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ORIGINAL PAPER

Phycoremediation Potential of *Botryococcus braunii*: Bioremediation and Toxicity of As(III) and As(V)

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Abstract Worldwide threats of climate change because of greenhouse gas emissions and fuel shortages in near future are posing considerate challenges and therefore it is vital for exploring viable ways of preventing the consequences. The dual usage of microalgae for phycoremediation and biomass production for ecological biofuels production is a practicable choice. Accumulation and toxicity of inorganic arsenic forms (As(III) and As(V)) to the green microalgae *Botryococcus braunii* depends on various environmental factors. This study examined the possibility of using living algae *B. braunii* for phycoremediation of arsenic-enriched media (As(III) and As(V)). As(V) was more harmful than As(III), particularly at pH 7.0, but it was reverse at pH 9.0. The phycoremediation efficiency of As(V) at pH 9.0 by algal cells was higher than that As(III). An increase in concentration of phosphate in growth medium reduced the toxicity and phycoremediation of As(III) and As(V). Value of μ_{max} remained almost constant after addition of arsenic (either As(III) or As(V)) in the media containing various concentration of phosphate ions, but the value of K_s increased. The microalgae *B. braunii* can be employed for tertiary treatment of wastewater and the producing biomass can be utilized as source of renewable energy.

Keywords Arsenic · Wastewater · *Botryococcus braunii* · Phycoremediation · Growth · Monod model

Introduction

Arsenic is the 20th most abundant element in the Earth's crust, 14th in seawater and 12th in the human body [1] and can be found in water because of natural processes such as weathering reactions, volcanic eruption, and biological activity [2] and also because of anthropogenic activities such as uses of fertilizers and pesticides, mining and smelting operations, and fossil fuel combustion [2–4]. Copper smelting creates a huge volume of wastewater containing large amounts of inorganic compounds, such as heavy metals like lead, copper, zinc, iron, cadmium, bismuth, etc. and highly carcinogenic metalloid-like arsenic species, poses a serious threat toward man and the flora and fauna of our ecosystem contaminating the natural water tables (ground water and surface water) in the vicinity. In copper smelting, wastewater concentration of arsenic is as high as 1979 mg/L [5]. With the aim of maintaining a good quality of fresh water resources, this wastewater must be treated so that the water can be reverted to the ecosystems. In natural waters, the inorganic form of arsenic mostly exists in trivalent (As(III)) and pentavalent (As(V)) oxidation states [6]. Short-term exposure to arsenic may lead to neuropathy, hyperkeratosis, and hypertension and prolonged exposure to higher doses may lead to lung, skin, and liver cancer and may be ultimately death [7]. On the basis of investigation of the fatal effect of arsenic on human body, the maximum contaminant level (MCL) of arsenic in drinking water has been revised from 50 to 10 μg/L by the World Health Organization (WHO) in 1993 [8] and the European Commission in 2003 [9]. Generally, both physical and chemical methods are costly. Moreover, most chemical methods

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raise the conductivity, pH, and total contents of dissolved matter in the wastewater and are not only eco-friendly, but also inexpensive [10]. In this respect, biological or biotreatment of wastewater is a better choice [11]. The use of microorganism for biosorption of arsenic ions from water is an enormously effective process, due to which it is becoming widespread day by day [12–14].

Phycoremediation is the use of macroalgae, microalgae and cyanobacteria for the elimination or biotransformation of contaminants, containing nutrients, heavy metals, and xenobiotics from wastewater and CO_2 from waste air (for environmental cleanup) [15]. Phycoremediation is a non-conventional wastewater treatment technology which is achieving attention because of its various benefits including it is non-invasive, cost-effective, and environmentally sound alternative to the currently existing physicochemical contaminant remediation methods [16]. The algae have various features which make them perfect contenders for the selective removal of heavy metals, which contain large surface area/volume ratios, high tolerance to heavy metals, ability to grow both heterotrophically and autotrophically, possible for genetic manipulation, phototaxy, and phytochelatin expression [17]. Ting et al. [18] described that the uptake of metal ions by microorganisms in batch systems occurs in two stages; an initial fast uptake (passive uptake) followed by a much slower one (active uptake). In the course of the passive uptake, the metal ions adsorb on the cell surfaces within a relatively few seconds or minutes. In the second uptake stage, the metal ions are transported across the cell membrane and into the cytoplasm. They also developed a mathematical description of these two processes.

Since microalgae uses CO_2 as a carbon source, they can grow photoautotrophically without the adding an organic carbon source [19]. The cultivation of algae in wastewater offers the combined advantages of mitigation of greenhouse gases, treatment of the wastewaters, and simultaneously producing algal biomass [20].

A significant concern related to the application of phycoremediation is handling and discarding of the obtained metal-enriched microalgal biomass wastes. At present, this is the bottle neck of the valorisation (breakthrough) of the obtained biomass/applied technique. Waste volume can be reduced by thermal, microbial, physical or chemical means This biomass can be exploited for multiple uses as bioenergy resources (biogas and biofuels), biofertilizer, bio-ore for precious heavy metals, and other valuable chemicals [21, 22].

Hossain and Anantharaman [23] studied the ability of *Bacillus subtilis* to remove As(III) from aqueous solution. Giri et al. [12, 14] reported the unexploited sorption properties of the *Bacillus cereus* for the removal of As(III) from aqueous solutions. Teclu et al. [24] and Vaxevanidou et al. [25] also studied the removal of arsenic using the microbial route. Recently, studies related to bioremediation of arsenic from aqueous solution by *Inonotus hispidus* [26], *Ulothrix cylindricum* [27], *Acidithiobacillus ferrooxidans* BY-3 [28], *Staphylococcus xylosus* [29], *Xanthoria parietina* [30], *Maugeotia genuflexa* [13], *Rhodococcus* sp.WB-12 [31], and *Arthrobacter* sp. [32] were carried out.

Botryococcus braunii is an autotrophic green colonial microalgae widespread in freshwater of tropical and temperate areas, occasionally in brackish lakes, reservoirs, ponds, and sea [33, 34]. *B. braunii* was used to remove As(III) (as well as Cd^{2+}, Cr^{6+}, and Pb^{2+}) from aqueous solution, but the arsenic elimination by this strain is not so far extensively verified [33]. Dayananda et al. [34] studied the growth of *B. braunii* in shake flasks by using different autotrophic media and found BG11 was the best medium for biomass and hydrocarbon production. So, in the present study the potential of microalgae *B. braunii* was investigated in autotrophic BG11 media.

Although a number of pioneering works have been reported on treatment of arsenic-containing wastewater by various techniques, only a few studies on a microbial route for detoxification of arsenic has been reported so far. The main objective of the present study was to investigate the potential of microalgae *B. braunii* for low-cost and eco-friendly wastewater treatment so as to implement in the management of municipal and industrial wastewater. Specific research goals included: (1) to perform the thorough kinetic study of living microalgae *B. braunii* by culturing in simulated solutions of arsenic (either As(III) or As(V)) in terms of dry biomass and chlorophyll content; (2) to optimize the growth rate and phycoremediation potential of the microalgal strain systematically under different experimental conditions, such as initial pH, inoculum size, contact time and initial concentration of arsenic (either As(III) or As(V)) ions; (3) to perform SEM-EDX and FTIR studies to analyze surface texture, morphology and element distribution of the biomass; and (4) to determine the effect of phosphate on toxicity and phycoremediation of arsenic (either As(III) or As(V)). Furthermore, the growth of the microalgae in arsenic-enriched media containing various levels of phosphate ions were modeled using the classical Monod equation to identify the effect of phosphate on the toxic nature of arsenic (either As(III) or As(V)) ions to microalgae *B. braunii* in terms of growth inhibition.

Materials and Methods

Materials

All the chemicals and reagents were of analytical reagent grade and used without additional purification. Standards, matrix modifier, and wash solutions were prepared with deionized double distilled water. All required chemicals utilized in the experiments were bought from Himedia Laboratories Pvt. Ltd., Mumbai, India. Glassware utilized for purposes of

experiments was washed in 10 % HNO_3 and rinsed with double distilled water.

Microalgae and Culture Medium

The pure strain of green microalgae *B. braunii* was collected from Department of Biotechnology, IIT, Roorkee. The compositions of the BG11 medium are given in Tables 1 and 2 as follows:

Stock Culture Maintenance

The pure culture of green microalgae *B. braunii* was transferred into separate acid-washed (1 % HNO_3) Erlenmeyer flasks containing 100 mL of fresh, sterilized (at 121 °C temperature, 15 psi pressure for at least 15 min) grown in BG11 medium photoautotrophically [34]. Cultures were maintained at 28 °C in a thermostatically controlled environmental chamber at approximately 2000 lux (Philips 40 W, cool daylight, 6500 K) on a 12:12 h light/dark cycle. To maintain the exponential growth, 1 mL of stock culture was aseptically transferred to fresh, sterile BG11 nutrient media every 7 days. To investigate the contamination of stock cultures, agar plates (12.0 g/L) were swiped with the culture medium under sterile conditions. Stock cultures were examined in triplicate and control plates were utilized to decide if any contamination was because of plating preparation. Petri plates were stored in an incubator under the same environmental conditions as the stock culture. Plates were observed for the presence of microalgal growth after 1 week.

Preparation of Arsenic-Enriched Water

Arsenic stock solution was prepared by dissolving salts of $NaAsO_2$ and Na_2HAsO_4, $7H_2O$ (analytical reagent grade) purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India, in double distilled water. BG11 media composition was also added into the solution. Then the prepared growth media was subjected to autoclave sterilization at 15 psi pressure and at 121 °C for 15 min. The pH of the phycoremediation medium was adjusted to the requisite value by dropwise addition of sterile 1 N HCl and 1 N NaOH solution.

