

Cadmium Tolerance by Haloarchaeal Strains Isolated from Solar Salterns of Goa, India

Adit Chaudhary, Imran Pasha M., Bhakti B. Salgaonkar, and Judith M. Braganca

Abstract—Haloarchaeal strains from solar salterns of Ribandar and Siridao, Goa, India were tested for tolerance to cadmium. The four strains selected named as BBK2, BK6, BS2 and BS17 belonged to different genera of halophilic archaea. An initial screening for various heavy metals [Zn, Cu, Ag, Co and Cd] was performed. Cadmium (Cd) concentrations of 0.5, 1, 2 and 4 mM Cd in complex and glucose synthetic media containing high sodium chloride concentrations were used. Growth was monitored by monitoring optical density (O.D.) values for each of the sample cultures in 24 hr intervals for an incubation period of 5-7 days. Pigmentation and growth was observed in test cultures in complex nutrient media containing cadmium. Interesting results for pigmentation scans of the tested strains having varying peak intensities and exhibiting spectrophotometric profiles associated with typical haloarchaeal pigments were obtained. Correlation between peak intensities of the tested strains and the amount of cadmium in test media was also observed. This investigation shows cadmium tolerance in haloarchaeal strains.

Index Terms—Cadmium, haloarchaea, metal tolerance, solar salterns.

I. INTRODUCTION

Heavy metals are metals which are of great concern to humans due to their impact on the animal or plant bio system when present in high concentration in the environment. Some of the heavy metals that are of interest to humans are cadmium, copper, zinc, lead, mercury, nickel, chromium, manganese and silver [1]. The sources of heavy metal discharge to the environment are natural (animal excretion, volcanic activity etc) as well as anthropogenic (agriculture, mining, fossil fuel burning etc) in nature [2]. While some heavy metals in low concentration are necessary for human bodies to function (cobalt, copper, chromium, nickel and manganese), some can cause serious toxic effects (mercury, lead, copper, cadmium, arsenic, chromium, nickel and manganese) which affect the kidney, liver, central nervous system, skin, teeth and bones [3]. These metals are a great

threat to both our land and water ecosystems as they cannot be biodegraded like organic matter and pose a major challenge in the form of bioaccumulation and biomagnifications because of their inherent quality to thrive in the environment. Hence there is a need to study these metals and look for sustainable ways to reduce the heavy metal concentration in the contaminated ecosystems.

Recent studies have shown that Indian sites such as coastal belt of Alang-Sosiya yard [4], Ranipet Industrial area, Chennai [5], Patancheru, Andhra Pradesh [6] and bed sediments of river Yamuna [7] have high concentration of heavy metals which can have a harmful effect on the surrounding environment. Also, the Indian marine water is highly prone to contamination by heavy metal from urban and industrial waste, gathering it and leading to bioaccumulation through food chain. These water bodies (average salinity 3.5%) are a natural resource; making it a necessity to use bioremediation for removal of heavy metal contaminants.

Haloarchaea or halophilic archaea are broadly classified as extremophiles which need high salt content (10% to 37%) in order to survive and are generally found in soda lakes and solar salterns [8]. In hyper saline or contaminated sea water, normal microorganisms cannot survive as the high salinity is detrimental to their growth. Also such an environment is generally contaminated with heavy metal due to urban and industrial discharge. It is here that halophilic archaea are of great importance to us, as they can thrive and function in such extreme environment. Haloarchaea are not only tolerant to high salt content but also to high temperatures (45°C to 122°C) as they can grow in extreme sunlight in hyper saline water making them polyextremophiles. These characteristics are highly desirable when we deal with heavy metal contamination in extreme natural environment (high salinity and high temperature) and hence this study focuses on metal tolerance (primarily cadmium) by four isolated haloarchaeal strains which may prove to be an important tool in bioremediation and biomonitoring of saline marine ecosystem [9], [10].

In this study, four haloarchaeal strains belonging to four different genera were used. They are BK6 identified as *Halococcus salifodinae*, BBK2 as *Haloferax volcanii*, BS2 as *Haloarcula japonica* and BS17 as *Halorubrum sp.* These strains were isolated and characterized in our previous study (Salgaonkar *et al.* 2012; Mani *et al.* 2012) [11], [12]. These strains were tested for their heavy metal resistance [zinc (Zn), copper (Cu), silver (Ag), cobalt (Co) and cadmium (Cd)]. The growth pattern in presence and absence of heavy metals (primarily Cd) of these halophilic archaeal isolates were also done.

