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Diversity of halophiles in Karak salt mine, KP, Pakistan and their ability to produce enzyme of industrial importance

Muzamil Shah[1], Fariha Hasan[1], Syed Salman Hashmi[2], Wali Muhammad[2*], Muhammad Zubair[1], Muhammad Asad Ullah[2]

[1]*Department of Microbiology, Quaid-i-Azam University 45320, Islamabad, Pakistan*

[2]*Department of Biotechnology, Quaid-i-Azam University 45320, Islamabad, Pakistan*

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Abstract

Halophiles are the microorganisms that possesses highly stable enzymes and can perform the cellular activities at extreme conditions of salinity. Enzymes and other functional proteins found in other groups of organisms are more susceptible to denaturation, aggregation and precipitation at such extreme condition. The current study explores the diversity and enzyme production of halophiles present in Bahadur Khel, situated in District Karak, Khyber Pakhtunkhwa (KP) Pakistan. Samples collection was done according to SOPs. The growth effects of isolated strains were determined on different temperature, salt concentration and pH. The serum was diluted for isolation of pure colonies followed by culturing and sub-culturing on agar. The range of temperature, salt concentration and pH for both types of strains isolated at 15°C and 37°C were 15°C, 28°C, 37°C,and50°C, Salt concentration 4-36% and 3, 5, 7, 9 and 11 pH respectively. The biochemical tests included Triple Sugar Iron (TSI), citrate utilization test, urease and oxidase test. Strains were evaluated for the production of different enzymes such as Amylase, Protease, Lipase, and catalase. Results indicated no significant production of lipase and c. The strains studied for enzyme assay results showed that the enzyme production increases with time. Protein estimation analysis revealed an increase in protein content with the passage of time. Our results indicated that isolates have the potential to produce valuable metabolites and can be exploited for high scale production, but it should be further investigated on a molecular level to elucidate their production potential.

* **Corresponding Author:** Wali Muhammad ✉ wali.biotech5511@gmail.com

Introduction

The word halophile is a combination of two words, “Halo” meaning salt and “Phile” meaning loving. Halophiles are classified into three main domains, Archaea, Bacteria, and Eucarya. Halophiles include oxygenic, anoxygenic, fermenters, methanogens and sulfatereducers etc. Slight halophiles are found in marine water while the moderate and extreme halophiles are found in the area having a higher salinity than the sea(Cho, Han *et al.* 2006).Halophiles have higher growing densities at 35% w/v salt but usually grow slowly(Pedrós-Alió, Calderón-Paz *et al.* 2000). Moderate halophiles can grow up to 8% while haloarchaea optimally at 10% salt concentration(Oren 2008). Soda lakes have Haloalkaliphilic haloarchaea (Grant, Gemmell *et al.* 1998). Halophiles have mostly low hydrophobicity. Media also affects the growth and depends on the constituents present in the media. Growth increases with the provision of rich nutritional conditions. The first isolating media for halophilic microorganism was synthesized and was comprised of more amino acids for their growth. Halophiles grow significantly at nutrient-rich medium or on the medium which supports growth at salt concentration. Halophiles also shows optimum growth in synthetic or complex medium containing casein hydrolysate and yeast extract (Ghasemi, Rasoul-Amini *et al.* 2011). However, most of the halophilic cell wall breaks in media containing yeast due to bile salts present to which halophiles are highly sensitive (Chelikani, Fita *et al.* 2004).The reported halophilic bacteria temperature is 4-50 ºC. The optimal salt concentration also increases with an increase in temperature to a certain limit. Most of the hypersaline environment contains psychro-halophilic bacteria. Halophiles have a diverse range of pH requirement ranging from acidic to basic pH. Most of the halophiles maintain their structure at low pH even if salt concentration is low.

The cell dissolves in a low ionic strength solution by raising pH (Shafiei, Ziaee *et al.* 2012). At pH below 3 and in the absence of salt, sphere formation takes place and the same occurs at 4.5M salt at pH 4 and 11 (Edgcomb, Orsi *et al.* 2009).The changes occur due to the changes in the cell membrane and not by osmotic pressure. Hypersaline environment has halophilic microorganisms which can be aerobic as well as anaerobic (Grant 2004). Due to the low solubility of oxygen in salt brines it can be a limiting factor for the halophiles. Some halophiles have aerotaxis sensors which help them to float toward the water surface for oxygen while, other grow in the absence of oxygen (Ng, Kennedy *et al.* 2000).Anaerobic halophiles use nitrate fumarate and Arginine as an electron acceptor (Oren 2013). In aerobic condition, Halorhabdus tiamatea has poor growth (Antunes, Taborda *et al.* 2008). Halophiles produce an antimicrobial substance known as halocins in hypersaline environments. Halophilicarchaea produce halocins as secreted compounds(Shand and Leyva 2008) but, no such evidence about halophilic bacteria in saline environments.