Table 1 Major elements

Composition	Quantity (per liter)
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4{\cdot}7H_2O$	0.075
$CaCl_2{\cdot}2H_2O$	0.036
Citric acid	0.006 g
Na_2EDTA	0.001 g
Na_2CO_3	0.02 g
Ferric ammonium citrate	0.006 g
Trace metal mix A5	1.0 mL

Table 2 Trace metal mix A5

Composition	Quantity (per 100 mL)[a]
H_3BO_3	2.86 mg
$MnCl_2{\cdot}4H_2O$	1.81 mg
$ZnSO_4{\cdot}7H_2O$	0.22 mg
$Na_2MoO_4{\cdot}2H_2O$	0.39 mg
$CuSO_4{\cdot}5H_2O$	0.08 g
$Co(NO_3)_2{\cdot}6H_2O$	0.05 g

[a] Only 1 ml from Trace metal mix A5 was added into the culture solution of major elements

Phycoremediation Experiments

The factors influencing the growth and phycoremediation efficiency of living microalgae *B. braunii* was investigated in 250 mL round bottom flask with 100 mL phycoremediation medium. BG11 medium was served as control. The pH of the medium was adjusted by dropwise addition of sterile 1 N HCl and 1 N NaOH. An optimum aliquot (10 %, volume of inoculum/volume of growth medium) of preculture was harvested aseptically during the exponential growth phase (OD value ~0.515 at 680 nm), and it was transferred to the fresh media (100 mL) supplemented with arsenic (either As(III) or As(V)) of requisite quantity for different studies and the cultures were grown in an environmental chamber for 16 days. Thermostatically controlled environmental chamber was sustained at 28 °C temperature. Illumination was supplied by continuous cool white fluorescent lamps at 2000 lux (Philips 40 W, cool daylight, 6500 K) with a dark/light period of 12:12 h. In preliminary control experiments, microalgae cultured 3 weeks under experimental conditions exhibited normal growth without distinguishable morphological changes and maximum growth of microalgae was found at 7 days and then the rate of increase in growth was not so insignificant. So, the experimental culture period of 7 days used in all experiments possibly had no negative effects.

Phycoremediation studies were performed in the batch reactors to determine the effect of initial pH, inoculum size, contact time, and initial arsenic (As(III)/As(V)) concentration on the growth of microalgae and the phycoremediation % of arsenic (As(III)/As(V)) with the range of operating parameters as mentioned in Table 3 (growth study) and Table 4 (phycoremediation study), respectively. The cultures were aerated with 2 % CO_2 in air at a flow rate of approximately 150 mL/min twice in a day for 30 min by bubbling through the growth medium using an air pump. Aeration of cultures with CO_2-enriched air is necessary to achieve high productivities for phototrophic microalgae and also

Table 3 Different process conditions used for the growth of microalgae

Type of experiments	Range of operating parameters				
	pH	Inoculum size (%, *v/v*)	Contact time (day)	Temp. (°C)	Initial conc. of As(III) or As(V) (mg/L)
Effect of pH	*2.0–12.0*	10	7	28	50
Effect of inoculum size	9.0 (As(III)) 9.0 (As(V))	*2–20*	7	28	50
Effect of contact time	9.0 (As(III)) 9.0 (As(V))	10	1–16	28	50
Effect of initial arsenic conc.	9.0 (As(III)) 9.0 (As(V))	10	7	28	*50–10,000*

In case of effect of pH, pH was varied from 2 to 12 and all other parameters are kept constant. So it was shown in italics. Other are simple.

Similarly, in case of effect of inoculum size, inoculum size was varied from 2 to 20 and all other parameters are kept constant. So it was shown in italics. Other are normal.

Similarly, in case of effect of contact time, contact time was varied from 1 to 16 day and all other parameters are kept constant. So it was shown in italics. Other are normal.

Similarly, in case of effect of initial arsenic conc., arsenic conc. was varied from 50 to 10000 mg/L and all other parameters are kept constant. So it was shown in italics. Other are normal.

Basically to highlight the parameter which is varying, it is written in italics.

helps in mixing and pH buffering [35]. Then the cultures were agitated for another 20 min to prevent algal cells sedimentation on the flasks bottom and to enhance the distribution of CO_2 in the growth medium [36].

Samples (5 mL) were withdrawn at certain time intervals and then centrifuged (Remi Instruments Ltd., Mumbai India) at 5000×*g* for 5 min, and the supernatant fraction was analyzed for residual concentration of arsenic (either As(III) or As(V)) ions in the medium using ThermoFisher Scientific iCE 3000 Series AA graphite furnace atomic absorption (GFAA) spectrometer (The detail procedure of residual arsenic concentration measurement is provided with supplementary materials). Cell growth was determined by measuring an optical density (OD) of the samples collected intermittently from the flask was at 680 nm. A correlation for converting OD680 values to microalgal dry weight was established from the calibration curve (The detail procedure of bacterial cell growth measurement is provided with Electronic Supplementary Materials). Microalgal mass was also analyzed in terms of chlorophyll content (The detail procedure of chlorophyll measurement is provided with Electronic Supplementary Materials). Arithmetic mean of results of two similar experiments was used to estimate data.

The phycoremediation % of metal ion is evaluated utilizing the following equation:

$$\text{Phycoremediation } \% = \frac{(C_0 - C_f)}{C_0} \times 100 \quad (1)$$

Characterization

The 7-day culture, grown in BG11 media in the absence and presence of 2000 mg/L arsenic (As(III)/As(V)) ions, was centrifuged at 5000×*g* for 5 min. The supernatant was discarded, and the cells were washed two times with phosphate buffer solution. The washed cells were resuspended in glutaraldehyde (2 %, *v/v*) for 2 h. The resuspended cells were centrifuged at 5000×*g* for 5 min. The supernatant was discarded and the cells were treated with serial dilution of ethanol. The samples were

Table 4 Different process conditions used for phycoremediation of As(III) and As(V) by microalgae

Type of experiments	Range of operating parameters				
	pH	Inoculum size (% v/v)	Contact time (h)	Temp. (°C)	Initial conc. of As(III) or As(V) (mg/L)
Effect of pH	*2.0–12.0*	10	144	28	50
Effect of inoculum size	9.0 (As(III)) 9.0 (As(V))	*2–20*	144	28	50
Effect of contact time	9.0 (As(III)) 9.0 (As(V))	10	*4–360*	28	50
Effect of initial arsenic conc.	9.0 (As(III)) 9.0 (As(V))	10	144	28	*50–2000*

To highlight the parameter which is varying, it is written in italics.

serially diluted to 30, 50, 70, 90, and finally 100 %. Every dilution of ethanol was applied on the cells for 10 min individually. The cells were allowed to evaporate ethanol under atmospheric conditions. The cells were subjected to gold sputtering using Sputter Coater, Edwards S150, which provides conductivity to the samples, under vacuum followed by degassing. Finally, the cells were characterized by scanning electron microscopy (Fe-SEM) and EDX. The measurements of SEM were done for observing the surface morphologies of the microalgae (SEM; LEO Electron Microscopy, England) [37]. The images were taken with an accelerator voltage = 15 kV and an emission current = 100 μA by the Tungsten filament.

The microalgal cells grown in BG11 media were centrifuged at 5000×*g* for 5 min. The cells obtained after centrifugation were washed twice with phosphate buffer solution and dried in oven at 353 K for 24 h. The dried biomass was finally grounded in mortar to obtain fine powder. The finely powdered cell biomass was mixed with photometric potassium bromide (KBr) to make a pellet of 1 % (*w*/*w*). The pellet was subjected to Fourier transform infrared spectrometer (NICHOLET 6700, coupled with OMNIC software version 6.2) spectrum between wave number 4000–400 cm^{-1} [37]. The infrared spectra of the unloaded and metal loaded *B. braunii* biomass were obtained using FTIR.

Modeling of Growth Kinetics and Determination of Kinetic Parameters for Growth of Algal Biomass in Pure and Arsenic Containing Media

The classical Monod equation was recommended for modeling the growth kinetics of the current microalgae *B. braunii* as follows [20]:

$$\mu = \frac{\mu_{\max} C_A}{(K_S + C_A)} \quad (2)$$

Results and Discussion

Effect of Initial pH on Growth Properties of *B. braunii*

One of the most significant factors in microalgal cultivation is pH because it controls the solubility and availability of CO_2 and essential nutrients and since it can have an important influence on algal metabolism [38]. Since the pH plays a major role in the growth of the microalgae *B. braunii*, so effect of initial pH on biomass concentration of the microalgae was explored in the pH range of 1.0–12.0 in the absence and presence of 50 mg/L of arsenic ions (either As(III) or As(V)).

The microalgae could not grow at extreme low pH 1.0 and also at extreme high pH 12.0 in the absence and presence of both As(III) and As(V) (Fig. 1) [39]. The highest biomass concentration achieved at pH 9.0 in the absence of arsenic ions was found to be 2.503 g/L, respectively. The highest biomass concentration was also determined at pH 9.0 as 2.34 and 2.377 g/L, respectively, in the media containing 50 mg/L As(III) and As(V) ions, respectively.

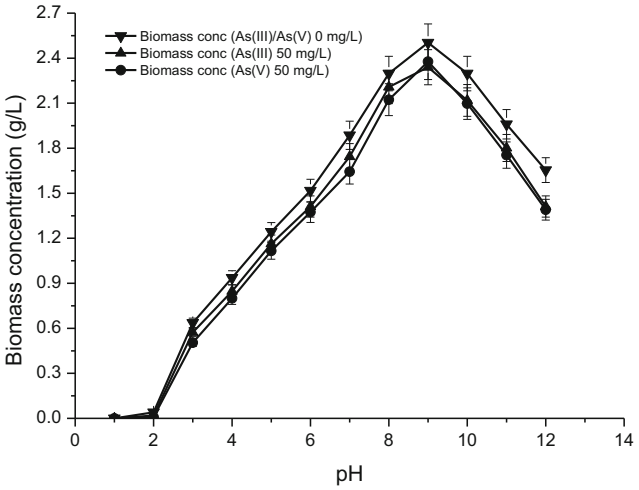

Fig. 1 Effect of pH on growth of *B. braunii* in absence and presence of As(III) and As(V) (inoculum size (%, *v*/*v*), 10; incubation time, 7 days; initial arsenic concentration, 50 mg/L; temperature, 28 °C; dark/light period, 12:12 h). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

Microalgae assimilate inorganic carbon in the photosynthesis. The inorganic carbon species normally used by microalgae are CO_2. The amount of CO_2 dissolved in water varies greatly with pH and addition of CO_2 results in a pH decrease [40]. The pH can increase significantly in microalgal cultures owing to the uptake of inorganic carbon by microalgae [38]. Remarkably, pH is the main influential factor of relative concentrations of the carbonaceous species in water [41]. The availability of carbon from CO_2 will be limited at higher pH, which successively inhibits the growth of microalgae [41]. The carbon for microalgae is available in form of carbonates at higher pH [42]. Higher pH also reduces the affinity of microalgae to free CO_2 [41]. The flexibility of the cell wall of mother cells increases at higher pH preventing its rupture and inhibiting the release of autospore, so the time for cell cycle completion increases [43]. At higher pH values, such as at pH greater than 9.0, most of the inorganic carbon is in form of carbonate (CO_3^{2-}) which cannot be assimilated by the algae acidic conditions can change nutrient uptake similar to alkaline pH [44]. So, the highest growth of microalgae was found to be at 9.0.

