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Carotenogenesis in *Haloferax* sp. Strain BKW301, a Halophilic Archaeon from Indian Solar Saltarns

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JB and FNH performed the experiments, analyzed the experimental results and prepared the draft manuscript. Author AKP designed the experiments and prepared the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In this study, attention has been focused on the production of carotenoid pigment by an extremely halophilic archaeon, *Haloferax* sp. BKW301 (NCBI Accession No. KT240044) isolated from multi-pond solar salterns of West Bengal, India.
Methodology: Nutritional and environmental factors influencing the growth associated carotenogenesis by the isolate have been optimized under batch cultivation. The identity of the pigment and its antioxidant activity has been determined by spectroscopic analysis and DPPH scavenging activity respectively.
Results: Growth associated production of carotenoid pigment by BKW301 has been optimized in MH medium under batch cultivation and maximum production of carotenoid pigment (1.59 mg/g) was achieved with 20% NaCl, 1% glucose, 0.5% peptone, 6% inoculum and at pH 8. Presence of light, a CVF ratio of 2.5:10, and continuous agitation at 120 rpm induced the pigment production significantly. The carotenoid pigment extracted from the cell mass showed distinct fingerprint absorption peaks at 469, 492, and 525 nm, and FTIR absorption spectra characteristic of bacterioruberin. Moreover, the purified bacterioruberin also showed high degree of DPPH

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scavenging activity.
Conclusion: Production of significant amount of bacterioruberin under optimized cultural conditions by *Haloferax* sp. BKW301 and its strong DPPH scavenging activity indicated the biotechnological potential of the haloarcheaon strain for mass production of bacterioruberin and effective utilization in pharmaceutical industry.

Keywords: Haloferax; multi-pond solar salterns; carotenogenesis; bacterioruberin; DPPH scavenging activity.

1. INTRODUCTION

Carotenoids, the yellow to orange-red pigments produced by a wide variety of organisms including bacteria, fungi, algae and higher plants [1] are commercially exploited for their biological activities and potential health benefits. Carotenoids have been proved to be important nutraceuticals, efficient antioxidants, antitumor and heart disease preventing agents [2]. Moreover, they are widely applied in food, medical, pharmaceutical, and cosmetic industries as dyes and exhibit free radical scavenging activity [3] and significant anti-proliferative activity against human cancer cell lines [4].

Extremely halophilic microorganisms particularly the members of the family Halobacteriaceae, which grow in environments with high salinity (15-25% NaCl) are reported to produce β-carotene, lycopene and derivatives of acyclic C_{50} bacterioruberin [5-7] while members of *Salinibacter* are characterized by the production of salinixanthin [8-9]. Bacterioruberin as the major carotenoid has been detected in different members of *Halobacterium, Haloarcula, Halorubrum* [10,3] and *Halococcus* [11] and attracted great interest as alternative commercial sources over that of the chemically synthesized ones.

Moreover, sustainable large scale production of carotenoids by the extremely halophilic archaea has number of advantages. They could be grown under non-aseptic conditions with high salinity and the pigment produced could be directly extracted from the cell mass by solvent extraction without any mechanical disintegration [12]. Following the optimization of cultural conditions using conventional one factor at a time and response surface methodology (RSM), the total carotenoid production of *Halorubrum* sp. TBZ126 has been evaluated [13-14]. Apart from these, the elucidation of carotenoid biosynthetic pathways [15] in extreme halophilic strains has proved useful for efficient production of bacterioruberin by employing genetically modified haloarchaeal strains [16].

In this study, attention has been focused on the production of bacterioruberin by a Gram-negative, extremely halophilic archaeon, *Haloferax* sp. BKW301 isolated from multi-pond solar salterns of West Bengal, India. Effect of the environmental conditions on growth and production of bacterioruberin by this archaeon has been investigated under batch culture. The identity and the antioxidant activity of the pigment have been confirmed by spectroscopic analysis and DPPH scavenging activities respectively.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Culture Conditions

An extremely halophilic archaeon, *Haloferax* sp. BKW301 (GenBank Accession No. KT240044) isolated from multi-pond solar salterns of West Bengal, India was used throughout this study. The isolate was maintained on the slopes of MH agar medium supplemented with 10% NaCl by repeated subculturing at monthly intervals.

