC test, Starch hydrolysis, Gelatin liquefaction, Nitrate reduction, TSI, Catalase, Oxidase and Indole production test were studied for their characterization.

Fig. 1. Situation of Miankaleh Wetland showing the sites (I, II, III) used sampling in this study.

Investigating the production of hydrolytic activities

The presence of amylolytic activity on plates was determined (Amoozegar et al., 2003; Vidya *et al*, 2011). Using starch agar medium (Sigma) contains 5% (w/v) salts. After incubation at 35 °C for 1 week, the plate was flooded with 0.3% I_2- 0.6% KI solution; a clear zone around the growth indicated the hydrolysis of starch. Proteolytic activity of cultures was screened in skim milk agar containing 10% (w/v) skim milk, 2% (w/v) agar, supplemented with 5% (w/v) salt. Clear halos around the growth after 7 days were taken as evidence of proteolytic activity (Amoozegar et al, 2008). DNase activity of the isolated was determined using 42 g/l of DNase test agar medium (Sigma). Supplement with 5% (w/v) salt and 0.008 g/l toluidine blue. After incubation at 35 °C for 7 days, the plates flooded with 1 N HCl solution. Clear halos around the colonies showed DNase activity (Onishi *et al*, 1983). The presence of pectinolytic activity on the plates was identified using a medium,

including pectin 10 g/l, $(NH_4)_2SO_4$ 1.4 g/l, K_2HPO_4 2 g/l, $MgSO_4.7\ H_2O$ 0.02 g/l, $FeSO_4.7H_2O$, 5mg/l, $MnSO_4.H_2O$ 1.6 mg/l, $ZnSO_4.7H_2O$ 1.4mg/l, $CaCl_2$ 2 mg/l, agar 20 g/l, 5%(w/v) salt. After incubation at 35 °C for 7 days, the plates were flooded with 0.3% (w/v) I_2-0.6% (w/v) KI solution. A clear zone around the growth exhibited pectinolytic activity (Soares *et al.*, 1999).

Production and assay of lipase

For enzyme production, bacteria were cultivated in 250 ml Erlenmeyer flasks containing 50 ml medium composed of (% w/v): peptone 0.5, yeast extract 0.5, NaCl 7, $CaCl_2$ 0.005, and olive oil pH 7.5. The production broth (50 ml) was incubated at 37°C under shaking (150 rpm, 48 h) conditions. Cells were separated from the cultivation medium after centrifugation at 4800 rpm for 20 min at 4°C and the supernatant was used as the source of the extracellular crude enzyme (Kumar *et al*., 2005). Lipase activity was assayed by alkali titration using olive oil as substrate, as described by Pignede *et al*, with some modifications (Pignede *et al*., 2000). The assay mixture consisting of 5 ml of olive oil, 2 ml of phosphate buffer (0.05 M, pH 7.0), and 1 ml of the crude enzyme preparation was incubated for 20 min at 37°C. The reaction was terminated by adding 4 ml ethanol-acetone (1:1), and the amount of liberated fatty acids during incubation was tittered with 50 mL KOH in the presence of 0.09% phenolphthalein as an indicator. One unit of lipase activity was defined as the amount of enzyme that liberated 1 mole of free fatty acids per ml per minute under the assay conditions.

Characterization of lipase

To investigate the effect of pH, lipase activity was assayed at various pH values (5.0-10.0). Media were inoculated for 20 min at 37 °C temperature. The enzyme activity was measured in the range of 15-45 °C using the standard activity assay procedure at related temperatures. The reaction mixture incubated at 37 °C was maintained as a control. Effect of salinity on lipase activity was studied by incorporating 2.5%, 5%, 7.5%, 10%, 15% and 20% (w/v) of NaCl in lipase production. Media were inoculated for 20 min at optimum temperature.

