

Optimization of environmental conditions for the growth and bioaccumulation of As(III) and As(V) ions by growing *Corynebacterium glutamicum* MTCC 2745: inhibition kinetic study

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Abstract The significant factors affecting simultaneously the growth and bioaccumulation of arsenic [As(III) and As(V)] ions by living cells of bacteria, *Corynebacterium glutamicum* MTCC 2745, were explored in growth media under various experimental environmental conditions such as pH, inoculum size, contact time, temperature, concentrations of peptone and arsenic [either As(III) or As(V)] ions. Three kinetic models namely competitive, noncompetitive and uncompetitive models were verified with the aim of deciding the toxic effects of arsenic on specific growth rate of *C. glutamicum* MTCC 2745. As(III) was observed to be more toxic than As(V). Competitive inhibition was found in either As(III) or As(V) systems which was suggested by almost same maximum specific growth rate (μ_m) value and an increase in the saturation constant ($K'_{S,app}$) value that were evaluated from Lineweaver–Burk plots. A simultaneous reduction in the inhibition constant recommended that inhibitory effect increases with the increase in arsenic concentration.

Keywords As(III) and As(V) · *Corynebacterium glutamicum* MTCC 2745 · Bioaccumulation · Inhibition model

Nomenclature

C_0 Initial metal concentration (mg/L)
 C_f Final metal concentration (mg/L)

I Concentrations of the inhibitory component (mg/L)
 K_I Inhibition constant (mg/L)
 K_S Half velocity constant or saturation constant or Monod constant (g/L)
 $K'_{S,app}$ Apparent saturation constant (mg/L)
 M Concentrations of microorganism (g/L)
 S Concentration of substrate (g/L)
 S_0 Initial peptone concentration (g/L)
 t Incubation time (h)
 T Absolute temperature (°C)
 X Concentrations of dried cell mass (g/L)
 X_m Maximum biomass concentration (g/L)
 X_1 Initial concentration of peptone (g/L)
 X_2 Initial concentration of arsenic [either As(III) or As(V)] ions (mg/L)

Greek symbols

μ Specific growth rate of the microorganisms (h^{-1})
 μ_m Maximum specific growth rate of the microorganisms (h^{-1}) when $S \gg K_S$
 $\mu_{m,app}$ Apparent maximum specific growth rate of the microorganisms (h^{-1})

Introduction

Arsenic is ubiquitous in the environment and highly toxic to all forms of the life (Singh et al. 2015; Podder and Majumder 2016a, b). The word arsenic has been derived from the Greek word arsenikon, meaning potent which is known as king of all poisons (Rahim and Haris 2015). Arsenic is categorized as a category 1 and group A human carcinogen by the International Association For Research on Cancer (IARC 2004) and the US Environmental Protection Agency (US EPA 1997), respectively.

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Contamination due to arsenic is rising all over the world because of natural processes such as geochemical reactions, volcanic eruption and increased anthropogenic activities such as mining of arsenopyrite gold ores, which constitute about one-third of world gold reserves, non-ferrous smelting, industrial waste discharges, combustion of arsenic-bearing coal and even the agricultural use of arsenical pesticides (Su et al. 2009; Podder and Majumder 2015a, b, 2016a, b). Copper smelting creates a huge volume of wastewater containing large amounts of inorganic compounds, such as heavy metals like lead, copper, zinc, iron, cadmium and bismuth, etc., and highly carcinogenic metalloids like arsenic species, poses a serious threat towards 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 (Basha et al. 2008a). 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. Wastewater from the processing of arsenic-bearing ores may contain varying amounts of As(III) (arsenite) and As(V) (arsenate) oxyanion (Basha et al. 2008b). At the pH range of most natural and wastewater sources As(III) is more toxic, mobile and less efficiently removed than As(V) (Podder and Majumder 2015a, 2016a, b). Arsenic is a potent gene and chromosomal mutagen, able to generate intercellular oxidative DNA damage (Su et al. 2009). The human toxicity of arsenic varies from skin lesions, hyperpigmentation, and hypertension to cancer of lung, kidney, stomach, liver and brain (Podder and Majumder 2015a, b, 2016a, b). Because of the harmful effects of arsenic on people's health, the maximum contaminant level (MCL) of arsenic in drinking water has been revised to 10 from 50 $\mu\text{g/L}$ by the World Health Organization (WHO 1993) and the European Commission in 2003 (European commission Directive, 98/83/EC 1998).

To date, conventional methods applied for removing As(III) and As(V) ions from water and wastewater include precipitation (Nishimura and Robins 1998), electrochemical treatment (Basha et al. 2008a), electrocoagulation (Basha et al. 2008b), and adsorption (Podder and Majumder 2016a). However, these high-technology methods have considerable drawbacks, such as, operational complexity, high capital and operational cost, high reagent or energy necessities, necessities for costly equipment and monitoring systems, incomplete metal removal or production of contaminated sludge or other waste products that require disposal and are not appropriate for small-scale industries and are not eco-friendly (Podder and Majumder 2015a, 2016b). So, there is an emergent need for the improvement of unique, effective, eco-friendly and cost-effective approach for the remediation of the

environmental contaminants arsenic. Bioremediation is a treatment technology that employs biosorbents or plants or indigenous, naturally occurring, or bioaugmented microorganisms or to decrease, remove, and/or immobilize contaminants (Reddy et al. 2003). Biological method has many benefits over the chemical ones in the aspects of environmental friendliness, ease in application, more efficiency and cost reduction (Su et al. 2009; Podder and Majumder 2015a, 2016b, c). Several microorganisms retain their vitality in heavy metal-polluted environments resulting from industrial waste and these organisms obtain resistance to the metals. Moreover, many of those microorganisms can bioaccumulate the toxic metals (Açikel and Erşan 2010).

The bioaccumulation of heavy metals has received a huge attention in current centuries, not only as a technical innovation, but also for its possible use in industry. In the course of the metal uptake by growing cells or living cells, both passive and active metal uptake mechanisms are simultaneously active. Metabolism-independent (i.e. passive) uptake is termed "biosorption." In this process, cations and anions are entrapped on the cellular surface via electrostatic attraction, ion exchange, complexation, physical adsorption and chemical adsorption (Açikel and Erşan 2016). Surface adsorption is done by the functional groups such as $-\text{OH}$, $-\text{NH}_2$, $-\text{COOH}$ and $-\text{SH}$ on microbial cell walls. After the metals being transferred into the cells, they can bind to the functional groups of the intracellular native molecules (Su et al. 2009). Metabolism-dependent (i.e. active) uptake is achieved only by growing cells or living cells, and it transferred metal ions across the cellular membrane. In bioaccumulation processes, metal ions accumulate inside the cell to use in some metabolic cycles and essential cellular activities. So, more fast and effective metal uptake is attained by growing cells or living cells than by dead cells (Açikel and Erşan 2016).