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II. MATERIALS AND METHODS

A. Source of Metals

Metal salts used in the study were CuSO₄, AgNO₃, CdCl₂, CoCl₂ and ZnCl₂. These were obtained from SD-Fine Chemicals Ltd.

B. Haloarchaeal Strain Selection and Cultivation

BK6 identified as *Halococcus salifodinae*, BBK2 as *Haloferax volcanii*, BS2 as *Haloarcula japonica* and BS17 as *Halorubrum sp.* (as characterized by Mani *et al.*) were used in the study (Fig. 1).

Available samples of haloarchaeal strains were cultivated in two plates each. For all the strains, streak plate method was used in which strains were subcultured from existing stock cultures onto new plates containing the required growth medium. Two media were used in the study, NaCl Tryptone Yeast Extract (NTYE) medium and NaCl Tri- Na-citrate (NT) medium [13]-[15], both containing 25% NaCl. The main difference between the two media is the presence of trisodium citrate in NT medium, which can support the growth of fastidious organisms in comparison to NTYE medium [11]. Strains BK6 and BBK2 were grown in NTYE medium. And BS17 and BS2 cultures were grown in NT medium. Plates were incubated at room temperature (30 °C) for 12-14 days until red-orange pigmented colonies appeared. Fig. 2 shows the photographs of culture plates for the different strains with the pigmented colonies visible.

C. Testing Halophilic Organisms for Metal Tolerance

The four haloarchaeal organisms were tested for metal tolerance using available metal salts. A preliminary screening test was performed for metal salts CuSO₄, AgNO₃, CdCl₂, CoCl₂ and ZnCl₂ by inoculating strains BBK2 and BS17 in 0.1mM concentration of each of the metal salts along with the appropriate complex media (NT/NTYE). The test tubes containing only media and culture (i.e. without Cd), were taken as the control. The test media were observed after a period of 48-72 Hours of incubation at 37 °C for any pigments/turbidity. Both strains BS17 and BBK2 showed growth in presence of most of the metals. Since all the 4 strains were isolated from similar environments i.e. solar salterns of Goa, all of them were selected for further metal tolerance tests and analysis.

Strain BS17 was tested for tolerance to Cu and Co by inoculating the cultured strain (two loopfuls for 50 ml growth medium) in glucose synthetic medium (NGSM) [11]-[13] containing various metal concentrations (0.5, 1, 2 and 4 mM CuSO₄ and CoCl₂). Test was conducted in two sets of Control, Cu and Co solutions for the haloarchaeal strain (2 Sets of test media were prepared: Control 1, CuSO₄ 1, CoCl₂ 1; Control 2, CusO₄ 2 and CoCl₂ 2). Culture flasks were incubated in rotary shakers (Labtop) at 37 °C and 100.8 rpm for a period of 5-7 days and microorganism growth was monitored by measuring test media optical density (O.D.) values at 600 nm using spectrophotometer (Shimadzu, Japan) in 24 hr intervals. Similarly, strain BBK2 was tested for tolerance to Zn and Co (using salts ZnCl₂ and CoCl₂) in NGSM medium and monitored for growth by measuring O.D. values at 600 nm in 24 hr intervals. For both the strains BS17 and BBK2, very faint visible pigmentation was observed and on analyzing the

growth curves for the strains plotted using the O.D. values, no significant growth could be inferred. Further tests for tolerance to cadmium for strains BS17, BBK2, BK6 and BS2 were carried out in complex media (NT/NTYE). Strains BBK2 and BK6 were inoculated in NTYE medium in 5 flasks each, one for control and 4 for the different CdCl₂ concentrations (0.5, 1, 2 and 4 mM). Similarly BS17 and BS2 were inoculated in NT medium for Cd tolerance. Culture flasks were incubated as before in rotary shakers and O.D. values were measured in 24 hr intervals at 600nm for the incubation period.

D. Pigment Extraction and Analysis

Pigment extraction was carried out from 10 ml of 7 day old test cultures, i.e. when the cells reached stationary phase of growth. The cells were harvested individually by centrifugation at 8000rpm for 10 minutes. To the cell pellet thus obtained, a mixture of chloroform: methanol in the ratio 2:1 was added and vortexed (REMI CM101) for 5 minutes. The chloroform: methanol fraction containing the pigment was separated from the cell debris by centrifugation at 8000 rpm for 10 minutes. The supernatant was then scanned between 350 – 650 nm using a UV Vis spectrophotometer (Shimadzu, Japan).

