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Antifungal Properties and Purification of Chitinase from *Haloferax mediterranei* Isolated from Lake Urmia in Iran

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Abstract

Background and Objective: Chitin and its hydrolyzing enzyme, chitinase, are produced by a variety of organisms and microorganisms. Production chitinase with Archaea are more concern today, for easy to extraction and contamination control. Therefor in this research the production of this enzyme is reported by Archaea.

Materials and Methods: 60 samples were collected from different saline areas of Iran. After enrichment and qualitative screening, chitinase-producing archaea were isolated. The isolate was identified biochemically and molecularly and its optimal growth conditions were determined.

Results and Conclusion: Halophilic Archaea strains were isolated on chitin as the only sources of carbon for chitinase-producing archaeal strains. The result showed, a pink colony strain from Lake Urmia with chitinase activities of 5.1875 IU/ml was selected for maximum enzyme production and identified as *Haloferax mediterranei* by polymerase chain reaction reaction test. The enzyme was concentrated and partial extracted. Enzyme production from archaea can be useful for industries. The hydrolysis of chitin, the second biopolymer after cellulose, which also leaves a lot of waste every year, is of great importance, therefore, with high chitinase activity from haloarchaea, it can be used in industry without bacterial contamination science this microorganism is stable only in high salt solution.

What is “already known”:	• Bacterial produced chitinase are known • study in Archaea is important due to their tolerance to moisture and stress conditions
What this article adds:	• An Archaea enzyme resistant to salinity and temperature is obtained. • Presence of a specific band for chitinase was demonstrated by zymogram in SDS gel.

1. INTRODUCTION

Chitin $(C_8H_{13}O_5N)_n$ is the most abundant biopolymer after cellulose which is present in the cell wall of fungi, the exoskeleton of insects, invertebrates [1], arthropods [2], marine organisms (molluscs, marine invertebrates, crustaceans, algae) and etc [3]. Polysaccharide insoluble in water and other organic solvents [3,4], non-toxic, biocompatible and biodegradable [5], but it is almost resistant to degradation [1]. This resistance, especially in terms of the decomposition of pests and waste, has led to paying attention to ways to destroy it [1]. Chitin-degrading enzymes (chitinases) are a group of hydrolytic enzymes that hydrolyze β-glycosidic bonds (1-4) and form monomeric N-acetylglucosamine and oligomeric units [5]. They can be found in many organisms and microorganisms, including animals (for nutrition and defense against pathogens containing chitin), fungi, bacteria, plants, insects, archaea. This enzyme has many applications in waste disposal and management of chitinous waste, agricultural industries, as well as its potential use in the food, fishing, cosmetic, SCP production, biological control of harmful and pathogenic fungi and insects, etc. [1].

According to BRENDA database reports, chitinases are in a range of molecular weight from 20 to 90 kD and optimal temperature includes 90-18 C° and pH 1-5 [5]. Based on the location of the enzyme act on the chain, chitinases are divided into endo & exochitinases; Based on the similarity of their amino acid sequence and also their gene sequence, placed in 4 families of glycosyl hydrolases (GH 18, 19, 23, 48) and 6 gene classes, respectively [1, 3]. The physicochemical characteristics and source of chitinase, are the main bases that determine the functional characteristics and hydrolyzed products of enzyme activity [6]. Although chitinases have been reported to be present in many organisms and even a few extremophilic chitinases have been reported in fungi and plants. But chitinases of microbial origin, especially archaea and bacteria, are the main sources of extremophile chitinases and are more suitable and stable for use in various industries [3]. Although chitinases are abundant in archaea, but unlike bacteria, there are not many studies in this field [1]. The use of mesophilic enzymes in chemical reactions is limited due to the harsh and unfavorable conditions in the industry, at the same time, thousands of enzymes have been identified and found applications in the industry [7].

For the first time, Takeshi Tanaka et al in 1999 characterized chitinase (ChiA) from the hyperthermophilic archaeon Thermococcus codocarensis (Tk-ChiA). Also, chitinase was expressed through the cloned gene in Escherichia coli cells and the recombinant protein was purified. Because no structural information was available on archaeal chitinases, they analyzed the gene and its protein product. This early work greatly contributed to basic studies (structure-function analysis) and industrial applications (biomass recycling) of thermostable chitinases [8].

Microorganisms present in saline environments adapt not only to high salinity but also to other extreme conditions such as high or low pH, temperature, and the presence of toxic substances including heavy metals. These characteristics lead to downstream fermentation processes, without contamination and under non-sterile conditions, to be continuously implemented [1]. On the other hand, archaeal enzymes are important in industrial biotechnology [9] and are considered safe sources because no archaeal pathogen has been found to date [10], and they do not cause any diseases in humans, animals, plants, bacteria and other archaea [11]. Advantages such as minimal steps in purification, recovery and affordability, metabolic adaptations to withstand harsh industrial conditions and also adaptations such as the production of several vesicles, granules, primary and secondary metabolites, make haloarchaea and their enzymes appropriate and compatible for the conditions in the industry [12].

In this study, an archaeal strain with high potential for chitinase enzyme production was isolated and identified from Lake Urmia in Iran. The growth and enzyme production conditions of isolate were investigated and optimized. Then, the enzyme was relatively purified and its antifungal properties were investigated on a number of specific plant pathogenic fungi.

2. MATERIALS AND METHODS

2.1. Sample collection and isolation of halophilic microorganisms

60 samples were collected from different saline areas of Iran and stored at ambient temperature. In order to isolate haloarchaea, Erlenmeyer flasks containing 100 ml of DSM liquid culture medium

(12.5% salt, 0.013% calcium carbonate ($CaCl_2$), 1.6% magnesium chloride ($MgCl_2$), 0.1% peptone, 0.1% yeast extract, 0.5% glucose, 0.5% potassium sulfate (K_2SO_4) and 0.002% chloramphenicol -after autoclaving) were prepared for each sample and after inoculation of the samples, they were transferred to an incubator at 37°C. After 14 days, the contents of the Erlenmeyer flasks were cultured in plates containing DSM agar medium by surface culture method and transferred back to the incubator at 37°C. All isolation processes and subsequent purification of the grown colonies were repeated three times. The first stage of qualitative screening was performed to isolate chitinase-producing strains by replacing colloidal chitin as the sole carbon source instead of glucose.

