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Original Article

Isolating Two Native Extreme Halophilic Bacterial Strains Producing Bacteriorhodopsin Protein from Aran-Bidgol Lake

Sahar Shakuri[1], Ali Mohammad Latifi[1*], Morteza Mirzaei[1*], Samaneh Khodi[1]

Abstract

Bacteriorhodopsin operates as a light/proton transfer pump which converts the light energy into a proton gradient. The energy stored in the proton gradient can be used in a variety of ways. The main source of Bacteriorhodopsin are some *Halobacterium* species such as *Halobacterium sodomense* and *Halobacterium salinarum* which grow in harsh and salt-saturated conditions. In order to isolate strains from Aran-Bidgol Lake, two red pigment (IRLS.1) and orange pigment (IRLS.2) strains were isolated. Spectroscopy reviews and the results of SDS-Page of membrane proteins of two isolated strains as well as Iranian native *Halobacterium salinarum* showed that bacteriorhodopsin protein presents in the collected sample. Spectroscopic studies showed that *Halobacterium salinarum* produces the maximum amount than IRLS.1 and IRLS.2 produces less and lesser amount of bacteriorhodopsin respectively. The results of biochemical and molecular identification based on the 16srRNA of both mentioned strains indicated their highest similarity with *Natrinema* sp. XA3-1 and *Archaeon* RC34, respectively. In this study, the presence of bacteriorhodopsin protein in Iranian native strains was examined for the first time and the local strains were isolated purely from *Halobacterium salinarum* membrane by gel filtration chromatography that given the widespread use of bacteriorhodopsin protein, it will be so effective.

1. Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

*** Corresponding Authors**
Ali Mohammad Latifi
Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
E-mail: amlatifi290@gmail.com

Morteza Mirzaei
mraga85@chmail.ir

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Introduction

Rhodopsin is a biological pigment in retinal receptor cells responsible for the first event, which occurs in the process of receiving light. This protein belongs to a family of coupled G-protein receptors and is extremely sensitive to the light, so that enables the sight in lower light conditions [1-3]. This protein was isolated from a prokaryote *Halobacterium halobium* membrane by Oesterhelt and Stoeckeniusin (1971) for the first time. Further studies on the structure and function of this protein revealed more details [4]. Bacteriorhodopsin is produced under certain conditions such as the presence of light and oxygen. When the dissolved oxygen concentration is less than adequate, the organism creates a region in plasma membrane, which converts the energy from visible light into energy, which is stored by proton pumps along the plasma membrane. That is, the light energy is used by ATP synthase to produce ATP. Bacteriorhodopsin absorbs the light at 570 nm wavelength (visible green light) and then reflects it as red and blue light with a change in bacteriorhodopsin structure. Hence, this special area is called the purple membrane [4, 5]. Purple membrane comprises 75% bacteriorhodopsin and 25% lipid with an approximate ratio of 10 lipid molecules per bacteriorhodopsin monomer [6, 7]. Bacteriorhodopsin-producing gene has 786 nucleotides coded into 248 amino acids with an approximate molecular weight of 26 kDa. Because this protein is located inside membrane completely, only a small portion with a molecular weight of 1.5 kDa in the C-terminal is available for proteolytic enzymes [8-10]. This protein in bacteria is called bacteriorhodopsin. The mentioned bacteria are usually bar-shaped, Gram-negative, oxidase and catalase positive species, which are observed in red, orange and pink colors due to the presence of pigment [11].

These bacteria cannot break down starch, but degrade casein and gelatin. Among major properties of these bacteria is that they live in salt marshes and stagnant waters [11-13]. These bacteria also produce other products beside bacteriorhodopsin, including: ATP, new extracellular polysaccharides, extracellular enzymes such as amylase, cellulose, xylose, lipase and protease, and poly-β-hydroxyalkanoate which is used for analyzing plastic products [14]. Bacteriorhodopsin has many applications due to its unique properties, such as light sensors in optical real-time processing and as light modulators for other optical processing, as well as in light hard, artificial retina, photon exchange, Fourier-plane spatial filter, optical limiter, holographic interferometry, holographic real-time, etc. [15]. Because of these many practical applications, studying bacteriorhodopsin light cycle takes on a substantial significance [16].