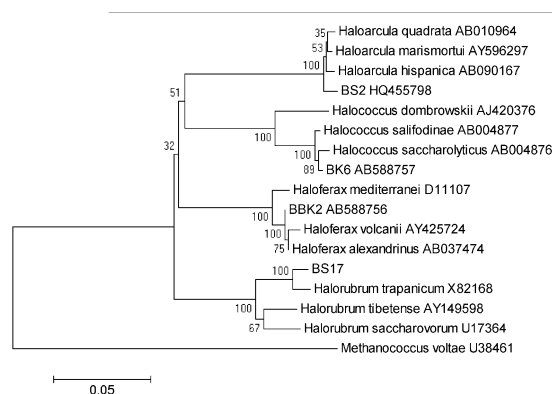


Fig. 1. Phylogenetic tree showing the positions of isolated haloarchaeal strains.

III. RESULTS AND DISCUSSION

A. Strain Selections and Cultivation

Haloarchaeal strains BBK2, BS17, BS2 and BK6 were selected among the strains isolated from solar salterns of Goa. These strains were selected as they represented different genera in the haloarchaea phylogenetic tree (see Fig. 1) and hence would provide diversity in the samples being tested for metal tolerance. The strains when maintained in NT/NTYE plates grew in incubation period of 12-14 days, the results for which are shown in Fig. 2.

B. Growth Kinetics of Haloarchaeal Strains in Presence and Absence of Cadmium.

Based on observations from the preliminary test, the four haloarchaeal strains were selected for further metal tolerance tests. The microorganisms when tested for metal tolerance (metals such as zinc, copper, cobalt) in NGSM medium did not exhibit significant growth and there was very faint pigmentation observed. Resistance profile of halophilic archaea belonging to the genera *Haloferax* (BBK2),

Haloarcula (BS2), *Halococcus* (BK6) and *Halorubrum* (BS17) was evaluated for cadmium (Cd). Growth kinetics and metal tolerance studied in complex media NTYE/NT containing 20% NaCl and 2% MgSO₄. The minimal

inhibition concentration (MIC) of Cd for haloarchaeal cultures BK6, BBK2, BS2 and BS17 is 2mM, 1mM, 2mM and 0.5mM, respectively.

TABLE I: METAL TOLERANCE TEST FOR HALOARCHAEAL STRAINS (*G AND C IN COLUMN THREE STAND FOR GROWTH AND COLOR OF PIGMENT, RESPECTIVELY)

Strains	Growth medium	Heavy Metal (Cadmium) concentration in the growth media					Remarks	
		Control	0.5 mM	1 mM	2 mM	4 mM		
BBK2	NTYE	G*	+	+	+	+	+	At concentrations higher than 0.5 mM cadmium, reduction in pigment intensity and reduced growth observed.
		C*	Red-Orange	Red-Orange	Very Faint Orange	Very Faint Orange	Very Faint Orange	
BS17	NT	G	+	+	-	-	-	Strain could not grow in concentrations of cadmium higher than 0.5 mM. No turbidity or culture pigmentation observed at 1, 2 and 4 mM Cd.
		C	Red-Orange	Red-Orange	-	-	-	
BK6	NTYE	G	+	+	+	+	+	Strain showed consistent growth at all concentrations of cadmium, with a gradual reduction in growth and pigment intensity with increasing cadmium concentration.
		C	Red-Orange	Red-Orange	Orange	Orange	Orange	
BS2	NT	G	+	+	+	+	+	Strain showed consistent growth at all concentrations of cadmium, with pigment intensity and growth significantly reducing at 4 mM cadmium.
		C	Red-Orange	Red-Orange	Orange	Orange	Faint Orange	

