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Production and Optimization of Alkaline Lipase by a Novel Psychrotolerant and Halotolerant Strain *Planomicrobium okeanokoites* ABN-IAUF-2 Isolated from Persian Gulf

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ABSTRACT

lipases have many different applications in detergents, cleaners, food industry, pharmaceutical industry, pulp and paper production and leather industry, but the extremophile lipases have more range applications. These enzymes are resistant to the high salty, temperature and alkaline conditions. Halophiles were isolated from Persian Gulf, Iran at 20°C in the presence of 10% NaCl. For screening the lipase producing bacteria, Rhodamine B agar and minimal medium were used. Then in order to find the best growth condition for the production of lipase, single-factor optimization was carried out. The best environmental conditions and their interactions for lipase production were obtained using 16 levels Taguchi statistical test. The WS4 isolate indicated a good lipase activity. The 16s-rDNA sequencing revealed that the WS4 isolate was Planomicrobium okeanokoites. We named this novel strain Planomicrobium okeanokoites ABN-IAUF-2 and its 16s-rDNA sequence was deposited in GenBank, NCBI, under accession number of KP403724. The most enzyme production was measured after 72 hours incubation at 20°C in the presence of hazelnut oil as carbon source and yeast extract as nitrogen source and pH 7. The analysis of Taguchi test showed that the most effective factors in enzyme production were carbon source with 54.65% and nitrogen source with 19% of effectiveness. This is the first report of alkaline lipase production by Planomicrobium okeanokoites. This lipase was resistant to low-temperature and 15% saline, so it has wide applications in medical as well as microbial biotechnology.

Key words: Alkaline Lipase, Halotolerant, Optimization, Psychrotolerant, *Planomicrobium okeanokoites*, Taguchi Statistical Test

INTRODUCTION

Marine facies which comprise more than 70% of the earth surface, are not only abundant in terms of biodiversity, but also are useful as a source of microorganisms with industrial potential and biotechnological applications. These habitats are really expanded in terms of microbial diversity [1]. Because most of the international waters are salty, these environments are useful for finding halophilic microorganisms [2]. Halophiles are aerobic microorganisms that their existence and growth are defined in saline media. The salt concentration of halophilic medium is 10 times more than oceans concentration. Halophilic microorganisms are categorized into 3 groups as: 1) Slight halophiles in which the optimum condition of growth is carried out in the salt concentration of 0.2-0.85 (M/l), equal to 2-5% (w/v) of NaCl. 2) Moderate halophiles in which the optimum condition of growth is carried out in the salt concentration of 3.4-5.1 (M/l), equal to 5-20% (w/v) of NaCl. 3) Extreme halophiles in which the optimum condition of growth is carried out in the salt concentrations over than 3.4-5.1 (M/l), equal to 20-30% (w/v) of NaCl.

Almost all of the halotolerant microorganisms could grow in a wide range of 0.5-21% of saline [3, 4]. Lipase (EC1.3.3.1) is an enzyme that capable of hydrolyzing the ester bond of triglyceride in order to produce glycerol and fatty acids [5]. Although lipases have many different applications in detergents, cleaners, food industry, pharmaceutical industry, pulp and paper production and leather industry, the extremophile lipases have more range applications. These enzymes are resistant to the high salty, temperature and alkaline conditions. So far, the production of lipase by halophilic microorganisms of *Salinivibrio* and *Natronococus* has been reported [6, 7]. Halophiles use different methods to resist the NaCl bactericidal effect. There are a lot of similar fundamental methods to send sodium ions into the outside of the cell. The usual method includes the accumulation of ion K^+ and Cl^- inside the cell to keep osmotic balance [8]. Another method to keep the turgescence pressure in the hyper saline habitat is the accumulation of the compatible dissolved compounds. These are molecules with low molecular weight which are accumulated in high salty concentrations and control osmotic pressure. Carbohydrates, alcohols, amino acids, betaine, ectoine and the other combinations which are made of these materials are the usual dissolved and compatible ingredients that are synthesized by halophiles [9]. The aims of this study were isolation and identification of halotolerant and psycrotholerant bacteria from marine environment, feasibility of alkaline lipase production by isolated halophiles and optimization of enzyme production using Taguchi statistical test.