Anever-increasing interest in microorganisms from hypersaline environments have resulted in the discovery of several new species and genera in bacteria and archaeadomains. District Karak of KP province in Pakistan is well known for salt mines like the Jatta Ismail Khel and Bahadur Khel with reservoirs of approximately 52,563 tons of salt as per the Pakistan mineral development corporation (Edbeib, Wahab *et al.* 2016).

Up to date, the isolation and identification of halophilic strains from salt mines of District Karak have been extensively studied (Bangash, Ahmed *et al.* 2015). However, no comprehensive study is available on enzyme production from halophilic strains isolated from District Karak. The current study focuses on the diversity and enzyme production of halophiles of Bahadur Khel Karak salt mine KP.

Materials and methods

Sampling

Four different samples (Functional salt mine, non-functional salt mine, soil and water) were collected aseptically from Karak salt mines, Bahadur Khel, KP, Pakistan. The different samples were collected in sterile zipper bags and bottles. While taking the

samples in sterilized bottles and zipper bags standard microbiological procedure and protocol were used. The collected samples were kept at -4 ºC in the freezer.

Cell count (CFU/ml)

For all samples, CFU/ml was calculated to know the cell count. In autoclaved distilled water each sample was diluted 3 times in a separateplate, 150 µl was spread and then incubated at a specific temperature. At temperature 15ºC and 37ºC, the CFU/ml was determined. After one day ofincubation, the colonies were counted and CFU/ml was determined.

Bacterial diversity

Nutrient agar, LB agar and in some cases Yeast extract and starch with the desired amount of salt were used. Strains having different colonies and cultural characteristics were selected and subcultured on the above-mentioned mediums for confirmation.

Sub-culturing

In a sterile way, different bacterial colonies were selected and then sub cultured on their specific medium. With the help of loop in aseptic conditions, the bacterial colonies were isolated and streaked on the specific medium in a new plate.

Isolate characteristics

Media

Different media like nutrient agar, LB agar and yeast extract with the required amount of salt for the isolated strains were used. Media was prepared according to the standard recipe and then autoclaved at 121ºC and pressure of 15 lb for 20 min. In a laminar flow, the sterilized media was poured into sterilizingPetri plates. To check the sterility of the media the plates were incubated at 37ºC for overnight.

Then the bacterial isolated colonies were streaked in the Petri plates in the laminar flow hood by means of a sterile loop. The streaked plates were incubated at temperature 37ºC and 15ºC. After 24 and 48 hours growth characteristics such as color, shape, size, and colony morphology were recorded. For each medium and isolate the same procedure was followed.

Temperature

To observe strains characteristics and growth pattern various temperature were used for the isolates obtained from 15ºC and 37ºC. The used temperature was 15ºC, 28ºC, 37ºC and 50ºC. The change in temperature and its effect on the isolates were recorded.

Salt concentration

Different salt concentration (w/v) were used in the media i.e. 4%, 8%, 12%, 16%, 20%, 24%, 28%, 30%, 32%, 34% and 36% to study the growth patterns and characteristics of the isolates. The desired amount of salt was added to the medium and isolates were streaked to grow at different incubation temperatures (15ºC, 28ºC, 37ºC and 50ºC). With each salt concentration, the growth pattern and characteristics were recorded.

pH

Isolates were then grown on different pH media for further characterization. The different pH media used were pH3, pH5, pH7, pH9 and pH11. Using 1M HCl and 1M NaOH the pH of the media was adjusted. At low or extreme pH (pH 3) gel rite was used as a solidifying agent in the media. As a basic medium nutrient agar and LB agar were used in the experiment. The desired strains were streaked on the plates having specific pH.

The plates were incubated at 15ºC and 37ºC for several days. After the specific incubation period growth was recorded with no growth, normal growth and maximum growth.

Enzyme assay

Reagents

Maltose stock solution (250 mg or 0.25 g/100 ml), analytical reagents, a) DNS, b) 0.02M phosphate buffer.