Bacteriorhodopsin light cycle involves a series of steps in which several intermediates are produced. The possibility of M-phase half-life control with different genetic, physical or chemical methods has made bacteriorhodopsin promising for technical applications [13, 17]. Studies of bacteriorhodopsin light cycle have permitted to produce optical sensors which are on the basis of artificial visual receptors. Biological production of bacteriorhodopsin from photo physical materials has been examined as a critical issue.

Despite that, a high level of bacteriorhodopsin is demanded for research and industry, but the current available amount is very below sufficient which arises from the problems in production and purification of the protein [18].

The process of bacteriorhodopsin production from cells needs multiple stages including cells collection, protein concentration, polishing, purification of final products and removing salts and impurities [19]. The purpose of this study is to investigate the protein production in Iranian native *Halobacterium* and optimize the protein extraction.

Materials and Methods

Bacterial strains

In this study, two native isolates named as IRLS.1 and IRLS.2 from Aran-o-Bidgol Lake (N: 34°30′33.9″, E: 51°49′34.9″) and *Halobacterium sodomense* (PTCC 1653) strain which was previously isolated from the same location and has been registered in Microbial Collection of Iranian Research Organization of Science and Industry and *Halobacterium salinarum* which was prepared by University of Tehran (isolated and characterized from natural resources of Iran). *Halobacterium sodomense* PTCC 1653 and *Halobacterium salinarum* strains were used as control (control prepared from faculty of Science in Tehran University).

Culture media and growth conditions

In this study, GMS medium in screening phase, specific medium of *Halobacterium (Halobacterium salinarum and Halobacterium sodomense)* in the presence of sea salt and *Halobacterium salinarum* medium for bacterial growth were used, also 250 g/L NaCl; 2 g/L KCl; 20 g/L $MgSo_4$; 3 g/L Tri-Na-Citrate; 0.02 g/L $FeSO_4.7H_2O$; 5 g/L casamino acid; 0.026 g/L $MnCl_2.4H_2O$; 5 g/L Yeast extract; and 20 g/L agar were used for medium solidification. Bacterial growth was assessed in different salt concentrations and pH in order to optimize the medium. The medium pH was adjusted to 7.3 before being autoclaved.

Sampling and screening (Collection of Bacterial Strains)

Aran-o-Bidgol Lake was selected for sampling because of its extremophilic conditions given that the production of bacteriorhodopsin is one of the defense mechanisms against extreme environmental conditions. Samples were directly collected mainly from the areas of Salt Lake where suffered the color change because of the biological interactions of organisms turned into orange and red (Fig. 1); then, were transferred to the laboratory in sterile bottles. For screening bacterial strains, 200 µl from each sample were inoculated in a 250 ml flask with 50 ml of GMS medium. Flasks were incubated in shaking incubator at 37°C and 150 rpm for 9 days. Later, 10 ml of medium was transferred to a new flask with 250 ml of GMS medium. After three times subculture, 100 µl of sample was spread on GMS agar and incubated at 37°C for 9 days. At the end of incubation duration, different colonies had been purified on GMS agar medium [11].

Figure 1. Alteration in Lake's water color due to the function of isolated red and orange bacterial strains.

Bacteriorhodopsin protein assay methods

In order to review the bacteriorhodopsin production, bacteria were harvested from 2 ml culture (OD_{600}=1) by centrifugation. Then a lysate (0.01 mg DNase in 1 ml distilled water), 4M NaOH, and 4 M NH_4OH were added to the debris with the ratio of 9.0:0.5:0.5. Immediately after addition of the buffer, absorption at a wavelength of 568 nm in the darkness was measured by using spectroscopic technique Then, samples were exposed to the light in order to remove the retinal of purple membrane and absorption was read with time intervals of 24, 48 and 96 hours, respectively. Results showed that the longer the bacteria are exposed to the light, the more the OD decreases. For each sample, the obtained value for OD was put in the following formula and absorption curve was plotted [20]:

M: Amount of protein bacteriorhodopsin in purple membrane

$$M = \frac{26000 \times [A_{568}(0) - A_{568}(24)]}{63000}$$

This step of study was performed for the both isolates IRLS.1 and IRLS.2, as well as the standard strain of *Halobacterium salinarum* as the control sample.

