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Screening and Characterization of an Alkaline Protease Isolated from Hot Springs in North IRAN

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ABSTRACT

Alkaline proteases are an important group enzyme to commercially biology that are used as additives in detergents In this study, samples of hot springs in Ramsar, and the purpose of the identification of alkaline protease-producing bacteria native was isolation and identification as a new species and strain. Samples isolated from a hot spring to transfer a laboratory and in medium supplemented with Skim milk has enriched in temperature of 55 ° C. And then cultured on plates supplemented with Skim milk and colony were isolated by a halo. Then did measured isolated production rate according to conventional techniques (Lowry's method). Finally morphological and biochemistries characteristics of isolates were identified by API kit and the final Identification of bacteria was also sequenced 16s r RNA. In this study, two protease-positive bacteria were isolated. The new two strain have the ability to produce alkaline protease high temperature above 55 ° C and pH 8. The genus Bacillus is isolated from family and Bacillaceae. After the initial identification sequencing have relationship 99% to Bacillus sp. SGD-03 and Bacillus sp. PH-2. The new two strain of alkaline protease production at 60 ° C after 48 h to reach their Maximum. Since the consumption is very high alkaline protease in the industry. Therefore isolation of native strains producing heat-stable alkaline protease is important. In addition to this study two isolates high levels of protease producing, the able to have produce protease at high temperature. It should be noted that this isolates are wild strains and in their Protease produced naturally habitat, so no optimization has been done on protease production. Optimization of culture conditions on protease production could be this isolates to considered by a producer industry.

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INTRODUCTION

Generally thermophiles microorganisms with the ability to grow in the temperature range 70-45° so far, more than 450 strains of the identification [1]. Biodiversity among microorganisms in many parts of the world including hot springs has been the attention of many researchers [2]. Some of this microorganism can grow in very difficult circumstances, such as the simultaneous growth in acidic conditions [3, 4]. These bacteria often produce high capacity of such important enzyme lipase protease and amylase and DNA polymerase, in the biotechnology and industries have many uses [5]. Of all the alkalophilic microorganisms used in industrial applications are separate genus *Bacillus* are the dominant source of proteases [6]. Alkalophile microorganisms like alkaline protease production - they need optimal growing conditions for these organisms accompanied by increased production of enzymes, are provided. Culture conditions that promote the production of protease,and the conditions of cultivation, cell growth is different [7]. In the industrial production of alkaline protease, commonly used selective medium that contains very high concentration of complex, carbohydrates, proteins and other components of the culture medium [8]. Hot springs and mineral, phenomena are signs have emerged in geographic areas City Ramsar is beautiful cities in the North West province is located in the extremegeological structure of the site of the creation of such a lots of hot mineral water andthen there are several sulfur hot springs that their number is listed more than 50 springs. East Grand Hotel in Ramsar has 9 hot springs of sulfur and radium which studies showed that they have the sulfur compounds. Temperature properties of the source of alkaline protease-producing bacteria were studied.

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MATERIAL AND METHODS

Sampling and Culture:

Hot springs were sampled from depth of 30cm.Samples collected were cultured in two broth (Thermophile Bacillus Medium) TBM containing g/l: peptone 8 g, yeast extract 4 g,NaCl 3 g Skimmed milk Medium, containing g/l: Skimmed powder 10 g and spring water filtered then at 50 and 55 ° C for 24 to 48 hours at a speed of 180 rpm was incubated under same conditions. Isolated colonies showing clear zones on the Skimmed milk medium. At this stage the enzymatic activity of the protease-producing colonies were tested.The final confirmation of the identification and molecular techniques, 16sr RNA sequencing bacteria were finally identified.

Protease Activity Assays:

Alkaline protease activity was estimated by the method of Lowery [9]. Colonies in 100 ml LB broth culture and incubated at 50 ° C and for 36 hours.Then Solution centrifuged at 7000 rpm for 30 min and was separated the supernatant.1 ml substrate containing 1% casein in phosphate buffer (pH:7) was added to 1 ml of diluted enzyme and was incubated at 55 ° C for 3 hours.In the next step stop the reaction by adding 2 ml TCA (5 % trichloroacetic acid). Mix was incubated for 20 min. The precipitated protein was then passed through a No. 1 Whatman paper to 1 ml filtered material was added 0.5 ml solution of sodium bicarbonate. The final step isadd 1 ml Folin reagent and incubated for 20 min at 55 ° C until color developed the green color was measured at 660 nm wavelength.