The use of non-living cells has benefits over living cells because of the absence of both toxicity constraints and necessities of growth media and nutrients. Moreover, adsorbed metal ions can be readily desorbed as well as the regenerated biomass can be reused. However, the major constraint of employing non-living biomass is that since the cells are dried, biochemical cell energetic reactions are not carried out for longer time, while living cells can be preserved biochemically active (Gönen and Aksu 2008). Furthermore, using living cultures are more beneficial compared to using the non-living cells because the simultaneous metal removal is acquired during the microbial growth and it also escape the necessity for a separate processes of biomass production (for example, cultivation, harvesting, drying, processing and storage before usage) (Gönen and Aksu 2008; Das et al. 2010). However, sensitivity of living cells to extremes of pH or high salt

concentrations or high metal concentration and necessity to provide metabolic energy are some of the major limitations of using growing cells for bioremediation. The efforts to meet such challenges via isolation of metal-resistant bacterial/algal/fungal strains which can survive in high concentrations of metals and have the capacity to accumulate various metal ions and also the use of organic wastes as carbon substrates have started (Malik 2004; Gönen and Aksu 2008). The living cells, however, have the potential for mutant isolation or generic recombination to enhance the metal-accumulative strain. The vital features of a living biomass used in a metal ion removal process are tolerance and uptake capacities (Dönmez and Aksu 1999). So, in addition to these varieties of mechanisms, cells can be genetically manipulated to alter morphological and physiological features (Srinath et al. 2002).

Few reports have discussed the feasibility of using living bacterial cells for the heavy metals bioaccumulation in a single system (Dönmez and Aksu 1999; Preetha and Viruthagiri 2007; Gönen and Aksu 2008; Giri et al. 2013; Açikel and Erşan 2010, 2016; Podder and Majumder 2015c). Bioaccumulation of arsenic mainly includes the biosorption of arsenic by microbial biomass and its byproducts and physiological uptake of arsenic by microorganisms through metabolically active and passive processes (Mahimairaja et al. 2005). As(V) uptake by living organisms is through phosphate transporter, whereas As(III) in water is an inorganic equivalent of nonionized glycerol and can be transported across cell membranes by glyceroporin membrane channel proteins (Rosen 2002).

Corynebacterium glutamicum MTCC 2745 species is of specific attention due to its high capability for abatement biologically. Bacteria can depollute arsenic containing wastewater, by accumulation outside the cells and/or biosorption of the ion on their surface (Mateos et al. 2006) as was defined earlier for *E. coli* (Kotrba et al. 1999) and *Ralstonia eutropha* (Valls et al. 2000). *C. glutamicum* MTCC 2745 does not have an outer membrane, it contains a typical cell-surface S-layer formed by a protein encoded by *cspB* (Peyret et al. 1993).

The objective of the current research were (1) to investigate the effect of various process parameters such as pH, inoculum size, contact time, temperature and initial arsenic [either As(III) or As(V)] concentrations systematically on the growth and bioaccumulation properties of *C. glutamicum* MTCC 2745 evaluating the optimum operating conditions; (2) to explore the effect of initial peptone concentration on the growth of *C. glutamicum* MTCC 2745; (3) to find the combined effects of initial concentrations of peptone and arsenic [either As(III) or As(V)] ions on the growth and bioaccumulation properties of *C. glutamicum* MTCC 2745, and (4) to characterize the

inhibition of arsenic [either As(III) or As(V)] ions on the bacterial growth.

Theory

Kinetic approach

Appropriate mathematical models are developed to define the inhibition of metal and kinetics of growth of microorganism is quite beneficial to design the bioaccumulation systems utilizing living cells. The specific grow rate of microorganisms is defined as (Dursun et al. 2003):

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad (1)$$

μ can be estimated from the slope of the plot of $\ln X$ versus t at the exponential growth region. For modelling the specific growth rate, the maximum extensively employed model is the Monod equation for the substrate limited growth as follows (Bailey and Ollis 1977; Shuler and Kargi 1992; Berg et al. 2006; Nelson and Cox 2008):

$$\mu = \frac{\mu_m S}{K_S + S} \quad (2)$$

μ_m is the maximum specific growth rate of microorganism (h^{-1}) when $S \gg K_S$. The constant K_S is the half velocity constant or saturation constant or Monod constant (g/L). While the specific growth rate (μ) is the same as one-half of the maximum, K_S is the same as the rate limiting substrate concentration, i.e. $K_S = S$ while $\mu = \frac{1}{2} \mu_m$ (Nelson and Cox 2008). Equation (2) is linearized in double reciprocal form (Lineweaver–Burk plot) as follows:

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{K_S}{\mu_m S} \quad (3)$$

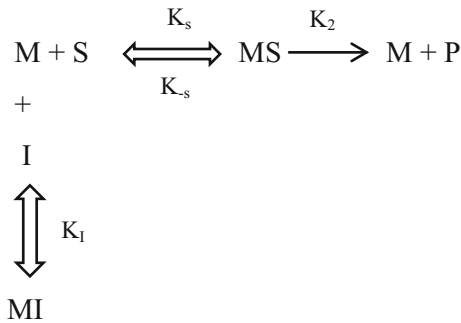
Values of μ_m and K_S can be evaluated from $1/\mu$ versus $1/S$ plot (supposing $S = S_0$ at the start of exponential growth) returns a straight line with a y-axis intercept of $1/\mu_m$ and a slope of K_S/μ_m .

As(III) has a great affinity towards thiol (–SH) groups, because it forms kinetically stable bonds to sulphur readily. Since thiol groups are vital to the functions of many enzymes, so the reaction with As(III) tempts inactivation of enzyme. As(V) has a poor affinity for thiol groups, occasioning in very fast excretion from the body. Though it is a molecular analogue of phosphate (PO_4^{3-}), it can disjoin mitochondrial oxidative phosphorylation, causing energy metabolism system failure (Andjelkovic et al. 2014). So, the toxicity of arsenic is credited to affinity of As(III) for protein thiol groups (R–SH), DNA–DNA cross-linking and protein–DNA and the replacement of As(V) for phosphate (Kostal et al. 2004).

So the microbial growth becomes prohibited in the presence of inhibitory (toxic) substances such as heavy metals in nutrient media and specific growth rate is governed by the concentration of inhibitor. Consistent with the effects of toxic components on the specific growth rate (μ) and saturation constant (K_S) as demarcated in the literature, inhibition models namely, competitive, noncompetitive and uncompetitive are categorized (Bailey and Ollis 1977; Shuler and Kargi 1992). For three types of inhibitions, the expressions of specific growth rate were derived with the resemblance of inhibition of enzyme as follows (Shuler and Kargi 1992; Açikel 2003; Açikel and Alp 2009).