Identification of bacterial strain

The genomic DNA of the isolate was extracted by DNA extraction kit (Thermo Scientific) according to the manufacture's recommended producer and the 16S rRNA gene was amplified using the universal primers (Weisburg *et al*., 1991) 27F (5′-AGAGTTGATCMTGGCTCAG) and 1492R (5′-TACGGTTACCTTGTTACGACTT). PCR cycle amplification: initial denaturation at 95 °C for 5 min, followed by cycles of 95 C for 1 min, 58.5 °C for 45s, 72 °C for 1 min, with final 10 min extension at 72 °C. Purification and sequencing of the PCR production to compile by Microgen Company (South Korea). The phylogenetic relationship of the isolates was determined by comparing the sequencing data with the related 16S rRNA gene sequence in the GenBank database of the National Center for Biotechnology Information, via BLAST search. The phylogenetic tree was drawn by Mega 6 software. The 16S rRNA gene sequences of the isolated strain were published in NCBI under the following accession numbers: KY038871.

Results

Isolation and characterization of the bacterial strain

A total of 30 halophilic bacteria strains were achieved from water, saline soil, and sediment of Miankaleh Wetland. Among them were 15-gram positive rods, 10 gram negative rods, 4-gram positive cocci, and 1 gram negative cocci. (Table 1). Three halophilic strains (AR11, AR18, and AR28) were isolated from the Miankaleh Wetland and were screened for the production of hydrolytic enzymes and lipolytic activity. The results showed that only the AR11 strain could produce all four hydrolytic enzymes (Table 2). Based on morphological, physiological, and biochemical characterization, the one isolate AR11 was Gram-positive, non-spore-forming, rod-shaped, and non-motile bacterium. Colonies are cream. Optimal bacterial growth was observed at pH 8.0 to 35°C and 2.5% (w/v) NaCl. H_2S production, methyl red, nitrate reduction, and Voges-Proskauer tests were negative, while, oxidase, hydrolysis of gelatin was positive (Table 3).

Production of lipase and lipase assay

All three positive isolates were confirmed lipase production by duplication on 1% (w/v) tributyrin agar plate. After incubating for 48 h at 37°C, the plates were checked for a clear halo around the colony. The most effective lipase-producing bacterium isolated AR11, which gave the largest clear zone occurring by hydrolysis ester bond of triglyceride from lipase enzyme (Fig. 2).

Table 1. Sampling sites, conditions, and some physiologic characteristics of halophilic isolates in Miankaleh Wetland.

Location		pH	Gram-positive rod	Gram-negative rod	Gram-positive cocci	Gram-negative cocci
Sit I	Sediments	8	5	1	3	0
	Water	8.2	3	2	0	0
	Saline soil	8	3	1	0	0
Sit II	Sediments	8.1	4	1	0	1
	Water	8.6	2	0	0	0
	Saline soil	8.3	1	0	1	0
Sit III	Sediments	7.9	2	1	0	0
	Water	8.4	1	0	0	0
	Saline soil	8.2	0	2	0	0

Table 2. The hydrolytic activity of halophilic culture collection strains, the lipase activity (U/mL).

Enzyme	AR11	AR18	AR28
Amylase	+	+	–
Protease	+	–	+
DNase	+	+	–
Pectinase	+	–	+
Lipase activity(U/ml)	1.82±0.1	1.51±0.4	1.63±0.09

Table 3. Biochemical characteristics of isolated AR11.

Biochemical tests	AR11
Glucose utilization test	+
Maltose utilization test	+
Sucrose utilization test	+
Fructose utilization test	+
Indole production test	–
Methyl red test	–
Voges - Proskauer test	–
Citrate utilization test	+
Nitrate reduction test	–
Gelatin hydrolysis test	+
Catalase test	+
Oxidase test	–
TSI test	–

Lipase activity, and the effects of different parameters

Lipase activity of the isolates was investigated by growing in nutrient broth with olive oil for 48 h at 37°C. AR11 isolate showed a high activity of 1.82 U/ml lipase activity more than other isolates; therefore, this isolate was used to determine the optimum conditions for lipase production (Table 2). The temperature profile of enzyme activity is illustrated in Fig. 3.