2.2. Extraction of chitin from shrimp shell

10 g of shrimp shell was mixed with 100 cc of 37% hydrochloric acid (HCL) and placed on a shaker for 2h at room temperature. Then, 500 ml of 96% ethanol was added and the pH of the medium was measured, which should be in the neutral range. Next, centrifugation was performed for 20 min at 3800 rpm and the bottom contents were washed several times with PBS after centrifugation to wash away any remaining acid and base. Finally, the washed bottom contents were placed under a temperature of 50°C to dry. In order to examine the production rate of chitinase enzyme of each strain and reduce the costs of enzyme production by them, instead of colloidal chitin shrimp shell (powdered), which leaves a lot of chitin waste annually, was used as the sole carbon source in DMS agar culture medium. However colloidal chitin was used as substrate for enzyme assay.

2.3. Measuring the amount of total protein

To select the best strain among all strains, the amount of enzyme and total protein production was examined. In measuring the total protein content of each strain, the Bradford protocol was used, whose standard curve was initially drawn using bovine serum albumin and at a wavelength of 595nm. According to the number of strains, 5 cc of colloidal chitin broth liquid media were prepared in tubes, and then each strain was inoculated with a concentration equivalent to half McFarland's concentration in these broth culture media, and after the growth period, and the total protein content of each strain after centrifugation, was measured according to the Bradford protocol (800 µL mixture of reagent + 200 µL of microbial sample) at a wavelength of 595 nm.

2.4. Quantitative measurement of chitinase enzyme

The test and standard curve of N-acetylglucosamine sugar were used. First, 5 concentrations of 1,2,3,4,5 mmol of this sugar were prepared and 750 µL of each concentration was combined with 1 cc of Schale reagent (0.5 M sodium carbonate and 1.5 mM potassium ferrocyanide) and boiled in a water bath at 80°C for 15 min. Their optical absorption was read at a wavelength of 585 nm and a standard curve was obtained.

Colloid chitin liquid culture medium was prepared in tubes in a volume of 5-10 ml and 1 ml of each of the strains, which had been made at a concentration equivalent to half McFarland's concentration, was added and placed in a 37°C incubator. After the growth period, centrifugation was performed for 10 min at 3800 rpm, and then 500 µL of colloidal chitin solution, 450 µL of acetate buffer pH 5 were mixed with 50 µL of each sample and placed in a 37°C incubator for 1 h. After that, 200 µL of sodium hydroxide was added to them and centrifuged again for 10 min at 3800 rpm (centrigugal radius = 7 cm). Next, 750 µL of the supernatant was combined with 1 cc of Schale's reagent and placed in a water bath at 80°C for 15 min, and then the optical absorption of each was read and recorded at a wavelength of 585 nm. All these steps were repeated to quantitatively measure. The activity of the chitinase enzyme was determined using a standard curve of different concentrations of N-acetylglucosamine released over a period of one hour and is reported in units of IU/mL. One unit of chitinase enzyme activity (IU/mL) is the amount of enzyme that releases 1 micromol of N-acetylglucosamine in one hour under the conditions studied. The production of chitinase enzyme, using powdered shrimp shell as the only carbon source, which is cheap, abundant, and available, and their optical absorption values were recorded.

2.5. Antifungal properties

A number of strains with higher absorption values were selected. In order to identify the superior strain, in a preliminary study, on the same DSM archaeal culture medium, the effect of the produced chitinase

enzyme on specific fungi including *Aspergillus niger* ATCC 6275, *Alternaria alternate* PTCC: 5224 and *Fusarium solani* PTCC 5284 was measured. The fungi were completely cultured in the plates and then a hole was created in the medium and the liquid culture prepared from each of the strains was poured into them. Then the plates were transferred to an incubator at 25 °C and were examined every day to measure the zone of inhibition and the effect of the enzyme.

2.6. Molecular and biochemical identification of the selected strain

All strains were cultured in TSA medium and examined for halotolerant or obligate halophile. For the best strain in terms of chitinase enzyme production, biochemical tests (citrate test, nitrate reduction, consumption of different sugars, methyl red/Voges-Proskauer), as well as microscopic examinations and Gram staining [13] were performed according to the Bisal method. The isolated selected strain was sent to the Iranian Genetic Resources Center for molecular identification, sequencing, and identification of the genus and species. Total DNA was extracted according to the modified Marmur method (1) and polymerase chain reaction reaction was performed using primers 27F and 1492R.

2.7. Investigation of chitinase activity and strain growth curve growth curve

Twelve tubes each containing 10 ml of DSM medium with colloidal chitin as a carbon source were prepared and a concentration equivalent to half the McFarland concentration was made from the selected strain (strain I) and inoculated into the tubes and then placed in a 37°C incubator. Growth and chitinase enzyme production were measured and recorded for 9 sequential days.

2.8. Optimization of compounds and environmental conditions

Various components and process parameters involved in chitinase enzyme production by the *Haloferax mediterranei* ASFM403 strain were optimized using a one-factor-at-a-time approach. All experiments were conducted in triplicate, and the obtained results were expressed as mean values. The inoculation and cultivation conditions were maintained according to those described in the previous section. The investigated variables included the following. By replacing glucose and a number of different compounds containing chitin such as wheat straw, wheat bran, rice straw, rice bran, colloidal chitin, shrimp shells, powdered ants, the best carbon source for the growth and production of chitinase enzyme by the selected strain was evaluated. Ammonium chloride, yeast extract, cysteine, casein, tryptose were also investigated to select the best nitrogen source, as well as the values of 5, 10, 12.5, 15, 20, and 25%, to determine the best salt concentration level required by the strain. Environmental conditions such as temperature and pH were among the conditions that were investigated. Temperatures of 4, 15, 25, 35, 45 °C and pH of 2.8, 3.6, 4.4, 5.2, 6, 6.8, 8.4, 9.2, 10, 10.8 were investigated. Growth and enzyme production were measured on the sixth day according to the growth curve.

2.9. Extraction and parcial purification of enzyme and anti-fungi activities

250 ml of colloidal chitin liquid medium was prepared and after inoculation of the selected strain, it was placed in a shaking incubator at 37°C and 120 rpm for 1 week. The supernatant obtained from centrifugation at 3800 rpm for 10 min was mixed with 2-butanol in a ratio of 1 to 2 and placed in a shaking incubator for 2 h. After 2 h, 2-butanol was removed and the liquid was dialyzed. The supernatant obtained from centrifugation of the colloidal chitin liquid medium inoculated with the selected strain I was placed in a dialysis bag with a pore diameter of 5 Daltons from Biogen Company after removing 2-butanol. Then, it was immersed in distilled water at room temperature and after changing the distilled water several times, it was placed for 24 h at 4°C. Finally, the liquid was removed from the dialysis bag and heated at 60°C until it was completely concentrated. This liquid was used to perform sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) and investigate the antifungal effect of the chitinase enzyme of this strain.