Biomass concentration of microalgae was found to be less in the presence of arsenic (either As(III) or As(V)) ions compared with control owing to suppression of the growth of microalgae. In batch cultures, the cells grow exponentially until restricted by a nutrient or some other growth factor or until an inhibitor accumulates sufficiently to stop further algal growth [45]. In the current investigation, it was agreed that

inorganic arsenic (either As(III) and As(V)) ion was toxic to algal cells; so the growth decreased with an increase in arsenic concentration in the culture medium.

The availability and toxicity of inorganic arsenic to *B. braunii* depend on the arsenic concentration, its chemical valence, and environmental factors as pH during the exposure. However, the biouptake and toxicity of inorganic arsenic species remains controversial. Although As(III) was considered to be more toxic than As(V) in animals and marine phytoplankton, their toxicity was reversed in freshwater microalgae [46, 47]. However, many exceptions to this rule were also reported. As(V) and As(III) exerts equal toxicity to freshwater algae *Stichococcus bacillaris* at pH 8.2 with phosphate levels between 0.03 and 0.3 mg P/L [48]. Lower pH increased As(V) toxicity to *S. bacillaris* [48]. They reported that induced thiol-peptides–short-chain phytochelatins (PC_{2-3}) are produced in response to arsenic exposure. As(V) was more toxic than As(III), especially at the near neutral pH 6.8.than at pH 8.2 because more phytochelatins (PC_{2-3}) were produced in response to As(V) and As(III) under exposure conditions. However, the intracellular arsenic concentration was higher than the concentration of phytochelatins able to detoxify the metal under the identical conditions. Equal toxicity of As(III) and As(V) was also found from the 72 h growth inhibition tests for the freshwater algal specie *Chlorella* (pH 7.6) [46], whereas As(V) was more toxic than As(III) for *Monoraphidium arcuatum* (pH 7.6). Karadjova et al. [47] also demonstrated that inorganic arsenic species (As(III) and As(V)) exhibit equal toxicity toward *C. salina* in seawater (pH 8.1).

The optimum pH for the growth evaluated in the present investigation is in agreement with the optimum pH for the growth of cyanobacterial strain reported by Kushwaha et al. [20]. They reported that the cyanobacterial growth is very sensitive to pH and the optimal pH for the growth of cyanobacterial consortium of *Oscillatoria subbrevis* and *Gloeocapsa atrata* was 9.0 in the Chu–10 growth media. Generally, the higher toxicity of As(V) than As(III) toxicity to *S. bacillaris* is in agreement with reports of toxicity of arsenic toward natural algal communities in fresh and marine waters [49].

Effect of Initial pH on Phycoremediation Properties of *B. braunii*

The pH is one of the significant factors that considerably influences the adsorbate ion speciation, the chemistry of solution, interaction between adsorbate and biosorbent, and surface charge of biosorbent surface [50]. In the current study, the effect of pH on phycoremediation of both As(III) and As(V) ions were performed in range of 2.0–12.0 and the results are shown in Fig. 2. In the present study, the term phycoremediation is used to describe any of these possible modes of interactions

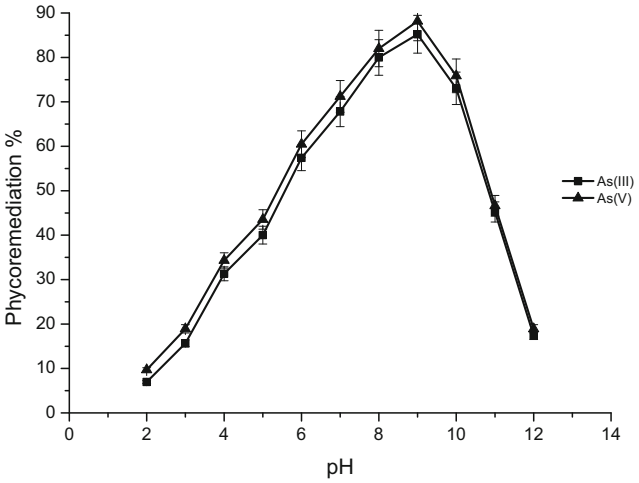

Fig. 2 Effect of initial pH on phycoremediation of As(III) and As(V) by *B. braunii* (inoculum size (%, *v*/*v*), 10; contact time, 144 h; initial arsenic concentration, 50 mg/L; temperature, 28 °C). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

(passive uptake, i.e., sorption and active uptake, i.e., accumulation) between the metal ion and the surface of the cells without distinction.

From Fig. 2, it was evident that the phycoremediation % of both As(III) and As(V) were higher in basic media than in acidic media and the highest removal was found at pH 9.0. This may be attributed to the favorable growth of *B. braunii* biomass in alkaline media pH (i.e., pH 9.0).

The phycoremediation of both As(III) and As(V) varied significantly depending upon the arsenic species also. The difference in the phycoremediation % of As(III) and As(V) could be elucidated on the basis of the charge on the species of arsenic and the surface charges of the algal biomass.

It was agreed from Fig. 2 that the phycoremediation of both As(III) and As(V) ions (15.652 and 18.923 %, respectively) were very poor in the pH range <3.0. With the rise in pH from 3.0 to 9.0, there was a significant increase in the phycoremediation of both As(III) and As(V) ions. The highest phycoremediation % of As(III) and As(V) ions were achieved as 85.217 and 88.154, respectively, at pH 9.0. Then a sharp decrease in the phycoremediation was found and As(V) exhibited more phycoremediation than As(III). These results can be understood from the following explanations.

In the pH range of 2.0–9.0 and 10.0–12.0, As(III) exists generally in neutral (H_3AsO_3) and anionic ($H_2AsO_3^-$) forms, respectively. Reports also approve that As(V) exists mostly in the monovalent form of $H_2AsO_4^-$ in the pH range 3.0–6.0, yet at pH near 2.0, a small extent of H_3AsO_4 also remains. However a divalent anion $HAsO_4^{2-}$ prevails at higher pH values (>8.0); both species co-exist in the intermediate region of pH 6.0–8.0 [51]. The dissociation constants of these As(III) and As(V) are shown in Table 5 [52].

Also at low pH 1.0–6.0, the density of hydrogen ion was quite high against As(III) and As(V) ions, which resulted in

Table 5 Dissociation constants of arsenic species

Forms of arsenic species	Dissociation reactions	Dissociation constants
Arsenious acid	$H_3AsO_3 \rightarrow H^+ + H_2AsO_3^-$	9.23
	$H_2AsO_3^- \rightarrow H^+ + HAsO_3^{2-}$	12.13
	$HAsO_3^{2-} \rightarrow H^+ + AsO_3^{3-}$	13.40
Arsenic acid	$H_3AsO_4 \rightarrow H^+ + H_2AsO_4^-$	2.22
	$H_2AsO_4^- \rightarrow H^+ + HAsO_4^{2-}$	6.98
	$HAsO_4^{2-} \rightarrow H^+ + AsO_4^{3-}$	11.53

protonation of the components of the cell wall, i.e., the surface of microalgae *B. braunii* biomass. Thus, the amine and hydroxyl group in the surfaces of algal biomass were vastly protonated in acidic conditions. The protonation of algal cell wall moieties reduced the phycoremediation efficiency because there was a strong electrostatic interaction remains between positively charged surface of the biomass and oxyanions [53]. Comte et al. [54] described that the deprotonated form of the reactive sites in cell wall, generally amino, phosphoric, and carboxylic groups, is mainly responsible for the metal ions binding to EPS. Chojnacka et al. [55] also stated that various functional groups of cell wall of microalgal biomass are responsible for binding of metal ions. The solution pH influences the ionization state of these functional groups. Anions could be anticipated to interact more strongly with cells as the concentration of positive charges rises.

The surfaces of microalgal biomass are vastly protonated in extreme acidic conditions and such a condition is not so encouraging for removal of As(III) and As(V) due to the presence of neutral As(III) and As(V) species in this range, resulting in virtually less change in the phycoremediation within the pH range 2.0–4.0. Surface sites are positively charged at lower pH of the medium and therefore attract negatively charged As(III) and As(V) by an electrostatic interaction or columbic force [56, 57]. The surface of *B. braunii* biomass fulfills the coordination shells with the prevailing OH group with the materials under hydration. On the variation of pH, these surface active OH groups may further bind or release H^+ where the surface remains positive because of various reactions provided in the Electronic Supplementary Materials. Though many microorganisms have been revealed to volatilize arsenic, exploring the potential of microbial volatilization or vaporization for bioremediation is still under considerable debate due to their low efficiency. Maximum studies of microbial communities on volatilizing arsenic have concentrated on archaea, bacteria, and fungi with little attention to other eukaryotic microorganisms, such as aquatic alga and protozoans. A few eukaryotic microorganisms have been proved to volatilize arsenic such as a Yellowstone thermoacidophilic eukaryotic alga, *Cyanidioschyzon* sp., [58], a marine green microalgae *Ostreococcus tauri* [59]. Studies exhibited that arsenic volatilization had a direct relationship with microbial growth and nutrient levels [60]. In the experiments on removing arsenic compounds, while organic matter was added, small amounts of innate arsenic present in retorted shale could be volatilized [61, 62]. In the present study, no such organic matter was added. So *B. braunii* could not vaporize or volatilize arsenic (As(III)/As(V)) rom wastewater.

The degree of protonation of the surface decreases progressively, with the increase in pH of the system. The highest phycoremediation of As(V) was found at pH 9.0 where the prevailing species of As(III) was only non-ionic species H_3AsO_3 [2], might be attributed to several products of undetermined reaction during the process of phycoremediation. The neutral (H_3AsO_3) and monoanionic ($H_2AsO_3^-$) species are thus considered to be responsible for the phycoremediation of As(III), also due to the substitution of hydroxyl ions or water molecules. The neutral species (H_3AsO_3) cannot undergo electrostatic interaction with the microalgal biomass. However, such species can interact with the unprotonated amino groups [29, 32]. The dominant species of As(V) in the above-mentioned pH range are $H_2AsO_4^-$ ions, which can be phycoremediated on the microalgal biomass by substituting hydroxyl ions or coordination of hydroxyl groups with the microalgal biomass [32, 53]. At pH 5.0–9.0, anionic species of As(V) ($H_2AsO_4^-$ and $HAsO_4^{2-}$) exists and the surface of microalgal biomass is also protonated and so a strong electrostatic interaction remains between positively charged microalgal biomass surface and oxyanions and as a result the removal improved in this pH range because of the increase in $HAsO_4^{2-}$ species with the rise in pH of the solution. The dominant species of As(V) in the above-mentioned pH range are $H_2AsO_4^-$ ions, which can be phycoremediated on the microalgal biomass by substituting hydroxyl ions or coordination of hydroxyl groups with the microalgal biomass [32, 53]. So, the highest removal of As(V) was found to be at pH 9.0.