The optimum temperature for the enzyme activity was found to be 45 °C and can thus be classified as moderately thermoactivated lipases. The maximum pH for the enzyme activity was obtained around 9 (Fig. 4).

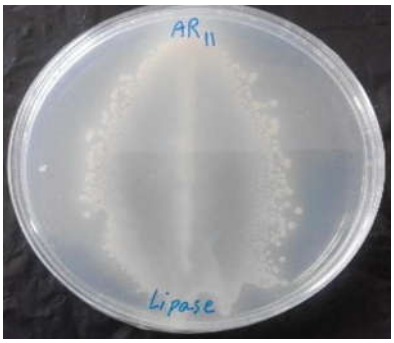

Fig. 2. Lipase produced by isolated AR11

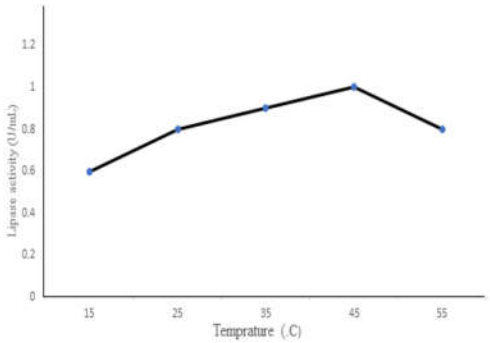

Fig. 3. Effect of temperature on lipase activity producing by AR11 in a medium containing 5% (w/v) NaCl (pH9). Results represent the means of three separate experiments, and bars indicate ±1 standard deviation

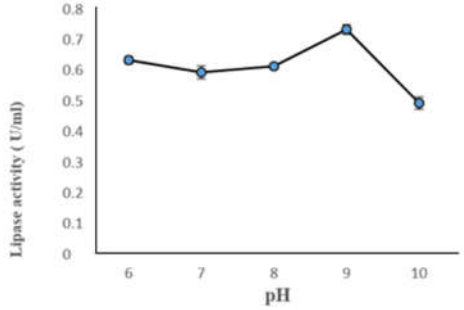

Fig. 4. Effect of pH on lipase activity producing by AR11 in a medium containing 5 % NaCl. Results represent the means of three separate experiments, and bars indicate ±1 standard deviation.

The effects of different salts on lipase activity are presented in Fig. 5. The results are similar with lipase from *B.pumilus* LV01, which gives maximum activity at pH 9 (Mariana *et al*., 2008) and halotolerant *Staphylococcus warneri* PB233 (Kanlayakri and Boonpan, 2007), active maximally at 40˚C.

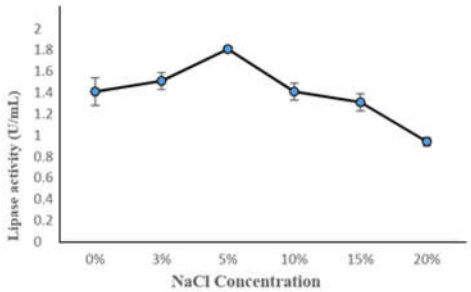

Fig. 5. The effect of NaCl concentration on lipase activity producing by AR11 in a medium with (pH 9). Results represent the means of three separate experiments, and bars indicate ±1 standard deviation.

Identification of the Isolate AR11 Based on 16S rRNA

The 16S rRNA gene sequence showed that the AR11have an above of 99.66% homology with the *Halobacillus trueperi*. The phylogenic tree showed the relationship between the isolate AR11 and the same group (Fig.6). Therefore, the isolate AR11 was identified as *Halobacillis truperi* AR11.