Zymogram analysis was performed to evaluate chitinase activity. Protein samples were mixed with SDS–PAGE sample buffer containing 60 mM Tris–HCl (pH 6.8), 15% glycerol, and 0.1% bromophenol blue, followed by heat treatment. Proteins were separated by electrophoresis on a 10% polyacrylamide gel

supplemented with 0.1% colloidal chitin as the substrate. Following electro-phoresis, SDS was removed by incubating the gel in washing buffer containing 0.4 M Tris–HCl (pH 7.0), 1% casein, 2 mM EDTA, and 0.02% sodium azide for 2 h with several buffer replacements. Subsequently, the gel was transferred twice into 50 mM Tris–HCl buffer (pH 7.0) for 15 min each and then rinsed with demineralized water, followed by treatment with 2-propanol for 5 min under shaking conditions. The gel was then incubated in digestion buffer containing 50 mM Tris–HCl (pH 7.0), 1 mM EDTA, and 0.1% colloidal chitin at 37 °C for 12 h to facilitate enzymatic hydrolysis of the substrate. Finally, the gel was stained with 0.1% Congo Red solution for 15 min and subsequently washed with 1 N NaCl solution to visualize zones of chitinase activity.

Following electrophoresis, the gel was immediately removed from the electrophoresis apparatus after the power was turned off. The gel was carefully separated from the glass plates, and two longitudinal strips were excised from both sides of the gel. One strip contained the protein molecular weight marker and one sample lane, while the other strip contained the corresponding sample lane. These strips were stained with Coomassie Brilliant Blue, whereas the remaining unstained portion of the gel was immersed in distilled water, sealed, and stored at 4°C until further processing.

After the gel strips were sufficiently stained, they were transferred to a destaining solution. Once the protein bands became clearly visible, the stained strips were aligned alongside the unstained gel. Using the stained strips as a guide, the corresponding protein bands were excised from the unstained gel. The excised gel bands were transferred into 2.5-mL microcentrifuge tubes.

The gel slices were washed three times with 250 mM EDTA (pH 7.4) for 5 min each, followed by three additional washes with distilled water, each lasting 5 min. The gel pieces were then cut into smaller fragments, and 1 mL of Tris buffer containing 1% (v/v) SDS (pH 7.4) was added to each tube.

Protein extraction was performed by sonication in an ice bath for 3 min using six 30-s pulses at an amplitude setting of 50. To separate the gel fragments from the extracted protein solution, Sephadex G-75 resin was used. chitinase activity was measured on the recovered eluate. Then its antifungal properties were examined.

The concentrated enzyme of the selected strain was treated on, *Aspergillus niger* ATCC 6275, *Alternaria alternate* PTCC: 5224 and *Fusarium solani* PTCC 5284. These fungi were cultured on PDA medium in a completely saturated state and then assayed by adding a drop of concentrated enzyme solution to a portion of the plates. Finally, the plates were transferred to an incubator at 25°C and observed after 2 days to examine the effect of the enzyme and the formation inhibition zone.

2.10. Statistical Analysis

All experiments were independently performed in triplicate, and the obtained data are expressed as mean ± standard deviation (SD). Statistical evaluation of the experimental data was carried out using one-way Analysis of Variance (ANOVA) to assess significant differences among treatment groups under various experimental conditions. Differences were considered statistically significant at a confidence level of 95% ($P < 0.05$). This analysis was employed to determine the influence of different experimental variables and their levels on the observed responses, thereby ensuring the validity, reproducibility, and statistical reliability of the findings.

3. RESULTS

In this study, 60 soil and sediment samples were collected aseptically from different areas and lake sediments. After being transferred to the laboratory, they were cultured in a medium containing 12% salt. Among them, 50 salt-tolerant strains were selected. After purification, the strains were transferred to a medium containing colloidal chitin as the only source of nitrogen and carbon. Among them, 49 strains grew on chitin and 49 strains were identified as archaea bacteria due to their sensitivity to chloramphenicol and resistance to Anisomycin. Out of all the strains, one showed high chitinase activity. Also, in the initial comparison of the effect of cell solution of strains with higher light absorption for preventing the growth of *Alternaria*, *Fusarium* and *Aspergillus* fungi, the strain with higher chitinase activities showed better anti-fungal activities too. Therefore, this isolated strain was selected as the further work in this study. Several

samples were obtained from different saline areas of Iran, the samples were cultured in media with chitin as the only sources of nitrogen and carbon. After isolation and purification stages, 50 strains were obtained.

Growth of all 50 strains in DSM culture plates with colloidal chitin instead of glucose is an indication of the presence of chitinase enzyme in all isolated strains. Also, 49 strains out of 50 strains were identified as chitinase-producing archaea, based on resistance to chloramphenicol and sensitivity to Anisomycin.

Chitinase enzyme assay of isolates and preliminary studies on a number of prepared plant pathogenic fungi such as *Aspergillus, Alternaria and Fusarium*, ultimately led to the selection of strain I as the superior and selected strain in this study according to the Schale protocol, this strain produces chitinase enzyme with an optical absorption of 0.079, equivalent to 5.1875 IU/mg. According to measurements based on the Bradford protocol, the optical absorbance of selected strain I was found to be 0.088, approximately 50 mg/ml, in terms of total protein content. This strain was observed with pink and convex round colonies in the plate with different shapes and the result of staining as gram-negative under the microscope with x100 magnification (Figure 1a, b). Also, due to the inability to grow in TSA, it was diagnosed as a halophilic strain. Its growth on culture medium containing shrimp shell as the only carbon source indicates the ability of this strain to consume chitin present in the shrimp shell structure (Figure 1c).

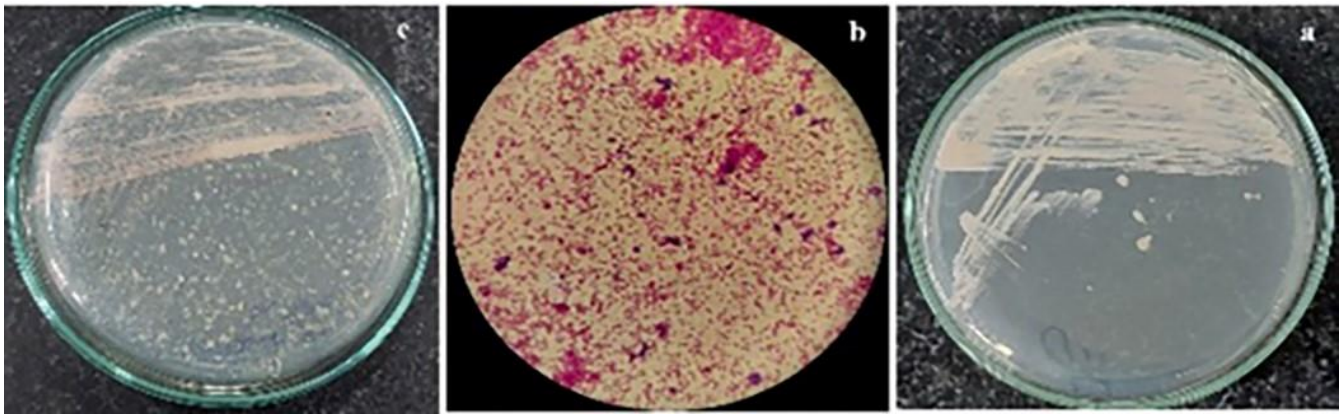

Figure 1. a) Culture of strain I on DSM; b) Microscopic image of strain I with x100 magnification; c) Cultivation of strain I on colloidal chitin