2.2 Time Course of Growth and Pigment Production

Time course of growth and production of carotenoid pigment by this strain was studied in MH medium [17] containing 10% NaCl, 1% glucose and was inoculated with freshly grown culture at 2% level (10^5 cells/mL). The inoculated flasks were incubated under continuous shaking (120 rpm) at 37℃ for 7 days. Samples were withdrawn at regular interval and evaluated for growth and pigment production. Growth was determined by measuring the optical density at 600 nm and the dry weight of cell mass after drying to constant weight at 80℃. Pigment from the freshly harvested cell mass was extracted in dark using acetone: methanol (7: 2) mixture and

quantified by measuring the optical density at 495 nm.

Total carotenoid content of bacterial cell mass was determined according to the following equation:

$$\text{Carotinoids}_{mg/g} = \frac{\text{A} \times \text{V(mL)} \times 10^4}{\text{E}^{1\%} \times \text{W(g)}}$$

Where, A= absorbance at 495 nm; V= volume of extract; W= dry weight of bacterial cells and 10^4 = conversion factor to obtain concentration in units of µg/g [18]. Archaeal bacterioruberin pigments were quantified based on an $E^{1\%}$ value of 2540 at 495 nm for α-bacterioruberin.

2.3 Thin Layer Chromatography

For thin layer chromatography, the harvested cell mass was extracted with different solvents like methanol, ethanol, propanol, acetone, butanol and acetone: methanol mixture (7: 2) under dark condition. The extracted pigment fractions were concentrated and subjected to TLC in silica gel coated aluminum sheets using different solvent systems such as petroleum ether: acetone (7: 3), petroleum ether: chloroform: acetone (50: 10: 17), diethyl ether: petroleum ether (3: 1), chloroform: hexane: methanol (20: 70: 5), petroleum ether: diethyl ether: acetic acid (80: 20: 1), methanol: water (9: 1), methanol: benzene (3: 97) and the R_f values were calculated.

2.4 Spectroscopic Analysis

Absorption spectrum of the pigment recovered from TLC plate was recorded at 200-700 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800 Series, Kyoto, Japan). The approximate content of total carotenoid was determined by measuring the optical density of the sample at 495 (λ_{max} of bacterioruberin).

2.5 DPPH Scavenging Activity

The free radical scavenging activity of the acetone: Methanol extract of bacterioruberin was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) according to the method of Braca et al. [19] with slight modification. To 0.5 mL of pigment solution in acetone: methanol, 3.0 mL of 0.01% (v/v) methanolic solution of DPPH was added. Absorbance at 517 nm was measured after 30 min of incubation. The scavenging activity of DPPH radical (%) was calculated according to the following equation:

$$\text{Scavenging (\%)} = \frac{\text{A}_{517}\text{ (Blank)} - \text{A}_{517}\text{ (Sample)}}{\text{A}_{517}\text{ (Blank)}} \times 100$$

Where A_{517} (blank) was the absorbance of the control (deionized water, instead of sample) and A_{517} (sample) was the absorbance of the test sample mixed with reaction solution.

3. RESULTS AND DISCUSSION

3.1 Time Course of Growth and Production of Pigment

The kinetics of growth and production of carotenoid pigment by the isolate BKW301 as determined in MH medium is shown in Fig. 1. Changes in the optical density and carotenoid pigment production by the isolate were more or less parallel till both parameters attained their maxima. Pigment production increased to a maximum of 1.48 mg/g of cell mass after 5 days of growth and was followed by a decrease in both growth and pigment production. It was evident that the phenomenon of carotenogenesis in isolate BKW301 was growth associated as reported by Asker and Ohta [5] for a halobacterial strain. Recently, Fang et al. [20] showed production of bacterioruberin and its derivatives by *Haloferax mediterranei* after 24 h of incubation in a defined medium. Studies performed by Mohanty and Mukherji [21] showed that maximum growth and pigmentation of *Chromobacter* sp. and *E. aurantiacum* were recorded after 2 days only.

3.2 Effect of Medium Composition

Most of halophilic strains are fastidious and need a complex media for growth and synthesis of carotenoids [22]. Similarly, growth and pigment production by the isolate BKW301 in complex media such as modified nutrient broth (NB), medium for halophiles (MH), and tryptone-yeast extract medium (TY) were much better than in synthetic media (Valera or Davis-Mingioli's media). The pigment production was maximum (1.48 mg/g) in MH medium and was followed by TY (1.47 mg/g) and NB (1.39 mg/g) (Table 1).

