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Enhancement of bacteriorhodopsin production from novel haloarchaea strains of Marakanam region of Tamil Nadu

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Haloarchaea from high saline habitats are known to contain industrially important value-added bioactive compounds such as bacteriorhodopsin, carotenoids, lipids, and proteins. The diversity of Haloarchaea from geographically less explored saline habitats has received less attention. In this study isolation and characterization of the biotechnological potential of novel haloarchaea strains from solar saltern, a hypersaline environment in Marakanam, Tamilnadu was carried out. The samples were collected during the pre-monsoon (July) and post-monsoon (January) season and ten different haloarchaea strains were isolated. UV-Vis spectroscopy analysis revealed the bacteriorhodopsin production by all the isolated strains. Further experiments were carried out to estimate the yield of bacteriorhodopsin and to assess their photocurrent activity and biosensor applications. The maximum yield of bacteriorhodopsin was 5.6 mg/L of cultured lysate. This study will contribute to a better understanding on effect of elicitors on bacteriorhodopsin producing different haloarchaea strains and growing knowledge on their biotechnological applications.

[**Keywords**: Bacteriorhodopsin, Elicitors, Haloarchaea, Purple membrane, Salt pan]

Introduction

Bacteriorhodopisin (BR) is a transmembrane integral protein found in certain halophilic microorganisms. It can function as proton pump[1]. Bacteriorhodopsin is present in two-dimensional hexagonal array of trimers in the Plasma membrane of the cell[2]. Green light is highly absorbed by this protein, which then pumps protons outward from the plasma membrane, converting light into an electrochemical gradient[3,4]. BR has high photochemical and photoelectric conversion efficiency and thermal stablilty. It helps to withstand extreme environmental conditions like, high temperature, high salinity and resource limited environment[5]. This ability of BR to convert light energy into chemical energy has taken as application in the field of optical electronic devices, chemical sensing, and drug delivery[6].

Members of archaea with high adaptability tend to dominate the habitats of extreme halophiles[7]. One of the most valuable byproducts of halophilic archaea is bacteriorhodopsin. It possesses a unique light absorbing quality that can be applied in biosensor applications. Knowing the BR molecule's technical properties has expedited studies targeted at isolating and identifying the halophilic archaeal strains that produce bacteriorhodopsin[8]. The most extensively researched archaea for producing BR is *Halobacterium salinarum*[9]. High-light, high salt and higher temperature is ideal for this microorganism's survival. High quantity of purple membrane from this microorganism is needed for the majority of applications. This has encouraged scientists to scale up cultivation and biochemical preparation to create affordable ways to produce large quantities of BR, which is a necessary feature for profitability[10].

Due to the difficulties in growing *Halobacterium salinarum*, a lot of research has been done in recent years to lower production costs and increasing the BR production yield[11-13]. Numerous techniques were tried, including mutation, gene expression, carotenoid removal, controlled stress, fermentation, light illumination, growth temperature, agitation speed, and synthetic medium[14-16]. Even though these initiatives have helped the production of BR to some extent, achieving cost efficiency remains a top priority.

In this case, bacteriorhodopsin-producing haloarchaea from Marakanam, Tamil Nadu were isolated and grown to examine the cultivable diversity of haloarchaea species and the yield of BR production.

Materials and Methods

Sample collection

Samples were collected from Marakanam, Tamil Nadu (12°18'69.52" N, 79°92'78.95" E) during two seasons namely pre-monsoon and post-monsoon. Totally 10 Samples were collected, brought into the lab, and stored at 4 °C until the isolation procedure. The salinity of the water sample was 280 ppt.

Isolation and screening of haloarchaea species

The samples were serial diluted to get pure isolated colonies. Dilution series were made in the concentration ranging from 10^{-1} to 10^{-5} and spread on Zobel marine agar plates with 20 % NaCl. Then the plates were incubated under light at 38 °C for 7 to 15 days. The distinct red coloured colonies were isolated and sub cultured on Zobel marine agar plates. The plates were incubated under light and temperature at 38 °C. This step was repeated until the pure isolates were obtained[17].

Cultivation of halophiles

The isolated pure colonies were inoculated into Zobel marine medium in 250 ml Erlenmeyer flask contains 100 ml media with 20 % NaCl, pH adjusted to 7.2 and incubated under light for 14 days for the growth. The identification of bacteriorhodopsin producing bacteria was done according to the spectrometric analysis. The growth rate was monitored at 660 nm and the presence of bacteriorhodopsin was observed at 560 nm[17].