Based on the observation of the results obtained (Figure 2a), strain I consumed glucose, xylose, arabinose, small amounts of fructose and rhamnose, but did not have the ability to use lactose, mannitol, sucrose and sorbitol sugars. The strain was motile and indole positive. Methyl red and nitrate + but Voges-Proskauer , H2S, citrate - (Figure 2).

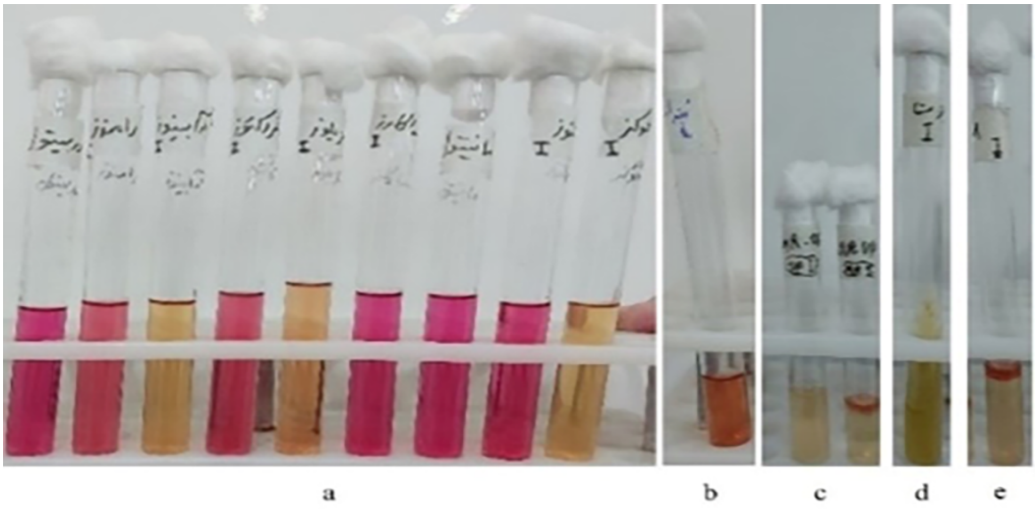

Figure 2. Biochemical results; a) consumption of different sugars, b) nitrate reduction test, c) methyl red/VP test, d) citrate test, e) SIM

The results of 16SrRNA gene sequencing in the EZBBioCloud database showed that the mentioned strain has phylogenetic affinity with 1179 nucleotides and 99.4% similarity with the strain *Haloferax mediterranei* CGMCC1.2087 (T) with accession number CP001868, and therefore this strain is reported as *Haloferax mediterranei* ASFM403 (Figure4). The strain was registered with accession number PQ327049 in National Center for Biotechnology Information (Figure3).

Tasks	Hit taxon name	Hit strain name	Accession	Similarity	Variation ratio	Hit taxonomy	Completeness (%)
⇄ O	Haloferax mediterranei	CGMCC 1.2087(T)	CP001868	99.41	7/1179	Archaea;Euryarchaeota;Halobacteria;Haloferacales;ae;Haloferax	100.0
⇄ O	Haloferax mucosum	ATCC BAA-1512(T)	AOLN01000016	98.98	12/1179	Archaea;Euryarchaeota;Halobacteria;Haloferacales;ae;Haloferax	100.0
⇄ O	Haloferax prahovense	TL6(T)	AB258305	98.47	18/1177	Archaea;Euryarchaeota;Halobacteria;Haloferacales;ae;Haloferax	100.0
⇄ O	Haloferax chudinovii	RS75(T)	JX669135	98.39	19/1179	Archaea;Euryarchaeota;Halobacteria;Haloferacales;ae;Haloferax	100.0
⇄ O	Haloferax sulfurifontis	ATCC BAA-897(T)	AOLM01000015	98.30	20/1179	Archaea;Euryarchaeota;Halobacteria;Haloferacales;ae;Haloferax	100.0
⇄ O	Haloferax denitrificans	ATCC 35960(T)	AOLP01000013	98.13	22/1179	Archaea;Euryarchaeota;Halobacteria;Haloferacales;ae;Haloferax	100.0

Figure 3. The highest percentage of similarity of other strains to the selected strain in the National Center for Biotechnology Information database

The phylogenetic tree of the isolated strains is shown in Figure 4. Referring to the growth curve in Figure 5, during a period of 9 days of continuous measurement, enzyme production reached its maximum level in 6 days and then remained at the same level until day 9 and then it created a variable and decreasing trend. This is while the growth rate continued to increase. Salt content between 12.5 and 15% and temperature of 35°C are more effective for optimal growth and enzyme production (Figure 6). Based on the measurements and results obtained (Figure 7), the growth rate and production of chitinase enzyme after using colloidal chitin and rice straw as a carbon source was higher and better than other compounds. Yeast extract with peptone as a nitrogen source also had a better effect on growth and enzyme level; while cysteine had the least effect.