Procedure

For the standard curve of maltose 0.2 g of maltose

was dissolved in 80ml dH_2O and the stock solution was prepared by making the final volume up to 100 ml. Different dilutions of about 1ml were prepared by mixing distilled water with the stock solution.

Then, in all tubes, 1ml of 0.02M phosphate buffer was added and incubated for 30 min at 50°C. It was followed by addition of 1ml of DNS to all test tubes and incubation for 10 min in water bath at 99.9 °C. After 10 min the tubes were well shaken and absorbance was determined with a spectrophotometer at 540 nm. The blank value was subtracted. A graph was plotted between the absorbance values and maltose concentration. The slope of the curve was calculated and the amount of sugar released was determined.

Protein estimation

For protein estimation, the following solutions were required.

Solution A= $NaCO_3$+NaOH, Solution B1= Na.K. tartarate, Solution B2= $CuSO_4$, Solution C= A (B1+B2), Solution D= Folin phenol (1:1).

We took the supernatant of the culture and added 1 ml of Sol C. We incubated it in dark for 10 min at room temperature. We then added 100µl Folin phenol (Sol D). Folin phenol is light sensitive. Again it was incubated for 30 min at room temperature in dark and optical density at 650 nm was recorded.

Along with the culture, we also took a blank reading which was a mixture of 1ml H2O+1ml Sol C. After 10min Sol D was added and kept it for 30 min. The solution inside was colorless and O.D was taken. The values were put in the formula.

Results

In the current study, we isolated different halophilic strains from functional and non-functional mines along with water from the same salt mines sites Karak KP.

The growth of isolates was checked by adding different salt concentrations (w/v) in agar culture medium. The highest growth was obtained in isolates KPS2, KPS4 and KRF1 showing growth at a maximum salt concentration of 36% (w/v), whereas partial growth of isolates KPS1, KPS3, KRF3 and KRF5 was observed on addition of more salt as shown in Table 1.

Table 1. The growth of isolates at different salt concentration.

Strains	Percentage of Salt concentration w/v										
	4%	8%	12%	16%	20%	24%	28%	30%	32%	34%	36%
KPS1	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
KPS2	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
KPS3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
KPS4	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
KPS5	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
KRF1	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
KRF3	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
KRF5	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-

Key: High growth (+++), Moderate growth (++), Partial growth (+), No growth (-)

Isolates were grown on different temperature range in order to optimize best growth conditions. Maximum growth was observed by KPS1, KPS2 and KRF1 at all temperature on agar medium. The rest of the isolates KPS3, KPS4, KPS5, KRF3,and KRF5 showed zero growth at elevated temperature (50°C). Interestingly at a lower temperature (15°C), KRF3 and KRF5 showed no growth at all. The optimum growth was noticed at temperature 37°C for all isolates. The growth curve shows that by increasing the temperature the growth decreases as shown in Table 2.

The overall result of the isolates showed that the growth of the isolates retarded by adding salt to the agar medium.

Biochemical tests

The following biochemical tests were performed.

Triple Sugar Iron (TSI)

The TSI was performed to check the utilization of Triple sugar and gas production.

The isolates KRF3, KPS1, KPS2, KPS4 and KPS5 result showed no utilization of sugar.

Table 2. The growth of bacterial isolates at a different temperature.

Strains	Growth at different temperature			
	15°C	28°C	37°C	50°C
KPS1	++	+++	+++	+
KPS2	++	+++	+++	+
KPS3	++	+++	+++	-
KPS4	++	+++	+++	-
KPS5	++	+++	+++	-
KRF1	++	+++	+++	+
KRF3	-	++	+++	-
KRF5	-	++	+++	-

Key: High growth (+++), Moderate growth (++), Partial growth (+), No growth (-)

To find the optimum growth at different pH, the isolates were grown at acidic, neutral and basic pH. The isolates KPS5, KRF3 and KRF5 showed maximum growth at all pH. Among these isolates KRF3 showed maximum growth and increase in growth can be seen at a different temperature. The isolates KPS1, KPS2, KPS3, KPS4 and KRF1 have no growth at acidic pH 3. The overall isolates have both ascending and descending growth at a different temperature as the growth of some isolates increases whereas other decreases as shown in Table 3.

The other isolates KRF1, KRF2, KRF4, KRF5 and KPS3 showed the utilization of glucose while among these isolates only KRF4 and KPS3 showed the utilization of all three sugar glucose, sucrose and lactose while KRF5 only utilized glucose and sucrose. All isolates failed to produce gas production as shown in Table 4.