Bacteriorhodopsin protein isolation (transmembrane)

Lee's method was used in order to investigate the presence of bacteriorhodopsin protein. According to this method, each four samples were suspended in PBS buffer containing 1 mM EDTA and 10 µg/ml lysozyme to each unit of cell concentration (OD_{600} = 1) and were then incubated at room temperature for 2 h. Cellular suspension was sonicated in 30 seconds and with a power of 70. Then centrifugation was done at 14,000 rpm for 2h. The collected sediment is the bacterial membrane that was studied by using SDS-Page technique.

Results

Sampling and screening (Collection of Bacterial Strains)

Two pigment producing isolated strains named as IRLS.1 and IRLS.2 were capable of producing red and orange pigments respectively, similar to *Halobacterium* species.

Optimization and growth conditions for bacteria

The first step of this study was optimizing the culture medium. Initially, all four samples were cultured in *Halobacterium sodomense* medium, but no growth was observed. The same result was obtained when NaCl was substituted with sea salt. Therefore, the culture medium was altered to HS medium (*Halobacterium salinarum*). For HS medium optimization, bacteria growth was studied in the present of 10%, 20%, 25%, 30%, 35%, and 40% NaCl. According to the results, bacterial growth showed the most favorable amount at 25% concentration of NaCl.

The growth of four bacteria was also examined in HS culture media with different pH values of 6.5, 6.8, 7, 7.3, 7.6, 8, and 8.5. Results showed that these strains were highly sensitive to pH level. Optimal bacterial growth was observed at pH 7.3. Consequently, HS medium with 25% NaCl and pH 7.3 was used with 150 rpm to culture all four strains at 37°C over a period of 9 days in the following laboratory process.

Bacteriorhodopsin protein assay

Bacteriorhodopsin is the only membrane protein that absorbs in 568 nm due to involving the retinal part. Retinal molecule is reduced in the presence of light and thus, the bacterial absorption at this wavelength decreases overtime. Accordingly, bacterial absorption rate was studied at OD_{568} during 96 h in order to examine the presence of bacteriorhodopsin (Fig.2). The absorption of isolated and controls (*Halobacterium salinarum and Halobacterium sodomense*) strains at OD_{568} decreases over time similar to that of *Halobacterium sodomense*. This result confirms the presence of bacteriorhodopsin in isolates.

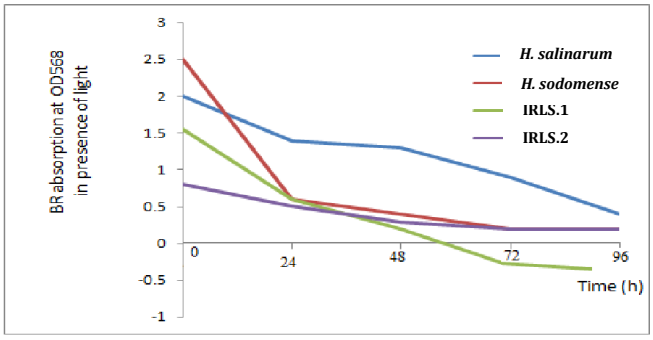

Figure 2. Bacteriorhodopsin absorption rate at OD_{568}. The decrease of bacteriorhodopsin absorption rate over the time indicates the presence of bacteriorhodopsin protein, which is decomposed in the presence of light.

Bacteriorhodopsin protein isolation from membrane

The separated membrane of four under-study strains were more investigated using SDS-PAGE techniques (Fig.3).

