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CHARACTERIZATION OF HIGHLY STABLE EXTRACELLULAR LIPASE FROM THE EXTREMELY HALOPHILIC ARCHAEON *Halolamina sp.*

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Abstract

Enzymes of the archaea living in extreme environments are resistant to the challenging conditions. Lipase is among the important enzymes used in the industry and agriculture. In this study, the extracellular lipase from extremely halophilic archaeon *Halolamina sp*. was characterized for the first time. Optimum temperature for the enzyme activity was determined as 70°C, optimum pH was 7.0, and the optimum salt concentration was 3.6 M. Additionally, more than 70% of the enzyme activity was remained between pH 3.0-10.0 for 48 h as well as incubation of the enzyme at 70°C for 30 min increased its activity for 44%, and no activity loss was observed after incubation at 80°C. Also, presence of the metals increased the enzyme activity up to 88%. The enzyme was highly resistant to the organic solvents acetone, methanol, and DMSO while strong inhibition was caused by *n*-butanol. Among the detergents, the enzyme kept its activity substantially in the presence of SDS; however, other detergents caused inhibition of the enzyme activity. This characterization study showed that the lipase from the haloarchaeon *Halolamina sp*. is highly stable at the wide ranges of temperature and pH values as well as in the presence of diverse inhibitors. This enzyme is promising to be used in biotechnological applications.

Keywords: Archaeabacteria. Enzyme Stability. *Halolamina*. Lipase.

1. Introduction

Archaea (archaebacteria) are the prokaryotes living in extreme environmental conditions such as high concentrations of salt or metals as well as high or low temperature and pH values (Swan et al. 2010). The halophilic archaebacteria (haloarchaea) are remarkable for their survival at saturation level of high salt concentrations. These microorganisms can be isolated from the natural environments such as alkaline salt lakes, hypersaline soils, or salt mines as well as from the sources with human intervention, such as foods with high salt or tanneries (Corral et al. 2020).

Extreme environments that haloarchaea live make them attractive for their physiological characteristics (Edbeib et al. 2016; Kurt-Kızıldoğan et al. 2017). For instance, many enzymes produced by haloarchaea require high salt concentrations for their activity (DasSarma and DasSarma 2015; Abanoz et al. 2017). Halophilic enzymes contain high amount of acidic amino acids mainly at their surface (Enache and Kamekura 2010), and generally keep their activity at wide ranges of temperature and pH values, in addition to salt concentrations, which is advantageous for various industrial and agricultural applications (Oren 2010; Shivanad et al. 2013; Corral et al. 2020) such as detergent, leather, and paper industries, as well as

production of soy and fish sauces (Gupta et al. 2016). These enzymes are also utilized for environmental bioremediation and treatment of wastes (Waditee-Sirisattha et al. 2016) such as biofuel production using agricultural and animal wastes (Amoozegar et al. 2019). Therefore, enzymes from the halophilic microorganisms isolated from diverse environments are characterized to find out the ones with desired features (Laye and DasSarma 2018).

Various halophilic hydrolases such as amylases, lipases, nucleases, and proteases are used for diverse biotechnological applications (Dumorné et al. 2017). Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are the enzymes hydrolyzing or synthesizing ester bonds in a variety of substrates having carboxylic and hydroxyl groups (Le et al. 2019). Lipases with high activity, wide substrate specificity, and stability at stringent conditions are used in a number of industrial applications such as processing of textile products, and as an additive for detergents (Sangeetha et al. 2011). Enzymes used in these industrial processes have been obtained from the organisms isolated from milder environments. However, halophilic lipases attract more attention recently for the applications involving high salt concentrations (Musa et al. 2018). For instance, the extracellular lipase from *Idiomarina* sp. isolated from Yuncheng Salt Lake in China was characterized and its usability for biodiesel production was investigated (Li et al. 2014). There are some other studies on the characterization of halophilic lipases (Martin del Campo et al. 2015; Samaei-Nouroozi et al. 2015); however, the lipase produced by genus *Halolamina* has not been characterized. In the present study, extracellular lipase produced by an extremely halophilic archaeon, *Halolamina* sp. isolated from a salt mine, was characterized in terms of diverse biochemical properties.