With the increase in pH of the system, the degree of protonation of the surface decreased gradually. As the pH of the solution increased more than 9.0 (alkaline medium), the negatively charged species $H_2AsO_3^-$ and $H_2AsO_4^{2-}$ started to govern in the medium and cell surface also inclines to gain negative charges (OH^-) resulting in the reduction of phycoremediation of both As(III) and As(V) [51]. This process can be defined in three stages: (1) the decline may be because of the negatively charged adsorbate by accumulating hydroxyl ions (OH^-) on the surface of microalgal biomass, (2) may be because of the ionization of very weak acidic functional groups of the microalgal biomass, or both at higher pH values, and/or (3) a repulsive force may exist between the anionic species and the negatively charged surface of the algal biomass [12, 14, 51]. This results in reduced As(III) and As(V) removal at higher pH values [51].

More attraction among the As(V) ions and H^+ ions on the surface of algal biomass may be the motive for the maximum As(V) phycoremediation at pH 9.0 compared with As(III).

Effect of Inoculum Size on Growth Properties of *B. braunii*

The volume of inoculum utilized for culturing the microalgae can influence the growth of *B. braunii* [63]. The fixed volume of a culture medium means that it can only contain limited nutrients for the microalgae. Moreover, the consumption of the nutrients mainly depends on the population of microalgae. The microalgal inoculum size should therefore be controlled to ensure a high growth of microalgae in the limited volume of medium [63]. To optimize the inoculum size for the algal growth, the inoculum concentration was in the flasks were varied from 2 to 20 % (*v*/*v*) both in the absence and presence of arsenic ions (either As(III) or As(V)) (Fig. 3).

An increase in biomass concentration from 1.111 to 2.308 g/L was observed in microalgal growth if the inoculum size was increased from 2 to 8 % in absence of arsenic ions. The maximum biomass concentration (2.503 g/L) was found for inoculum size of 10 % (*v*/*v*). Drop of biomass concentration from 2.313 to 0.889 g/L was found in microalgal growth if the inoculum size was increased from 12 to 20 % (*v*/*v*). The maximum biomass concentration was found to be for inoculum size of 10 % (*v*/*v*) for both presence of arsenic (either As(III) or As(V)) ions also. A higher inoculum of 20 % (*v*/*v*) was observed to decrease the algal growth at a higher extent than if the lower inoculum size of 2 % (*v*/*v*) was used. So, higher inoculum sizes did not essentially give higher growth of cell. It can be explained by the following explanations.

An improved distribution of dissolve CO_2 as well as more efficient nutrient uptake also leads to a higher growth of microalgae [63, 64]. At small inoculum volume microalgae would then to lead to high growth due to the higher surface area to volume ratio and more efficient uptake of nutrient [63, 65]. In the present study, when inoculum size is low (<10 % (*v*/*v*)), higher growth of microalgae was expected but optical density of cell suspension was found to be low. This may be because of overpopulated culture and fixed amount of nutrient with which the microorganisms begin to liberate proteolytic enzyme enhancing self-consumption [66].

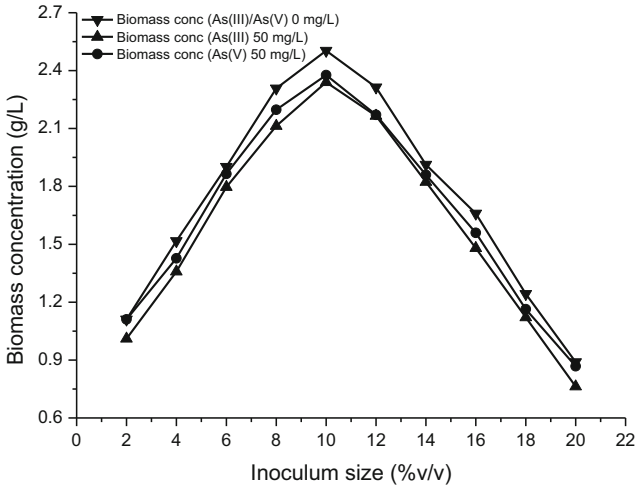

Fig. 3 Effect of inoculum size on growth of *B. braunii* in absence and presence of As(III) and As(V) (pH, 9.0; incubation time, 7 days; initial arsenic concentration, 50 mg/L; temperature, 28 °C; dark/light period, 12:12 h). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

This can also be explained the fact of cell-environment interaction. Two explanations seem possible: (1) that microalgae interact directly with one another for inhibiting the growth: such interaction is termed contact inhibition; and (2) that microalgae interact with the environment in such a way as to reduce it no longer conductive for further growth. Furthermore, if contact inhibition happened, one would not anticipate microalgae to produce large colonies while spreading on a nutrient agar plate. It therefore seems most possible that the event(s) instigating the cessation of growth in a batch culture reside in changes induced in the environment by the growth of the microalgae. It can also be explained by two possibilities: either growth leads to the depletion of some essential nutrient substances from the environment, or else growth leads to the accumulation of products that finally reach toxic concentrations [67, 68]. If the inoculum sizes are too small (<4 %), insufficient number of microalgae would lead to a reduced growth of microalgae [63, 64].

On the other hand, higher inoculum sizes (>10 % (*v*/*v*)) result in the lack of dissolve CO_2 and increased competition toward nutrient and depletion of nutrient in the culture media resulting in lower growth of microalgae [63–65]. High-density microalgae cultures grown under photoautotrophic method demand a large amount of CO_2 while the high concentrations of O_2 produced by the photosynthesis process can became an inhibitory condition for the growth of microalgae [69].

Other researchers have stated different optimum inoculum sizes for various bacteria: 5 % (*v*/*v*) for *B. subtilis* strain Rand [63], 3 % (*v*/*v*) for Z3—*Staphylococcus aureus*, KS1—*Escherichia coli*, and KHL2—*Pseudomonas aeruginosa* [66]. So, different microorganisms require various percentages of inoculum sizes for the highest cell growth. So, in the present study amount of inoculum size were optimized and 10 % (*v*/*v*) of inoculum size gave the best result than other size of inoculum.

Effect of Inoculum Size on Phycoremediation Properties of *B. braunii*

The influence of inoculum size on phycoremediation of *B. braunii* was performed by varying inoculum volume in the range of 2–20 % (*v*/*v*) in the growth media in the absence and presence of arsenic (either As(III) or As(V)) ions. For two

arsenic species (As(III) and As(V)), the phycoremediation % increased when the inoculum size was varied in the range of 1 to 8 % (*v*/*v*) and reached a maximum at inoculum size of 10 % (*v*/*v*) for both As(III) and As(V) ions. After that, a sharp decline in the phycoremediation % was observed (Fig. 4).

For 2 % (*v*/*v*) inoculum, phycoremediation % of As(III) and As(V) was 71.304 and 80.462, which increased to 85.217 and 88.154, respectively, at 10 % (*v*/*v*) inoculum of *B. braunii* microalgae and then reduced to 60.87 and 77.385, respectively, at an inoculum size of 20 % (*v*/*v*). It is found in the above study that the highest biomass concentration was found for 10 % (*v*/*v*) inoculum size. So, maximum phycoremediation of both As(III) and As(V) were found at an inoculum size of 10 %. Figure 4 also shows that the phycoremediation efficiency of As(III) was greater affected by the inoculum size. It was supported by greater toxicity of As(III) than that of As(V) which suppressed the growth of microalgae. The microalgae produced phytochelatins (PC_{2-3}) in response to As(III) and As(V), but the level of phytochelatins in cells was higher after exposure to As(V) than to As(III), signifying higher As(V) availability and accumulation in algal cells than that of As(III) at the same external concentrations [48].

Effect of Initial Arsenic Concentration on Growth Properties of *B. braunii*

Microalgal growth curves in the absence and presence of increasing concentrations of arsenic (either As(III) or As(V)) ions (50, 100, 500, 1000, 1500, and 2000 mg/L) in the BG11 growth media are shown in Fig. 5.

Growth of the microalga *B. braunii* was not inhibited by concentrations of arsenic ions (either As(III) or As(V)) up to 1000 mg/L, and the lag phase was approximately 1 day and the stationary phase starts after 14 days. While concentrations of As(III) and As(V) were at >1000 mg/L, microalgal growth was significantly inhibited with the lag phase increased to 4 days and the stationary phase starts after 14 days (Fig. 5). Thus to get an overall idea, experimentations was continued up to 16 days. Remarkably throughout the time span of 1–3 days, when exposed to 50–2000 mg/L of arsenic ions (either As(III) or As(V)), the growth rate was 2.85–14 % lower than that in arsenic-free medium, demonstrating that microalgal growth was encouraged by low concentrations of arsenic ions (either As(III) or As(V)) because of hormesis. For evaluating the toxicity of different inorganic arsenic species (either As(III) or As(V)) on *B. braunii*, the influence of increasing concentrations of arsenic (either As(III) or As(V)) on the microalgal growth rate inhibition after 16th day was investigated.

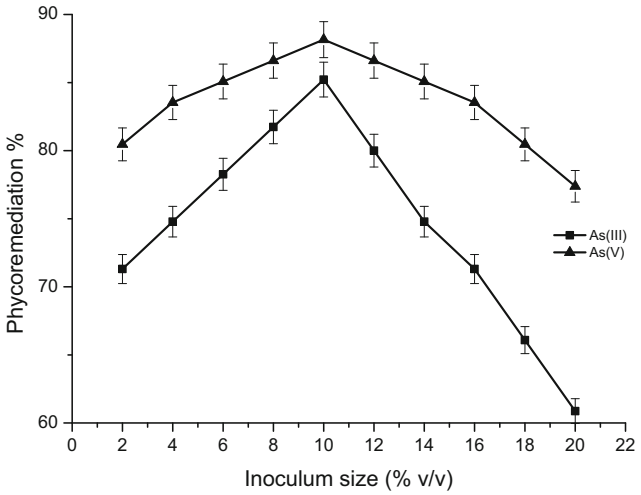

Fig. 4 Effect of inoculum size on phycoremediation of As(III) and As(V) of *B. braunii* (initial pH, 9.0; contact time, 144 h; initial arsenic concentration, 50 mg/L; temperature, 28 °C). (*Error bars* represent means ± standard errors from the mean of duplicate experiments).