DNA Extraction and PCR process:

The colonies have solved 1cc sterile distilled water at 1/5 micro tubeand were centrifuged 10 min at 3000 rpm.The supernatant was discarded and dry sedimentand 100 µl lysozyme and 75 µl SDS (10%) added after mixing with Vertex.The samples were incubated for 15 min at room temperature.Then, 150 µl phenol equilibrium and 300 µl chloroform added to it then mixture to become milky.Samples were centrifuged for 15 min at 10,000 rpm.Aqueous phase transferred to a new vial and the same volume was added chloroform to the mixture. Then Samples were centrifuged for 15 min at 10,000 rpm.Again, the aqueous phase transferred to a new vial and 2/3 volume was added of cold isopropanol and mixed.The samples for one hour at -20 °C was placed.After that, the samples were centrifuged for 15 min at 14,000 rpm. The supernatant discarded and 200 ml ethanol (70%) was added to sediment, and 1 min centrifuged at 14,000 rpm.Supernatant discarded incubated for 15 min at 37 °C Dry plate.The sediment added was 30 ml sterile distilled water and kept in the freezer -20 °C [10]. Polymerase chain reaction was used for Universal primers 16s rRNA, the sequence 27F 5'-AGAGTTTGATCMTGGCTCAG -3' and 1492 R '5-GGTTACCTTGTTACGACTT -3' PCR Cycles and temperatures are indicated in Table 1.

Table 1: PCR cycle time and temperature.

Number of cycles	Time(min)	Temperature(C °)	Stage
1	5	95	Initial denaturation
35	10	95	denaturation
	1	58.5	Annealing
	1	72	Extention
1	10	72	Final extention

RESULTS AND DISCUSSION

The study from Ramsar hot spring, two samples with high protease activity (code c2, zh5) was isolated. The optimum temperature for growth of isolated c2 and zh5 was observed60° C and the optimum pH:9. It were isolated from family *Bacillacae* genus *Bacillus* and morphology of the rod or oval are gram positive bacteria.So both strains were catalase positive, oxidase negative, gelatin hydrolysis and H2S production was negative (table 2).

Table 2: Test results of API in isolated.

Test	ONG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP
Zh_5	+	-	-	-	+	-	-	-	-	+
C_2	+	+	+	+	+	-	-	+	-	+
Test	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
Zh_5	+	+	+	-	+	-	+	-	+	-
C_2	+	+	+	+	+	-	+	-	+	-

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The sequencing of the two isolates c2, zh5 determinate the new strain of *Bacillus* genus names *Bacillus*.sp.ton1 (NCBI Code: KF730660), *Bacillus*.sp.ton 2 (NCBI Code: KF730661) were identified and were recorded NCBI. PH test results shows that the enzyme activity in the alkaline pH of 8 and 9 is very good for 72 hours (Fig. 1).Test results also indicate that the temperature of enzyme activity at 60 ° C for 48 hours to 96 hours (Fig. 2). Then sequenced, became clear that isolated from the genus *Bacillus* and close is to *licheniformis* species and then draw a phylogenetic tree determined that two new strains (Fig. 3).

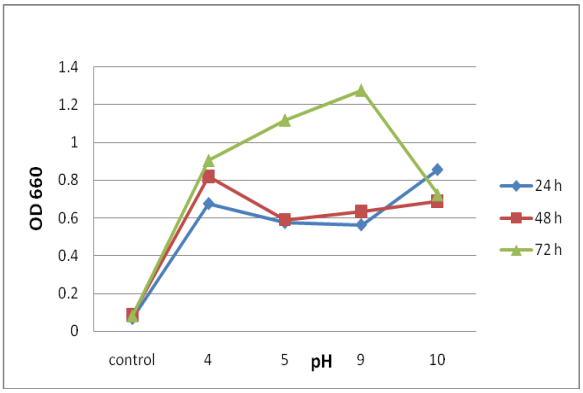

Fig. 1: Results of the protease enzyme activity at different pH.

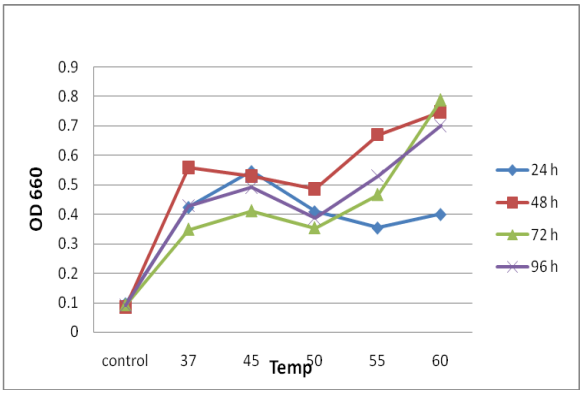

Fig. 2: Results of the protease enzyme activity at different temperature.