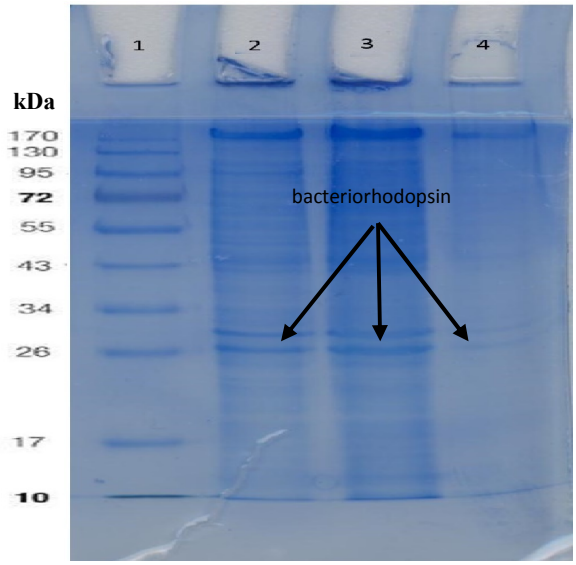

Figure 3. The protein band 26 kDa corresponds to bacteriorhodopsin molecular weight in test and control samples. Lane 1: protein size marker Sm0671; Lane 2: IRLS.1; Lane 3: IRLS.2; Lane 4: *Halobacterium salinarum*.

The band observed 26 kDa corresponds to bacteriorhodopsin and can be seen in both control sample of *Halobacterium salinarum* and test samples of isolated strains. This observe shows that bacteriorhodopsin is actually produced in studied isolates.

Bacteriorhodopsin protein concentration measurement

Bacteriorhodopsin production rate was calculated using the Lee's method. The results of bacteriorhodopsin concentration measurement are shown in grams per liter (Fig. 4). According to the results, $1x10^9$ cells/ml of *Halobacterium salinarum*, IRLS.1 and IRLS.2 produces 0.25, 0.19 and 0.15 g/L bacteriorhodopsin, respectively.

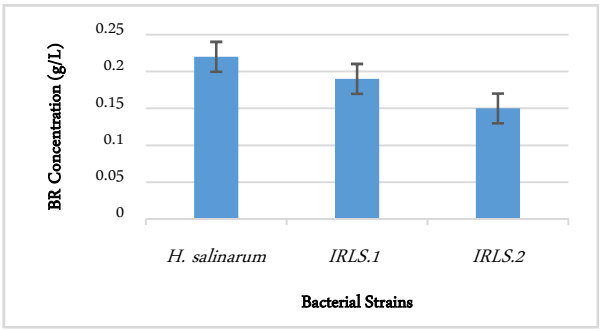

Figure 4. Bacteriorhodopsin protein rate (g/L). *Halobacterium salinarum* produces the maximum amount, than IRLS.1 and finally IRLS.2 with the lowest amount of bacteriorhodopsin.

Bacteriorhodopsin protein purification using FPLC technique

Based on the results of SDS-Page and calculation of protein production, *Halobacterium salinarum* produces the highest amount of bacteriorhodopsin. As mentioned before, the maximum absorption of bacteriorhodopsin occurs at 568 nm wavelength, while the maximum absorption of other proteins occurs at 280 nm. Therefore, it is expected that only bacteriorhodopsin absorbs light at 568 nm. FPLC result also showed only one peak in each injection and corresponding peaks to each injection were collected (Fig. 5).

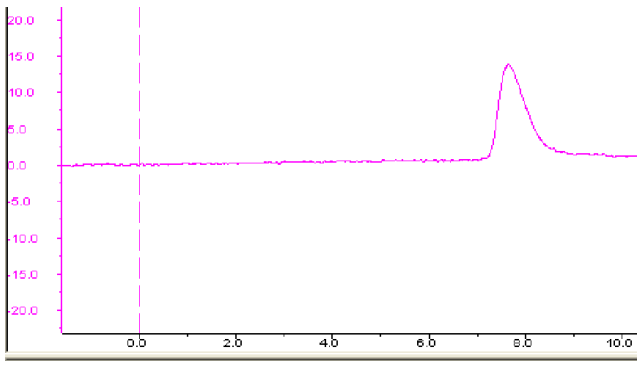

Figure 5. *Halobacterium salinarum* purple membrane chromatography, washed with flow rate of one ml/min. Light absorbance of samples was read at 280 nm.