MATERIALS AND METHODS

Culture media and Chemicals

The culture media applied in this study were enrichment culture medium [nutrient broth powder, 39 (g/l); NaCl, 10% (v/v); $MgSO_4$, 5% (v/v); $CaCl_2$, 5% (v/v); distilled water, 1000 ml; pH~8 adjusted with NaOH, 1M], screening culture media including olive agar A [olive oil, 10 ml; peptone, 5 (g/l); yeast extract, 3 (g/l); agar, 20 (g/l); NaCl, 100 (g/l); $MgSO_4$, 3 (g/l); K_2HPO_4, 1 (g/l); distilled water, 1000 ml; pH~8 adjusted with NaOH, 1M], olive agar B [olive oil, 10 ml; yeast extract, 2 (g/l); agar, 20 (g/l); NaCl, 100 (g/l); $MgSO_4$, 0.3 (g/l); K_2HPO_4, 0.1 (g/l); distilled water, 1000 ml; pH~8 adjusted with NaOH, 1M] and olive agar C [olive oil, 10 ml; yeast extract, 1 (g/l); NaCl, 100 (g/l); agar, 20 (g/l); $MgSO_4$, 0.3 (g/l); K_2HPO_4, 0.1 (g/l); distilled water, 1000 ml; pH~8 adjusted with NaOH, 1M] and Rhodamine B culture medium [olive oil, 1 ml; peptone, 5 (g/l); yeast extract, 3 (g/l); agar, 20 (g/l); NaCl, 100 (g/l); $MgSO_4$, 0.3 (g/l); K_2HPO_4, 0.1 (g/l); Rhodamine B 1% (w/v), 10 ml; distilled water, 1000 ml; pH~8 adjusted with NaOH, 1M]. For alkaline lipase production, oil broth medium [olive oil, 10 ml; yeast extract, 1 (g/l); NaCl, 100 (g/l); $MgSO_4$, 3 (g/l); K_2HPO_4, 1 (g/l); Arabic gum, 10 (g/l); distilled water, 1000 ml; pH~8 adjusted with NaOH, 1M] was used. The olive oil used was from Sigma-Aldrich, USA. The rest of chemicals were from Merck, Germany.

Sampling, enrichment and isolation of halophiles

For water sampling a 1000 ml sterile bottle was used. The surface water of Persian Gulf at Bandar Abbas harbor, Hormozgan province, Iran, was collected in the bottle and after capping immediately transferred to microbiology laboratory in Research Laboratories Complex, Falavarjan Branch, Islamic Azad University, Isfahan, Iran at 4°C. Ten milliliters of Persian Gulf water sample were transferred to enrichment culture medium using aseptic condition and incubated at 20°C and 140 rpm of aeration speed for 1 to 5 days [10].

The primary screening of the isolated bacteria

In this method, nutrients for bacterial growth were made accessible slowly. Therefore, bacteria were enforced to use olive oil as carbon and energy source in an adaptable manner. At first, 200 µl of Persian Gulf water was cultured on olive agar A and incubated at 20°C for 1 to 5 days. The bacterial colonies from olive agar A were cultured on olive agar B and incubated at 20°C for 1 to 5 days. Finally the bacteria of olive agar B were transferred to olive agar C and incubated at 20°C for 1 to 5 days. The latter had the more olive oil proportionally which resulted in the induction of alkaline lipase production and the use of olive oil as the main carbon and energy source by selected bacteria.

The secondary screening of the isolated bacteria

For screening the alkaline lipase-producing bacteria, the colonies from olive agar C were cultured in Rhodamine B culture medium using streak plate method and incubated at 20°C for 24-72 hours. Then the Rhodamine B agar was exposed to the UV wavelength of 350-370 nanometers. The formation of orange fluorescent around the individual colonies showed the lipase activity of isolated bacteria [11].

The alkaline lipase assay

In this research two different methods have been used to measure the activity of the alkaline lipase as follow.

1) Colorimetric method with nitrophenyl palmitate as the substrate

The first solution was included of 30 mg paranitrophenyl palmitate which was solved in 10 ml of propane-2-ole and the second solution was included 1 ml of triton 100x in 90 ml of phosphate buffer (0.5 M, pH~8). The two solutions

were mixed to prepare the mix solution. The bacterial culture media were centrifuged at 12000 RPM, 4°C for 10 minutes. Then 100 µl of supernatant was filter sterilized using 0.45 µm syringe filter, added to 900 µl of mix solution and incubated at 30°C, 160 RPM of aeration speed for 15 minutes. In the presence of alkaline lipase, the paranitrophenyl palmitate would be broken to paranitrophenyl and palmitate. The primary substrate is pale but the paranitrophenyl color is yellow. The intensity of the yellow color, indicative of alkaline lipase activity, was measured by spectrophotometer at 410 nm. One unit of alkaline lipase activity was defined as the amount of enzyme that could release 1 µl of paranitrophenyl from substrate, paranitrophenyl palmitate, at 30°C during 15 minutes of the reaction time [12].

2**) Titration method with olive oil as the substrate**

In this method the bacterial culture media were centrifuged at 12000 RPM, 4°C for 10 minutes. Then 1 ml of supernatant was filter sterilized using 0.45 µm syringe filter and added to 5 ml of substrate [olive oil, 5% (w/v), Arabic gum, 1% (w/v), phosphate buffer, 0.5 M, pH~8]. The mixture was incubated at 30°C, 160 RPM of aeration speed for 60 minutes. The 6 ml of mixture were transferred to a dry, clean 100 ml Erlenmeyer flask and 4 ml of ethanol-acetone mixture [(1:1), (v/v)] was added to the mixture to stop the enzyme reaction. Then 3-4 drops of phenolphthalein 1% were added to the flask and the contents were titrated against NaOH (0.05 M) until the disclosing of pale pink color in the flask contents. The amount of consumed NaOH, equal to fatty acid release, was measured. One unit of alkaline lipase activity was defined as the amounts of released fatty acid (mM) in one minute of reaction time and was reported by the scale of u/ml/m [13].

Optimization of alkaline lipase production in different conditions

Because the production of extracellular enzymes is severely affected by the environmental conditions and the precursors which are necessary for bacterial growth, several effective factors on bacterial growth and the alkaline lipase production by isolated bacterium were optimized. Optimization procedures were made based on single-factor method and multiple-factors method using Taguchi statistical examination to analyze the effects of variable environmental factors and their interactions on the production of alkaline lipase by the selected bacterial isolate [14, 15].