Maximum biomass concentration and the specific growth rate of microalgae *B. braunii* in pure media were 3.231 g/L and 0.148 day^{-1}, respectively. The maximum biomass concentration and specific growth rate of microalgae *B. braunii* in the presence of arsenic (either As(III) or As(V)) showed a declining trend, signifying that toxicity of media increased with increasing arsenic ion concentration.

With the increase in concentration of As(III) in the media from 50 to 2000 mg/L, maximum biomass concentration reduced from 3.068 to 1.949 g/L and the specific growth rate also reduced from 0.145 to 0.127 day^{-1} because of toxicity of media by As(III) ions.

Similarly, increasing the concentration of As(V) from 50 to 2000 mg/L, also led to decreased maximum biomass concentration from 3.062 to 1.955 g/L and specific growth rate from 0.145 to 0.127 day^{-1} also because of toxicity of media by As(V) ions.

The toxicity of As(III) and As(V) was found to be similar for the microalgae *B. braunii* because the maximum biomass concentration of *B. braunii* in the growth media was almost analogous in the presence of both As(III) and As(V) (pH 9.0). As(V) and As(III) showed similar toxicity to freshwater alga *S. bacillaris* at pH 8.2 with levels of phosphate between 0.03 and 0.3 mg P/L [48]. Equal toxicity of As(III) and As(V) was assessed from the 72 h growth inhibition tests for another freshwater algal species *Chlorella* sp. (pH 7.6), while As(V) was more toxic than As(III) for *M. arcuatum* (pH 7.6) [46].

Effect of Initial Arsenic Concentration on Chlorophyll Content

Carotenoids and chlorophylls, which are present in plants and algae, are very essential for the photosynthesis and growth of microalgae [70]. Figure 6 shows the changes in chlorophyll content over the days of growth of microalgae *B. braunii* during incubation period in the presence of various concentrations of arsenic (either As(III) or As(V)) ions. It was found that the total chlorophyll content increases significantly giving a

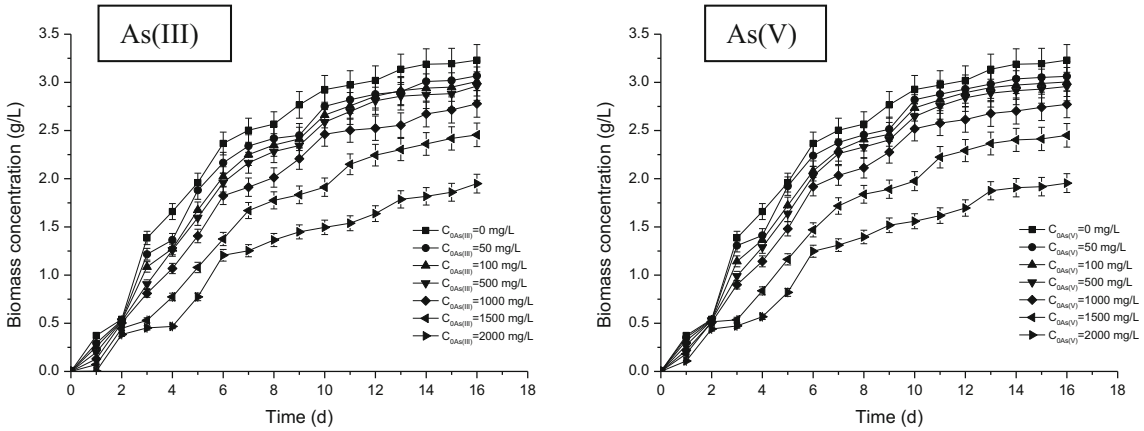

Fig. 5 Growth of *B. braunii* in absence and presence of increasing concentrations of biomass concentration of As(III) and As(V) (initial pH, 9.0; inoculum size (%, *v*/*v*), 10; initial arsenic concentration, 50 mg/L; temperature, 28 °C; incubation time, 16 days; dark/light period, 12:12 h). (*Error bars* represent means ± standard errors from the mean of duplicate experiments).

peak value on 14th day in all arsenic (either As(III) or As(V)) concentrations with the increase in days of incubation [20]. After the 14th day, there was no significant change in chlorophyll content in all the concentrations. Furthermore, the highest chlorophyll was recorded in absence of arsenic, followed by 50, 100, and so on up to 2000 mg/L. The lowest chlorophyll was recorded in the media containing 2000 mg/L.

Inhibited biosynthesis of chlorophyll and carotenoids and reduced phosphorylation are most commonly observed symptoms of metal toxicity [71, 72]. Overall, arsenic reduced the growth rate resulting in lower chlorophyll content in arsenic-exposed biomass than in the control. It can be due to the fact that the photosynthetic enzymes activities reduce with high concentration of arsenic (either As(III) or As(V)), which results in the decrease of chlorophyll content [73]. From the present study, it can be determined that certain concentration of arsenic (either As(III) or As(V)) inhibits the growth of microalgae, produces low chlorophyll in the microalgae, and disturbs the photosynthetic activities. Such decreases in the levels of photosynthetic pigments, including chlorophyll *a* and *b* and accessory pigments, such as carotenoids of some mosses, on exposure to heavy metals was also observed by Shakya et al. [74].

Effect of Contact Time on Phycoremediation of Arsenic

Figure 7 presents the effect of contact time on the phycoremediation % of As(III) and As(V) using microalgae. The phycoremediation % of both As(III) and As(V) was found to increase from 40 to 85.217 and 42 to 88.154, respectively, when contact time was varied from 4 to 144 h. Time required to for achieve equilibrium was 144 h for both As(III) and As(V) ions. Hence, further phycoremediation studies were continued for a contact time of 144 h.

The study reveals the high potential of the *B. brunii* in the elimination of both As(III) and As(V) from synthetic wastewater. An S-shaped growth curve was observed for both As(III) and As(V). This characterizes the effective growth of *B. brunii* biomass in synthetic wastewater. From the consequences, it is clear that in all the systems, the saturation time

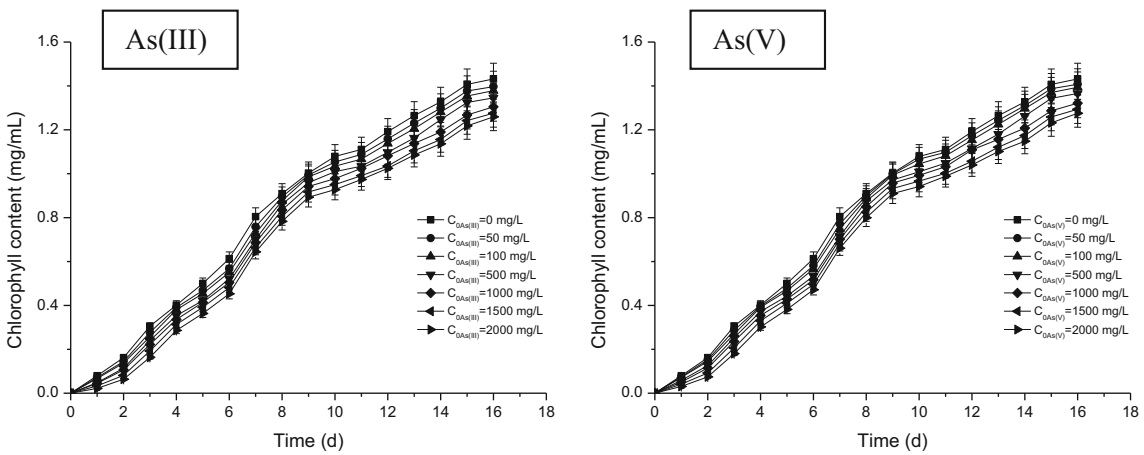

Fig. 6 Variation in chlorophyll content in *B. braunii* at different times of exposure under different concentrations of As(III) and As(V). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

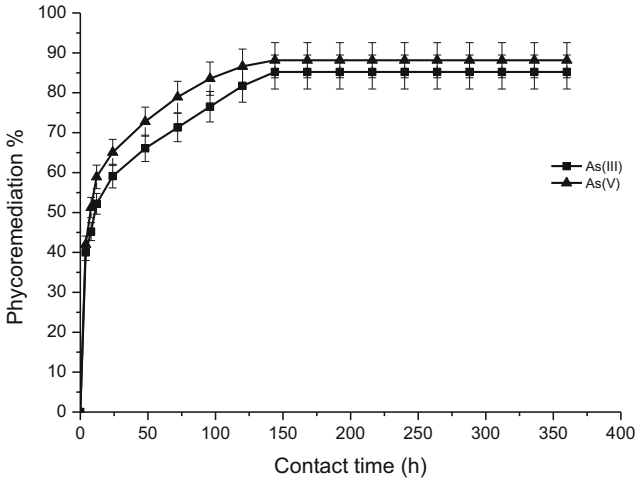

Fig. 7 Effect of contact time on phycoremediation of As(III) and As(V) of *B. braunii* (initial pH, 9.0; inoculum size (%, *v/v*), 10; initial arsenic concentration, 50 mg/L; temperature, 28 °C). (*Error bars* represent means ± standard errors from the mean of duplicate experiments).

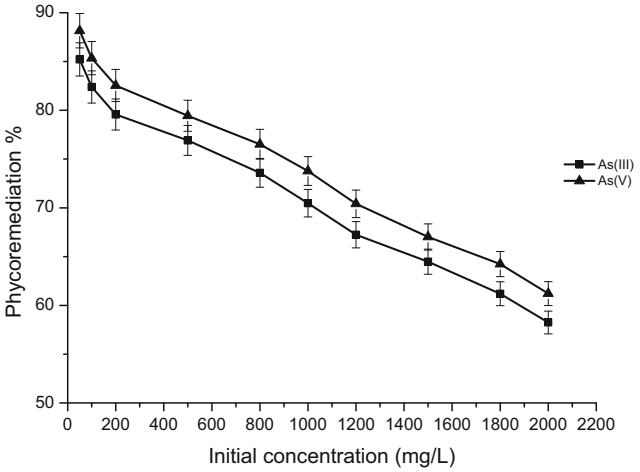

Fig. 8 Effect of initial arsenic concentration on phycoremediation of As(III) and As(V) of *B. braunii* (initial pH, 9.0; inoculum size (%, *v/v*), 10; contact time, 144 h; temperature, 28 °C). (*Error bars* represent means ± standard errors from the mean of duplicate experiments).

cannot be ruled by the adsorbate concentration in the solution. The change in the rate of phycoremediation might be owing to the fact that originally all sites of algal biomass surfaces are readily available and moreover the concentration gradient of adsorbate is very high. At optimum pH, the fast kinetics of interaction of adsorbate-algal biomass might be obvious to increase availability of the active sites of the algal biomass surface. Therefore, the phycoremediation of adsorbate was fast in the early stages and progressively decreases with the interval of time until equilibrium in each case. The reduction in elimination of metal ions at the later stage of the process was due to the falling in concentration of metal ions [75]. Consequently, the curves found were single, smooth, and continuous leading to equilibrium and suggested the possibility of phycoremediation of the adsorbate on the surface of microalgal cells [51].