Competitive inhibition

Competitive inhibitors contend with the substrates as well as with each other for binding to active sites on the cells of microorganism. In this type of inhibition, a microorganism cell can bind to the substrate (forming a MS complex) or inhibitor (MI) but not both (MSI). The competitive inhibitor frequently looks like the substrate and binds to the active site of the cells of microorganism. The competitive metal inhibition outline can be defined as follows (Shuler and Kargi 1992; Açikel 2003; Berg et al. 2006; Nelson and Cox 2008):



If the inhibitor competes with substrate in the growth medium, the specific growth rate of the microorganism supposing fast equilibrium can be defined as follows (Shuler and Kargi 1992; Açikel 2003; Berg et al. 2006):

$$\mu = \frac{\mu_m S}{K_S [1 + (I/K_I)] + S} \tag{4}$$

or

$$\mu = \frac{\mu_m S}{K'_{S,app} + S} \tag{5}$$

where

$$K'_{S,app} + K_S [1 + (I/K_I)] \tag{6}$$

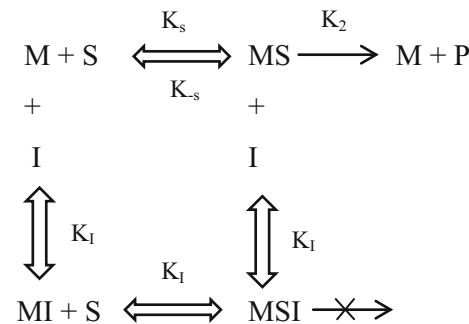
Equation (6) can be linearized as analogous to Eq. (3) as follows:

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{K'_{S,app}}{\mu_m} \frac{1}{S} \tag{7}$$

From the slope of the linear plot of $1/\mu$ versus $1/S$ at various initial concentrations of arsenic [either As(III) or As(V)], the values of $K'_{S,app}$ can be estimated and then K_I value can be estimated from Eq. (6). In the presence of competitive inhibitor, the slope of the plot is increased by a factor $(1 + (I/K_I))$. So the net result of competitive inhibition is a higher apparent saturation constant ($K'_{S,app}$) value. The competitive inhibitor so reduces the reaction rate to half at this concentration of substrate (Berg et al. 2006). The intercept is unchanged by the presence of competitive inhibitor. So the value of specific growth rate remains almost unaltered in the presence of competitive inhibitor (Berg et al. 2006; Nelson and Cox 2008).

Noncompetitive inhibition

In noncompetitive inhibition, the inhibitor combines with either the microorganism or the microorganism–substrate (MS) complex. Noncompetitive inhibitors bind on sites other than the active sites and decrease the affinity of microorganism to the substrate. In this type of inhibition, the substrate and the inhibitor can bind simultaneously to a microorganism cell at different binding sites. Noncompetitive inhibition scheme is presented as follows (Shuler and Kargi 1992; Açikel 2003; Berg et al. 2006; Nelson and Cox 2008):



If noncompetitive inhibitors exist in the growth medium, the specific growth rate of the microorganism can be defined as follows (Shuler and Kargi 1992; Açikel 2003; Berg et al. 2006):

$$\mu = \frac{\mu_m S}{(K_S + S)[1 + (I/K_I)]} \tag{8}$$

or

$$\mu = \frac{\mu_{m,app} S}{K_S + S} \tag{9}$$

where

$$\mu_{m,app} = \frac{\mu_m}{1 + (I/K_I)} \tag{10}$$

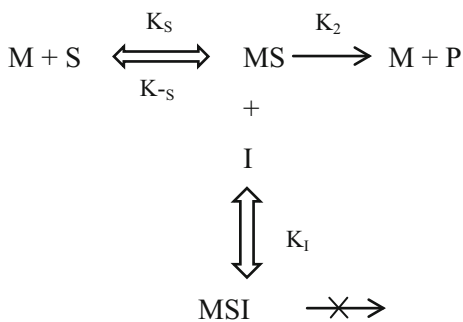
Equation (8) can be linearized as analogous to Eq. (3) as follows:

$$\frac{1}{\mu} = \frac{1}{\mu_{m,app}} + \frac{K_S}{\mu_{m,app}} \frac{1}{S} \tag{11}$$

From the intercept of the linear plot of $1/\mu$ versus $1/S$ at different initial concentrations of arsenic [either As(III) or As(V)] ions, the values of $\mu_{m,app}$ can be evaluated and then K_I value can be estimated from Eq. (10). So the net result of noncompetitive inhibition is a decrease in the value of maximum specific growth rate μ_m to a new value $\mu_{m,app}$ and thus the intercept of the vertical axis is increased. The new slope, which is equal to $K_S/\mu_{m,app}$, is higher by the same factor. In contrast to μ_m , K_S is not influenced by pure noncompetitive inhibition (Berg et al. 2006).

Uncompetitive inhibition

Uncompetitive inhibitor has no affinity for the microorganism itself and only combines with the microorganism–substrate (MS) complex. This type of inhibitor’s binding is generated only on interaction of the microorganism cell and substrate. The scheme for uncompetitive inhibition is shown as follows (Shuler and Kargi 1992; Açıklık 2003; Berg et al. 2006; Nelson and Cox 2008):



If uncompetitive inhibitors exist in the growth medium, the specific growth rate of the microorganism can be defined as follows (Shuler and Kargi 1992; Açıklık 2003; Berg et al. 2006):

$$\mu = \frac{\mu_m S}{K_S + S[1 + (I/K_I)]} \tag{12}$$

Equation (12) can be linearized as analogous to Eq. (3):

$$\frac{1}{\mu} = \frac{1}{\mu_{m,app}} + \frac{K_S}{\mu_m} \frac{1}{S} \tag{13}$$

where

$$\mu_{m,app} = \frac{\mu_m}{1 + (I/K_I)} \tag{14}$$

The slope of the plot of $1/\mu$ versus $1/S$ at different initial concentrations of either As(III) or As(V) ions, K_S/μ_m is the same as that for uninhibited microorganism. But the intercept on the y-axis will be increased by $(1 + (I/K_I))$. So the net influence of uncompetitive inhibition is drop in both maximum specific growth rate (μ_m) and the apparent saturation constant ($K'_{S,app}$) values. So the lines in double reciprocal plots will be parallel (Berg et al. 2006).

Materials and methods

Materials

All the chemicals and reagents were of analytical reagent grade and used without additional purification. The stock solutions of As(III) and As(V) were prepared by dissolving sodium arsenite (NaAsO₂) and sodium arsenate (Na₂HAsO₄·7H₂O), purchased from Himedia Laboratories Pvt. Ltd. Mumbai India, in double distilled water, respectively. All other necessary chemicals used in the experiments, were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Argon gas and ultra-pure grade HNO₃ used for the analysis of arsenic in the present study was purchased from Sigma gases, India and Merck, Germany, respectively. Glassware utilized for purposes of experiments was washed in 10% HNO₃ and rinsed with double distilled water for removing any probable interfering by other metals.

Microorganism and growth conditions

The arsenic resistant bacterium *C. glutamicum* MTCC 2745 was acquired from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Culture media was prepared as per the guidelines of microbial type cell culture (MTCC). Composition of growth medium and cultivation conditions are shown in Table 1. The pH of the growth medium was adjusted to 7.0 by adding 1 N NaOH and 1 N HCl.

Table 1 Composition of growth medium and cultivation conditions

Component (g/L) or condition	
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
NaCl	5.0
pH	7.0
Temperature (°C)	30

Preparation of metal-adapted living cells of *C. glutamicum* MTCC 2745

Microbial adaptation is demarcated as the capability of a microbial population for adjusting itself to an altering environment in the presence of either As(III) or As(V) ions as in the current investigation (Mondal et al. 2008a).