2. Material and Methods

Bacterial culture conditions

Halolamina sp. was isolated from Yozgat salt mine in Turkey (Kurt-Kızıldoğan et al. 2017). The haloarchaeon was cultured in SG medium involving (in g/l): KCl, 2; NaCl, 250; sodium citrate trisodium salt, 3; MgSO4·7H2O, 20; casamino acids, 7.5; FeSO4·7H2O, 0.0023; bacteriological agar, 15, yeast extract, 1; pH 7.55 (Sehgal and Gibbons 1960) at 37^{o}C for 7 days. The bacterial culture was centrifuged at 6000 rpm for 10 min, and the supernatant was used as the crude lipase source.

Determination of lipase activity

The lipase activity of *Halolamina* sp. culture supernatant was measured by a modified method of Malekabadi et al. (2018). Briefly, assay mixture included 10 µL of 10 mM *p*-nitrophenyl dodecanoate (*p*-NPD, Sigma Aldrich) dissolved in acetonitrile, 40 µL ethanol, 0.1 ml of 0.1 M phosphate buffer (pH 8.0), 10 µL supernatant, and 0.84 mL of 2 M NaCl. Mixtures were incubated at 50^{o}C for 30 min and the optical density values were measured at 410 nm (mentioned as the standard assay conditions). The samples were assayed as triplicates. One unit of lipase activity (U) was defined as the amount of enzyme that releases 1 µmol of *p*-NP per min under the mentioned experimental conditions.

Effects of temperature and pH on lipase activity

Effect of temperature on lipase activity was determined under the standard assay conditions except incubation temperature applied as 25, 30, 35, 40, 50, 60, 70, 80 and 90^{o}C. For the determination of thermostability, culture supernatant was incubated at 25, 30, 35, 40, 50, 60, 70, 80 and 90^{o}C for 30 min, and the lipase activity was measured under the standard assay conditions.

Effect of pH on lipase activity was investigated under the standard assay conditions changing the pH values as 3, 4, 5, 6, 7, 8, 9 and 10. To test the pH stability of lipase, culture supernatant was mixed with buffer having pH values of 3, 4, 5, 6, 7, 8, 9 and 10, then the mixture was incubated at 4^{o}C for 0, 12, 24, 36 and 48 h. The lipase activity was measured under the standard assay conditions.

Effects of NaCl, organic solvents, detergents and metals on lipase activity

Effect of NaCl on the lipase activity was determined under the standard assay conditions changing NaCl concentrations as 0,6 - 1,2 - 1,8 - 2,4 - 3,0 - 3,6 M. For the effect of organic solvents, 50 µl of isopropanol, n-butanol, hexanol, methanol, ethanol, acetone, glycerol or dimethyl sulfoxide (DMSO); for the effect of metals, 3 µM or 5 µM $CaCl_2$, $Cu(NO_3)_2$, $ZnSO_4$, $MgCl_2$ or $MnCl_2$; and for the effect of detergents 0.2% Tween-20, Tween-80, Triton X-100 or sodium dodecyl sulfate (SDS) was added to the assay mixture. The lipase activity was measured under the standard assay conditions.

Determination of lipase kinetics

The Michaelis–Menten constant (*K*m) and the maximum activity rate (V_{max}) of *Halolamina* sp. lipase were determined by a Lineweaver-Burk plot. Lipase activity was measured under the standard assay conditions changing the *p*-NPD concentrations (20–320 µM).

3. Results

Effects of temperature and pH on the lipase activity of *Halolamina* sp.

Effect of temperature on the extracellular lipase produced by *Halolamina* sp., a haloarchaeon isolated from a salt mine in Turkey, was found as a slight increment from 25°C to 60°C, followed by a sharp increase at 70°C, and a sharp decrease at 80°C and 90°C. Thus, the optimum temperature for *Halolamina* sp. lipase activity was determined as 70°C (Figure 1A). The thermostability of *Halolamina* sp. lipase was assayed incubating the crude enzyme at different temperatures between 25°C and 90°C. An increasing positive effect of incubation was observed on lipase activity from 25°C to 70°C (44% increment), the enzyme activity remained same at 80°C, and 12% decrease was occurred at 90°C (Figure 1B). Lipase of *Halolamina* sp. was shown to possess high thermostability.