The evaluation of incubation time on the production of alkaline lipase by WS4 isolate

In order to analyze the best incubation time for alkaline lipase production, 1 ml of WS4 (Water Sample 4) isolate broth medium (pH~8), with the turbidity of 0.5 McFarland standard (equal to 1.5×10^8/ml of bacteria) was transferred to oil broth culture medium and incubated at 20°C, aeration speed of 160 RPM for 96 hours. After 24, 48, 72, and 96 hours of incubation, 1 ml of broth culture was collected and the alkaline lipase activity was measured using titration method [16].

The effects of different carbon sources on the production of alkaline lipase by WS4 isolate

The oil broth culture media with different types of oils were prepared. The 7 different kinds of oil in the media were included olive, Tributyrin, tween 80, sesame, almond, hazelnut and coconut oil. The pH of all mediums was set to 8. One milliliter of WS4 isolate broth medium (pH~8), with the turbidity of 0.5 McFarland standard (equal to 1.5×10^8/ml of bacteria) was transferred to each oil broth culture medium and incubated at 20°C, aeration speed of 160 RPM for 72 hours. Then 1 ml of each broth culture was collected and the alkaline lipase activity was measured using titration method [16].

The effects of different primary pH on the production of alkaline lipase by WS4 isolate

The oil broth culture media with different pHs of 7, 8, 9 and 10 were prepared. One milliliter of WS4 isolate broth medium, with the turbidity of 0.5 McFarland standards (equal to 1.5×10^8/ml of bacteria) was transferred to each oil broth culture medium and incubated at 20°C, aeration speed of 160 RPM for 72 hours. Then 1 ml of each broth culture was collected and the alkaline lipase activity was measured using titration method [16].

The effect of different nitrogen sources on the production of alkaline lipase by WS4 isolate

Four different kinds of nitrogen sources including yeast extract, peptone, triptonee and $NaNO_3$ were selected. The oil broth culture media with these different types of nitrogen sources were prepared. The pH of all media was set to 8. One milliliter of WS4 isolate broth medium (pH~8), with the turbidity of 0.5 McFarland standard (equal to 1.5×10^8/ml of bacteria) was transferred to each oil broth culture medium and incubated at 20°C, aeration speed of 160 RPM for 72 hours. Then 1 ml of each broth culture was collected and the alkaline lipase activity was measured using titration method [16].

T**he effect of different temperatures on the production of alkaline lipase by WS4 isolate**

In order to analyze the best temperature for alkaline lipase production, 1 ml of WS4 isolate broth medium (pH~8), with the turbidity of 0.5 McFarland standard (equal to 1.5×10^8/ml of bacteria) was transferred to each oil broth

culture medium and incubated at different temperatures of 15, 20, 25 and 30°C, aeration speed of 160 RPM for 72 hours. Then 1 ml of each oil broth culture was collected and the alkaline lipase activity was measured using titration method [16].

Optimizing the production of alkaline lipase by WS4 isolate based on the statistical Taguchi method

The optimization of alkaline lipase production by WS4 isolate based on the statistical Taguchi method was carried out using Qualiteck-4 software. Before planning the tests, among diverse factors, a limited number of the most important factors were selected for the next studies. These variables were pH, in 3 levels, temperature, in 3 levels, and carbon source, nitrogen source and NaCl concentration each in 2 levels (Table 1). Using these variables and their levels, the Qualiteck-4 software designed 16 experimental conditions (Table 2). The designated experiments were fulfilled and the related results were analyzed by the software.

Table1. The factors and their levels used for the production of alkaline lipase by WS4 isolate in Taguchi experiment

Factor	Level-1	Level-2	Level-3
pH	7	7.5	8
Carbon source	Almond oil	Hazelnut oil	------
Nitrogen source	Yeast extract	Triptone	------
Temperature	10°C	15°C	20°C
NaCl concentration	5%	10%	------

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Table2. The L-16 platform of experimental conditions for the production of alkaline lipase by WS4 isolate designated by Qualiteck-4 software

Condition	pH	Temperature (oC)	NaCl Concentration	Carbon Source	Nitrogen Source
1	7	10	5%	Almond oil	Triptone
2	7	15	5%	Almond oil	Triptone
3	7	20	10%	Hazelnut oil	Yeast extract
4	7	10	10%	Hazelnut oil	Yeast extract
5	7.5	15	10%	Almond oil	Yeast extract
6	7.5	20	10%	Almond oil	Yeast extract
7	7.5	10	5%	Hazelnut oil	Triptone
8	7.5	15	5%	Hazelnut oil	Triptone
9	8	20	10%	Hazelnut oil	Triptone
10	8	10	10%	Hazelnut oil	Triptone
11	8	15	5%	Almond oil	Yeast extract
12	8	20	5%	Almond oil	Yeast extract
13	7.5	10	5%	Hazelnut oil	Yeast extract
14	7.5	15	5%	Hazelnut oil	Yeast extract
15	7.5	20	10%	Almond oil	Triptone
16	7.5	10	10%	Almond oil	Triptone