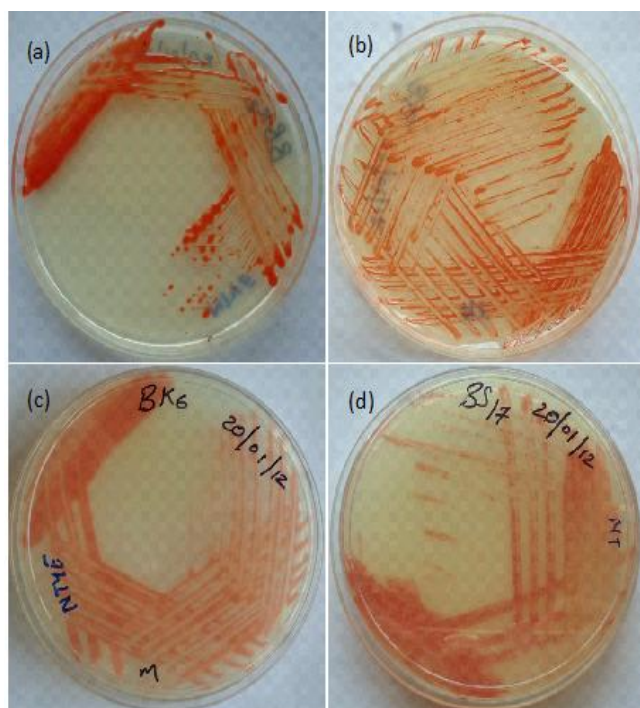


Fig. 2. The four grown cultures of haloarchaea: (a) BBK2, (b) BS2, (c) BK6, (d) BS17.

When these strains were tested in complex NT/NTYE media against various concentrations of cadmium metal salt (CdCl₂), pigmented cultures with color ranging from red-orange, orange to very faint orange were observed. Interesting thing to note was as the concentration of Cd for the metal tolerance test cultures increased, the pigment

intensity and color varied from red-orange in control and 0.5 mM Cd test culture to orange or faint orange for 1, 2, and 4 mM Cd cultures. For instance, BBK2 cultures had red-orange pigmentation for control and 0.5 mM Cd cultures, and very faint orange pigments for 1, 2 and 4 Cd mM cultures. BS2 cultures had red-orange pigmentation for control and 0.5 mM Cd cultures, and orange to faint orange pigments for 1, 2 and 4 mM Cd cultures. Culture flasks for BBK2 and BS17 haloarchaeal strains are shown in Fig. 3. *Haloferax* Strain BBK2 grew with a 3D log phase followed by stationary phase, with growth rate of 0.035 hr⁻¹ and generation time of 19.8 hr. However in the presence of 2 mM Cd growth rate was 0.04 hr⁻¹ and generation time was 17.3 hr. The growth of the BS17 (*Halorubrum*) increased steadily with a lag phase of 3D, log phase of 2D followed by stationary phase, with growth rate was 0.05hr⁻¹ and generation time was 13.86 hr. However in presence of 0.5 mM metal the culture showed 5D lag phase followed by log phase, with growth rate was 0.02 hr⁻¹ and generation time was 34.65 hr.

The Growth curves plotted using O.D. values showed a steady rise in absorbance for most of the test cultures. Growth curves for the 4 haloarchaeal strains are shown in Fig. 4. The curves also show the pattern of reduction in O.D. values for test cultures with rising cadmium concentrations, highlighting the effect of cadmium on haloarchaeal strain population in cultures. However, in the case of BS17, the results are a bit different. Since there was no turbidity observed in the case of 1, 2 and 4 mM Cd BS17 test cultures and the yellowness of the solution might have been due to the complex nutrient media, there might not have been any growth for the haloarchaeal strain in higher cadmium

concentrations of 1, 2 and 4 mM. The growth curve for the strain also shows that the curves for 1, 2 and 4 mM Cd test cultures have negligible O.D. values compared to the test cultures control and 0.5 mM Cd. Thus it may be inferred that BS17 strains are unable to tolerate cadmium in their environment in medium to high concentrations. The above discussed results are summarized in Table I.

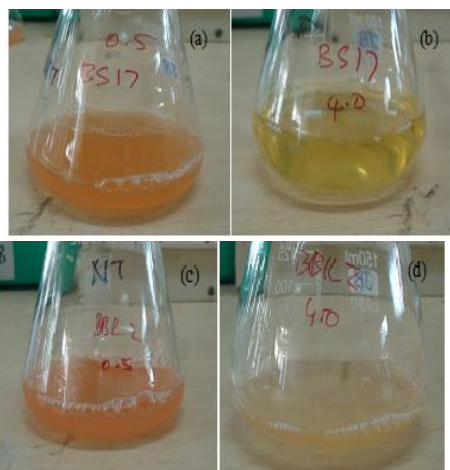


Fig. 3. BS17 and BBK2 test cultures with different Cd concentrations: (a) BS17 with 0.5 mM Cd, (b) BS17 with 4 mM Cd, (c) BBK2 with 0.5 mM Cd, (d) BBK2 with 4 mM Cd.