Discussion

According to the classification of halophilic bacteria, AR11 obtained in this study was slight halophiles, which exhibit good growth at NaCl concentrations 2.5% (w/v). In recent years, the ability of the halophiles to grow and produce enzymes over a very wide range of salinities makes them very attractive for research and for screening novel enzymes with unusual properties. The extracellular lipase can be classified as a moderately thermo active enzyme because of its optimal activity at 60°C. However, it was worth noting that the enzyme showed relatively high activity (50%) at 90°C. This characteristic made it similar to other lipases described previously and was usually inactive under temperatures higher than 80°C. Following our results, Mobarak-Qamsari *et al*, 2011 reported higher lipase activity values producing *Pseudomonas aeruginosa* KM110 at a temperature from 35 to 45°C. The optimal pH for the enzyme was found to be 9.0, indicating its alkali-stable nature (Mobarak-Qamsari *et al*., 2011). Similarly, Boutaiba *et al*, 2006 reported that lipase from *Natronococcus* sp. exhibited an optimum activity at pH: 7.0 (Boutaiba *et al*., 2006), while Ozcan et al, 2009 reported a range of pH of 8.0 to 8.5 for optimal esterase activities of five halophilic archaeal strains (Ozcan *et al*., 2009). In the present investigation, we described a moderately thermoactivated, alkali-stable, and halotolerant lipase from *Halobacilluse*. In this study, we screened lipase-producing bacteria from soil, water, and sediment samples in areas of the Miankaleh Wetland region. One of the isolates was observed for the clear zone on lipase media. The isolate AR11 was an effective lipase-producing bacterium, giving lipase activity at 1.82 U/ml using Olive oil as a substrate. The lipase production of the isolate AR11 had the

optimum temperature, NaCl concentration, and pH at 45°C and pH of 9, respectively. All of these results led us to conclude that the enzyme may have considerable potential for industrial application from the perspectives of its properties.