Molecular identification of WS4 isolate by 16s-rDNA analysis

An individual colony of WS4 isolate in Olive Agar C medium was cultured in 100 ml of oil broth medium and incubated at 30ºC, 160 RPM of aeration speed for 24 hours. Ten milliliters of oil broth medium was transferred to 15 ml sterile falcon and centrifuged at 3000g for 15 minutes. The supernatant was discarded and 10 mg of bacterial biomass was used for DNA extraction using DNA extraction kit (Bioneer, South Korea). The universal primers used for molecular identification of WS4 isolate, were RW01 as forward primer with the sequence of 5'AACTGGAGGAAGGTGGGGAT3' and DG74 as reverse primer with the sequence of 5'AGGAGGTGATCCAACCGCA3'. PCR was performed in an Eppendorf Thermal Cycler. The PCR program encompassed initial denaturation at 96°C for 4 minutes, followed by 30 cycles each of 94°C for 2 minutes, 55°C for 1 minute, 72°C for 1 minute, 72°C for 4 minutes and incubation at 4°C for 10 minutes. The expected molecular weight of PCR product using these primers was 370 bp [17]. The PCR product and primers were sent to Macrogene Co., South Korea, for DNA sequencing. The DNA sequence was reviewed using Finch TV V.1.4.0 and Mega4 software and its similarity to GenBank genomic sequences was investigated using BLASTN software (http://blast.ncbi.nlm.nih.gov). The isolated strain was identified after bioinformatics analysis.

RESULTS

The WS4 strain isolated from Persian Gulf, Hormozgan province, Iran had the good lipolytic activity in selective culture media of olive agar A, olive agar B and olive agar C and was selected for the subsequent studies. This bacterium was able to grow in different concentrations of salt ranging from 5 to 15% (w/v) and in alkaline conditions. The PCR of purified bacterial DNA with universal primers showed the 370 bp band in gel electrophoresis (Figure 1). The analysis of genomic sequence of 16s-rDNA using Finch TV and Mega4 and then BLASTN confirmed that the isolated strain from Persian Gulf water sample was related to species *Planomicrobium*

okeanokoites. This strain was named *Planomicrobium okeanokoites* ABN-IAUF-2 and its 16s-rDNA partial sequence was deposited in GenBank, NCBI under the accession number of KP403724. The phylogenic tree of this novel strain has been indicated in Figure 2.

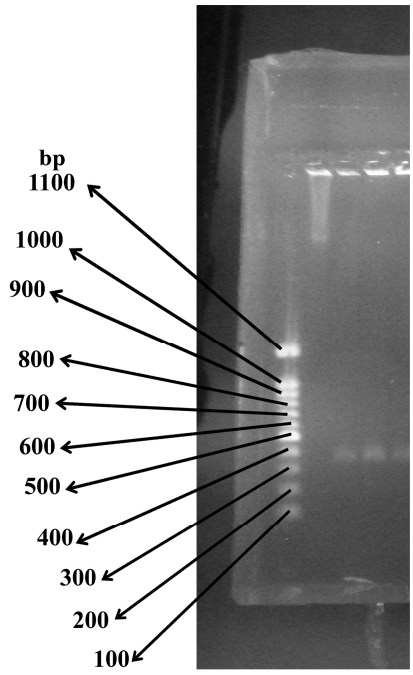

Figure1. The gel electrophoresis of the extracted DNA from WS4 isolate after PCR with 16s-rDNA universal primers. The MW of product is 370 bp