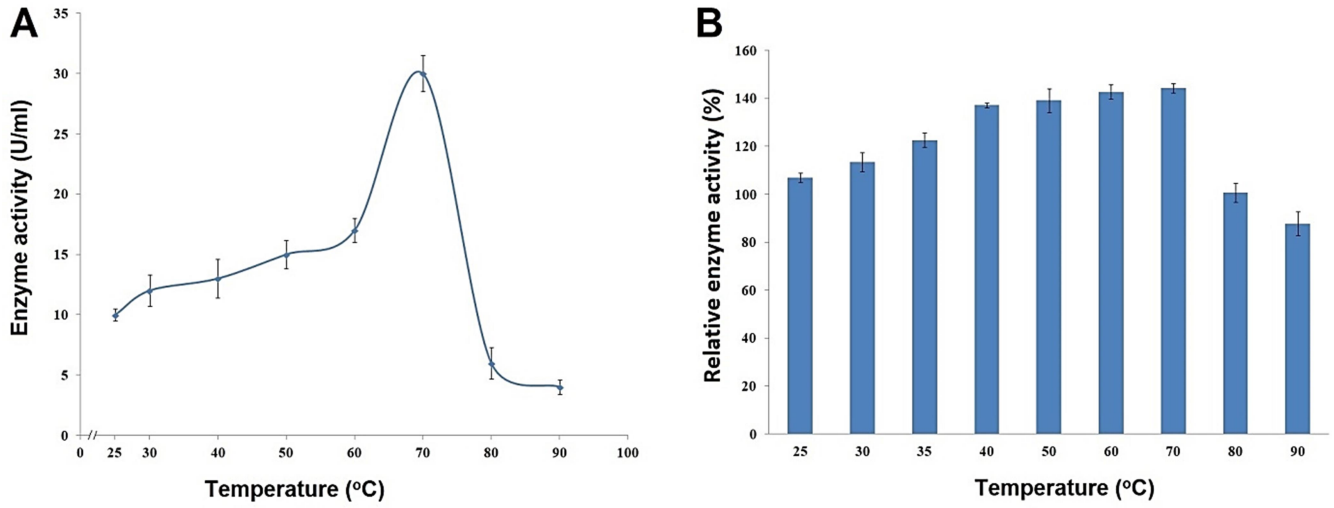

Figure 1. Effect of temperature on the extracellular lipase from haloarchaeon *Halolamina* sp. (A), and thermostability of *Halolamina* sp. lipase (B). The relative enzyme activity was calculated compared to the activity of enzyme assayed under the standard conditions.

The effect of pH on *Halolamina* sp. lipase was investigated via determination of enzyme activity between pH 3.0-10.0. Although a slight increase was observed at pH 4.0, the lipase activity was peaked at pH 7.0, and declined thereafter. The enzyme activity was determined to be optimum at pH 7.0 (Figure 2A). The stability of lipase was evaluated at different pH values and time points. The enzyme kept its activity same at pH 4.0, 72-88% at pH 6.0, and 80-99% for the rest (Figure 2B). The extracellular lipase from *Halolamina* sp. was found to be highly stable at diverse pH values.