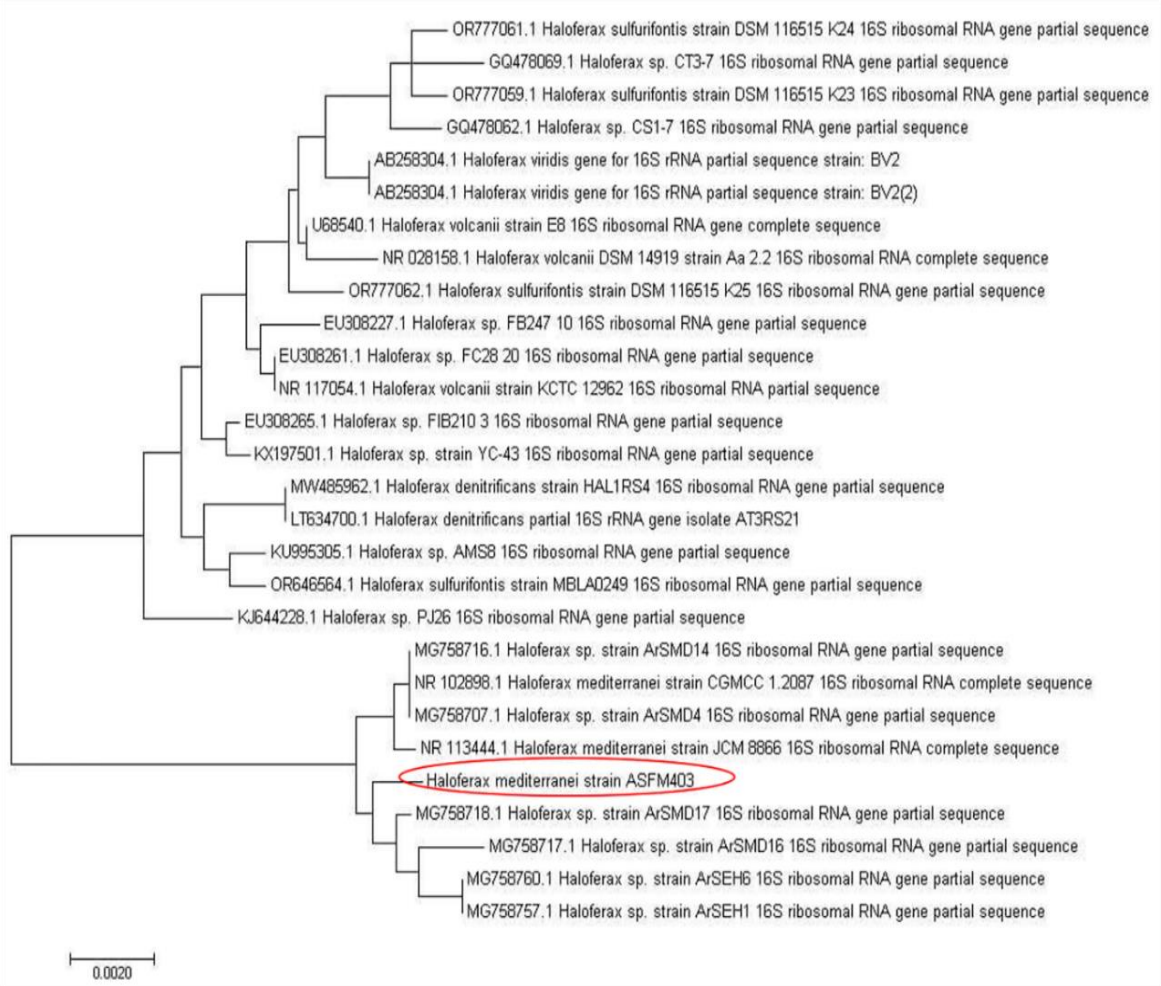

Figure 4. Evolutionary relationships of taxa

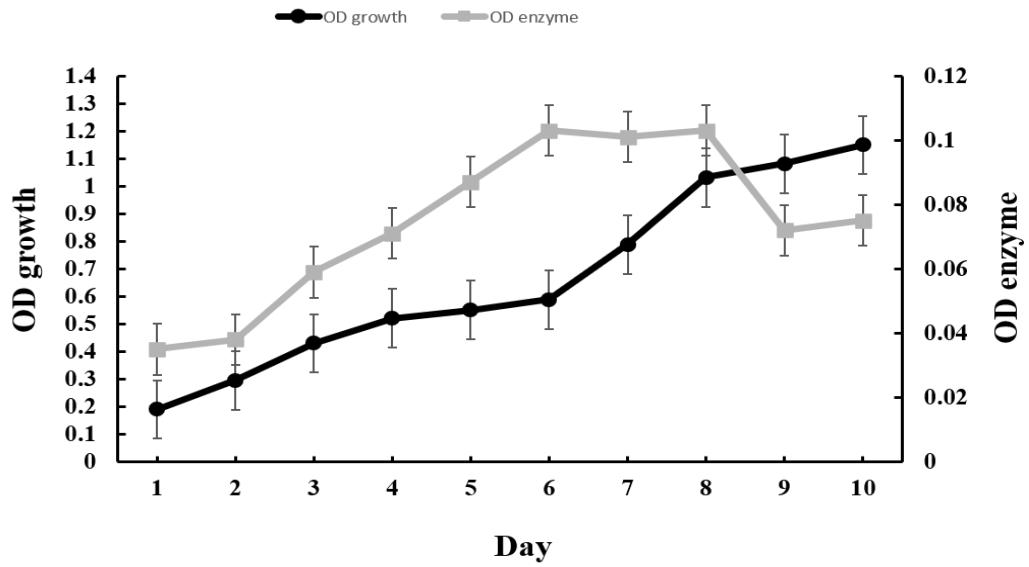

Figure 5. Growth curve and production of chitinase enzyme; The black curve shows the growth and the gray curve shows the production of chitinase enzyme.

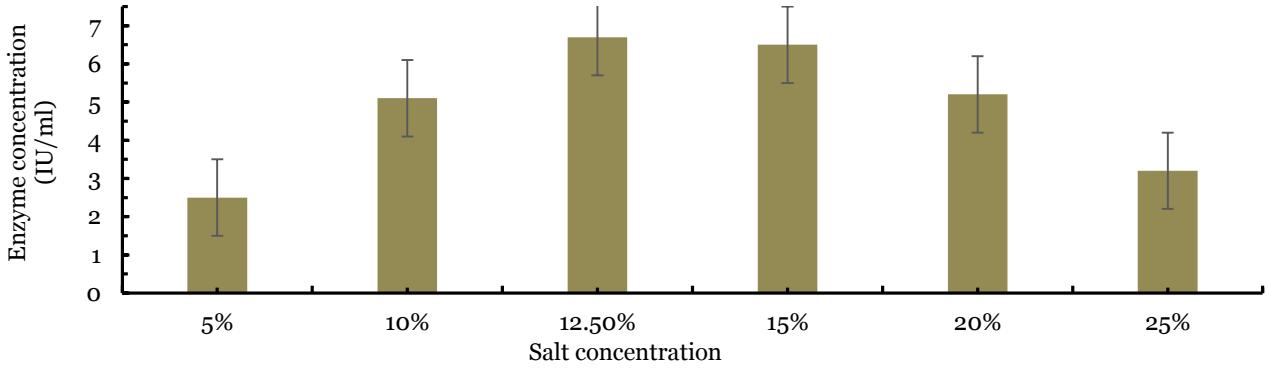

Figure 6. Effect of different concentration of NaCl on the production of chitinase enzyme by *Haloferax mediterranei* ASFM403.