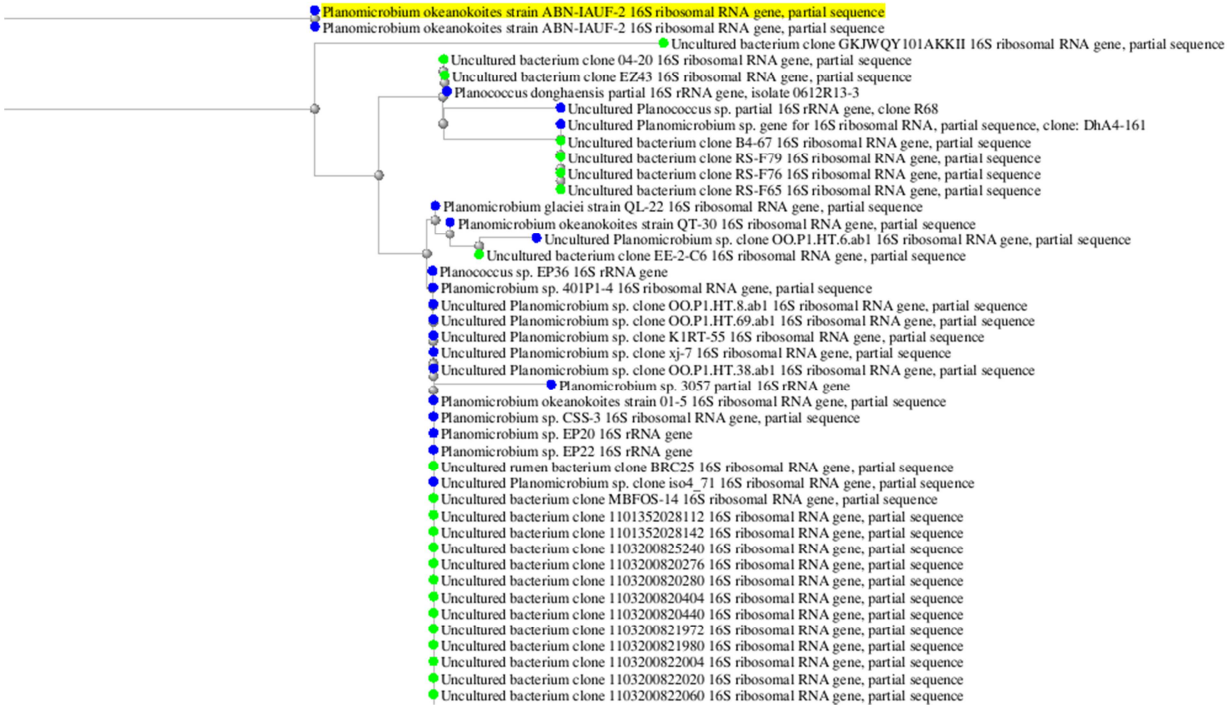

Figure 2. The phylogenic tree of *Planomicrobium okeanokoites* ABN-IAUF-2 isolated from Persian Gulf

The secondary screening method for disclosing the lipase-producing bacteria using Rhodamine B culture medium was positive for *Planomicrobium okeanokoites* ABN-IAUF-2. The *Planomicrobium okeanokoites* ABN-IAUF-2 individual colonies in Rhodamine B agar after exposing to the UV wavelength of 350-370 nm showed the orange fluorescent**.** In the evaluation of incubation time effects on the production of lipase by *Planomicrobium*

okeanokoites ABN-IAUF-2, the activity of lipase after 24, 48, 72 and 96 hours of incubation at 20°C and aeration speed of 160 RPM were measured as 9, 10, 10.80 and 10.35 (u/ml) respectively. It was indicated that the highest lipase activity was obtained after 72 hours of incubation (Figure 3).

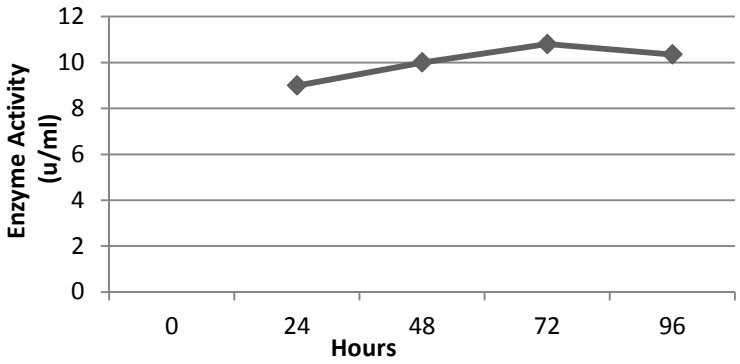

Figure 3. The effects of incubation time on the production of lipase by *Planomicrobium okeanokoites* ABN-IAUF-2 at 20°C and aeration speed of 160 RPM

In the evaluation of different carbon source effects on the production of lipase by *Planomicrobium okeanokoites* ABN-IAUF-2 at 20°C, aeration speed of 160 RPM after 72 hours incubation, the activity of enzyme in different oil broth media with carbon sources of olive oil, sesame oil, hazelnut oil, almond oil, tween 80**,** coconut oil and Tributyrin were measured as 10.80, 14, 17, 15, 14, 14.30 and 11 (u/ml) respectively. It was shown in Figure 4 that the maximum enzyme activity was obtained in the presence of hazelnut oil as the best source of carbon in the oil broth medium and was equivalent to 17 (u/ml).

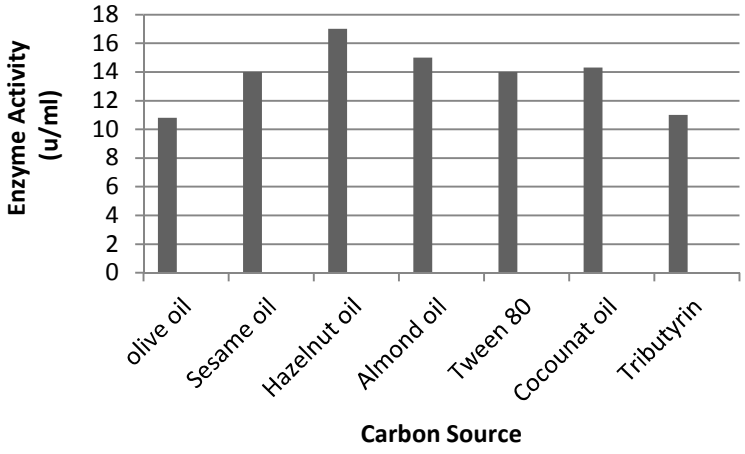

Figure 4. The effects of different carbon sources on the production of lipase by *Planomicrobium okeanokoites* ABN-IAUF-2 after 72 hours incubation at 20°C and aeration speed of 160 RPM

As it is drawn from Figure 5, in the evaluation of initial pH effects on the production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 after 72 hours incubation at 20°C and aeration speed of 160 RPM, the activity of lipase in oil broth media with initial pHs of 7, 8, 9, and 10 were equivalent to 10.8 (u/ml), 7 (u/ml), 4 (u/ml) and 4 (u/ml) respectively. So the highest enzyme activity was achieved in pH 7 and was equivalent to 10.8 (u/ml).