C. Pigment Analysis

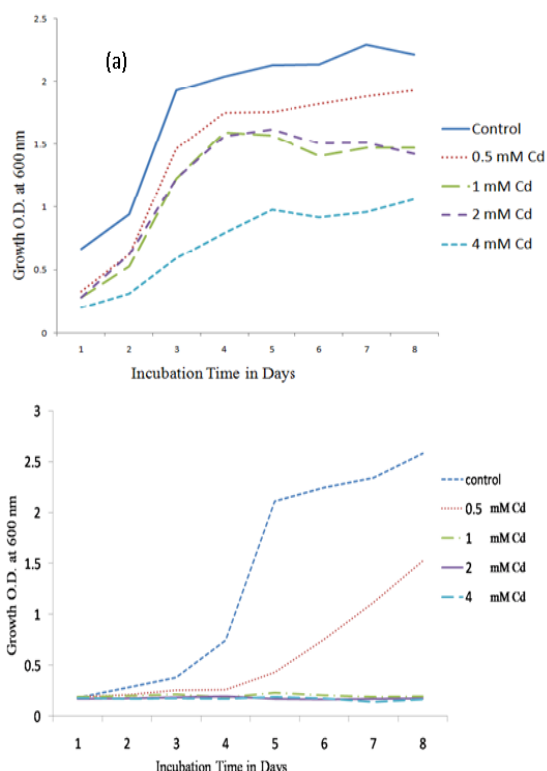


Fig. 4 (a). Growth curves for strains BBK2 (top) and BS17 (bottom).

Analysis of the pigments was done through spectrophotometric scans in 350-650 nm range. The resulting pigmentation curves for all the four strains are shown in Fig. 5. Two interesting inferences can be drawn from these curves. First, for most strains, the culture (apart from control) corresponding with lowest metal concentration in the medium exhibited the highest absorbance maxima in the

absorption spectrum (with the exception of BK6). For example, for BS2, the culture with 0.5 mM Cd concentration showed maximum peak (absorbance of 0.18 approximately) at around 500nm. Secondly, most of the pigments showed similar spectrophotometric profiles with shoulder peaks along with main peak, which have been known to be associated with haloarchaea [11]-[13]. From the above observations, one can speculate that the presence of cadmium in growth media of the haloarchaeal strains causes a change in their pigmentation intensity and spectrophotometric profile. Decrease of pigment intensity and change in visible color might be due to interference of heavy metal cadmium with pigment production and/or due to diminished haloarchaeal culture growth in presence of higher concentrations of cadmium as compared to growth in control and in lower cadmium concentrations.

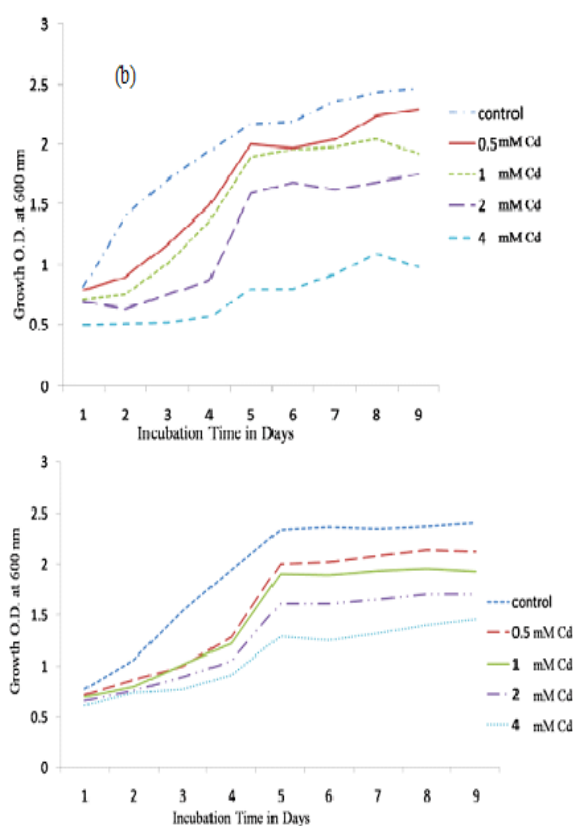


Fig. 4 (b). Growth curves for strains BS2 (top) and BK6 (bottom).