The bacterial strain was first grown in the round-bottomed flask. To do so, the bacterial inoculum was prepared by transferring a loop full of bacterial culture from the nutrient agar tubes to the flask containing growth media (sterilized at 121 °C temperature, 15 psi pressure for at least 15 min), incubated at 30 °C for 24 h with moderate agitation in an incubator cum orbital shaker. Then the bacterial strain was grown on a petri dish containing agar medium which consisted of beef extract (1.0 g), yeast extract (2 g), peptone (5.0 g), NaCl (5.0 g) and agar (15.0 g), in 1 L double distilled water and the pH was adjusted to 7.0 with 1 N NaOH and 1 N. After the incubation of cultures at 30 °C for 24 h in agar plates, the bacteria were inoculated from the plates on another fresh agar plate and stored at 4 °C until required for further experiments. Before bioaccumulation experiments were performed, *C. glutamicum* MTCC 2745 was adapted or acclimatized to both As(III) and As(V) ions separately in its culture medium.

Before the beginning of metal adaptation process, strains were enriched by transferring one loop of cells from the agar plates to 100 mL of previously sterilized liquid nutrient medium in 250 mL round-bottomed flasks and incubated at 30 °C for 24 h by agitating at 120 rpm in an incubator cum orbital incubator. After 24 h the synthetic medium in the flask had turned milky specifying substantial growth of bacteria in the flask. For this adaptation process bacterial cells were exposed to both As(III) and As(V) ions separately in the course of growth phase with the aim of increasing the bacterial metal resistance capacity. Subcultures were grown in growth medium containing various concentrations of arsenic [either As(III) or As(V)] ions in the range of 50–2000 mg/L. A culture of *C. glutamicum* MTCC 2745 resistant to 50 mg/L of arsenic [either As(III) or As(V)] ions was utilized to the first inoculation. While the metal stressed culture of *C. glutamicum* MTCC 2745 got its exponential growth phase (OD value ~ 2 at 600 nm), 5 mL of the culture medium was utilized for inoculating the next culture medium containing 100 mg/L of arsenic [As(III) or As(V)] ions. Metal adaptation process was sustained rising concentration of metal ion, i.e. the culture grown in the medium containing the lowest level was transferred to the next medium added with a higher concentration (200, 500, 800, 1000, 1200, 1500, 1800 and 2000 mg/L) and so acclimatized to higher concentrations of As(III) or As(V). The metal adaptation

experiments were performed at 30 °C with moderate agitation at 120 rpm in 250 mL flasks containing 100 mL of the sterilized growth medium. After the incubation of cultures at 30 °C for 24 h in the round bottom flask, the bacteria were inoculated from the flask on fresh agar plate and stored at 4 °C until necessary for further experiments.

Preparation of bioaccumulation medium containing metal ions

Requisite amount of arsenic [either As(III) or As(V)] salt was added to the prepared fermentation medium containing 1 g/L beef extract, 2 g/L yeast extract, 5 g/L peptone and 15 g/L NaCl. The range of metal ion concentrations in the prepared fermentation media varied between 50 and 2000 mg/L. Then the prepared growth media was subjected to autoclave sterilization at 15 psi pressure and at 120 °C for 15 min. The pH of the bioaccumulation medium was adjusted to the requisite value by dropwise addition of sterile 1 N HCl and 1 N NaOH solution.

Bioaccumulation experiments

The factors influencing the growth and bioaccumulation efficiency of living *C. glutamicum* MTCC 2745 examined in 250-mL round-bottomed flask with 100 mL bioaccumulation medium. The pH of the medium was adjusted by dropwise addition of sterile 1 N HCl and 1 N NaOH. An optimum aliquot (5%, volume of inoculum/volume of growth medium) of an adapted preculture was harvested aseptically during the exponential growth phase (OD value ~ 2 at 600 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. After the transfer of the bacteria from enrichment medium into the bioaccumulation medium, cultures were grown at 30 °C in an incubator cum orbital shaker at constant agitation speed 120 rpm.

Experiments were performed to investigate the growth of *C. glutamicum* MTCC 2745 in the varying pH from 1.0 to 12.0 at 30 °C in the absence and presence of 50 mg/L of arsenic ions [either As(III) or As(V)] in the bioaccumulation medium. The inoculated flasks were incubated in an incubator cum orbital shaker at 30 °C for 24 h shaking at 120 rpm.

To optimize the inoculum size, the inoculum concentrations in the shake flasks were varied from 1 to 10% (v/v). The influences of pH on bioaccumulation of arsenic were examined by changing the temperature of the bioaccumulation medium from 20 to 50 °C.

To determine the shortest exposure time for the respective metal-resistant bacteria for maximally bioaccumulating each of As(III) and As(V) ions (i.e. for

determining the equilibrium point/residence time of As(III) and As(V) in each metal-resistant bacteria), 5% (v/v) of the standard inoculum was contacted with 100 mL bioaccumulation medium containing 50 mg/L of arsenic [either As(III) or As(V)] in 250-mL round-bottomed flask. These flasks were then incubated at 30 °C for different time intervals ranging from 1 to 24 h.

The effects of initial concentration of arsenic [either As(III) or As(V)] on bioaccumulation of arsenic were investigated by changing the arsenic concentration from 50 to 2000 mg/L at a constant peptone concentration of 5 g/L under identical conditions. The inoculated flasks were incubated in an incubator cum orbital shaker at 30 °C for 24 h shaking at 120 rpm.

Experiments were also performed for studying the growth of *C. glutamicum* MTCC 2745 in the variable peptone concentration from 1 to 9 g/L at pH 7.0 at 30 °C temperature in the absence and presence of arsenic ions [either As(III) or As(V)] (50, 500, 1000, 1500, and 2000 mg/L) under same conditions.

The combined effect of media concentration and arsenic [either As(III) or As(V)] ion concentrations on bioaccumulation of arsenic was investigated by changing the arsenic concentration from 1000 to 2000 mg/L at constant peptone concentrations (1, 5 and 9 g/L).

Samples (5 mL) were withdrawn at certain time intervals and then centrifuged (Remi Instruments Ltd., Mumbai India) at 10,000 rpm for 10 min and the supernatant fraction was analysed for residual concentration of either As(III) or As(V) ions. The centrifuged cells were washed with 1 mL of double distilled water and resuspended in 2 mL of double distilled water and then measure the OD. Arithmetic mean of results of two similar experiments was utilized to estimate data.

Metal analysis

The analysis of total arsenic was conducted with a ThermoFisher Scientific iCE 3000 Series AA graphite furnace atomic absorption (GFAA) spectrometer, with Zeeman background correction (GF-AAS). Prior to the analysis, the samples were acidified with 10% HNO₃ of ultra-pure grade and suitably diluted to bring the concentration into the working range of the instrument. All measurements were based on integrated absorbance and performed at 193.7 nm using a hollow-cathode lamp (ThermoFisher Scientific) with a slit width of 0.5 nm. An arsenic high-intensity hollow-cathode lamp was utilized for determining As < 20 µg/L. Pyrolytic graphite coated tubes with forked pyrolytic platforms were utilized. Argon gas of ultra-high purity was used as a protective gas to sheath the atomizer and to purge internally. Pretreatment temperature of the furnace was kept at 1400 K and atomization temperature

was 2500 K. The calibration range was 5–20 µg/L. The detection limit of the instrument was <20 µg/L. To determine the dissolved arsenic, 8.0 mL of a centrifuged sample was added to 1 mL of 1% HNO₃ and 1.0 mL of chemical matrix modifier (50 g/L of nickel nitrate solution) in a flask. The prepared sample injected was 20 µL. All measurements were conducted at least in duplicate and on the basis of integrated absorbance. The procedure of detailed analysis for this fast and easy to operate method was defined by Podder and Majumder (2015b, 2016b).