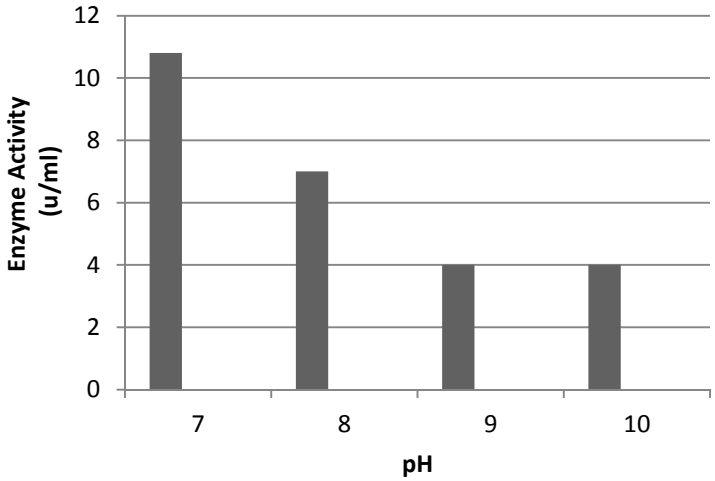

Figure 5. The effects of culture media initial pH on the production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 after 72 hours incubation at 20°C and aeration speed of 160 RPM

Figure 6 indicates that in the evaluation of different nitrogen source effects on the production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 at 20°C, aeration speed of 160 RPM after 72 hours incubation, the activity of enzyme in different oil broth media with nitrogen sources of yeast extract, peptone, triptone and $NaNO_3$ were equivalent to 10.8 (u/ml), 5.6 (u/ml), 8.9 (u/ml) and 5 (u/ml) respectively. It was indicated that the maximum of enzyme production was occurred in the presence of yeast extract as the nitrogen source and was measured as 10.8 (u/ml).

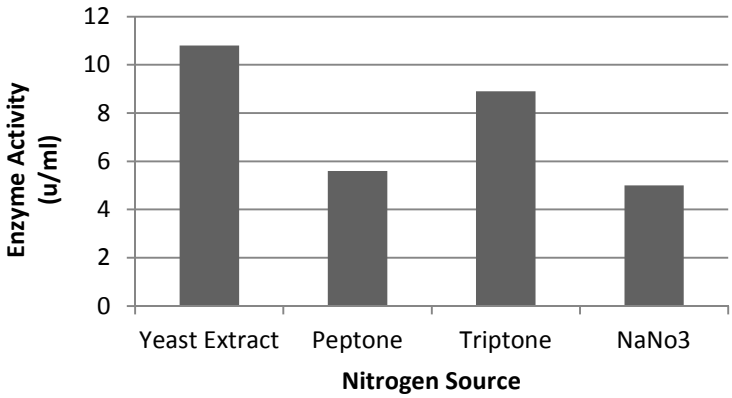

Figure 6. The effects of different nitrogen sources on the production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 after 72 hours incubation at 20°C and aeration speed of 160 RPM

As it is obtained from Figure 7, in the evaluation of different temperature effects on the production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 at the aeration speed of 160 RPM after 72 hours incubation, the enzyme activity in different incubation temperatures of 15, 20, 25 and 30°C were measured as 18 (u/ml), 20 (u/ml), 14 (u/ml) and 10.8 (u/ml) respectively. So it was shown that the maximum of enzyme activity was achieved at 20°C and measured as 20 (u/ml).

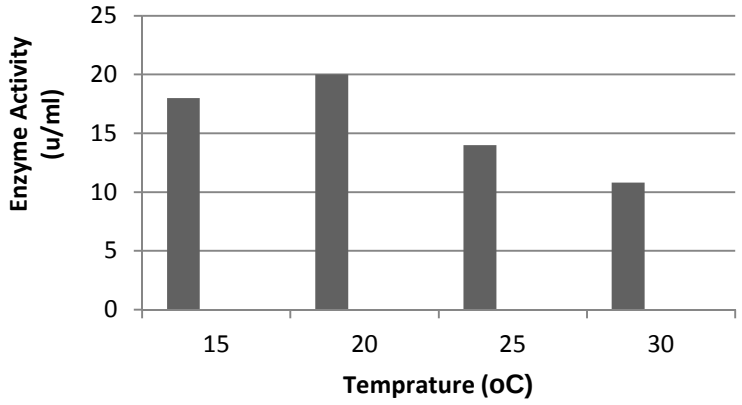

Figure 7. The effects of different temperatures on the production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 after 72 hours incubation at the aeration speed of 160 RPM

The results of statistical Taguchi experiment for production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 have been indicated in Table 3. This examination showed that the carbon source was the most effective factor in producing lipase with the level of 54.65%. The second and third effective factors were nitrogen source and temperature with the level of 19.00% and 11.78% respectively. The least effective factors in lipase production by *Planomicrobium okeanokoite*s ABN-IAUF-2 were pH and NaCl with the amounts of 11.54% and 0.46% respectively.