Illustrated on the FPLC fractions, the peak of gel filtration chromatography comprised a protein that corresponds in size with bacteriorhodopsin, indicating the accuracy of purification (Fig. 6).

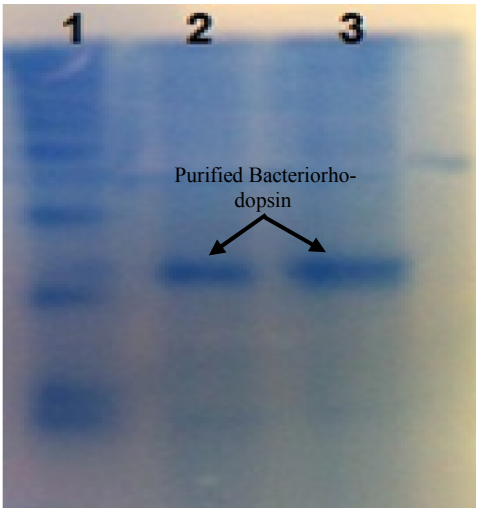

Figure 6. Evaluation of the presence of bacteriorhodopsin protein in fractions from gel filtration at 568 nm. Lane 1: protein size marker Sm0671; Lane 2 and 3fractions obtained by gel filtration.

Identification of degrading bacteria

Phylogenetic analysis was performed by using software MEGA4. The results showed that the strains of IRLS.1 and IRLS.2 had the most similarity to the strains of *Natrinema* sp. XA3-1 and *Archaeon* RC34, respectively (Fig. 7).

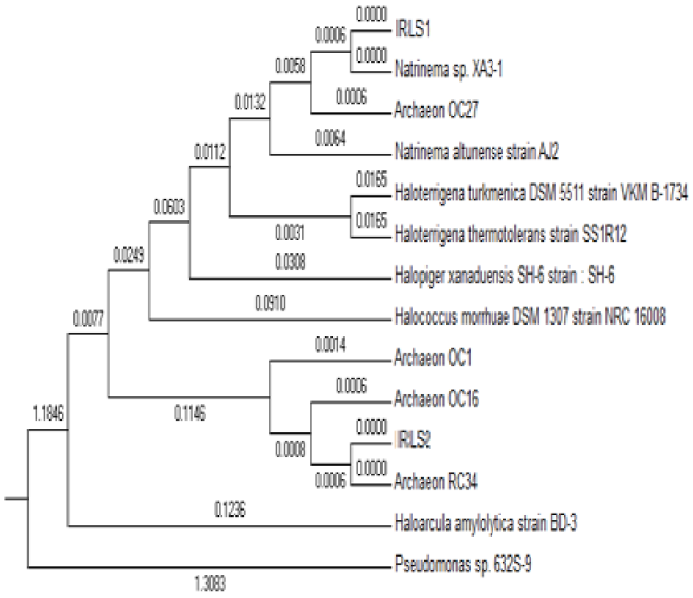

Figure 7. Dendrogram illustrating the isolated bacteria (RDP analysis and Fasta). Phylogenetic tree was prepared using the maximum composite likelihood algorithm and the UPGMA linkg method. A distance bar is illustrated.

Discussion

The first step of this study was to isolate the pigment producing isolates from Aran-o-Bidgol Lake and examine their ability of producing bacteriorhodopsin compared to Iranian native *Halobacterium salinarum. Halobacterium* genus, are usually isolated from salt lakes, static seas, saline lagoons and saline brines. Studies on the isolates obtained from saline lake showed that both bacilli are classified into Archaea [21].