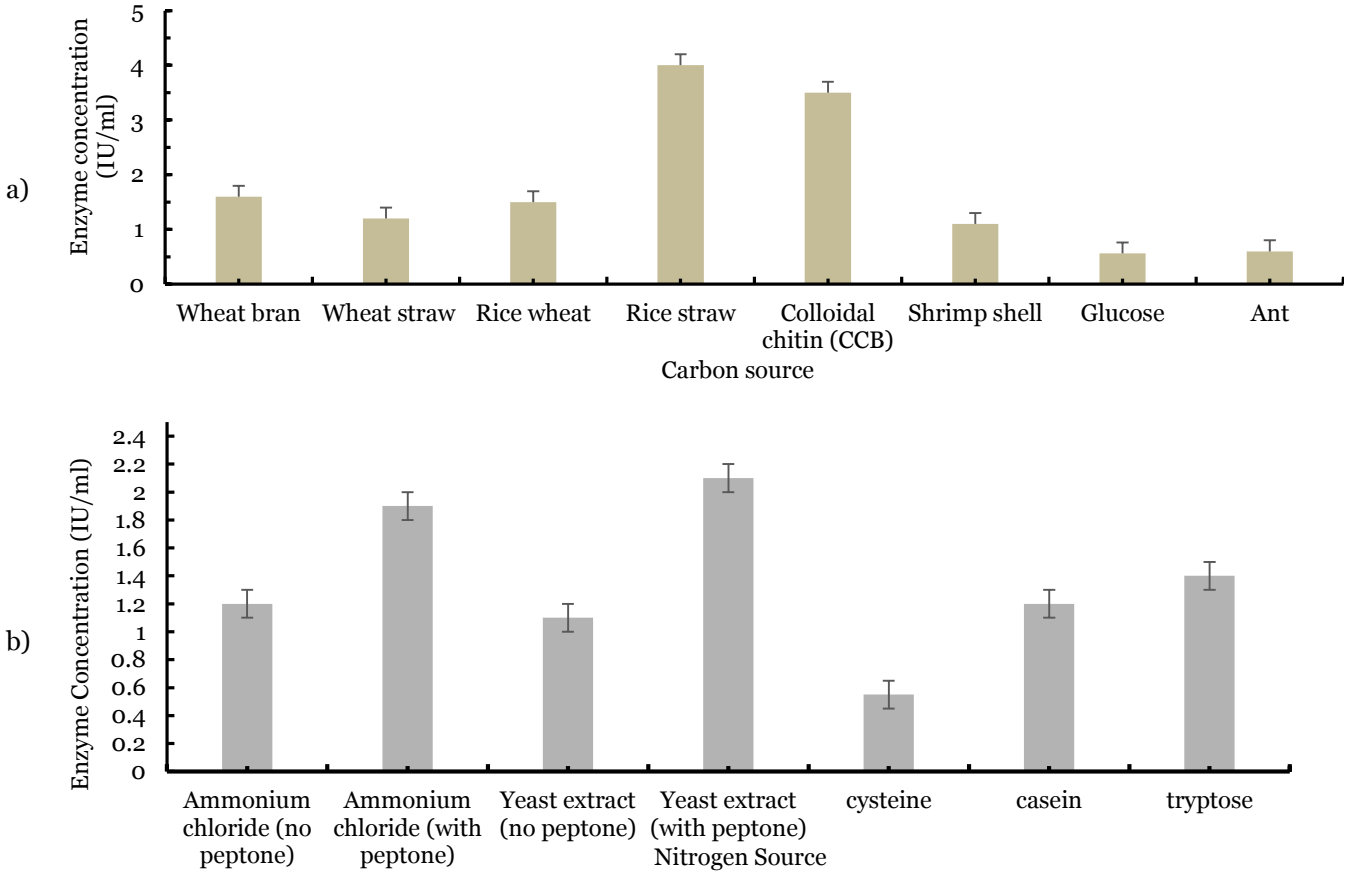

Figure 7. Effect of different a) carbon sources and b) nitrogen sources on the production of chitinase enzyme by *Haloferax mediterranei* ASFM403 in Archaea medium at 37°C for seven days.

The results of the culture conditions showed that the strain had the best enzyme activity in 12.5% salt, addition of yeast extract as a nitrogen source, temperature 35°C and pH 6.8. These optimal enzyme production results are shown in Figure 8.

The cell free concentrated supernatant was extracted by butanol and dialyses for salt removal. This fraction was concentrated at 37°C for one day and protein concentration were determined which was 10 mg/ml. Then the concentrated partial extracted loaded on SDS-page. Since the high concentration of colloidal chitin were used, the pure fragment of 22 kD seen on the SDS gel. The results SDS-page and zymogram are shown in Figure 8.