3.3 Effect of Carbon and Nitrogen Source

When glucose as carbon source was added to the medium, it gave the highest pigment yield

(1.48 mg/g) as a result of maximum biomass formation. Similarly, sucrose as carbon source has stimulated the pigment production in BKW301 as reported in other extreme halophiles [14]. Glycerol on the other hand has failed to support (Fig. 2a) bacterioruberin synthesis possibly by inhibiting both growth and supply of carbon units to the carotene chains [23]. The isolate showed requirement of complex organic nitrogen sources for the biomass production as well as carotenogenesis. The MH medium supplemented with 0.5% peptone showed maximum pigment production (1.44 mg/g) followed by tryptone (1.36 mg/g). Exceptionally, casein hydrolysate supported the highest biomass production (2.93 g/L) with low pigment yield (0.89 mg/g). The inorganic nitrogen sources neither supported growth nor the pigment production by the present isolate (Fig. 2b). These findings were in conformity with the observations made by Bhosale and Gadre [24]. However, an amplifying effect of NH_4NO_3 on growth and pigment production by *Halorubrum* sp. was reported by Khanaferi et al. [14].

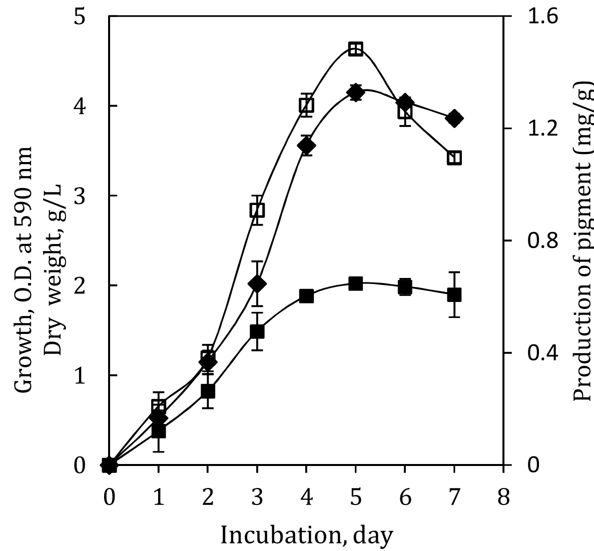

Fig. 1. Time course of growth [O.D. (♦) and dry weight (■)] and production of carotenoid pigment (□) by *Haloferax* sp. strain BKW301

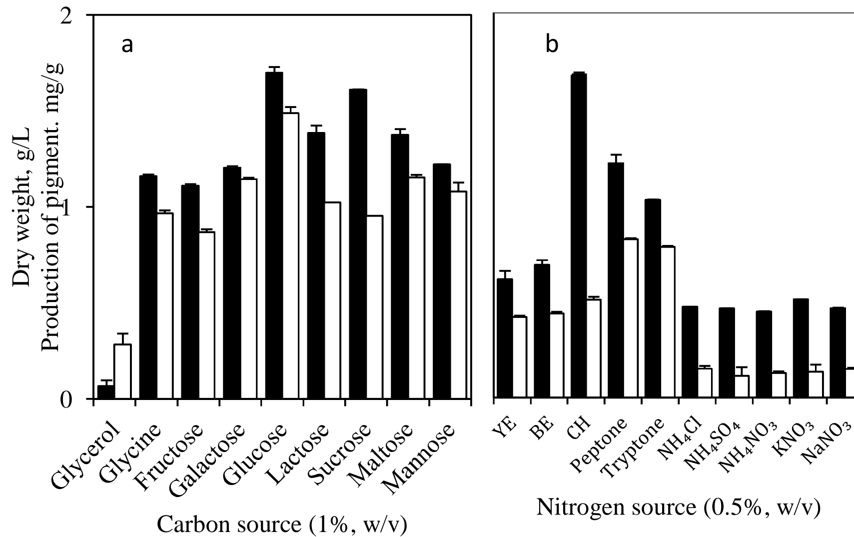

Fig. 2. Effect of different carbon (a) and nitrogen (b) source on production of biomass (■) and pigment (□) by *Haloferax* sp. strain BKW301 [YE, Yeast extract; BE, Beef extract; CH, Casein hydrolysate]