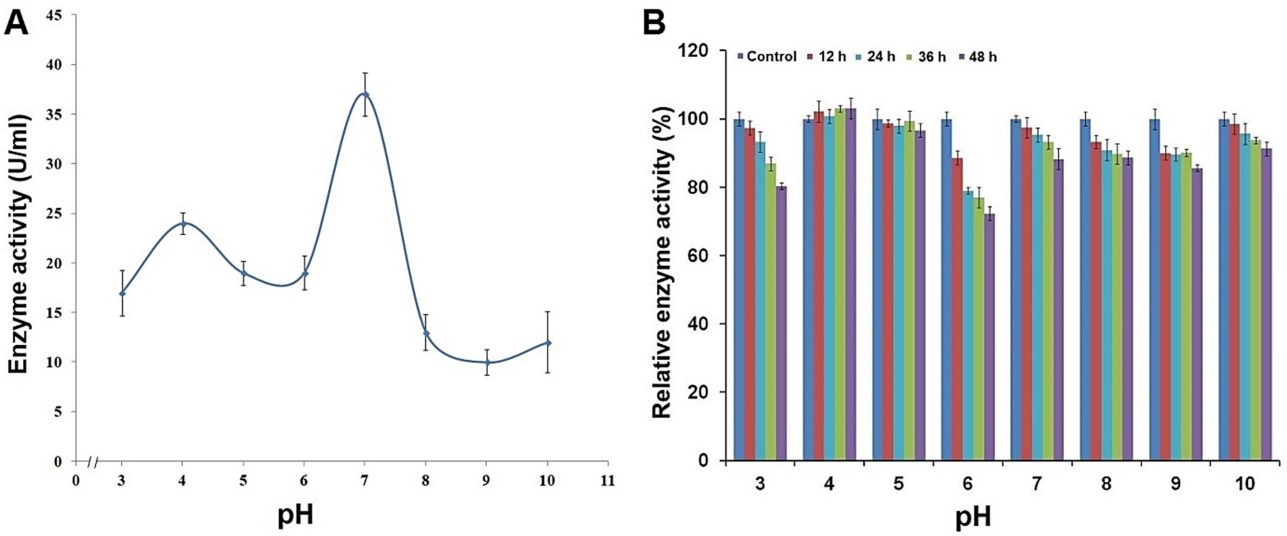

Figure 2. Effect of pH on the extracellular lipase from haloarchaeon *Halolamina* sp. (A), and pH stability of *Halolamina* sp. lipase (B). The relative enzyme activity was calculated compared to the activity of enzyme assayed under the standard conditions.

Effects of NaCl, metals, organic solvents, and detergents on lipase activity of *Halolamina* sp., and the lipase kinetics

Effect of NaCl on *Halolamina* sp. lipase was evaluated for 0.6 to 3.6 M salt concentrations. Elevated molarities of NaCl were accompanied by increased lipase activity. The highest enzyme activity was measured at 3.6 M NaCl, which is the saturation level for the buffer used (Figure 3). All metals used in this study (Ca^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}) had a positive effect on the activity of *Halolamina* sp. lipase. Increasing the concentration of metals from 3 µM to 5 µM led to an increase in enzyme activity from 28%-63% to 51%-88%. The highest increment was provided by $MnCl_2$ and $ZnSO_4$ at 3 µM and 5 µM concentrations, respectively (Figure 4).

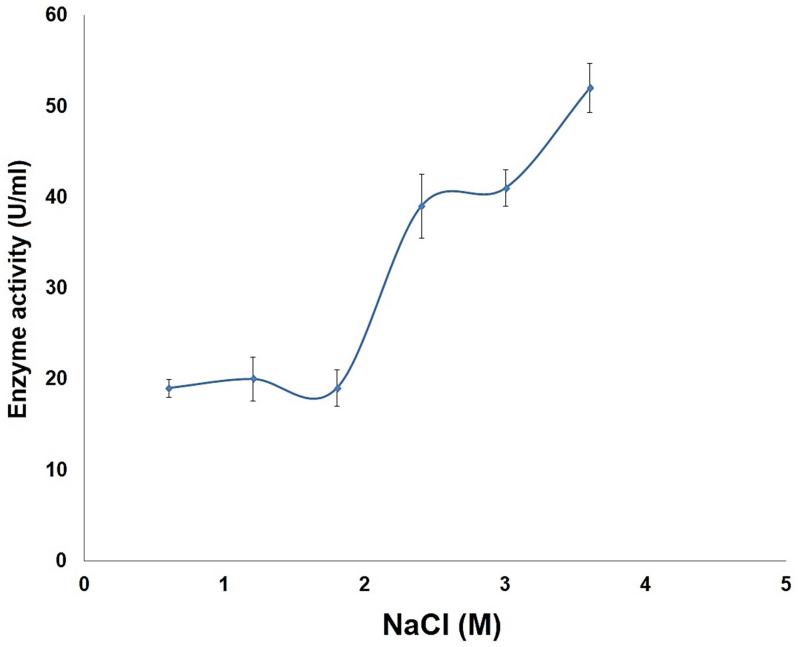

Figure 3. Effect of NaCl on the extracellular lipase from haloarchaeon *Halolamina* sp.