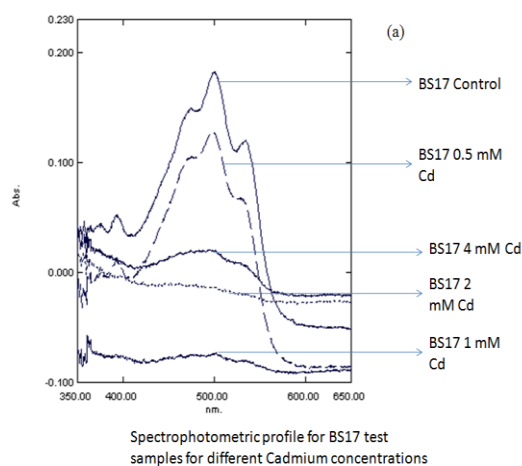


Fig. 5. (a). Spectrophotometric profile for BS17 haloarchaeal strain for different cadmium concentrations.

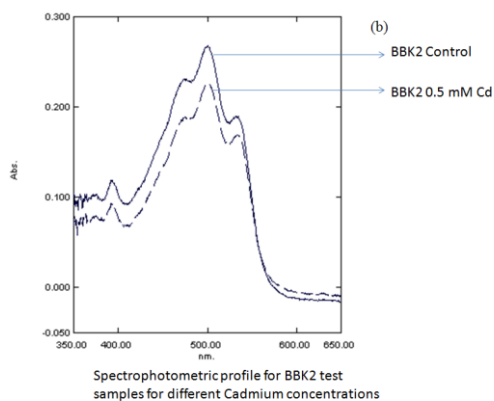


Fig. 5. (b). Spectrophotometric profile for BBK2 haloarchaeal strain for different cadmium concentrations.

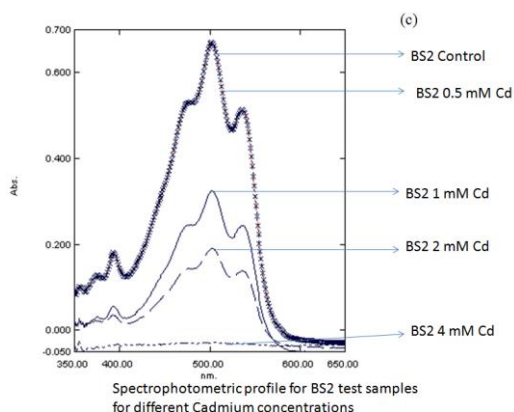


Fig. 5. (c). Spectrophotometric profile for BS2 haloarchaeal strain for different cadmium concentrations.

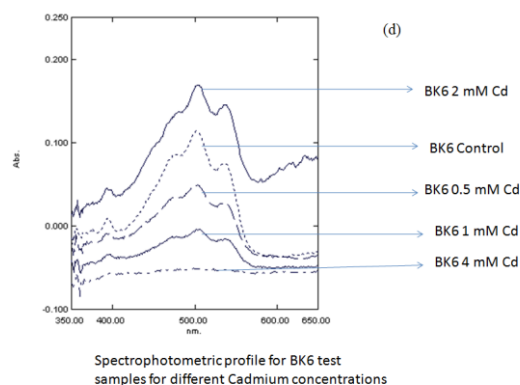


Fig. 5. (d). Spectrophotometric profile for BK6 haloarchaeal strain for different cadmium concentrations.

IV. CONCLUSION

This study highlighted the metal tolerating action of halophilic archaeal strains collected from natural solar salterns of Goa, India. The strains tested could grow and tolerate various cadmium concentrations (0.5, 1, 2 and 4 mM) in complex growth media (NT/NTYE), with the possible exception of BS17 strain which did not grow in medium to higher concentrations of cadmium (1, 2 and 4 mM) (see Table I). The Growth curves indicated that even though the test cultures grew in the presence of high cadmium concentrations such as 2 and 4 mM, the growth was less in comparison to control and 0.5 mM Cd cultures (see Fig. 4). Pigment analysis of the test cultures revealed that the

presence of higher concentrations of cadmium (1, 2 and 4 mM) might have an effect on the pigment intensity and spectrophotometric profile for the haloarchaeal strains (see Fig. 5). Decrease of pigment intensity and change in visible color might be due to interference of heavy metal cadmium with pigment production and/or due to diminished haloarchaeal culture growth in presence of higher concentrations of cadmium. These results obtained can be used for more intensive analysis of heavy metal tolerance mechanism in halophilic archaea which can further help build necessary scientific data for development of effective techniques in the domain of bioremediation of heavy metals from contaminated environments as well as in development of biosensors for detecting toxic concentrations of heavy metals in industrial effluents and other contaminated sites [16].

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