Table 1. Influence of different growth medium on production of biomass and pigment by *Haloferax* sp. strain BKW301

Medium	Dry weight, g/L	Production of pigment, mg/g
Malt extract-Yeast extract	1.047 ± 0.018	0.270 ± 0.007
Nutrient broth	1.792 ± 0.005	1.395 ± 0.010
Medium for halophiles	1.499 ± 0.033	1.483 ± 0.008
Tryptone-Yeast extract medium	1.425 ± 0.030	1.478 ± 0.027
Davis-Mingioli's medium	0.555 ± 0.004	0.461 ± 0.035
Valera synthetic medium	0.754 ± 0.070	0.391 ± 0.026

3.4 Effect of NaCl and Heavy Metals

Growth and carotenogenesis in the isolate BKW301 were studied in a wide range of NaCl (2.5-30%). The isolate was unable to grow and produce pigment in medium without NaCl. Pigment production, however, increased with increase in NaCl in the medium and produced maximum pigment (1.59 mg/g) at 20% NaCl (Fig. 3a). Such NaCl dependent carotenogenesis by extreme halophilic bacteria has been reported by several authors [25-26] and has been interpreted as an adaptive feature of halophiles to extreme salt concentrations [27].

Heavy metals like Zn^{2+}, Cu^{2+}, Hg^{2+} and Cd^{2+} were inhibitory to growth as well as the pigment production by the isolate BKW301 (Fig. 3b) although Zn^{2+}, Cu^{2+} or Fe^{2+} were reported to be important for carotenoid production by *Rhodotorula* sp. Y1621 [28]. With the present strain highest carotenoid production was obtained in MH medium containing $CrO4^{-2}$ followed by Mn^{2+} and Ni^{2+} at 1 mM concentration. The positive influence of Fe^{3+} on carotene production has been recorded and explained by Filotheou et al. [29].

3.5 Influence of Other Cultural Conditions

It is observed from Table 2 that maximum growth and pigment production was obtained at pH 8 (0.87 mg/g) by the isolate BKW301. No growth or pigmentation was observed below pH 6 and above pH 9. This is in accordance with the data recorded for *Serratia marcescens*, *Halorubrum sodomense* ATCC 33755, and *Halobacterium* sp. TM [14].

To elucidate the influence of light on pigment production, the isolate BKW301 was grown in MH medium supplemented with 1% glucose and 10% NaCl under continuous shaking at 37℃. In one set the flasks were illuminated with a 100W lamp from a distance of 8 inch, while the other set of flasks were wrapped with black paper to maintain dark condition. Growth and carotenoid production were significantly higher (1.47 g/L and

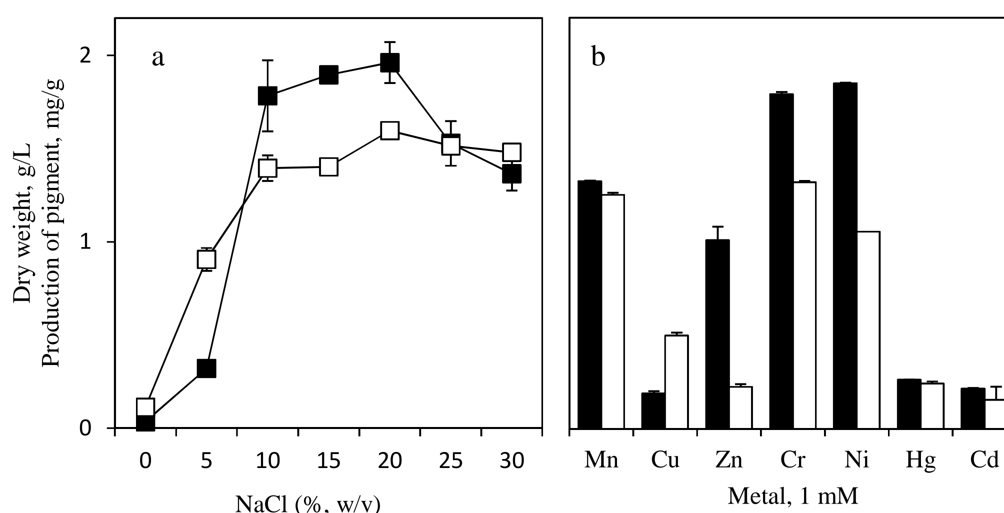

Fig. 3. Effect of NaCl (a) and metals (b) on production of biomass (■) and pigment (□) by *Haloferax* sp. strain BKW301

1.52 mg/g respectively) in light compared to those obtained in dark incubation (1.3 g/L, and 1.29 mg/g) (Table 2). This supported the previous studies that carotenogenesis is a photo-regulated process while, the reverse is not uncommon for a number of other organisms [26,30].