Table 3. The results of L-16 platform of experimental conditions for the production of alkaline lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 designated by Qualiteck-4 software

Condition Number	Lipase activity (u/ml)
1	18
2	17
3	16
4	16
5	16
6	15
7	15.5
8	15
9	17.5
10	18
11	21
12	18
13	15
14	16
15	18.5
16	16.5

DISCUSSION

In this study, WS4 strain isolated from Persian Gulf water, Hormozgan province, Iran was a gram positive, cocobacillus-shaped, non-spore forming bacterium that was able to grow in various concentrations of salt ranging from 5 to 15%. This isolate was identified as *Planomicrobium okeanokoite*s ABN-IUF-2 using 16s-rDNA analysis and its sequence was deposited in GenBank, NCBI under accession number of KP403724. There are no reports indicating the lipase production by *Planomicrobium spp* yet. In different studies, various culture media have been applied for screening of lipase-producing bacteria. Chakraborty et al. have used the tributyrin for screening lipase producing *Bacillus licheniformis* H1 strain [18]. The same culture medium has been used for isolating the *Staphylococcus* sp. capable of lipase production [19]. Wang et al have used Rhodamin B Agar for screening alkaline lipase producing *Pseudomonas* sp. [20]. In all mentioned researches, the carbon source has been a kind of oil. The presence of other hydrocarbons such as sugars has an inhibitory effect on the lipase production. If in Rhodamin B Agar, the glucose is used as the carbon source, the orange fluorescent will not be observed because the bacterium uses an easier carbon source and lipase production could be inhibited [18, 19, 20]. In this study, for the initial screening of lipase producing bacteria, a culture medium containing olive oil as the only source of carbon was used but the bacteria were not suddenly placed in this medium, rather the gradual loss of nutrients has led to the adaptation of isolated WS4 isolate and induction of lipase secretion. Since the growth of bacteria didn't give us a

criterion of lipase production, the bacteria were cultured on Rhodamin B Agar medium including 1% of olive oil. The tributyrin has been reported as the best carbon source for alkaline lipase production by *Acinetobacter* sp. Also the most appropriate carbon source for the production of this enzyme by *Rhizopus oligosporus* was Tween 80 [13]. The best carbon source for lipase production by *Pseudomonas aeroginosa* was olive oil and Tween 80-olive oil respectively [22]. In this research, to optimize the carbon source for production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2, seven kinds of oils were used. The results of single-factor optimization showed that the most appropriate carbon source for production of this enzyme by *Planomicrobium okeanokoite*s ABN-IAUF-2 were hazelnut oil and almond oil respectively. In different studies, a number of optimal pHs have been reported for alkaline lipase production by different spp. The best pH for lipase production was 7 for *Marinobacter Lipulyticus* [23]. In the present study, the initial pH of isolation and screening was set on 8. Therefore, only the alkalophylic and alkalotolerant bacteria were able to grow in culture medium. In order to obtain the most appropriate pH of medium for production of lipase, different pHs were evaluated. But the best initial pH of culture medium for production of lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2 isolated from Persian Gulf was pH 7. Also the best incubation temperature for producing lipase by *Planomicrobium okeanokoite*s ABN-IAUF-2, was 20°C that was fitted to the temperature of incubation from which isolated. The optimal temperature for production of alkaline lipase by *Marinobacter* SM19 was measured as 25°C. Also the appropriate temperature for production of this enzyme by *Acinetobacter johnsonii* LP28 was 30°C, for *Bacillus licheniformis* H1s, as 50°C and for *staphylococcus* sp. as 36°C [21, 24, 25]. Chakraborty and Raj have reported that the best nitrogen source for production of lipase by *Bacillus Licheniformis* H1 was yeast extract [18]. Prasanth Kumar and Valsa have indicated that the most appropriate nitrogen source for lipase production by *Bacillus coagulans* was triptone [26]. In this study, different nitrogen sources such as triptone, peptone, yeast extract and $NaNO_3$ in alkaline lipase production were evaluated. We suggested that the best nitrogen source for alkaline lipase production by *Planomicrobium okeanokoite*s ABN-IAUF-2 was yeast extract. So far there is no report indicating the optimization of alkaline lipase production by Taguchi statistical test. Taguchi test was designed for optimization of lipase production by *Planomicrobium okeanokoite*s ABN-IAUF-2 with 16-level platform. The analysis of statistical examinations showed that the most appropriate levels for production of the enzyme by *Planomicrobium okeanokoite*s ABN-IAUF-2 was level 11 with the maximum of lipase activity of 21 (u/ml). The carbon source had the most influence of 54.65%, in lipase production by *Planomicrobium okeanokoite*s ABN-IAUF-2. The nitrogen source with 19.00%, pH with 11.54%, temperature with 11.78% and NaCl concentration with 0.46% of influence were other effective factors in alkaline lipase production by *Planomicrobium okeanokoite*s ABN-IAUF-2.

CONCLUSION

This is the first report of production of lipase by a novel halotolerant and psychrotolerant bacterium, *Planomicrobium okeanokoite*s ABN-IAUF-2, that was isolated from Persian Gulf water, Hormozgan province, Iran. The maximum enzyme activity of 21 (u/ml) was measured in the presence of hazelnut oil as the carbon source in oil broth medium after 72 hours incubation at the pH 7 and 20°C. According to new advances in the production of industrial enzymes, the use of enzymes produced by extremophiles like *Planomicrobium okeanokoite*s could be an asset in the areas of Medical, Pharmaceutical and industrial Biotechnology.