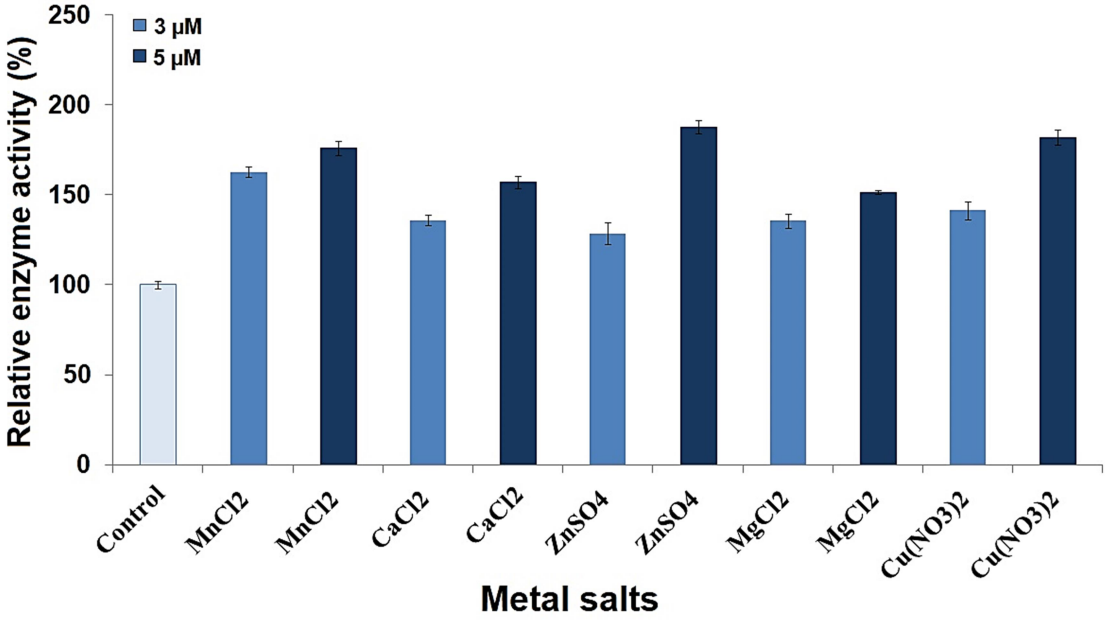

Figure 4. Effect of metals on the extracellular lipase from haloarchaeon *Halolamina* sp. The relative enzyme activity was calculated compared to the activity of enzyme assayed under the standard conditions (control).

The organic solvents used in this study showed inhibitory properties on the *Halolamina* sp. lipase at varying ratios. The lipase was highly resistant to acetone, methanol, and DMSO keeping it activity 90%. The highest inhibitory effect (67%) was observed in the presence of *n*-butanol. Detergents Tween-20, Tween-80, Triton X-100 and SDS decreased the lipase activity at 0.2% concentration. The least inhibitory activity (17%) was observed in the presence of SDS, and Tween-80 showed the highest inhibition (85%) (Figure 5). The kinetics of *Halolamina* sp. lipase was determined by a Lineweaver-Burk plot. K_m value of the lipase was calculated as 0.041 mM, and the V_{max} value was 0.0123 U/ml (Figure 6).

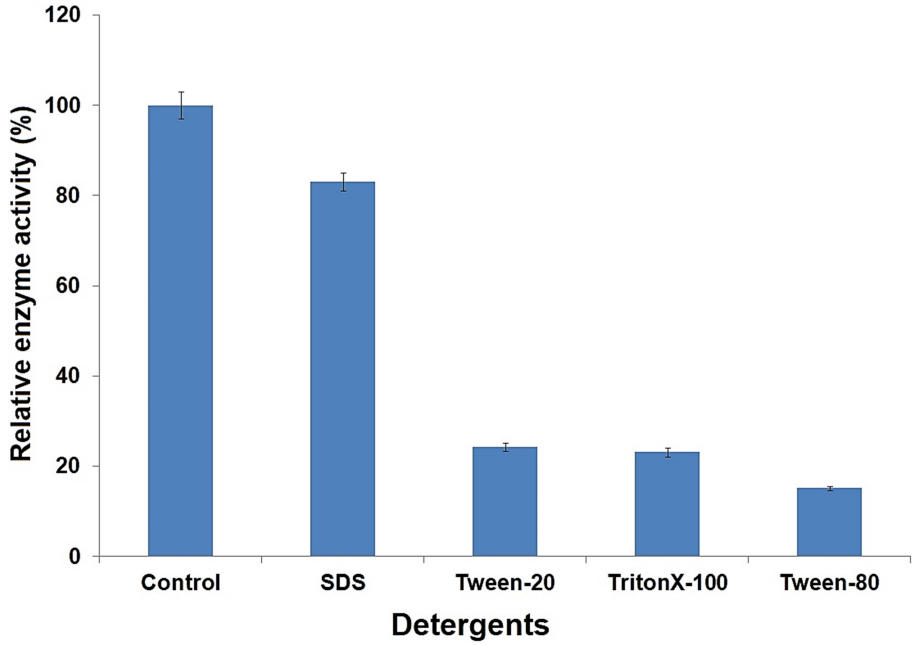

Figure 5. Effect of detergents on the extracellular lipase from haloarchaeon *Halolamina* sp. The relative enzyme activity was calculated compared to the activity of enzyme assayed under the standard conditions (control).