The bioaccumulation % of heavy metal is evaluated utilizing the following equation:

$$\text{Bioaccumulation \%} = \frac{(C_0 - C_f)}{C_0} \times 100. \quad (15)$$

Biomass analysis

The growth of both As(III) and As(V) resistant bacteria was checked by measuring the optical density of small volume of samples withdrawn from the cell culture periodically. The optical density was measured at 600 nm, against double distilled water, which served as reagent blank (Mondal et al. 2008a; Ergul-Ulger et al. 2014; Podder and Majumder 2015c).

The values of optical density were measured by serially diluting overnight cultures with known OD600 values. By oven drying an aliquot in a preweighed aluminium foil container to a constant weight at 80 °C and measuring their mass (Puranik and Paknikar 1997) and then correlated with the concentrations of cells, in terms of dry weight of cells per litre of suspension (g/L) by appropriate calibration curves (Podder and Majumder 2015c). A correlation for converting OD600 values to bacterial dry weight was established from the calibration curve. These correlations were utilized all through the research for determining the biomass concentration of both As(III) and As(V) resistant bacteria.

Results and discussion

Calibration curve

A typical calibration curve, utilized for correlating the optical density of As(III) and As(V) resistant *C. glutamicum* MTCC 2745 cell suspensions with known cell concentrations, is exhibited in Fig. 1a, b.

Correlations for converting OD600 values to bacterial dry weight were established from the calibration curves. The correlations used for the As(III)-resistant *C. glutamicum* MTCC 2745 and As(V)-resistant *C. glutamicum* MTCC 2745 can be expressed by Eq. (16) as follows:

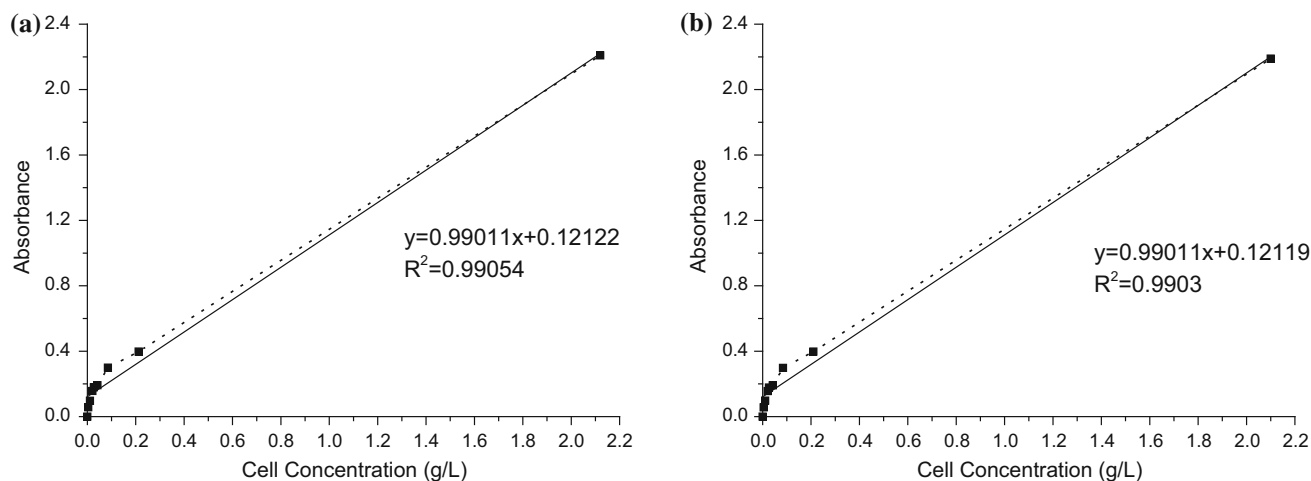


Fig. 1 Typical calibration curve used to correlate optical density with biomass concentration of **a** As(III)-resistant bacteria, **b** As(V)-resistant bacteria

$$\text{Cell concentration (g/L)} = 1.0099 \times \text{OD600} - 0.1224. \quad (16)$$

Effect of initial pH on growth properties of *C. glutamicum* MTCC 2745

pH plays a main role for the growth properties of *C. glutamicum* MTCC 2745. It also greatly influences the integration of the metals into the bacteria (Su et al. 2009). The results reported by Su et al. (2009) and Teclu et al. (2008) indicated that acidic conditions were in favour of As(III) integration, whereas Shivaji et al. (2005) reported that the bacterial growth is very sensitive to pH and the encouraging pH for the growth of *B. arsenicus* MTCC 4380 is 5.5–8.0. So, effect of initial pH on biomass concentration of *C. glutamicum* MTCC 2745 was investigated in the pH range of 1.0–12.0 in the growth media free from arsenic ions or containing arsenic [either As(III) or As(V)] ions. The growth of *C. glutamicum* MTCC 2745 is strongly inhibited in extreme lower pH 1.0 and at extreme higher pH 12.0 for both As(III) and As(V) resistant bacteria (Fig. 2a, b). The maximum absorbance (OD at 600 nm) and biomass concentration achieved at pH 7.0 in the absence of As(III) and As(V) ions was determined as 2.189 and 2.088 and 2.202 and 2.102 g/L, respectively. The growth of bacteria in an acidic medium at pH 6.0 was higher as compared to that in alkaline at pH 8.0. Biomass concentration was more suppressed in the media containing 50 mg/L As(III) ions, compared to the media containing 50 mg/L As(V) ions. The maximum absorbance and biomass concentration was also determined at pH 7.0 as 2.128 and 2.027 g/L and 2.168 and 2.067 g/L, respectively, in the media containing 50 mg/L As(III) and As(V) ions, respectively. The optimum pH for the growth determined

in this study is in agreement with the optimum pH for the growth of *Bacillus subtilis* strain Rand (Abusham et al. 2009).

Effect of initial pH on bioaccumulation properties of *C. glutamicum* MTCC 2745

pH is one of the significant parameters that significantly influences adsorbate ion speciation, the chemistry of solution, interaction between adsorbate and adsorbent and surface charge of adsorbent surface (Podder and Majumder 2015a, 2016d). The influence of initial pH on arsenic [either As(III) or As(V)] bioaccumulation of *C. glutamicum* MTCC 2745 was examined taking 50 mg/L of either As(III) or As(V) in the growth medium changing the pH in the range of 1.0–12.0 at a temperature of 30 °C and contact time of 12 h. The concentration of peptone in the growth medium was kept constant at 5 g/L. The initial pH substantially influenced both As(III) and As(V) bioaccumulation properties of *C. glutamicum* MTCC 2745 and the highest bioaccumulations were noted at pH 7.0 for both As(III) and As(V) ions. Figure 3 shows that As(III)-resistant and As(V)-resistant *C. glutamicum* MTCC 2745 could accumulate As(III) and As(V) to various levels at pH 7.0, respectively, and the bioaccumulation of both As(III) and As(V) varied significantly depending upon the arsenic species. The difference in the bioaccumulation % of As(III) and As(V) could be clarified on the basis of the charge on the species of arsenic and the cellular surface charges of the bacterial biomass.