Effect of elicitors on growth and pigment production

The isolated strains were grown at six different concentrations of elicitors (EDTA and $MnCl_2$) *viz*. 0.01, 0.02, 0.03, 0.05, 0.07, and 0.1 M in 50 ml of Zobel marine broth at the optimal NaCl concentration (20 %) in order to examine their effects on growth and BR production. The effect of elicitors on the cell growth and BR production was checked on every alternative day by measuring optical density (OD) 660 nm and OD at 560 nm for BR production.

BR concentration measurement

Optical Density (OD) at 600 nm was used to monitor cell growth over time (OD 600). The absorbance at 560 nm was used to measure PM synthesis throughout the experiment. To measure the concentration of BR[18], 2 ml culture samples were centrifuged for 15 minutes at 4 °C at 10,000 rpm. The particle was then thoroughly mixed in 1 mL of distilled water that contained 30 µl of DNase. Then freshly prepared 4 M NaOH and NH_4OH 9:0.5:0.5 (v/v) was added to the sample in the dark. A spectrophotometer was used to measure absorbance at 560 nanometres (A_{560}). The samples were bleached (retinal removal from purple membrane) by exposure to light for 48 h before measuring the absorbance of the solution (OD 560). The amount of BR was measured as the difference between bleached and unbleached absorption at 560 nm. The molecular weight of BR is 26 kDa and the molar extinction coefficient is 63000/M cm, The BR concentration was calculated as:

$$\text{Bacteriorhodopsin (g/l)} = 26,000 \times (A_{560} - A^{48}_{560} / 63,000)$$ (ref. 19)

Biochemical characterisation of isolated strains

Gram staining

The isolated strains were subjected to gram staining. The gram staining was performed using a gram staining kit from HI media. A thin smear was prepared with a loopful of bacterial culture on a glass slide. Then, the glass slide was air-dried, heat-fixed, and staining was performed according to the procedure[20]. After staining, the slide was air-dried and observed under light microscope at a magnification of 100 X. The bacterial culture was obtained from the colony of previously streaked plates.

Biochemical characterization

The isolated strain was characterised biochemically using the Bergey's manual of systematic bacteriology. Totally, 12 biochemical tests (Himedia biochemical test kit (KB001) were performed such as Indole, Methyl red, Voges Proskauer's, Citrate utilization tests and 8 different carbohydrates utilization tests such as glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose, and sucrose for all isolates. As per the standard protocol, a 50 µl of broth inoculum (OD = 1.8) was used for all the biochemical tests and incubated at room temperature under light (1000 lux) for 24–48 h in order to interpret the results. After incubation, based on the colour changes, the results were interpreted accordingly.

Results and Discussion

Following the isolation steps in the current study, 10 different haloarchaeal strains were found in the samples based on colony size and morphology and colour. In each sampling period 5 of BR producing strains were isolated. Afterward, the ability to produce bacteriorhodopsin was assessed in these isolated strains, and 10 of them were found to have this potential.

Biochemical characterization

The morphology of the isolated strains was identified using gram staining (Table 1). Further, the biochemical characterisation of all the strains were also analysed (Table 1). Most of the isolated strains showed positive result for glucose and sucrose utilization test. The carbohydrate utilisation study shows that most of the isolated halophile's strains can utilize glucose, sucrose and mannitol as a carbohydrate source. Hence, further study using this source for the bacteriorhodopsin optimization will be useful in industrial applications.

Cultivation and BR production capacity

The cell culture experiments were preceded for 14 days and samples were collected on alternative days for analyses of growth and bacteriorhodopsin concentration. The bacterial growth curve and BR concentration is shown in Figure 1.

The M4 strain produces maximum bacteriorhodopsin production (5.74 mg/l) compared to other isolates. All the ten isolates have the great potential to synthesise bacteriorhodopsin.