Table 3. The growth of bacterial isolates at different pH.

Strains	The growth of isolates at different pH				
	pH 3	pH 5	pH 7	pH 9	pH 11
KPS1	-	++	+++	+++	++
KPS2	-	+	+++	+++	+++
KPS3	-	++	+++	+++	+
KPS4	-	++	+++	+++	+++
KPS5	+	++	+++	+++	++
KRF1	-	+	+++	+++	+++
KRF3	+	++	+++	+++	+++
KRF5	+	+++	+++	+++	++

Key: High growth (+++), Moderate growth (++), Partial growth (+), No growth (-).

Citrate utilization test

In citrate utilization test Simon citrate agar was used as a medium. Strains were cultured on Simon citrate agar having 10% NaCl and incubated for 24 hours at 37°C. In this test, the citrate is used as a carbon source by the organisms. Citrate utilization is shown when the bluecolor appears and no citrate is used when the color remains green.

Table 4. Different strains were checked for the utilization of 3 types of sugar. The list is given in the table.

Strains	Sugar fermentation			Gas product	H_2S
	Glucose	Lactose	Sucrose		
KRF1	+	-	-	-	-
KRF2	+	-	-	-	-
KRF3	-	-	-	-	-
KRF4	+	+	+	-	-
KRF5	+	-	+	-	-
KPS1	-	-	-	-	-
KPS2	-	-	-	-	-
KPS3	+	+	+	-	-
KPS4	-	-	-	-	-
KPS5	-	-	-	-	-

Key: More growth (+), No growth or gas production (-).

Urease test

Urease broth medium having a small amount of yeast extract was autoclaved and urea was added by syringe filter method. The culture was added and then incubated for 24 hours. When the color changes pink it shows positive urease activity while no color changes show negative activity.

Oxidase test

Oxidase test is performed to check oxidase enzymes. Microbes are applied in oxidase reagent i.e. N, N, N`, N`-tetramethyl-p-phenylenediamine (TMPD). Within 15 sec if the colony appears then blue color indicates cytochrome oxidase positive.

The production of indophenol changes the color blue indicates the presence of oxidase enzyme. The isolates KPS2, KPS4, KRF1 and KRF2 showed citrate utilization while two different color changes appeared in all the isolates. Only KRF2 and KRF3 showed a positive result in oxidase test as shown in Table 5.

Table 5. Different strains show growth and color changes while performing the biochemical tests.

Strains	Growth	Colour	Growth
	Citrate utilization	Urease test	Oxidase test
KPS1	++	Yellow	_
KPS2	+++	Y	_
KPS3	++	Pink	_
KPS4	+++	P	_
KPS5	++	Y	_
KRF1	+++	Y	_
KRF2	+++	Y	++
KRF3	++	Y	+
KRF4	++	Y	_
KRF5	+++	P	_

Key: High growth (+++), Moderate growth (++), Yellow (Y) Pink (P), No growth (-).

Enzymes production

The isolates were checked for the production of different enzymes at agar medium. All isolates showed production of catalase enzymes while none of the isolates showed the production of either Cellulase or lipase. Some of the isolates showed partial enzymes production of protease and amylase. The isolate KPS3 and KRF3 showed maximum production of protease, amylase and catalase enzymes as shown in Table 6.

Enzyme assay

Enzyme assay was done after reading for 96 hours (4 days). The graph shows that with the passage of time the isolates produced more enzymes. The graph also indicated that enzyme production at pH 5 and 9 was

similar whereas at neutral pH the production was high. Enzyme production at 48 hours and neutral pH was far better than pH 5 and 9 as shown in Fig 1.

Protein estimation

Proteins of the isolates were estimated by the Folin-Lowry method. In this method, the copper and nitrogen make complexes first in the protein and then the complexed tryptophan and tyrosine react with Folin-Ciocalteu phenol reagent.

Table 6. Isolates show the production of different enzymes.

	Protease	Amylase	Cellulase	Catalase	Lipase
KRF1	+	-	_	+++	_
KRF2	+	+	_	+++	_
KRF3	+	++	_	+++	_
KRF4	+	+	_	+++	_
KRF5	+	-	_	+++	_
KPS1	-	++	_	+++	_
KPS2	-	-	-	+++	_
KPS3	+	++	-	+++	_
KPS4	-	+	-	+++	_
KPS5	+	+	-	+++	_

Key: High growth (+++), Moderate growth (++), Partial growth (+), No growth (-).