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REFERENCES

[1] Bruni V, Maugeri T, Alonzo V. Lipolytic activity of marine bacteria influence of NaCl and $MgCl_2$. Marine Biol. 1982;67:113-119
[2] Kumar SR, Kapoor S, Singh SP, Khare SK. Screening and isolation of halophilic bacteria producing industrially important enzymes. Braz J Microbiol. 2012;43:1595–1603
[3] Ventosa A, Nieto JJ, Oren A. Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev. 1998;62:504–554
[4] Ventosa A, Sánchez-Porro C, Martin S, Mellado E. Halophilic archaea, and bacteria as a source of extracellular hydrolytic enzymes. In: Simerman G. High Salt Concentration in Archaea, Bacteria and Eukaria, Springer, 2005;2nd edition: 337–355.
[5] Jaeger KE, Reetz MT. Microbial lipases form versatile tools for biotechnology. Trd Biotechnol. 1998;16:396-403
[6] Amoozegar MA, Salehghamari E, Khajeh kabiri M . Production of an extracellular thermohalophilic lipase from a moderately halophilic bacterium, *Salinivibrio* sp. strain SA-2. J Bas Microbiol. 2008;48:160–167

[7] Boutaiba S, Bhatnagar T, Hacene H, Mitchell DA, Baratti JC. Preliminary characterization of a lipolytic activity from an extremely halohilic archaeon, *Natronococcus* sp. J Mol Cat Enzm. 2006;41:21–26
[8] Kushner D, Kamekura, M. Physiology of halophilic eubacteria. In: Rodriguez-Valera F, Boca Raton FL. Halophilic Bacteria, CRC Press, 1998;1st edition: 109-140.
[9] Bursy J, Kuhlmann AU, Pittelkow M, Hartmann H, Jebbar M, Pierik J, Bremer E. Synthesis and uptake of the compatible solute ectoine and 5-hydroxyectoine by Streptomyces coelicolor A3(2) in response to salt and heat stresses. Appl Environ Microbiol. 2008;74:7286-7296
[10] Kouker G, Jaeger KE. Specific and sensitive plate assay for bacterial lipases. Appl Environ Microbiol. 1987;53:211-213
[11] Vorderwülbecke T, Kieslich K, Erdmann H. Comparison of lipases by different assays. Enz Microbiol Technol. 1992;14:631–639
[12] Singha M, Singhb RS, Banerjeea UC .Stereoselective synthesis of (R)-1chloro-3(3,4-difluorophenoxy)-2-propanol using lipases from *Pseudomons aeruginosa* in ionic liquid-containing system. J Mol Catal Enz B. 2008;56:294-299
[13] Nahas E. Control of lipase production by *Rhizopus oligosporus* under various growth conditions. J Gen Microbiol. 1998;134:227–233
[14] Sugihara A, Tani T, Tominaga Y. Purification and characterization of a novel thermostable lipase from *Bacillus* sp. J Biochem. 1991;109:211-215
[15] Sirisha E, Lakshmi Naras MU, Rajasekar N. Isolation and optimization of lipase producing bacteria from oil contaminated soils. Adv Biol Res. 2010;4:249-252
[16] Mahler G, Kok, R, Cordenons A, Hellingwerf K, Nudel, B. Effects of carbon sources on extracellular lipase production and lipA transcription in *Acinetobacter calcoaceticus*. J Ind Microbiol Biotechnol. 2000;24:25–30
[17] Bickley J, Short JK, McDowell DG, Parkes HG. Polymerase Chain Reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. Lett Appl Microbiol. 1996;22:153– 158
[18] Chakraborty K, Paul Raj R. An extra-cellular alkaline metallolipase from *Bacillus licheniformis* MTCC 6824: Purification and biochemical characterization. Food Chem. 2008;109:727–736
[19] Thomson CA, Delaquis PJ, Mazza G. Detection and Measurement of Microbial Lipase Activity: A Review. Crit Rev Food Sci Nut. 1999;39:165-187
[20] Wang SL, Lin YT, Liang TW, Chio SH, Ming LJ, Wu PC. Purification and characterization of extracellular lipases from *Pseudomonas monteilii* TKU009 by the use of soybeans as the substrate. J Ind Microbiol Biotechnol. 1999;36:65-73
[21] Wang HK et al. Novel Alkaline Lipase from *Acinetobacter johnsonii*. Food Technol Biotechnol. 2011;49:96–102
[22] Singh S, Banerjee U. Purification and characterization of trans-3-(4-ethoxyphenyl) glycidic acid methyl ester hydrolyzing lipase from *Pseudomonas aeruginosa*. Proc Biochem. 2007;42:1063-1068
[23] Perez D, Kovacic F, Wilhelm S, Jaeger KE, Garcia MT, Ventosa A, Mellado E. Identification of amino acids involved in the hydrolytic activity of lipase LipBL from *Marinobacter lipolyticus*. Microbiol. 2003;158:2192-2203
[24] Pérez D, Martín S, Fernández-Lorente G, Filice M, Guisán JM, Ventosa A, García MT, Mellado EA. A novel halophilic lipase, LipBL, with applications in synthesis of Eicosapentaenoic acid (EPA). 2011; 0023325.
[25] Khyami-Horani H. Thermotolerant strain of *Bacillus licheniformis* producing lipase. W J Microbiol Biotechnol. 1996;12:399-401
[26] Prasanth Kumar MP, Valsa AK. Optimization of culture media and cultural conditions for the production of extracellular lipase by *Bacillus coagulans*. Ind J Biotechnol. 2007;6:114-117