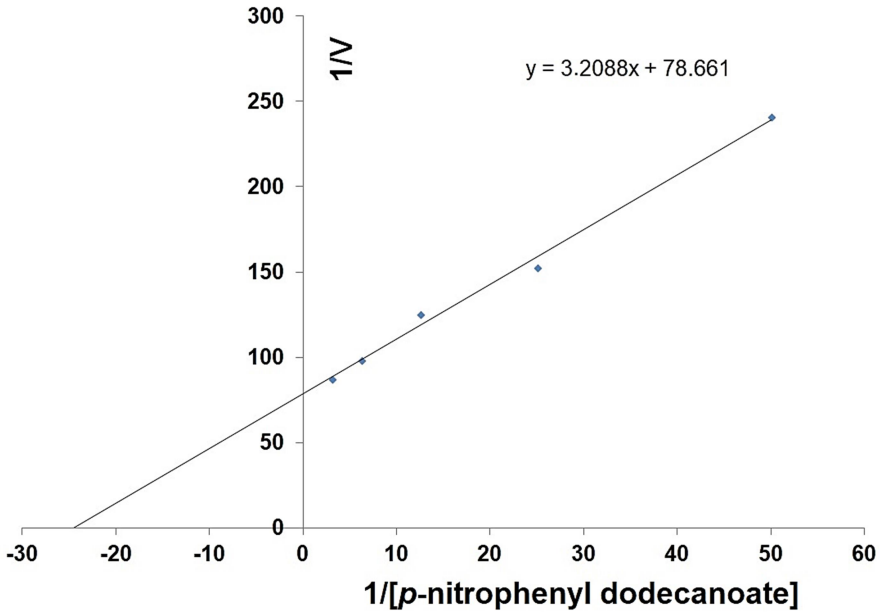

Figure 6. Effect of substrate on the kinetics of extracellular lipase from haloarchaeon *Halolamina* sp.

4. Discussion

Biotechnologically important enzymes from the extremophiles possess high activity and stability properties as reported for the protease produced by the haloarchaeon *Haloarcula* sp. isolated from a Salt Lake (Abanoz et al. 2017). Lipases are subclass of esterases, and involved in various industrial applications such as hydrolysis, alcoholysis, acidolysis, interesterification, esterification, and aminolysis (Rajendran and Thangavelu 2013). They are crucial enzymes especially for food, detergent, leather, pharmaceutical, cosmetic, textile, paper, and biodiesel industries. Such lipases are mainly obtained from mesophilic and thermophilic microorganisms. However, hydrolytic enzymes from extremophiles gained high attention in recent years. Among the extremozymes, lipases possess improved activity, stability, and broad substrate specificity at high or very low temperatures in non-aqueous or water/solvent environments as well as high thermodynamic stability in harsh industrial applications (Woodley et al. 2013; Febriani et al. 2020). Additionally, the chemical processes for the treatment of lignocellulosic materials occur at high temperature and under acid-base conditions. The neutralization process produces salt that should be further removed by the use of high water and energy. Utilization of halophilic lipases overcomes this extra energy consumption for the removal of excess salt (Şafak et al. 2020). Thus, salt tolerance adaptations of haloarchaea coupled with robust nature of lipases result in halophilic lipases as impressive candidates for various industrial and biotechnological applications (Schreck and Grunden 2014).

Halophilic lipases and esterases show optimum activity at the temperature range between 50-80^{o}C, pH values of 7.0-8.5, and NaCl concentrations of 0-4.5 M (reviewed by Schreck and Grunden 2014). In the present study, optimum temperature, pH, and NaCl concentration for the *Halolamina* sp. lipase were found as 70^{o}C, pH 7.0, and 3.6 M, respectively, under the experimental conditions. The activity of lipase increased 44% after incubation at 70^{o}C for 30 min and kept its activity 88% at 90^{o}C. Also, the lipase activity remained more than 70% after incubation at the pH values of 3.0-10.0 for 48h.