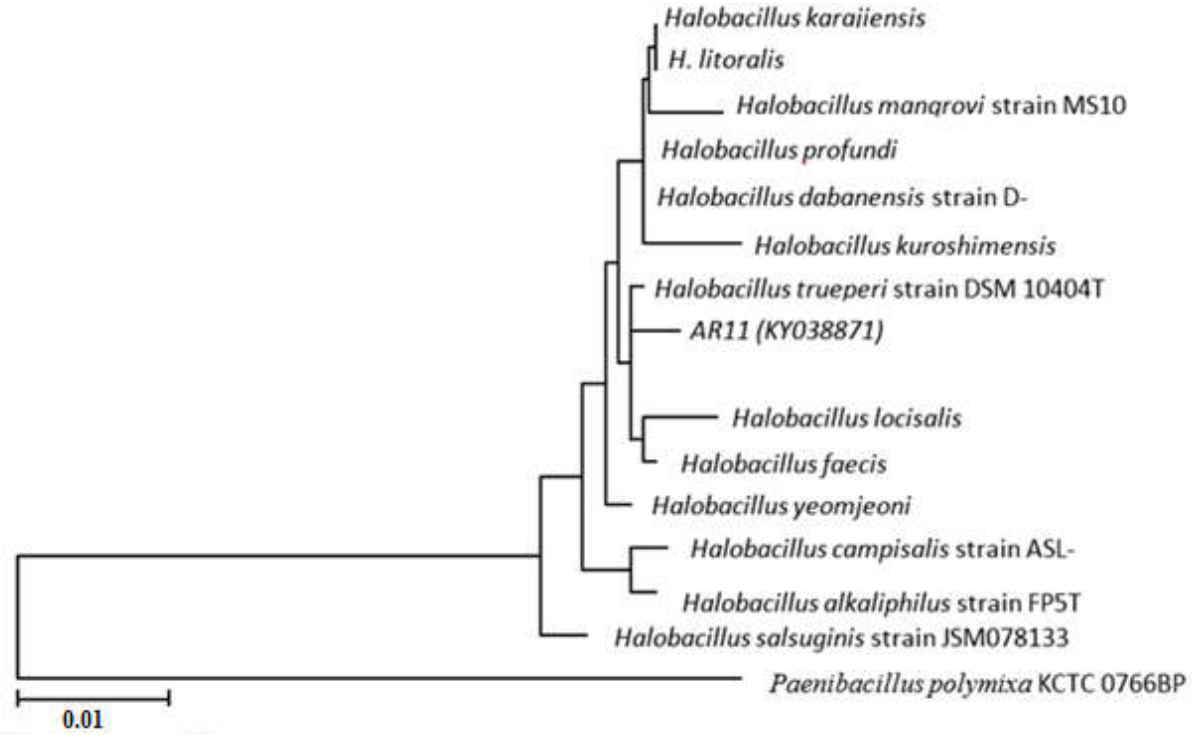

Fig 6. Phylogenetic tree based on 16S rRNA sequences, showing the relationship of the isolate AR11 to other members of the genus *Halobacillus.*

In this study lipase-producing, halophilic bacteria were isolated from sediment, water, and soil of Miankaleh Wetland in Mazandaran, Iran. One of them, genetically identified by 16S rRNA sequencing as *Halobacillus* sp. AR11 turned out to have an exceptional ability to grow and to produce lipases in saline environments, so it was selected for further optimization of the operating conditions of pH and temperature. *Halobacillus* sp. AR11 lipase is a potential alkaline lipase. The enzyme exhibited maximum activity and stability between pH 9, but the stability was low at 7 pH. The remarkable stability of *Halobacillus* sp. AR11lipase in this range has proved it to be a potential alkaline lipase similar to others, and a candidate for industrial applications such as detergent, leather, and fine chemical industries. In this research, strain AR11 was considered a salt-loving bacterium with the ability to produce several hydrolytic enzymes. The lipase produced by this strain, due to its significant stability at high temperatures, alkaline pH, and salt concentration, can be a good choice for improving the quality in industrial and commercial applications.

Acknowledgments

We thank the Pasture Institute for molecular analysis and the Department of Biology, the University of Mazandaran (Babolsar, Iran) for their support of this study, which is a section of the M.Sc. thesis of S. Rajaei-Maleki.

Conflicts of interest

The authors have no conflict of interest.

References

Amoozegar MA, Malekzadeh F, Malik KA. 2003. Production of amylase by newly isolated moderate halophile Halobacillus sp. Strain MA-2. *J Microbiol Method* 52; 353-359.

Amoozegar MA, Schuman P, Hajighasemi M, Fatemi AZ. 2008. *Salinivibrio proteolyticus*

sp. nov., a moderately halophilic and proteolytic species from a hypersaline lake in Iran. *Int J syst Evol Microbiol* 58: 1159-1163.

Boutaiba S, Bhatnagar T, Hacene H, Mitchell DA, Baratti JC. 2006. Preliminary characterization of a lipolytic activity from an extremely halophilic archaeon, *Natronococcus* sp. *J Mol Catal B Enzym* 41: 21-26.

Castro-Ochoa L, Rodriguez-Gomez C, Valerio-Alfaro G, Oliart Ros R. 2005. Screening, purification and characterization of the thermo alkalophilic lipase produced by *Bacillus thermoleovorans* CCR11. *Enzyme Microb Technol* 37(6): 648-654.

Ghasemi Y, Rasoul-Amini S, Ebrahiminezhad A, Zarrini G, Kazemi A, Mousavi-Khorshidi S, Ghoshoon MB, Raee MJ. 2010. Halotolerant amylase production by a novel bacterial strain, *Rheinheimera aquimaris*. *Res J Microbiol* 5(2): 144-149.

Govender L, Naidoo L, Setati ME. 2009. Isolation of hydrolase producing bacteria from Sua pan solar salterns and the production of endo-1, 4-β-xylanase from a newly isolated haloalkaliphilic *Nesterenkonia* sp. *Afr J Biotechnol* 8: 5458-5466.