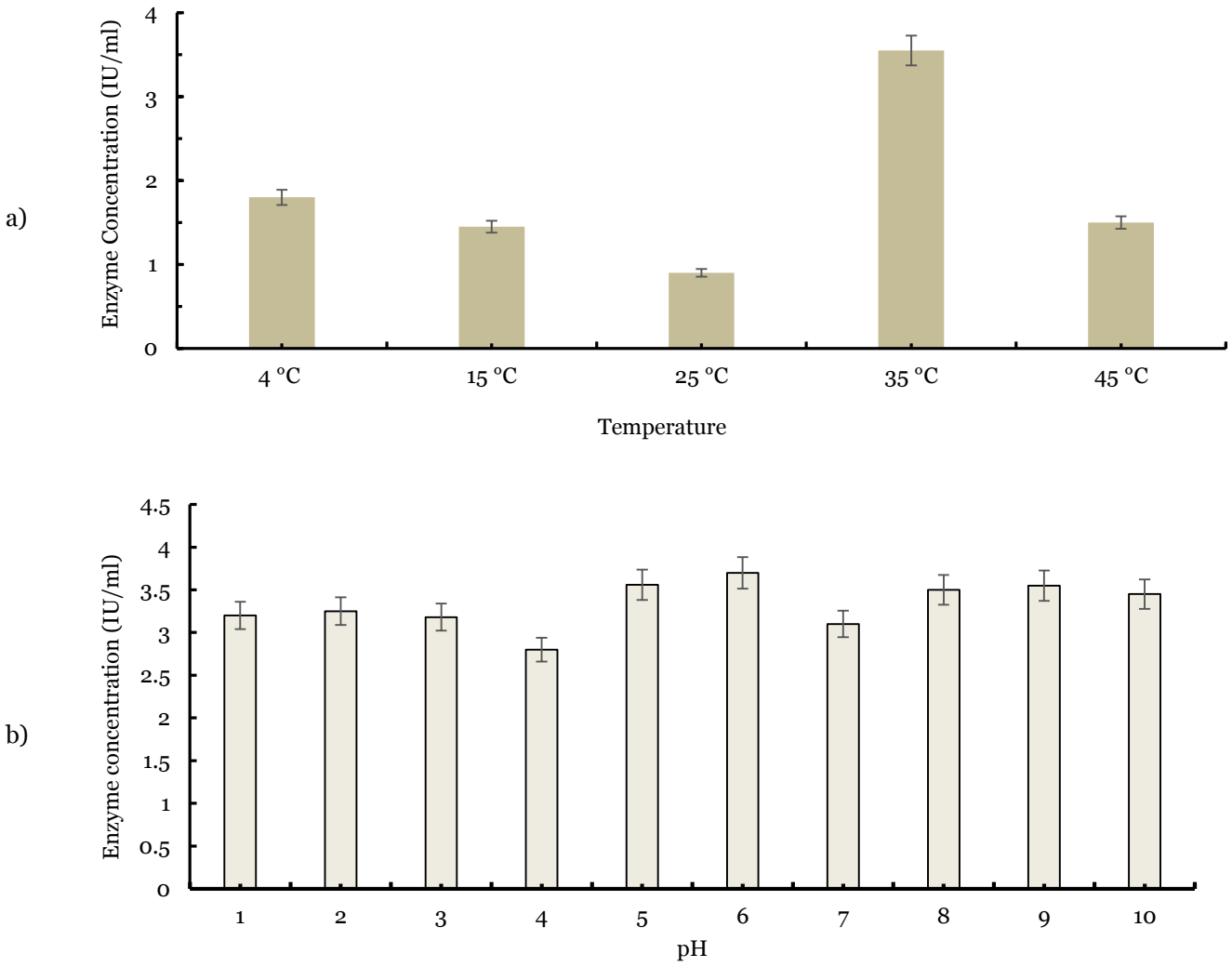

Figure 8. Effect of a) temperature changes and b) pH changes on the production of chitinase enzyme by *Haloferax mediterranei* ASFM403 in Archaea medium for seven days.

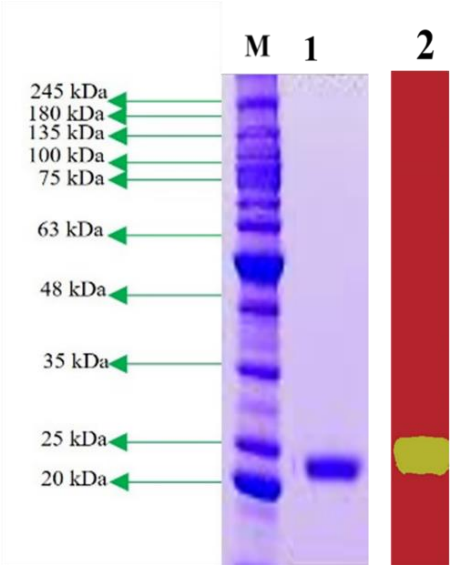

Figure 9. SDS-page result; 1) concentrated and extracted enzyme samples. Enzyme band observed in the 22 kD range, 2) Zymogram enzyme. M: Ladder

The concentrated and extracted chitinase enzyme of the isolate were experienced on the growth of three fungi strains, *Aspergillus*, *Fusarium*, and *Alternaria*. As shown in the figure10, the crude enzyme was effect on all 3 studied fungi while the zone of inhibition for *Alternaria* were more than *Aspergillus*. This not only show the antifungal activities of the isolated strain but showed the crude extract was mostly chitinse which disturbed chitin cell wall of examined fungi and inhibit the growth of fungal.

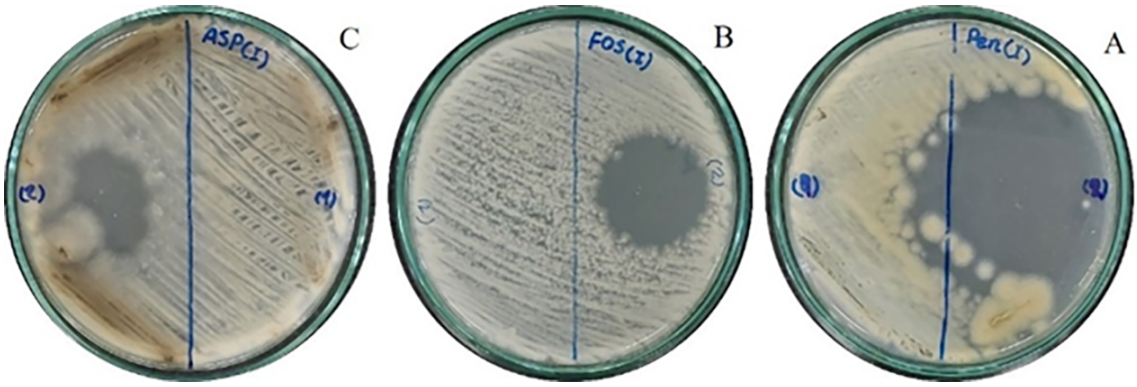

Figure 10. Diameter zone of inhibition created by the concentrated enzyme solution from *Haloferax mediterranei* on PDA: a) *Alternaria* 50 mm, b) *Fusarium* 30 mm, c) *Aspergillus* 30 mm.

The purification process resulted in a gradual increase in enzyme specific activity accompanied by a reduction in total protein content. The crude extract showed a total protein concentration of 50 mg with an enzyme activity of 881 U, whereas after subsequent purification steps, the protein content decreased to 26.5 mg and finally to 1.5 mg with corresponding enzyme activities of 622 U and 106 U, respectively. The increase in specific activity across purification steps indicates successful enrichment of the target enzyme, while the reduction in total activity reflects the expected loss of enzyme during purification procedures(table 1).

Table 1. Summary of the steps in chitinase purification from one liter of *Haloferax mediterranei* ASFM403.

Purification step	Total Protein (mg)	Activity (U)	Specific activity (U.mg-1)	Purification (n-fold)	Yield (%)
Culture filtrate	50	881	17.62	1	100
NH4(SO4)2 precipitate	26.5	622	23.47	1.33	70.60
Sephadex G-100	1.5	106	70.67	4.01	12.03

4. DISCUSSION

In saline and highly saline environments, halophilic microorganisms are the dominant groups of extremophiles, which have found many potential applications in biotechnology due to their metabolic and other abilities to tolerate harsh conditions. Among the domains of life, archaea constitute an important and large group of extremophiles that are able to grow and live in saline and hypersaline environments [1].