The results of the effect of contact time on removal identified that the respective algae had an optimum residence time for As(III) and As(V) and when this time passed, the removal either become almost constant or diminished slightly. The constant nature of the phycoremediation % curve after 144 h for both As(III) and As(V) may be owing to initiation of the stationary phase of the microalgae *B. braunii*.

Effect of Initial As(III) and As(V) Concentration on Phycoremediation of *Arsenic*

The effect of initial metal ion concentration on phycoremediation of arsenic using *B. braunii* was carried out by varying phosphate concentrations (50, 100, 200, 500, 800, 1000, 1200, 1500, 1800, and 2000 mg/L) in the growth media, at an initial optimized pH value of 9.0 are shown in Fig. 8.

The phycoremediation % decreased with the increase in arsenic (As(III)/As(V)) concentration from 50 to 2000 mg/L. It was supported by the decrease in microalgal growth. The reduction in phycoremediation % of As(III) and As(V) was caused by the toxicity of As(III) and As(V) at higher concentrations which decreased the biomass concentration. The maximum biomass concentration was found to decrease from 3.068 to 1.949 g/L with the increase in As(III) concentration from 50 to 2000 mg/L. Varying the concentration of As(III) from 50 to 2000 mg/L resulted in an increased phycoremediation levels from 42.609 to 1165 mg/L.

Similarly, the maximum biomass concentration was found to decrease from 3.062 to 1.955 g/L with the increase in As(V) concentration from 50 to 2000 mg/L. Varying the concentration of As(V) from 50 to 2000 mg/L also resulted in an increased phycoremediation levels from 44.077 to 1224.192 mg/L.

The elimination of As(V) is higher than As(III) for all concentrations due to more production of phytochelatins in cells after exposure to As(V) than to As(III) [48].

Effect of Phosphate Concentration on Growth Properties of *B. braunii* in Arsenic-Free Media

Macronutrient such as phosphorous also plays a major role to control the metabolism and finally the growth of microalgae [20, 76]. The effect of phosphate ion concentration on the growth rate of microalgae *B. braunii* in arsenic-free media was explored in the phosphate concentration range of 0.005–0.05 g/L keeping other compositions constant as prescribed in the BG11 medium, at pH 9.0 and at a temperature of 28 °C. As presented in Fig. 9, the relationship of biomass concentration to phosphate ion concentration for microalgae *B. braunii*, often adopted the saturation kinetics form. It was observed that

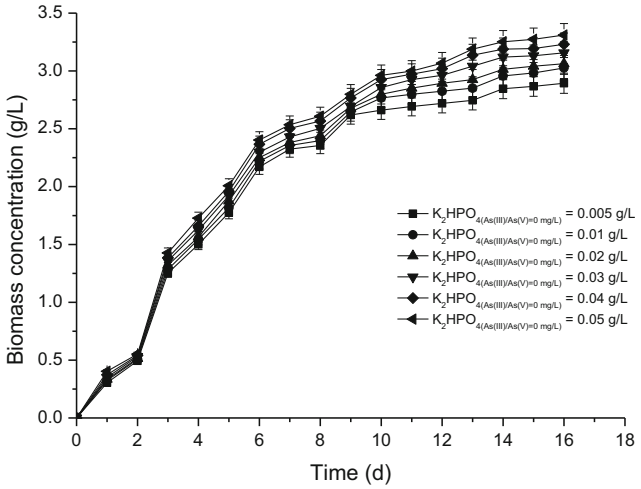

Fig. 9 Effect of phosphate concentration on growth of microalgae *B. braunii* in arsenic free media (initial pH, 9.0; inoculum size (%, *v/v*), 10; temperature, 28 °C; incubation time, 16 days; dark/light period, 12:12 h). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

biomass concentration increased with increasing initial phosphate ion concentration from 0.005 to 0.5 g/L. Phosphorus typically constitutes 1 % of dry weight of algae [77], but it may be essential in significant excess because not all added phosphate is bioavailable because of the formation of complexes with metal ions [78]. Immediate effects of phosphorus limitation include a decrease in the synthesis and regeneration of substrates in the Calvin-Benson cycle and a significant decrease in the rate of light utilization necessary for carbon fixation [79]. Phosphorus starvation reduces chlorophyll *a* and protein content [38]. Phosphate deficiency was revealed to result in accumulation of astaxanthin and an overall reduction in cell growth [80]. It was observed that the specific growth rate increased from 0.146 to 0.148 day^{-1}, with an increase in phosphate concentration from 0.005 to 0.05 g/L and biomass concentration of microalgae *B. braunii* increased from 2.893 to 3.31 g/L.

Effect of Phosphate Concentration Microalgal Growth in Arsenic-Containing Media

The effect of increasing phosphate concentrations on growth properties of microalgae *B. braunii* in 50 mg/L of arsenic (either As(IIII) or As(V)) containing media was studied in the range from 0.005 to 0.05 g P/L under same experimental conditions.

The present studies supported the result that phosphate limited cells were more sensitive to arsenic exposure, possibly because arsenic toxicity interfered with phosphate metabolism, leading to depletion of phosphate or inhibition of adenosine triphosphate production (ATP) [46]. As(V) enters the microalgal cell via phosphate transporters due to its chemical similarity to phosphate [81]. Thus, under low phosphate condition, the smaller amount of phosphate may not be adequate to compete with As(V) in the outer cell transporting system [72]. However, increasing phosphate concentrations reduced the toxicity of both As(III) and As(V) in a similar way (Fig. 10).

The growth rate and maximum biomass concentration of microalgae *B. braunii* increased from 0.14 day^{-1} and 2.698 g/L to 0.145 day^{-1} and 3.136 g/L (As(III) and from 0.139 day^{-1} and 2.793 g/L to 0.145 day^{-1} and 3.126 g/L (As(V)), respectively with increasing initial phosphate concentration from 0.005 to 0.05 g/L in the presence of arsenic ions (either As(III) or As(V)). It was clear from the results of the present study that initial nitrate concentration played a major role in the microalgal growth and decreased the inhibitory effects of arsenic on the microalgal growth.

The reduction in inorganic arsenic toxicity was perhaps related to the reduced intracellular As(III) and As(V) contents in cells grown in phosphate-enriched BG11 growth media. The addition of 0.04 mg P/L as phosphate to BG11 growth media resulted in a 12 % increase in biomass concentration in algae exposed to 50 mg/L of arsenic (either As(III) or As(V)). Karadjova et al. [47] described that seawater enrichment with phosphate (up to 1.3 mg P/L) resulted in a remarkable reduction of toxicity because of As(III) and As(V). A reduction in the As(V) toxicity was observed for freshwater algae *Chlorella* sp. and *M. arcuatum* in culture medium containing 0.5 mg P/L [46]. On the other hand, extremely high phosphate concentrations (9.1 mg P/L) did not protect the cells of *Chlorella* sp. isolated from arsenic contaminated sites from arsenate stress [82].

Modeling of Growth Kinetics of Algal Biomass in Presence Various Concentration of Phosphate Ions

The growth of the microalgae was investigated in batch culture by varying the quantity of phosphate ions (K_2HPO_4) in the range of 0.005−0.05 g/L in pure and 50 mg/L of arsenic-containing (either As(III) or As(V)) media. The experimental values of dry biomass obtained all through the experimentation period for different values of K_2HPO_4 in pure and 50 mg/L of arsenic-containing (either As(III) or As(V)) media have been plotted in Fig. 11. The classical Monod equation was employed for representing the growth of the microalgae *B. braunii* in pure and arsenic (either As(III) or As(V)) media during exponential phase only. Maximum specific growth rate and saturation constant have also been estimated by non-linear regression analysis utilizing professional graphics software package OriginPro (8.5.1 version) for fitting the experimental data obtained during the batch study. In case of pure media, the value of μ_{max} was 0.148 day^{-1} and that of K_s was 7.01E−05 g/L (correlation coefficient is equal to 0.982). The plot showed that moderate addition of arsenic increased K_s to 2.162E−04 g/L (correlation coefficient is equal to 0.98) for

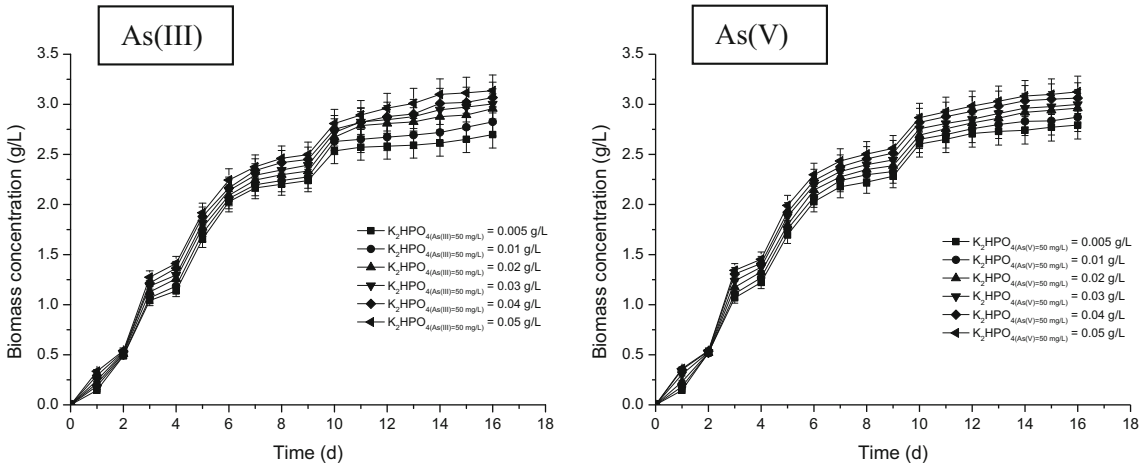

Fig. 10 Effect of phosphate concentration on growth of microalgae *B. braunii* in arsenic-enriched media (initial pH, 9.0; inoculum size (%, *v*/*v*), 10; initial arsenic concentration, 50 mg/L; temperature, 28 °C; incubation time, 16 days; dark/light period, 12:12 h). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

As(III) and that to 2.534E−04 g/L (correlation coefficient is equal to 0.997) for As(V), without significantly influencing μ_{max} (0.146 for As(III) and 0.146 for As(V), respectively. The specific growth rate showed little variation in growth rates in the presence of arsenic (As(III)/As(V)). It indicates that the toxicity of arsenic (both As(III) and As(V)) was reduced by increased concentrations of phosphate. This is similar to results described by Sanders [83] for the diatom *Skeletonema costatum*, that exhibited that growth rates were not affected by phosphate concentrations over a range of arsenic concentrations from 5 to 25 μg/L. Foster et al. [84] also reported that *P. tricornutum* showed small deviation in growth rates over the three phosphate concentrations (3, 1.2, and 0.5 mg/L) in the presence of 2 μg/L As(V) ions.