It can be understood from Fig. 3 that in the pH range <3.0, the bioaccumulation of both As(III) and As(V) ions (17.39 and 20.46%, respectively) was very poor. With the increase in pH from 3.0 to 7.0, there was a noteworthy increase in bioaccumulation % of both As(III) and As(V) ions. Maximum bioaccumulation % of As(III) and

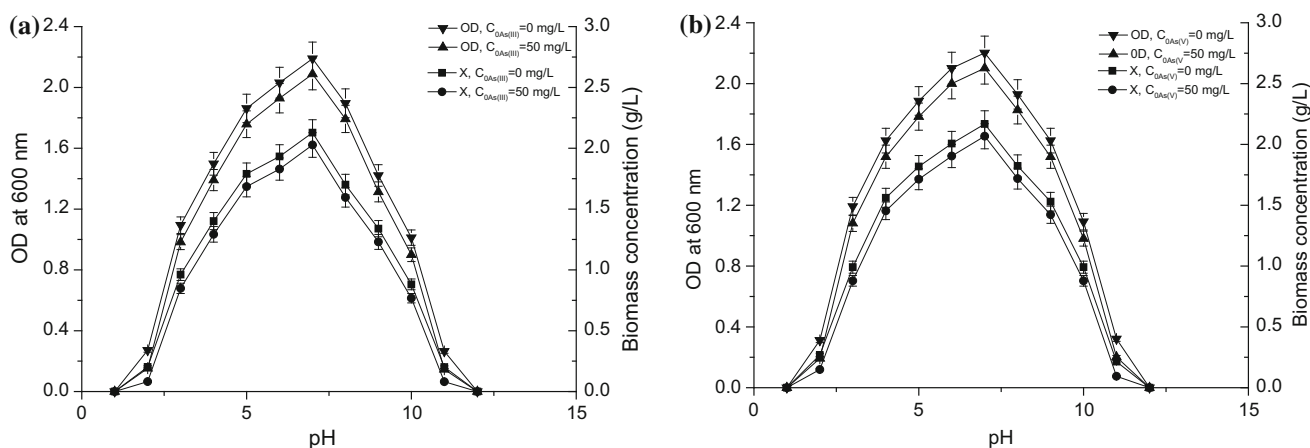


Fig. 2 Effect of pH on growth of *C. glutamicum* MTCC 2745 in absence and presence of **a** As(III) and **b** As(V) (C_0 : 50 mg/L; S_0 : 5 g/L; T : 30 °C; agitation speed: 120 rpm; incubation time: 24 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

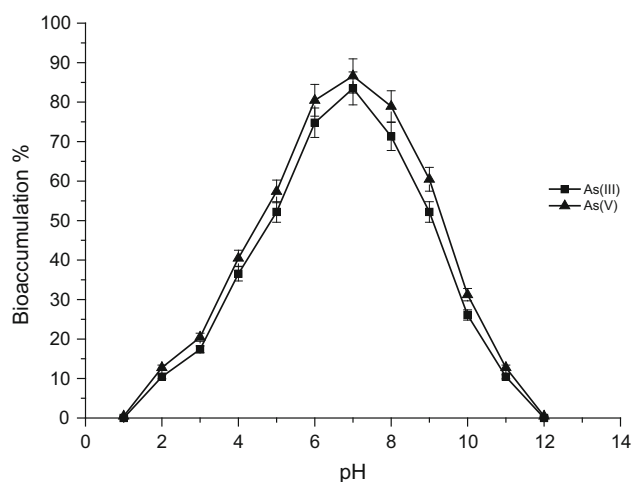


Fig. 3 Effect of initial pH on bioaccumulation of As(III) and As(V) by *C. glutamicum* MTCC 2745 (C_0 : 50 mg/L; S_0 : 5 g/L; pH: 7; T : 30 °C; agitation speed: 120 rpm, t : 10 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

As(V) ions were achieved as 83.48 and 86.62, respectively, at pH 7.0. Then, a sharp decrease in the removal was observed and As(V) showed more bioaccumulation than As(III). These results can be understandable from the following reasons.

In the current study the maximum biomass concentration *C. glutamicum* MTCC 2745 for both As(III) and As(V) was found at pH 7.0. The bacteria *C. glutamicum* MTCC 2745 used in the study stops to grow in extreme acidic and extreme alkaline conditions (Mondal et al. 2008a; Sengupta and Balomajumder 2014). So, the maximum bioaccumulation of As(III) and As(V) ions in bioaccumulation process was attained at pH 7.0. The growth of the bacterial cells reduced with the reduction in pH, which reduced the bioaccumulation % of both As(III) and As(V). At pH 4.0, the bacterial growth was less, as a

result the bioaccumulation % of both As(V) and As(III) was slightly greater than that of the consistent values achieved by physicochemical adsorption under the experimental conditions. The growth of bacteria was detected very less at pH < 3.0.

In the pH range of 2.0–9.0 and 10.0–12.0, As(III) exists mainly in neutral (H_3AsO_3) and anionic ($H_2AsO_3^-$) forms, respectively. Reports also approve that As(V) occurs mostly in the monovalent form of $H_2AsO_4^-$ in the pH range 3.0–6.0; however, at pH near 2.0, a small extent of H_3AsO_4 also remains. Whereas 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 (Ranjan et al. 2009).

Also at low pH 1.0–6.0, the density of hydrogen ion was quite high against the As(III) and As(V) ions, which instigated the protonation of the components of the microbial cell wall, i.e. cellular surface of living *C. glutamicum* MTCC 2745 biomass. The protonation of bacteria cell wall moieties affected the bioaccumulation capacity because a strong electrostatic interaction remains between positively charged cellular surface of the biomass and the oxyanions (Boddu et al. 2008; Velásquez and Dussan 2009). Comte et al. (2008) have recognized that the deprotonated form of the reactive sites in cell wall, usually amino, phosphoric and carboxylic groups, is typically liable for the metal ions binding to EPS.

Virtually no change in the bioaccumulation % was seen in the pH range of 2.0–5.0 in the case of As(III) and then removal increased sharply and achieved an extreme at pH 7.0. After that, a sharp decrease in the bioaccumulation was found. The cellular surfaces of bacterial biomass are immensely protonated in extreme acidic conditions and such a condition is not so promising for removal of As(III) because of the presence of neutral As(III) species in this range, affecting virtually no change in the degree of bioaccumulation within the pH range 2.0–5.0. With the

increase in pH of the system, the degree of protonation of the cellular surface reduces gradually. The highest bioaccumulation of As(III) was at pH 7.0 where merely non-ionic species H_3AsO_3 are dominant. The further decrease in bioaccumulation of both As(III) and As(V) with the increase in pH (7.0–12.0) may be due to the fact that, at higher pH the substrate may be negatively charged by adsorbing hydroxyl ions (OH^-) on the cellular surface or by ionization of very weak acidic functional groups of the living *C. glutamicum* MTCC 2745 biomass. A repulsive force may develop between the anions and negatively charged cellular surface (Giri et al. 2011, 2013). The negatively charged H_2AsO_3^- species starts to dominate in the alkaline medium and so the cellular surface also tends to gain negatively charge hydroxyl ions (OH^-). This trend of biosorbent surface and adsorbate species will continue to increase with the increase of pH influencing an ongoing increase in the repulsive forces between the adsorbate species and biosorbent surface instigating a decrease of biosorption (Ranjan et al. 2009).