The total protein concentration was observed with UV absorption. The protein absorbance was found at 280nm while tryptophan and tyrosine were found at 250nm. The result of the isolates showed that with time the protein estimation also increases and was found highest at 96 hours as shown in Fig 2.

Specific Activity (S.A) = Enzyme Assay (E.A)/Protein Estimation (P.E)

S.A= μ/ml×Ex²Rx/mg/ml×Ex²

Discussion

In Pakistan, halophiles are less studied compared to the other parts of the globe. Pakistan has a number of salt mines like Khewra salt mine, Karak salt mine, Warcha and Kalabagh. Karak Bahadurkhel (Salt mines) is a village of District Karak KP. Salt mines are located with GPS coordinates at 33°10'57N 70°57'15E with an altitude of 548 meters. Karak salt mines are highly saline and the microbial communities have not been explored yet (Roohi, Ahmed *et al.* 2012). Karak salt mines have a great number of halotolerant/ halophilic bacteria having different bacterial populations (Roohi, Ahmed *et al.* 2014) and can be used in various industries after identification and screening.

This is first studied conducted in Quaid-i-Azam University, Islamabad on Karak Salt mines. The strains were studied at different salt concentration, pH and temperature. The tolerance of the halophilic bacteria was also analyzed. The strains were grown at 36% salt concentration under different temperature and pH to check the isolates tolerance. Microbes ability to grow under such harsh conditions gives some important understandings regarding their ability to increase under the ecological limitations of the environment of salt mines and their possible biological role (Grant, Gemmell *et al.* 1998).Nutrients enrich media such as LB. agar and nutrient agar were used for the growth of isolates. Diverse colony morphologies were observed based on the results of basic characterization. We checked the growth effect on different culturing media of the halophilic isolated bacterial strains, the result showed by all these strains shows that certain strains show one type character and growth texture and on other media show different texture and morphology. According to Duckworth *et al.*, (1996)study gram staining is not a

reliable procedure in several cases prove unsatisfactory(Duckworth, Grant *et al.* 1996). In general, the results obtained from the present research agree with similar studies on bacteria from the hypersaline environment. All of the identified species were truly halophilic because they present widespread growth on salt media. A few species can grow up to 36% salt concentration. Roohi *et al.*, (2012) evaluated the growth of halophilic bacteria at 5 to 40 % salt concentration (Roohi, Ahmed *et al.* 2012).

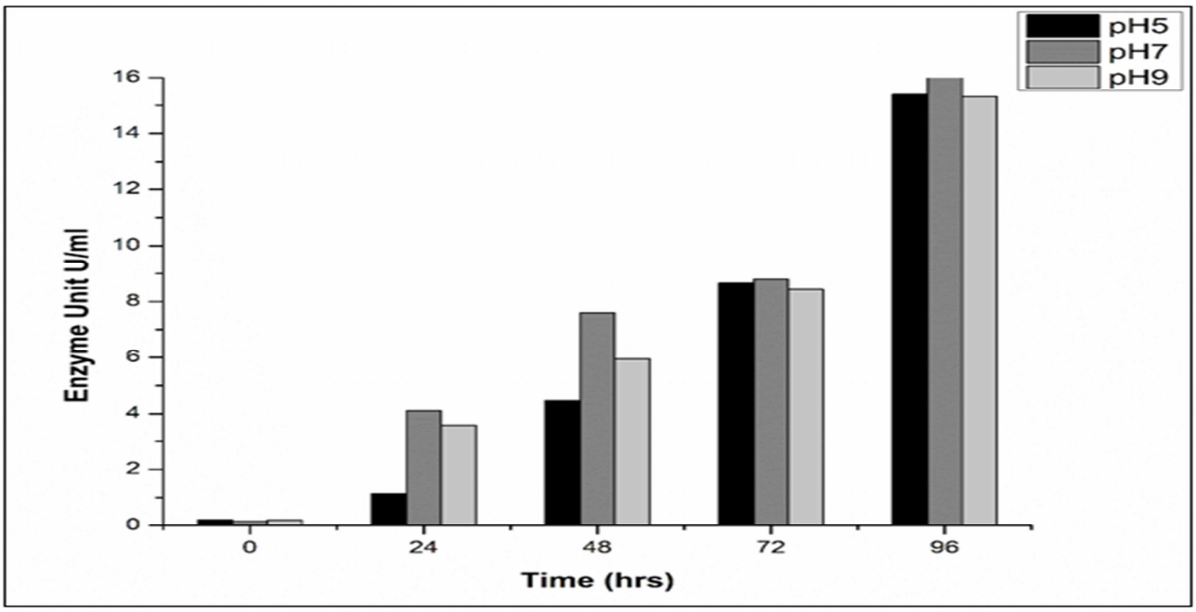

Fig. 1. The increase in the production of an enzyme with increase in time.