The optimum temperature and pH for thermophilic and halophilic esterase from *Janibacter* sp. R02 were determined as 80^{o}C and pH 8.0-9.0, respectively (Castilla et al. 2017). This enzyme kept its activity after incubation at boiling temperature for 1h and found more stable at alkaline pH values than acidic ones. The lipase from *Haloarcula* sp. G41 showed maximum activity at 70^{o}C and pH 8.0 in the presence of 15% NaCl. The enzyme activity remained high at the pH values of 7.0-11.0 (Li and Yu 2014). In another study, *Marinobacter lipolyticus* SM19 lipase had optimum activity at 80^{o}C and pH 7.0 but the enzyme activity decreased approximately 80% in the presence of 1-3 M NaCl and after incubation at 50^{o}C for 2 h, and 90% after incubation at 60^{o}C for 1h (Perez et al. 2011). Li et al. (2014) reported the optimum temperature, pH, and NaCl concentration for *Idiomarina* sp. W33 as 60^{o}C, pH 7.0-9.0, and 10%, respectively, keeping its activity until 65^{o}C and at the pH values of 7.0-11.0. Additionally, the lipase from

Salinivibrio sp. strain SA-2 showed optimum activity at 50°C and pH 7.5. This enzyme kept its stability at pH 7.5-8.0 and after incubation at 80°C for 30 min; however, 2 M KCl, NaCl and $NaNO_3$ inhibited 50-60% of the enzyme activity (Amoozegar et al. 2008). Ozcan et al. (2009) characterized lipase and esterase activities belonging to five isolates of archaea, and reported the optimum conditions as 45-65°C, pH 8.0-8.5, and NaCl concentrations of 3-4.5 M. As compared to the halophilic lipases previously studied, the extracellular lipase from *Halolamina* sp. was found to be active at high temperature, at high concentrations of NaCl, but at moderate pH value (pH 7.0).

Presence of various metals was reported to inhibit lipases from diverse organisms. $MgCl_2$, $MnCl_2$, $FeSO_4$, $CaSO_4$, $NiSO_4$ and $CuSO_4$ at 1mM concentration inhibited the activity of *Janibacter* sp. R02 lipase (Castilla et al. 2017). Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, especially Hg^{2+} at 1 mM concentration inhibited the activity of lipases from *Idiomarina* sp. W33 (Li et al. 2014) and *Haloarcula* sp. G41 (Li and Yu 2014). However, Ca^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+} at 5 µM concentrations increased the activity of *Halolamina* sp. lipase at a ratio of 51-88%.

Organic solvents, methanol, acetone, and DMSO, inhibited the extracellular lipase activity of *Halolamina* sp. approximately 10%, also 27% and 33% inhibition were caused by ethanol and butanol, respectively. Methanol, acetone, DMSO, ethanol and butanol inhibited the lipase activity from *Haloarcula* sp. G41 by 52%, 32%, 20%, 75%, and 19% (Li and Yu 2014) as well as from *Idiomarina* sp. W33 by 32%, 31%, 14%, 38%, and 26% (Li et al. 2014), respectively. On the other hand, 30% methanol and ethanol increased the activity of lipase from *Marinobacter lipolyticus* SM19 by 20% and 8%, respectively, yet this enzyme was also inhibited by acetone and DMSO less than 10% (Perez et al. 2011). Lipase from *Halolamina* sp. was found to be highly resistant to SDS, keeping 83% of its activity. The *K*m and V_{max} values for lipases from five isolates of archaea were reported as 0.052-0.167 mM and 0.053-0.092 U/ml, respectively, which were 0.041 mM and 0.0123 U/ml, respectively for *Halolamina* sp. lipase. The kinetics of extracellular lipase from *Halolamina* sp. was found to be lower.

5. Conclusions

The extracellular lipase from *Halolamina* sp., isolated from a salt mine in Turkey, was determined to be highly stable at a wide range of temperature and pH values as well as NaCl concentrations. Metals have a positive effect on the lipase activity, and it is resistant to methanol, acetone, DMSO, and SDS. This enzyme is promising to be used in biotechnological applications such as biodiesel production.

Authors' Contributions: OKAY, S.: conception and design, analysis and interpretation of data, drafting the article; ADEM, Ş.: conception and design, acquisition of data, analysis and interpretation of data; KURT-KIZILDOĞAN, A.: interpretation of data and drafting the article. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

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