Experiments also proved the essentiality of oxygen for growth and pigment production, and showed that shaking condition was more preferable for pigmentation in bacteria than the static condition. The isolate BKW301 exhibited an increase in growth as well as in pigment production as the incubation condition changed from static to shaking (0.077 to 1.48 mg/g). Results revealed (Table 2) that both the biomass and pigment production did not take place at static condition and cultures shaken at 120 rpm showed maximum growth and pigment production (1.49 mg/g). Similar results were also observed in pigment production by *Halorubrum* sp. SH1 [26]. This could be assigned to the aeration which was beneficial for the growth and performance of microbial cells by improving the transfer of substrates and oxygen to microbial cells [31].

The effect of different volume of culture medium/flask volume (CVF ratio) on pigmentation of the isolate BKW301 was also studied and the results revealed that maximum growth and pigment production (1.54 mg/g) were obtained when the isolate was incubated in 100 mL flasks containing 25 mL of culture medium (Table 2). Further increase in culture volume/flask volume were inhibitory to both biomass and pigment production. This could be explained by the decrease in amount of dissolved oxygen with increasing culture volume leading to a decline in growth and pigment production by strict aerobic isolates [32].

The influence of inoculum size on the pigmentation of the isolate BKW301 was tested and it was noticed that 6% inoculum was conducive to maximum growth and pigment production (1.59 mg/g) by this isolate (Table 2). Reduction in pigment production at higher inoculum supplementation in the medium may be due to the quick depletion of nutrients which causes inhibition of carotenogenesis. Ji et al. [33] and Babitha et al. [34] have pointed out that high inoculum sizes may increase biomass with increased pigment production.

3.6 Extraction and Purification of Carotenoid

Of the different solvent and solvent mixtures used for pigment extraction, acetone: methanol (7: 2, v/v) mixture gave the highest pigment yield (1.63 mg/g) and was followed by ethanol and acetone. The simplicity of pigment extraction with acetone: methanol at room temperature gives the strain an added advantage over those of others and therefore, was found to be most effective (Fig. 4).

Table 2. Effect of pH, inoculum, light, agitation and aeration on production of biomass and pigment by *Haloferax* sp. strain BKW301

Cultural condition		Dry weight, g/L	Production of pigment, mg/g	Cultural condition		Dry weight, g/L	Production of pigment, mg/g
pH	5	0.21 ± 0.81	0.180 ± 0.00	Inoculum	1	1.02 ± 0.12	1.054 ± 0.007
	6	0.35 ± 0.11	0.027 ± 0.02	(%, v/v)	2	1.22 ± 0.23	1.346 ± 0.016
	7	1.12 ± 0.09	0.231 ± 0.00		4	1.34 ± 0.05	1.393 ± 0.002
	8	1.53 ± 0.05	0.874 ± 0.07		6	1.48 ± 0.19	1.589 ± 0.002
	9	1.23 ± 0.14	0.572 ± 0.05		8	1.13 ± 0.23	1.490± 0.003
Light		1.47 ± 0.09	1.525 ± 0.05	Dark		1.30 ± 0.11	1.297 ± 0.007
Agitation	0	0.102 ± 0.81	0.077 ± 0.01	CVF ratio	1.0:10	1.31 ± 0.15	0.778 ± 0.038
(rpm)	80	0.35 ± 0.11	0.800 ± 0.02		1.5:10	1.51 ± 0.16	1.090 ± 0.029
	100	1.12 ± 0.09	0.882 ± 0.00		2.0:10	1.61 ± 0.15	1.157 ± 0.058
	120	1.53 ± 0.05	1.492 ± 0.07		2.5:10	1.86 ± 0.11	1.535 ± 0.053
	140	1.23 ± 0.14	1.304 ± 0.05		3.0:10	1.76 ± 0.31	1.406 ± 0.023
	160	1.56 ± 0.31	1.203 ± 0.02		4.0:10	1.46 ± 0.17	0.777 ± 0.020
	200	1.51 ± 0.11	0.587 ± 0.07		5.0:10	1.35 ± 0.41	0.629 ± 0.019