In case of temperature, the bacterial isolates were cultured at different temperatures. The isolates were checked at temperature 15°C, 28°C, 37°C and 50°C. The results showed that all of the strains are truly mesophilic while some isolates are psychrophilic and can grow at temperature 15°C. The results also showed that some of the strains are psychrophilic, mesophilic and the rmophilic as well. Only a few of the strains showed no growth at 15°C and most of the strains do not grow at 50°C. None of the strains showed growth at 55°C. Our results are in accordance with previous studies conducted by Roohi *et al* (Roohi *et al* 2014).

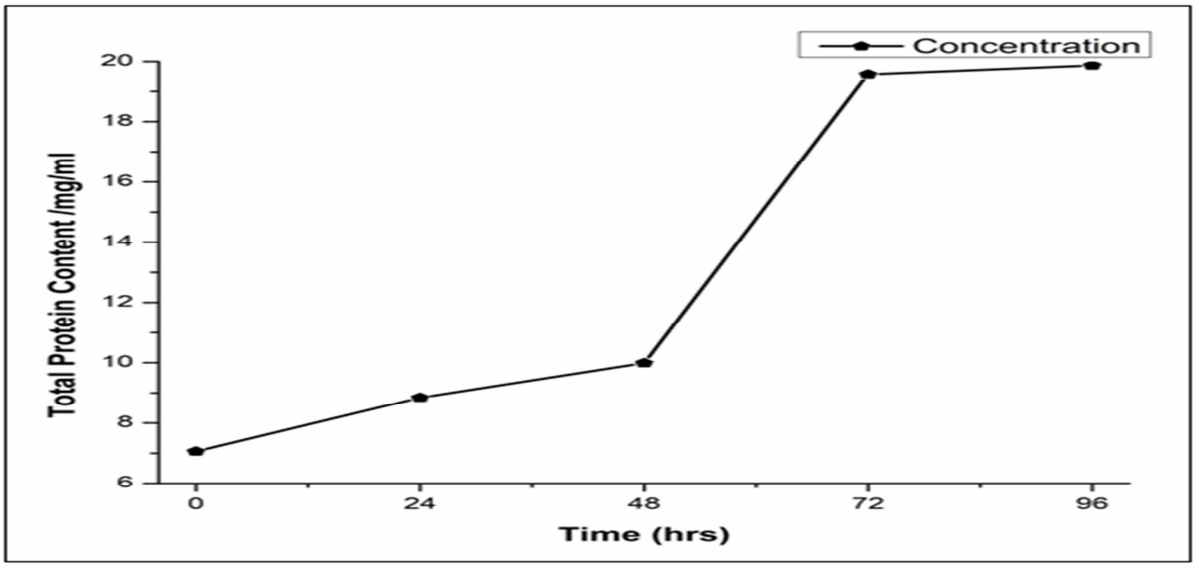

Fig. 2. The graph shows that slowly and gradually the proteins content increases with time.

The isolates were also checked at different pH and incubating them at a different temperature. The different pH values were 3, 5, 7, 9 and 11. The result showed that all strains grow well at pH 7 with temperature 37°C. Some strains showed growth at pH 3 while most of them grow at pH 5 keeping temperature constant. Strains also grow well at pH 9 at temperature 37°C some few strains on pH 11.Roohi *et al.*, (2014) also studied the growth of halophilic bacterial strains on a wide range of pH 5-9 (Roohi, Ahmed *et al.* 2014). Halophiles produce different enzymes in various conditions and about 60% of the total enzymes sales in the world is microbial protease produce from halophiles. For enzymes, the world market is estimated to grow 7.6% per year to $6 billion (Sehar and Hameed 2011).

In the present study, enzyme production from halophiles were also studied and assay of amylase enzyme along with protein estimation was also done. The production of amylase was checked at different p-H 5, 7 and 9. After 96 hours of incubation, maximum enzyme production was observed at pH 7 with enzyme activity of about 16.22 U/ml.

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Declaration of interest

None of the authors of this paper had any personal or financial conflicts of interest.

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