Microbial growth in the medium containing no arsenic ions (either As(III) or As(V)), As(III) ions and As(V) ions was described with the following Monod Eq. (3), Eq. (4), and Eq. (5), respectively:

$$\mu = \frac{0.148C_A}{(7.01\text{E}{-}05 + C_A)} \quad (3)$$

$$\mu = \frac{0.146C_A}{(2.162\text{E}{-}04 + C_A)} \quad (4)$$

$$\mu = \frac{0.146C_A}{(2.534\text{E}{-}04 + C_A)} \quad (5)$$

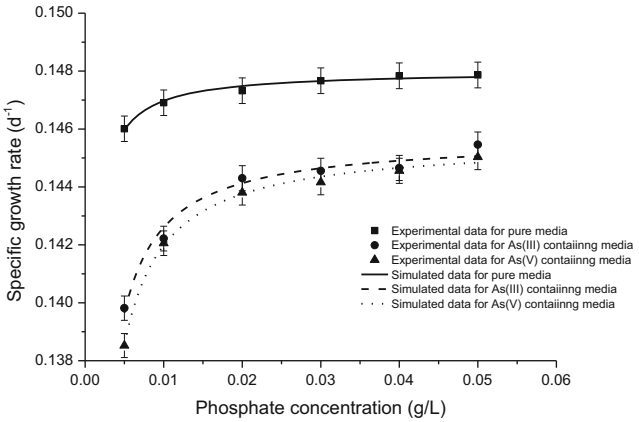

Fig. 11 Effect of phosphate concentration on growth kinetics of microalgal biomass (initial pH, 9.0; inoculum size (%, *v*/*v*), 10; temperature, 28 °C; incubation time, 16 days; dark/light period, 12:12 h). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

Effect of Phosphate Concentration on Phycoremediation of Arsenic

The effect of phosphate on phycoremediation of arsenic using *B. braunii* was carried out by varying phosphate concentrations (0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 g/L) in the growth media containing 50 mg/L of arsenic (either As(IIII) or As(V)), at an initial optimized pH value of 9.0 at 28 °C temperature under the same experimental conditions are shown in Fig. 12.

The phycoremediation % of both As(III) and As(V) increased with the increase in phosphate concentration from 0.005 to 0.5 g/L. It was supported by the increase in microalgal growth. The increase in phycoremediation % of As(III) and As(V) was caused by the reduction of toxicity of As(III) and As(V) at higher phosphate concentration which increased the biomass concentration. The maximum biomass concentration was found to increase from 3.068 to 1.949 g/L in the presence of As(III) and also from 3.068 to 1.949 g/L in the presence of As(V) with the increase in phosphate concentration from 0.005 to 0.5 g/L. It can also be explained by the following fact.

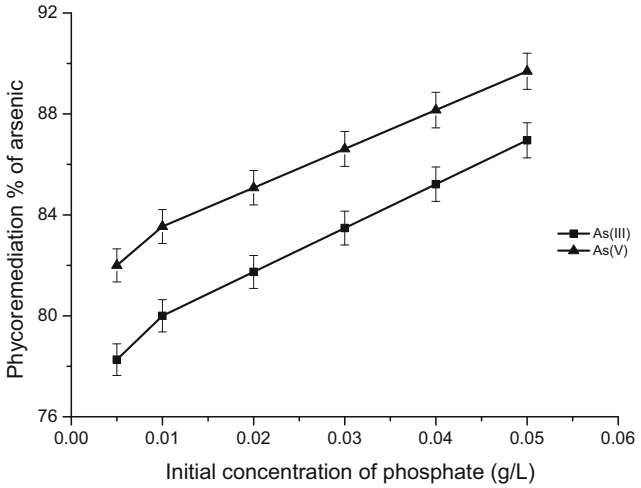

Fig. 12 Effect of initial phosphate concentration on phycoremediation of As(III) and As(V) of *B. braunii* (initial pH, 9.0; inoculum size (%, *v*/*v*), 10; contact time, 144 h; temperature, 28 °C). (*Error bars* represent means ± standard errors from the mean of duplicate experiments)

Both phosphate and arsenic are located in the same main group, and the molecular structure of the phosphate ion is very similar to that of the arsenic ion. So phosphate caused a higher reduction in arsenic phycoremediation, because the existing phosphate ions must strongly contend with arsenic ions for active binding sites on the surfaces of microalgae [85]. Though both divalent As(V) and phosphate anions are capable to form hydrogen bonding with MOH_2^+ and $M–NH^{3+}$, the basicity of divalent As(V) is very similar to that of divalent phosphate since both parent acids have very close values of pK_{a1}, pK_{a2}, and pK_{a3}. Then the noteworthy difference does not exist in strength of the hydrogen bonding of each anion with MOH_2^+ and $M–NH^{3+}$, between $HAsO_4^{2-}$ and HPO_4^{2-} species. Conversely, significant difference can be observed between ionic sizes and charge density of both anions. In $HAsO_4^{2-}$, lengths of three equivalent As–O bonds are 0.1654–0.1671 nm and As–O bond length in As–OH is 0.1742 nm [86]. Similarly of HPO_4^{2-}, lengths of three equivalent P–O bonds are 0.1510–0.1524 nm and P–O bond length in P–OH is 0.1551–0.1564 nm [87]. Then again the ionic radii of phosphate (0.17 Å) are also smaller than As(V) (0.335 Å) [88]. Thus crystallographic size or ionic radii of divalent phosphate anion is smaller than that of divalent As(V) anion and bear high charge density of divalent phosphate anion [89]. Below pH 5.0, the monovalent phosphate species ($H_2PO_4^-$) was prevailing and above pH 5.0, the dominant species of divalent phosphate species (HPO_4^{2-}) raised and phosphate phycoremediation also increased [90]. So phycoremediation of As(V) decreased in this pH range. Furthermore, it is assessed that Coulombic interaction between phosphate and positive sites is more than that in case of As(V), resulting in stronger interaction of phosphate with positive site than As(V). So phosphate might overcome As(V) in competition for overall binding with positive sites [90]. The results in Fig. 12 exhibited As(III) removal was also influenced to a similar extent by phosphate oxyanions [91].

Characterization of Microalgae

SEM-EDX

Figure 13a–c shows the scanning electron microscopy (SEM) images of native microalgal biomass and As(III)- and As(V)-loaded microalgal biomasses, respectively. In both images, two types of structure have been seen. The spherical shape represents *B. braunii* [92]. By comparing Fig. 13a–c, it is found that in native microalgal biomass, the surface is smooth while after arsenic (either As(III) or As(V)) treatment, the surface becomes rough in both the microalgal structures. Such roughness of the surface may be because of the phycoremediation of arsenic (either As(III) or As(V)) over the surface that makes the surface coarser than its original form. It has also been seen that there has been very little or no change in the fraction of spherical shape of *B. braunii* before and after arsenic (either As(III) or As(V)) removal. It recommends that the presence of arsenic (either As(III) or As(V)) does not make the medium selective toward any of the strains and the biological nature of the consortium remains fairly constant. Densities of the nodules also seem to be unaffected by the presence of arsenic (either As(III) or As(V)) signifying that the growth kinetics of the consortium remains unaffected in the presence of arsenic (either As(III) or As(V)) in simulated wastewater. But, the nodules are not clearly visible in the SEM. The cells seem to be glued to each other. It was because of more EPS production, which is one of the well-known responses against stress.

The corresponding EDX spectra of the unloaded and loaded microalgae was collected and given in Fig. 9a–c. The presence of arsenic on the loaded microalgae surface was exposed evidently. This outcome again established the occurrence of phycoremediation of arsenic by the microalgae.

FT-IR Analysis

The Fourier transform infrared (FT-IR) spectra of the *B. braunii* biomass with and without As(III) and As(V) ions loaded which were achieved to determine the possible functional groups, may have contributed to the phycoremediation of As(III) and As(V) ions, are presented in Fig. 14 and Table 6. The FT-IR spectra of the *B. braunii* biomass without As(III) and As(V) ions loaded exhibited a number of absorption peaks, indicating the complex nature of the microalgal biomass (Fig. 14). The spectra of unloaded and loaded with either As(III) or As(V) ions are compared and observed the