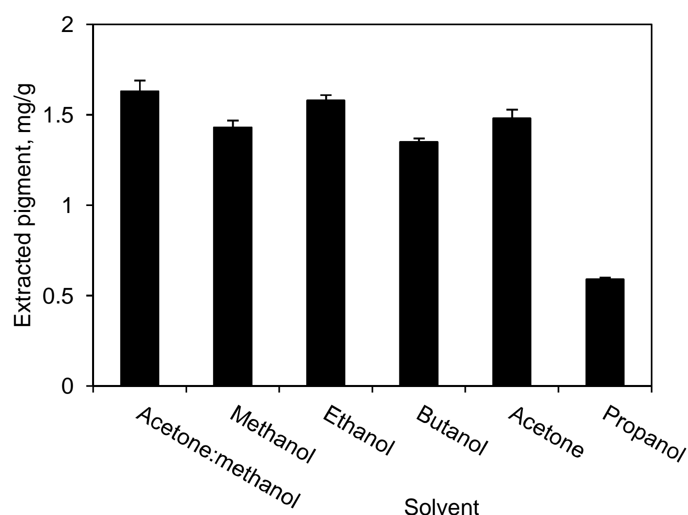

Fig. 4. Efficiency of different solvents and solvent mixture in the extraction of pigment from *Haloferax* sp. cell mass

Table 3. Thin layer chromatographic separation of the extracted pigment of *Haloferax* sp. in different solvent systems

Solvent system	R_f value of pigment	Pigment assigned
Methanol: water (9: 1)	0.47	Bacterioruberin
Petroleum ether: acetone (7: 3)	0.45	Bacterioruberin
Petroleum ether: chloroform: acetone (50: 10: 17)	0.57	Bacterioruberin
Diethyl ether: petroleum ether (3: 1)	-	-
Chloroform: hexane: methanol (20: 70: 5)	-	-
Petroleum ether: diethyl ether: acetic acid (80: 20: 1)	-	-
Methanol: benzene (3: 97)	-	-

The pigment extracted from *Haloferax* sp. strain BKW301 exhibited dark blue coloration upon the addition of sulfuric acid indicating the presence of C_{50} carotenoids [35]. Among the different solvent systems used for separation of pigments in TLC, methanol: water, petroleum ether: acetone and petroleum ether: chloroform: acetone showed distinct band of the pigment extracted in acetone: methanol with R_f values of 0.47, 0.45 and 0.57 respectively which correspond to bacterioruberin (Table 3).

3.7 Absorption Spectra

The purified pigment extracted in all the solvents and solvent mixture showed characteristic absorption peaks at 469, 492 and 525 nm indicating bacterioruberin as the main component in the extracted sample (Fig. 5). Britton [36] described that bacterioruberin and its derivatives exhibited the characteristic spectral peaks of red carotenoids at nearly identical absorption maxima at 469, 492, and 525 nm for three fingered peaks and at 370 and 386 nm for two cis peaks. Similarly following Resonance Raman Spectroscopy, Jehlicka et al. [7] and Abbes et al. [14] have identified bacterioruberin as the major carotenoid in the halophilic archaea *Halobacterium salinarum*, *Haloarcula sodomense, Halorubrum vallismortis* and *Halobacterium halobium.*

3.8 FTIR Spectrum

The purified carotenoid pigment obtained from *Haloferax* sp. BKW301, however, produced fingerprint peaks at 1730, 1653, 1540, 1521, 1458, 1397, 1340, 1290, 1121, 1057, 670 cm^{-1} as depicted in Fig. 6. The representative band at 1458 cm^{-1} appears to be the bending vibration of methylene ($–CH_2$) group while peaks between 1397 and 1340 cm^{-1} of the purified pigment could be attributed to C-H, and ($–CH_3$) symmetrical bending of beta-carotene [37-38].