The results obtained in this study indicates that the enzymatic activity of bacteria isolated from hot spring at high temperature and alkaline pH is strong protease.Since the consumption is very high alkaline protease in the industry, the native strain producing alkaline protease and heat resistance is important. In this study two isolatesnot only are able to produce high levels of protease and but alsocan produce protease at high temperature, which is one the valuable benefits of this producers. Should be noted that these are isolated from wild strains produce proteases and their natural habitat and no optimization has been done on protease production.Optimization of culture conditions on protease production in the isolated they could consider for an Industrial producers. According to the majority of subjects including *licheniformis* species, our findings after sequencing also showed that they are closely relate to *licheniformis* species (Fig. 3).

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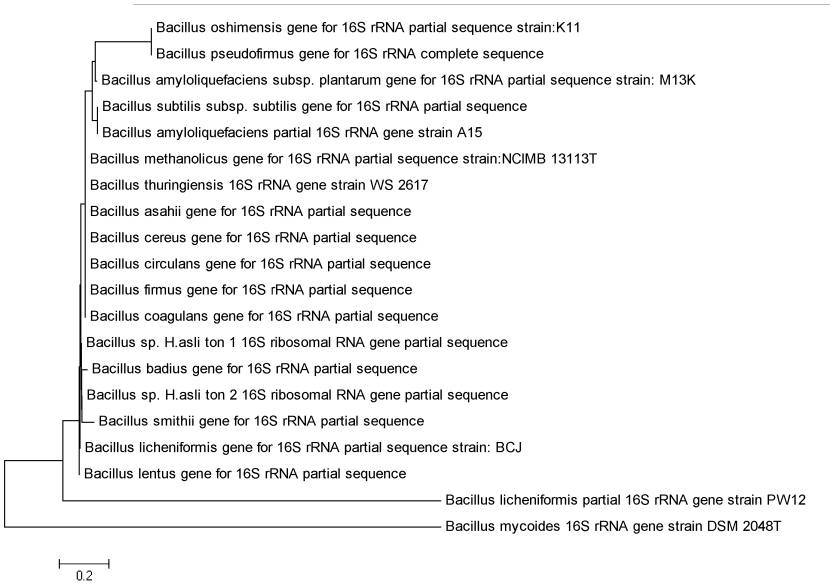

Fig. 3: A phylogenic tree of isolates (*Bacillus*sp.H.asli ton1, 2).

According to research done the proteasesinvestigated this study by research of other researchers: Shivangi and colleagues in 2013 have researched inprotease producing bacteria from wastewater sources Soap. They isolated a species of *Bacillus* Enzymatic activity in pH: 8 and temperature 65 ° C .The temperature enzymatic activity was very good, but the pH enzymatic activity was weak in comparison with this study and other researchers [11]. Anupama and associates in 2012 has worked on *Bacillus licheniformis* KBDL4 producing protease from Lonar soda lake in India and the results of this study showed that the enzymatic activity was the bacteria at 60 ° C and pH: 10 the highly valued was enzyme alkaline protease and compared with the results of this study, enzyme activity was favorable [12].

Mukesh and colleagues were studied in 2012, on the proteases produced by *Bacillus* sp. MPTK472 from dairy sludge. According to results obtained this bacteria at 55 °Cin pH: 9the with isolated is same strain of *Bacillus* sp.H.asli.ton 1 [13]. Suganthi and colleagues was studied in 2012 on *B.licheniformis* alkaline protease isolated from salt mine sediment .The results showed that this bacterium had a good enzymatic activity in pH: 8 than the weaker results obtained in this study [14]. Mohsin and colleagues have been studied in 2011 on protease-producing bacteria isolated from different soil, this study isolated alkaliphile *Bacillus* that its enzymatic activity was in pH: 11.5temperature 50 ° C.The results of this researchers showed that alkaline protease was good, but the temperature of the results in this study in lower group would not be a good thermophilic protease [15]. IzraelZivkovic and colleagues work in 2010 on *Pseudomonas aeruginosa* ATCC 27853 alkaline producing- protease. The results shows that the best enzymatic activityis in pH: 7.1 and temperature 60 ° C in compared with these results for both factors did not contain a good protease and it is not at neutral pH and alkaline protease [16].

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