The maximum BR producing strain (M4) was chosen for the next experiment. The effect of elicitors

Table 1 — Biochemical characterisation of the isolated 10 halophilic strains

Biochemical tests	Results [Positive (+) /Negative (-)]								
	M1	M2	M3	M4	M5	Hak1	Hak2	Hak3	Hak4
Indole test	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-
Voges Proskauer's	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	-	+	-
Adonitol	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	-	+	-	+	-	+
Sorbitol	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	-	+	+	+	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-
Gram staining	-	-	-	-	-	-	-	-	-
Shape	Short rod	Round	Round	Short rod	Round	Round	Round	Short rod	Round

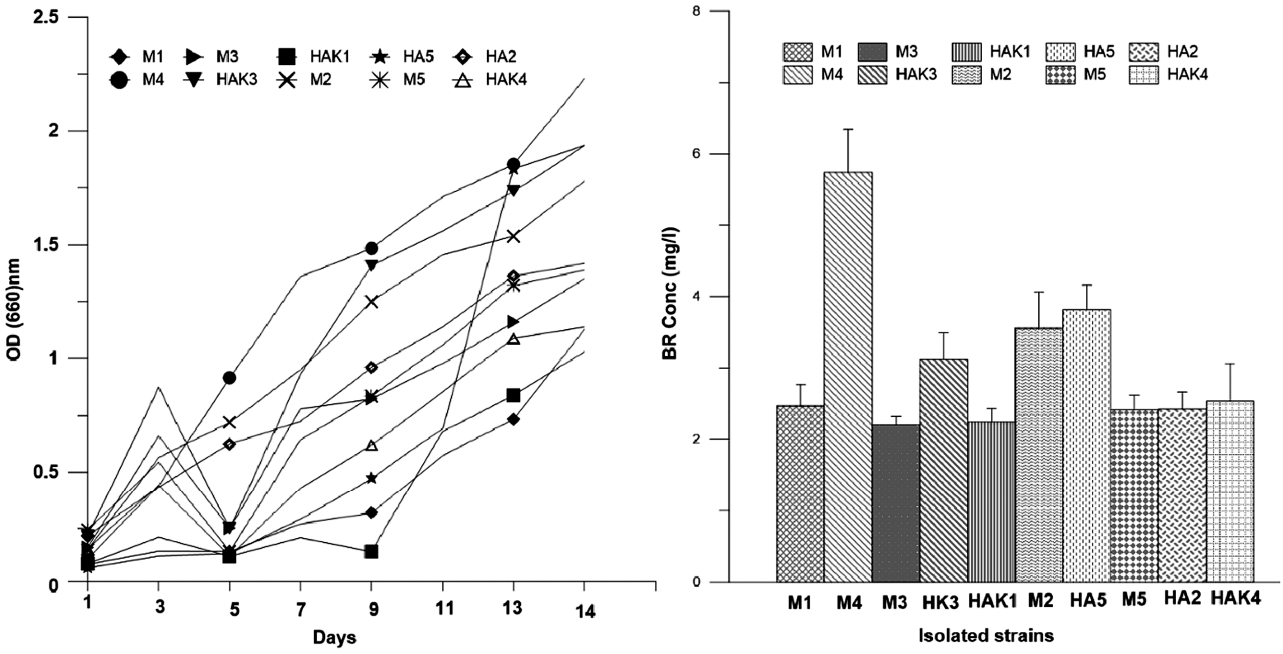

Fig. 1 — Growth curve of 10 haloarchaea isolates and their BR concentration at exponential phase