The first haloarchaeal isolates described in the literature more than a hundred years ago were strains of the genera *Halobacterium* and *Halococcus*, which were obtained from salted fish, salted meat, and salted skin, and their quality was negatively affected by the growth of red microorganisms [14]. The first isolated species and the most important species for chitin degradation is *Serratia marchiscens*, which is also considered a model bacterium for studying enzymatic activities. In an attempt to develop superior chitinase efficiency by *Bacillus thuringiensis*

NM101-19, Goma and Al-Mahdi (2018) exposed the strain to different doses of gamma radiation [6].

This study focused on the isolation and screening of halophilic archaeal strains that can produce chitinase, an enzyme with enormous industrial and biotechnological applications. Chitin, the second most abundant biopolymer on Earth after cellulose, has attracted much attention due to its potential applications in various industries. However, the degradation and degradation of chitin poses challenges, making the discovery of effective chitinase-producing microorganisms a priority.

Given the harsh reaction conditions required in industrial processes, enzymes used in industry must have characteristics that make them suitable for this task. Hence, the best approach is to use enzymes from extremophilic microorganisms [4]. It has been stated that most members of extremophiles are also archaea [7]. It has also been mentioned that archaea are considered safe sources due to their non-pathogenicity in humans, animals, plants, bacteria, and other archaea [10,11].

One of the most important problems is maintaining enzyme activity in high concentrations of organic solvents and low water activity, but haloarchaeal enzymes can work properly in addition to high salt, under other harsh conditions such as very low water activity, activity in a number of organic solvents, activity in extreme temperature and pH ranges, etc. In general, inorganic solvents offer enzyme catalysis advantages such as increased stability, changes in substrate and enantiomeric properties, and increased product yield [1,7].

Despite various reports of chitinase-producing organisms in different regions, this type of research in the field of archaea is less than other microorganisms; especially in Iran, it is much less. Here, samples from extreme environments in Iran, including Hormuz Island, Lut Desert, Lake Urmia, etc., were examined to isolate halophilic archaeal strains capable of producing chitinase; then, the effect of their enzymes on a number of specific plant pathogenic fungi was investigated. This was the first time that the potential ability of chitinase to kill plant pathogens was assessed in Iran. The use of a specialized medium containing high salt concentrations, along with the addition of chloramphenicol to inhibit bacterial growth, increased the possibility of successful isolation of haloarchaeal strains.

Extraction and preparation of colloidal chitin from shrimp shells, a readily available natural source, provided a suitable platform for evaluating the chitinase activity of isolated haloarchaeal strains. Qualitative screening for chitinase production and assay using colloidal chitin is a common method [1], which we also used in this study and several haloarchaeal strains with the ability to hydrolyze chitin were isolated. This is a promising result, as it indicates the presence of active chitinase enzymes secreted by extremophilic archaea. Subsequent screening using an anti-archaeal antibiotic (anisomycin) confirmed the isolation of chitinase-producing haloarchaea that were sensitive to this compound. The use of powdered shrimp shells was also practical in line with our goal to investigate the potential of these extremophilic microorganisms for cost reduction and practical applications in waste management, agricultural industries and other sectors where chitin degradation is critical.

Archaea, often overlooked compared to their bacterial counterparts, have emerged as a rich source of novel and highly specialized enzymes that can grow under harsh industrial conditions. The ability of isolated haloarchaeal strains to produce chitinase, an enzyme with diverse applications, highlights their potential applications.

Mina et al. reported a thermostable chitinase from the bacterium *Brevibacillus formosus* BISR-1 with a half-life of more than 5 h at 100 °C. Li et al. also identified a thermophilic chitinase from the marine bacterium *Microbulbifer* sp BN3 stable at 60 °C with maximal activity [3].

The method and introduction chosen to measure the chitinase enzyme was Schale, which directly and only measures the concentration of the sugar N-acetyl-D-glucosamine, unlike the Dinitrosalicylic acid method, which generally measures the concentration of reducing sugars, such as the study Investigation of chitinase gene activity in the genus *Natrinema* using polymerase chain reaction and quantitative studies are reported by Yavari et al. [1]. The chitinase-producing activity and catalytic efficiency of the

abyssal fungus Purpureocillium lilacinum FDZ8Y1 under extreme conditions e.g. high hydrostatic pressure, low temperature, and high salinity is investigated [15]. The research uses transcriptomic analysis to reveal that high hydrostatic pressure enhances chitinase activity and activates key metabolic pathways related to chitin degradation and utilization, including glycolysis, fatty acid β-oxidation, and nitrate metabolism. The findings provide insights into the functional adaptability of deep-sea fungi and their potential as a source of novel extremozymes. Also the antifungal potential of a marine-derived chitinase as a biocontrol agent has been reported. The research focuses on the purification, characterization, and application of this chitinase to combat Colletotrichum gloeosporioides, a fungal pathogen responsible for anthracnose disease, thereby providing a sustainable green defense strategy for agriculture [16]. The purification and characterization of a chitinase from the marine bacterium Bacillus haynesii has been also studied. The research evaluates the enzyme's properties and its antifungal potential, highlighting its application for sustainable chitin bioconversion, a process that can convert chitin waste into valuable products [17].

5. Conclusion

In conclusion, the successful isolation and screening of chitinase-producing haloarchaeal strains from extreme environments in Iran demonstrates the untapped potential of these fascinating microorganisms. These findings pave the way for further research and development on practical applications of extremophile archaea and their enzymes, especially in the fields of chitin degradation, waste management, plant pathogens in agricultural industries, and sustainable industrial processes. The focus of this study on halophilic archaea is in line with the growing understanding of the importance of these microorganisms in addressing challenges posed by conventional industrial processes. The haloarchaeal chitinase enzymes identified in this work were recognized for their stability and activity under extreme conditions, making them attractive candidates for various industrial applications. The archaeal strain selected in this study, which had the highest enzyme production and effect on plant pathogenic fungi in this study, was isolated from Lake Urmia; this strain is able to tolerate high temperatures of about 60-65°C, relatively acidic pH, and consume materials such as rice straw as a carbon source.The ability of the selected strain to utilize rice straw as a carbon source makes it economically very suitable for industrial use.

6. DECLARATIONS

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6.2. Conflict of Interest

The authors declare no conflict of interest.

6.3. Authors Contributions

Farhanaz Sadat Mirlohi participated in conducting the initial experiments and studies and in preparing the main draft of the article. Soheila Abbasi as Corresponding author for the study design, methodology, data analysis, participated in data collection, laboratory or experimental procedures, and interpretation of results. Maryam Jalili Tabaii participated in the critical revision of the article. Giti Emtiazi supervised the study, participated in the development of the conceptual framework, and critically reviewed and edited the article. All authors read and approved the final version of the article.

6.4. Using Artificial Intelligent chatbots

No AI chatbots or tools were used in this research.

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