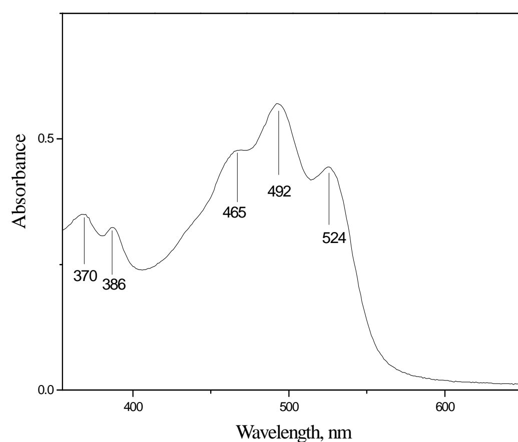

Fig. 5. UV-Vis absorption spectrum of the carotenoid from *Haloferax* sp. strain BKW301 extracted in acetone: methanol mixture

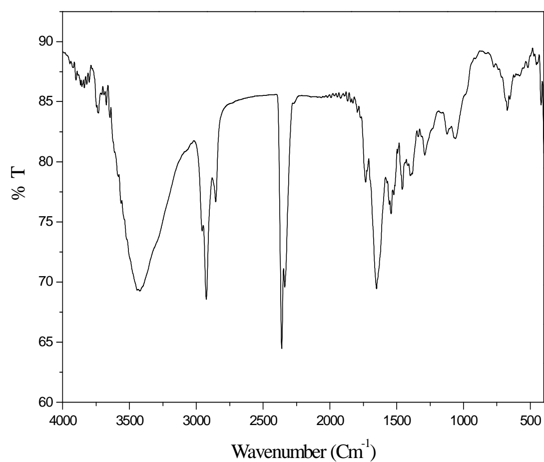

Fig. 6. FTIR spectrum of the purified carotenoid obtained from *Haloferax* sp. strain BKW301

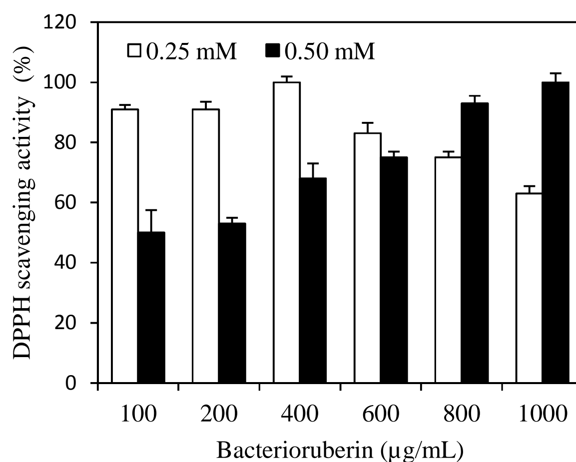

Fig. 7. DPPH scavenging activity of the carotenoid extracted from *Haloferax* sp. strain BKW301

3.9 Antioxidant Activity of Bacterioruberin

DPPH, the stable free radical donor is widely used to test the free radical scavenging effect of natural antioxidants. It involves the scavenging of a performed stable radical by an electron transfer mechanism from the bacterioruberin to the radical, generating a bacterioruberin radical cation [39]. Fig. 7 showed that the DPPH scavenging capacity of bacterioruberin was increased with increase in pigment concentration. While 400 µg/mL of bacterioruberin of *Haloferax* sp. BKW301 was enough to scavenge 100% of 0.25 mM DPPH, 1 mg/mL pigment was required for complete scavenging of 0.5 mM DPPH. Similarly, antioxidant activities of bacterioruberin extracted from *Halobacterium* were reported against 60 µM arachidonic acid and 50 µM H_2O_2 [14]. The antioxidant capacity of bacterioruberin has been correlated with its number of conjugated double bonds (CDB) and the presence of functional hydroxyl (OH^-) groups [40-41] and identified as an efficient DPPH radical scavenger.

4. CONCLUSION

Under optimized cultural conditions the extremely halophilic archaeon *Haloferax* sp. strain BKW301 produced significant amount (1.596 mg/g) of carotenoid in MH medium. The extracted and purified pigment showed a typical UV-Vis absorption spectrum of bacterioruberin and also revealed carotenoid specific peaks in the FTIR spectrum. The strong DPPH scavenging activity of the pigment indicated its potential as an effective antioxidant for future exploitation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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