For As(V), firstly the removal improved from pH 2.0 to 7.0, achieved an extreme at pH 7.0 and then declined with further increase in the pH up to 12.0. At pH 2.0–7.0 neutral (H_3AsO_4) and anionic (H_2AsO_4^-) species exist; however, the governing species is H_2AsO_4^- . The cellular surface of bacterial biomass is extremely protonated at low pH (2.0–4.0) and so a strong electrostatic interaction remains between positively charged cellular surface of bacterial biomass and oxyanions and as a consequence the removal enhanced in this pH range due to the increase in H_2AsO_4^- species with the increase in pH. The further decrease in bioaccumulation of As(V) with the increase in pH (7.0–12.0) may be clarified as, the cellular surface of bacterial biomass may be negatively charged by accumulating hydroxyl ions (OH^-) on the cellular surface or by ionization of very weak acidic functional groups of the cellular surface of bacterial biomass, or both at higher pH values. A repulsive force may exist between the anionic species and negatively charged cellular surface. These result in reduced As(V) removal at higher pH values (Ranjan et al. 2009).

More attraction among the As(V) ions and H^+ ions on the cellular surface of bacterial biomass may be the reason for the maximum As(V) bioaccumulation at pH 7.0 compared to As(III). Su et al. (2009) reported that the bioaccumulation of As(III) by *Escherichia coli* expressing human metallothionein was higher at 3.0, whereas Kostal et al. (2004) reported that the highest bioaccumulation of As(III) by *E. coli* expressing ArsR occurred at pH 7.4. Takeuchi et al. (2007) studied that the maximum uptake of As(III) by *Marinomonas communis* was found at neutral pH. The highest removal of As(III) by sulphate-reducing bacteria was higher at pH 2.0 reported by Teclu et al. (2008).

Effect of inoculum size on growth properties of *C. glutamicum* MTCC 2745

The volume of inoculum used for culturing the bacteria can affect the growth of *C. glutamicum* MTCC 2745 (Abusham et al. 2009). Inoculating different fermentation media, with different inoculum sizes (from 1 to 10%) of the *C. glutamicum* MTCC 2745, could affect the bacterial growth. The result indicated that bacterial growth of both As(III)-resistant bacteria and As(V)-resistant (Fig. 4a, b) were optimum when 5.0% (v/v) of bacterial inoculum was used. Increase in optical density (OD600) from OD 0.792–1.918 [for As(III)] and from OD 0.691–1.717 for As(V) were seen if the inoculum size was increased from 1 to 4% (v/v). The maximum OD [2.189 for As(III) and 2.202 for As(V)] was found for inoculum size of 5% (v/v). Drop of optical density (OD600) from OD 1.921–0.331 [for As(III)] and from OD 1.682–0.321 for As(V) were observed in bacterial growth if the inoculum size was increased from 6 to 10% (v/v). So, higher inoculum sizes do not essentially give higher growth of cell. Same trend was followed in the presence of both As(III) and As(V). A higher inoculum of 10% (v/v) was observed for decreasing the bacterial higher than if the lower inoculum size of 1% (v/v) was utilized. It can be explained by the following reasons.

At inoculum volume <5% (v/v), an improved distribution dissolves oxygen in addition to more efficient nutrient uptake also lead to a higher growth of bacteria when inoculum size is low, although optical density of cell suspension was found to be low. This may be owing to the overpopulated culture and fixed amount of nutrient with which the microorganisms begin to liberate proteolytic enzyme enhancing self-consumption (Srinath et al. 2002; Jayanthi et al. 2013). So fixed amount of inoculums were optimized and 5% (v/v) of inoculum volume gave the best result than other size of inoculum.

On the other hand, higher inoculum sizes [$>5\%$ (v/v)] could result in the lack of oxygen and depletion of nutrient in the culture media. Other researchers have stated different optimum inoculum sizes for different bacteria: 5% (v/v) for *B. subtilis* strain Rand (Abusham et al. 2009), 3% (v/v) for Z3—*Staphylococcus aureus*, KS1—*E. coli* and KHL2—*Pseudomonas aeruginosa* (Jayanthi et al. 2013). So, different microorganisms need different percentages of inoculum sizes for the highest cell growth.

Effect of inoculum size on bioaccumulation properties of *C. glutamicum* MTCC 2745

The effect of inoculum size on bioaccumulation of *C. glutamicum* MTCC 2745 was performed by changing inoculum volume in the range of 1–10% (v/v) in the growth media containing 50 mg/L of arsenic [either As(III) or

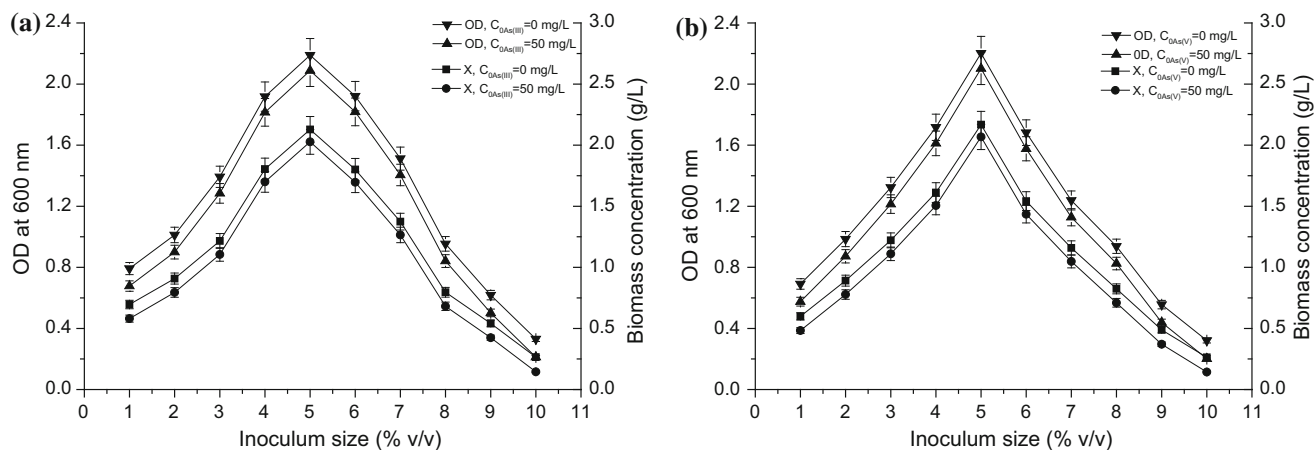


Fig. 4 Effect of inoculum size on growth of *C. glutamicum* MTCC 2745 in absence and presence of **a** As(III) and **b** As(V) (C_0 : 50 mg/L; S_0 : 5 g/L; T : 30 °C; agitation speed: 120 rpm; incubation time: 24 h)

As(V)], at an initial optimized pH value of 7.0 and temperature 30 °C. The concentration of peptone in the growth medium was kept constant at 5 g/L.