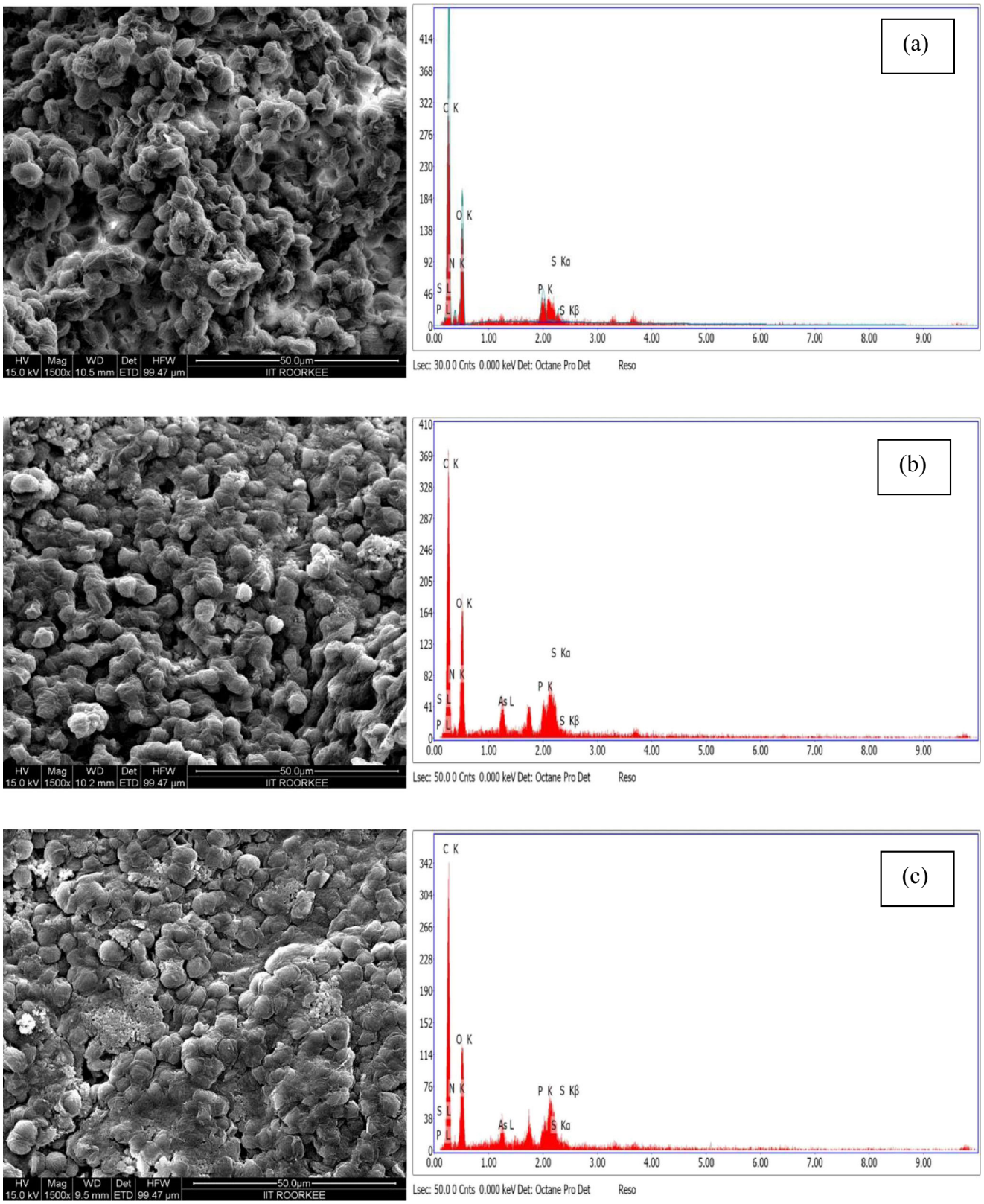

Fig. 13 Scanning electron micrographs (SEM; ×1500) and EDX of **a** native *B. braunii* biomass, **b** As(III)-loaded biomass, and **c** As(V)-loaded biomass

following shifting (Fig. 14; Table 6). The spectra of biomass exhibited an absorption band at 3370 cm^{-1} because of bonded –OH and –NH stretching vibration which was shifted to 3430 cm^{-1} (As(III)) and 3380 cm^{-1} (As(V)) which may be

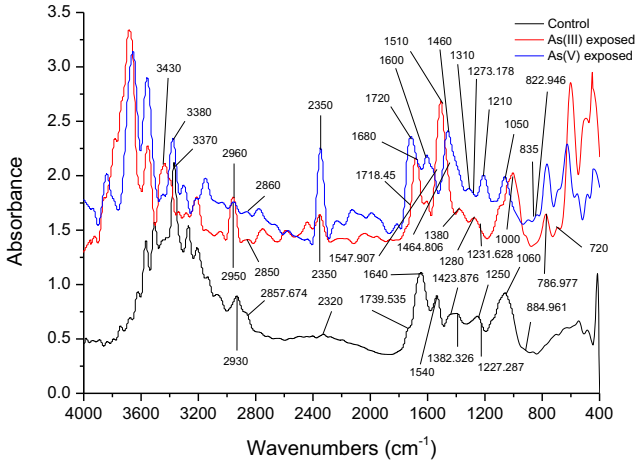

Fig. 14 FT-IR spectra of *B. braunii* control, As(III) ions exposed, and As(V) ions exposed

possibly because of the complexation of –OH and –NH groups with As(III) or As(V) ions [93, 94]. Aliphatic C–H stretching may be responsible for phycoremediation of As(III) and As(V) on the microalgal biomass as wavenumber shifted from 2930 to 2960 and 2950 cm^{-1}, respectively, possibly because of the complexation with As(III) and As(V) ions [93]. Aldehyde C–H stretching may be responsible for As(III) and As(V) phycoremediation on *B. braunii* biomass as wavenumber shifted from 2857.674 to 2850 and also to 2860 cm^{-1}, respectively [94]. The next absorption peaks at 2320 cm^{-1} has been shifted at a lower frequency to 2350 cm^{-1} (As(III)) and 2350 cm^{-1} (As(V)), probably because of the complexation of –CH stretching vibration of alkyl chains [12]. Table 6 also shows the responsibility of aliphatic acid C=O stretching for As(III) and As(V) phycoremediation by shifting the wavenumber from 1739.535 to 1718.45 and to 1720 cm^{-1}, respectively [94]. The next adsorption peaks at 1640 cm^{-1} shifted to 1680 cm^{-1} for As(III) and 1600 cm^{-1} for As(V), perhaps because of the complexation of amide group (N–H stretching and C=O stretching vibration) with As(III) and As(V) ions [12, 93]. Wavenumber shifted from 1540 to 1510 cm^{-1} (As(III)) and 1547.907 cm^{-1} (As(V)), probably because of the complexation of secondary amine group with As(III) and As(V) ions [93]. Another shift was found from 1423.876 to 1464.806 cm^{-1} (As(III)) and 1460 cm^{-1} (As(V)), possibly due to the complexation of nitrogen with As(III) and As(V) ions of the N–H group [95] and also due to the complexation with carboxyl groups [93]. Wavenumber shifted from 1382.326 to 1380 cm^{-1} (As(III)) and 1310 cm^{-1} (As(V)) assigned the reactivity of carboxylate anion C=O stretching for the phycoremediation process [96]. Wavenumber shifted from 1250 to 1280 cm^{-1} (As(III)) and 1273.178 cm^{-1} (As(V)) assigned the symmetric bending of CH_3 group [93]. Wavenumber 1227.287 cm^{-1} shifted to 1231.628 cm^{-1} (As(III)) and 1210 cm^{-1} (As(V)) assigned for $–SO_3$ stretching for the phycoremediation process [93]. The peaks at 1060 cm^{-1} may be attributed to the C–N stretching vibrations of amino groups which was shifted and appeared at 1000 and 1039.460 cm^{-1}, respectively, due to the interaction of nitrogen from the amino group with As(III) and As(V) ions [12]. The other weak adsorption peak shifted from 885.18 to 814.264 cm^{-1} (As(III)) and 896.744 (As(V)), corresponding to the O–C–O scissoring vibration of polysaccharide [97]. The presence of As(III) and As(V) on the microalgal biomass can be assured from the bands appeared at 721.24 and 819.845 cm^{-1}, respectively [29, 98]. It has to be cited here that a clear band was very hard to be got in the case of both As(III) and As(V). This may be because of different mechanisms involved in As(III) and As(V) phycoremediation. It

Table 6 Wavenumber (cm^{-1}) for the dominant peak from FT-IR for phycoremediation of As(III) and As(V)

Functional groups	Native biomass	As(III) loaded biomass	As(V) loaded biomass
Surface O–H and N–H stretching	3370	3430	3380
Aliphatic C–H stretching	2930	2960	2950
Aldehyde C–H stretching	2857.674	2850	2860
–CH stretching vibration of alkyl chains	2320	2350	2350
Aliphatic acid C=O stretching	1739.535	1718.45	1720
Amide group (N–H stretching and C=O stretching vibration)	1640	1680	1600
Secondary amine group	1540	1510	1547.907
N–H group and carboxylate anion	1423.876	1464.806	1460
Carboxylate anion	1382.326	1380	1310
Symmetric bending of CH_3 group	1250	1280	1273.178
$–SO_3$ stretching	1227.287	1231.628	1210
C–N stretching vibrations of amino groups	1060	1000	1050
O–C–O scissoring vibration of polysaccharide	884.961	786.977	835
As(III)–O	×	720	×
As(V)–O	×	×	822.946

should be distinguished that the As–O band after phycoremediation of arsenic was not clearly observed because of the broad overlapping peaks in this region [99].

Conclusions

Fuel shortage in the near future poses a serious challenge; hence, a renewable energy resource having less environmental effect is necessary. Application of microalgae for such a purpose is an effective step. In the present study, an efficient microalgae *B. braunii* was found suitable for the elimination of both As(III) and As(V) from wastewater. A kinetic study has been performed in pure media as well as in simulated wastewater. The microalgae *B. braunii* removes 85.217 and 88.154 % of As(III) and As(V), respectively, from the growth media supplemented with arsenic (either As(III) or As(V)) after 144 h of treatment with initial arsenic concentration of 50 mg/L at pH 9.0 and inoculum size of 10 % (*v*/*v*). As(V) was more toxic than As(III), particularly at the near-neutral pH 7.0, but at higher pH (pH 9.0) As(III) was much more toxic than As(V) to fresh microalage. A rise in phosphate concentration in the growth medium from 0.005 to 0.05 g P/L reduced the toxicity of As(III) and As(V). It was also supported by measuring the kinetic growth parameters. Value of μ_{max} remained almost constant after addition of arsenic (either As(III) or As(V)), but the value of K_s increased. Thus it can be stated that the microalgae *B. braunii* can be potentially employed as an effective biomaterial for arsenic remediation. The spent biomass can be applied for different aims, including the production of renewable energy source such as a number of biofuels like biomethane, bioethanol, biohydrogen, biobutanol, and the extraction of added value products like carotenoids or other bio-molecules for commercialization. However, this is a preliminary work involving a simulated solution of either As(III) and As(V) and a comprehensive study with real industrial wastewater encompassing a detailed parametric study in a continuous reactor system is needed.

Nomenclature

C_0	initial concentration of arsenic in the solution (mg/L)
C_f	final concentration of arsenic in the solution (mg/L)
μ	the specific growth rate (day^{-1})
μ_{max}	the maximum specific growth rate (day^{-1})
C_A	the limiting substrate concentration at time t (g/L)
K_s	the substrate saturation constant (g/L)

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