Gutiérrez-Arnillas E, Rodríguez A, Sanromán MA, Deive FJ. 2016. New sources of halophilic lipases: isolation of bacteria from Spanish and Turkish saltworks. *Biochem Eng J* 109: 170-177.

Hasan F, Sanh A, Hameed A. 2005. Industrial applications of microbial lipases. *Enzyme Microb Technol* 39: 235-251.

José Murillo P, Barbosa Ranyere L, Souza Cláudia Moura de Melo Alini T, Mara F, Soares e Álvaro S. 2012. Biochemical characterization of lipase from a new strain of *Bacillus* sp. ITP-001. *Quim Nova* 35(6): 1173-1178.

Kushner DJ. 1985. The Halobacteriacea, In the Bacteria, Vol.8, eds Woese .C.R. & Wolfe, R. S. Londen Academic Press. 171-214.

Kumar S, Kikon K, Upadhyay A, Kanwar S, Gupta R. 2005. Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. *Protein Expr Purif* 41(1): 38-44.

Mariana NG, Virginia A, Vargas HA, Michal S. 2008. Lipolytic enzyme production by halophilic/halotolerant microorganisms isolated from laguna verde, Bolivia. *Rev Bol Quim* 25: 14-23.

Mobarak-Qamsari E, Kasra-Kermanshahi R, Moosavi-nejad Z. 2011. Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110. *Iran J Microbiol* 3(2): 92-98.

Niyonzima F, More S. 2014. Biochemical properties of the alkaline lipase of *Bacillus flexus* XJU-1 and its detergent compatibility. *Biologia* 69(9): 1108-1117.

Onishi H, Mori T, Takeuchi S, Tani K, Kobayashi T. 1983. Halophilic nuclease of moderately halophilic *Bacillus* sp .production purification and characteristic. *Appl Environ Microbiol* 45: 24-30.

Oren A. 2006. Life at High Salt Concentrations. Prokaryotes 2: 263-282.

Oren A. 2002. Molecular ecology of extremely halophilic archaea and bacteria. *FEMS Microbiol Ecol* 39: 1-7.

Ozcan B, Ozyilmaz G, Cokmus C, Caliskan M. 2009. Characterization of extracellular esterase and lipase activities from five halophilic archaeal strains. *J Ind Microbiol. Biotech* 36: 105-110.

Pignede G, Wang H, Fudalej F, Gaillardin C, Seman M, Nicaud J. 2000. Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*. *J Bacteriol* 182: 2802-2810.

Paul S, Bag SK, Das S, Harvill ET, Dutta C. 2008. Molecular signature of hyper saline adaptation: insights from genome and proteome composition of halophilic prokaryotes. *Genome Biol* 9: 1-19.

Rohban R, Amoozegar MA, Ventosa A. 2009. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *Ind Microbiol Biotechnol* 36: 333-340.

Sahay H, Singh S, Kaushik R, Saxena AK, Arora DK. 2011. Characterization of halophilic bacteria from environmental samples from the brackish water of Pulicat Lake, India. *Biologia* 66 (5): 741-747.

Schreck SD, Grunden AM. 2014. Biotechnological applications of halophilic lipases and thioesterases. Appl. Microbiol. Biotechnol 98: 1011-1021.

Soares MM, Silva RD, Gomes E. 1999. Screening of bacterial strains for pectinolytic activity: Characterization of the polygalacturonase produce by *Bacillus* sp. *Rev Microbiol* 30: 229-303.

Vidya J, Swaroop S, Singh SK, Alex D, Sukumaran RK, Pandey A. 2011. Isolation and characterization of a novelα-amylase from a metagenomic library of Western Ghats of Kerala, India. *Biologia* 66 (6): 939-944.

Weisburg WG, Barns SM, Pellriter DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173: 697-703.

Kanlayakrit W, Boonpan A. 2007. Screening of halophilic lipase-producing bacteria and characterization of enzyme for fish sauce quality improvement. *J Natural Sci* 41: 576-585.