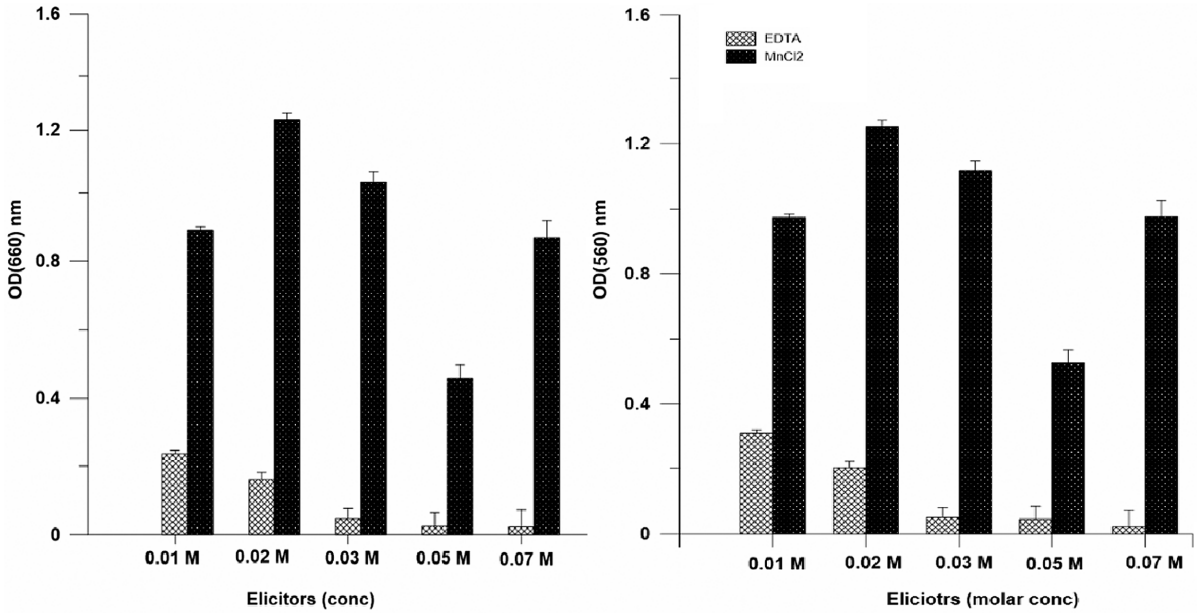

Fig. 2 — Effect of elicitors (EDTA, $MnCl_2$) on optical density (660 nm) and BR (560 nm) production in M1 strain on the 14th day of incubation

on growth and BR production were analysed by spectrophotometrically. Figure 2 shows the bacterial growth curve and the BR production. The strain M4 shows maximum growth and BR production in presence of 0.02 M concentration of $MnCl_2$ with comparative to Elicitors EDTA.

Furthermore, a number of studies have demonstrated that salt lakes, as hypersaline habitats, are naturally occurring sources of halophilic archaea that produce the bacteriorhodopsin that gives the water and shoreline their pink colour. For instance, two of the largest hypersaline lakes in the world, Lake Magadi (Kenya) and the Great Salt Lake (USA), are recognised as major producers of bacteriorhodopsin[21]. Current study area is a significant research site that may contain a wide range of microbes with biotechnological significance. A small number of recent studies have focused on the identification and culture of biotechnologically important microorganisms from salt pans of Tamil Nadu. In similar manner, isolation and cultivation of bacteriorhodopsin producing haloarchaea from Marakanam saltern, Tamil Nadu was done in current study and a total of 10 strains were identified as bacteriorhodopsin producers.

The current studies about bacteriorhodopsin-producing haloarchaea isolates, thought it to be endemic hypersaline microbial diversity; however, according to the prior studies each hypersaline habitat has highly specific ecological features and biodiversity[22-25].

Conclusion

The current study revealed valuable knowledge on Marakanam biodiversity as well as a diverse bacterial source for industrial research on bacteriorhodopsin in the future. This study was conducted to characterise bacteriorhodopsin producing haloarchaea from salt pan of Tamil Nadu. The study isolated 10 halophilic strains from Markanam salt pan and optimised the growth and bacteriorhodopsin production in order to use it for the bioelectronic and other applications. To increase the production of bacteriorhodopsin and growth several optimization strategy were used like elicitors. Further, the effect of elicitors on the pigment production and growth of 10 isolates were studied. The total amount bacteriorhodopsin concentration in cells were analysed at the end of the experiment. The result shows that all the isolates were able to produce higher concentration of bacteriorhodopsin. In the presence of 0.02 M concentration of $MnCl_2$ in Zobel medium shows maximum growth and bacteriorhodopsin production in M4 isolate. The maximum bacteriorhodopsin producing strain was M4 (5.6 mg/L) compared to other halophilic strains at the exponential phase. Hence, the study suggests that the halophilic strains from Marakanam salt pan have great potential for producing large amount of bacteriorhodopsin in the presence of elicitors. Further studies are required to find out the suitability of the supplementation of medium nutrients (carbon source and nitrogen source) to the isolated haloarchaea strains for the better yield of growth and bacteriorhodopsin.

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Conflict of Interest

There is no conflict of interest.

Ethical Statement

The study is the original work and not submitted for any other mean of publications. There are no endangered or live organisms used in the study.

Author Contributions

SJ: Experiment analysis, data interpretation and manuscript preparation; LSA: Conceptualization, experiment design and manuscript correction; DI & GD: Experiment suggestions; and KG: Experiment analysis.

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