To optimize a specific medium for the growth of *Halobacterium*, the growth of bacteria was assessed at different concentrations of NaCl, different pH and temperatures. The effect of NaCl concentration on the growth *Halobacterium salinarum* was studied in a study by Zeng *et al*., They found that 25% NaCl is the best concentration for the growth of *Halobacterium salinarum* [22]. In another study by Hassan Shahian [11], 22% salt concentration was reported as the optimal concentration for growth of the *Halobacterium salinarum* isolates obtained from Iran's Qom Salt Lake. In this study, four studied species showed the best grow that a concentration of 25% salt. They investigated the effect of the supplementations such as peptone and corn powder on the base medium of *Halobacterium salinarum*. They showed that adding peptone and corn powder could increase the production of bacteriorhodopsin 1.25 fold and 2.12 fold, respectively. In the current study, each four samples were cultured at temperatures of 30, 35, 37 and 40 in order to optimize the growth temperature and the result for optimal growth temperature was obtained as 37°C. This is while Hassan Shahian and his colleagues examined the temperatures of 35, 36, 37, 38, 39 and 40 and reported 38°C as the best growth temperature for *Halobacterium salinarum*. In addition, their research group examined the pH levels of 5.5, 6, 6.5, 7, 7.5, and 8, reporting pH 7.5 as the optimum pH for bacteriorhodopsin production, whereas, the present data showed that isolates show the best growth at pH 7.2.

The study of purple membrane synthesis in parallel to bacterial growth is an approach to identify the bacteriorhodopsin-producing isolates. For this purpose, spectroscopic technique was used. During a study, the presence of bacteriorhodopsin in *Halobacterium sodomense* isolated from Qom Salt Lake has been investigated by studying the absorption at 570 nm wavelengths [11]. In another study, the increase in the light absorption of isolate at the wavelength of 570 nm over time along with the increase in bacterial concentration (increase in absorption at 600 nm) showed that purple membrane are emerging with the growth of isolate. Ghasemi and colleagues have calculated the concentration of bacteriorhodopsin in *Halobacterium salinarum* PTCC 1658 based on Shand–Betlach method in 2008 [23]. According to their findings, *Halobacterium salinarum* grown on base medium of *Halobacterium salinarum* supplemented with 1 g/L is able to produce 191.7 mg/L bacteriorhodopsin, while in our study, the bacteriorhodopsin concentration of *Halobacterium salinarum* grown on base medium of *Halobacterium salinarum* is estimated 200 mg/L.

In a different study, the bacteriorhodopsin produced by *Halobacterium sodomense* was calculated by Shand–Betlach method from a salt marsh. Its results showed that this isolate can produce 11.3 mg/L bacteriorhodopsin during 11 days [24], while the findings of the present study showed that *Halobacterium sodomense* PTCC 1653 could produce about 800 mg/L bacteriorhodopsin during 9 days, which is several times greater. Results of separating purple

membrane fractions shows that gel filtration is a useful tool to separate the purple membrane fractions and this technique can be used for the purification of bacteriorhodopsin existing in the purple membrane in order to evaluate its various features and functions.

Although, further reviews and studies in this area is subject to further research work. In this study according to the above subjects, FPLC device was adjusted for purification of bacteriorhodopsin. The fraction of the resulted peak at the wavelength of 568 nm was collected and checked by SDS-PAGE technique. Gel chromatography showed that the collected fraction has about 27 kDa molecular weight purified from *Halobacterium salinarum* isolate directly for the first time.

Conclusion

Despite the strains used in this study were screened for carotenoids production at first, their bacteriorhodopsin production was confirmed. The hopes for producing bacteriorhodopsin came from the presence of carotenoids within bacteriorhodopsin proteins.

In the following, the conditions required for a proper bacteriorhodopsin production in native strains were optimized using experimental and computational methods and the amount of production in these conditions was calculated. Although, it was found that *Halobacterium salinarum* which was used as control strain had higher rates of bacteriorhodopsin production. IRLS.1 strain produced higher rates of bacteriorhodopsin compared to IRLS.2, however still it was lower than *Halobacterium salinarum*.

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