For the two arsenic species [As(III) and As(V)], virtually no change was found in the bioaccumulation % for the inoculum size in the range of 1–4% (v/v) and subsequently removal increased sharply and achieved the highest at inoculum size of 5% (v/v). After that, a sharp drop in the bioaccumulation % was found (Fig. 5).

It is observed in the above study that the biomass concentration rose while the inoculum size increased from 1 to 5% (v/v). For 1% (v/v) inoculum, the As(III) bioaccumulation % was 50.43, which was increased to 83.48 at 5% (v/v) inoculum of As(III)-resistant bacteria and then reduced to 38.26 at an inoculum size of 10% (v/v). It was supported

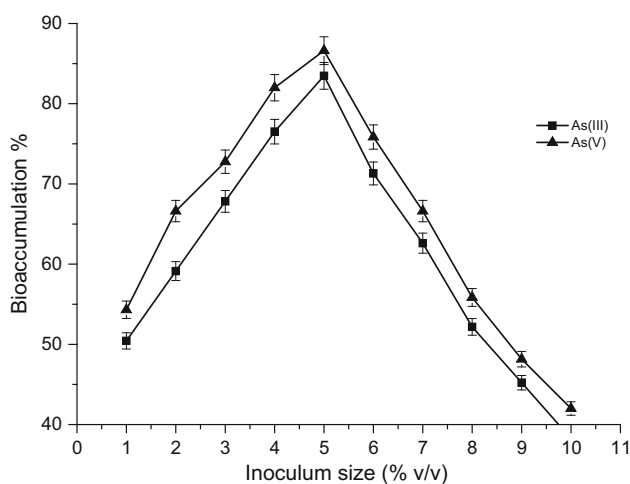


Fig. 5 Effect of inoculum size on bioaccumulation of As(III) and As(V) of *C. glutamicum* MTCC 2745 (S_0 : 5 g/L, pH 7.0; T : 30 °C; agitation speed: 120 rpm; t : 10 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

(error bars represent means \pm standard errors from the mean of duplicate experiments)

by the alteration in biomass concentration from 0.581 to 1.7 g/L from 1 to 4% (v/v) and from 1.697 to 0.145 g/L from 6 to 10% (v/v). The highest biomass concentration 2.027 g/L was found for 5% (v/v) inoculum size.

Increasing the concentration of As(V) also follows the same trend. For 1% (v/v) inoculum, the As(V) bioaccumulation % was 54.31, which was increased to 86.62 at 5% (v/v) inoculum of As(V) resistant bacteria and then decreased to 42 at an inoculum size of 10% (v/v). It was supported by the change in biomass concentration from 0.483 to 1.506 g/L from 1 to 4% (v/v) and from 1.435 to 0.143 g/L from 6 to 10% (v/v). The highest biomass concentration 2.067 g/L was found for 5% (v/v) inoculum size.

Effect of initial ion concentration on growth properties of *C. glutamicum* MTCC 2745

Bacterial growth curves in the absence and presence of increasing concentrations of arsenic [either As(III) or As(V)] (50, 100, 500, 1000, 1500 and 2000 mg/L) in the growth media, initial concentration of peptone was kept constant at 5 g/L at an initial optimized pH value of 7.0 are presented in Fig. 6a, b.

In the present case, the bacteria was previously acclimatized up to 2000 mg/L arsenic (As(III) and As(V) separately) containing growth media. Therefore, the lag phase did not increase while arsenic had been supplemented in the growth media. Presence of arsenic [As(III)/As(V)] in the growth media extended the stationary phase for the As(III) resistance bacteria and also for As(V) resistance bacteria. It is an established fact that the carbon source in the media raises the cell numbers while nitrogen source raises the cell mass of bacteria (Lengeler et al. 1999). In the current investigation, yeast extract acts as carbon source and peptone acts as nitrogen source for all the cases.

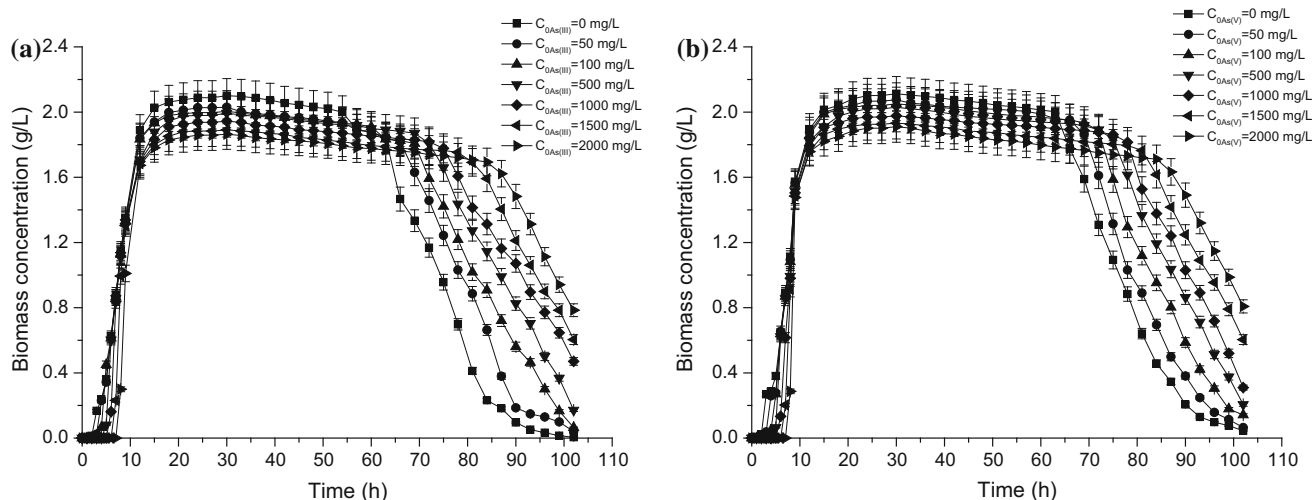


Fig. 6 Growth of *C. glutamicum* MTCC 2745 in absence and presence of increasing concentrations of **a** biomass concentration of As(III) and **b** biomass concentration of As(V) (S_0 : 5 g/L; pH: 7; T :

30 °C; agitation speed: 120 rpm; incubation time: 102 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

Furthermore, in the current study, the arsenic concentration is same as maximum arsenic resistance concentration (2000 mg/L) of the above As(III) and As(V) resistant bacteria. Owing to these reasons, the highest OD value of the media in absence as well as in presence of arsenic is almost analogous for the above two bacteria. But, the uptake rate of carbon is slower in the presence of arsenic than in the absence of arsenic. This prolongs not only the log phase, but also the stationary phase. In the current study, since the bacteria have previously been acclimatized in 2000 mg/L arsenic [As(III)/As(V)] containing media, the lag phase does not increase while arsenic is added to the growth media.

It is possible that the rate of substrate consumption in absence of arsenic [As(III)/As(V)] was high while as the arsenic [As(III)/As(V)] concentration increased the rate of substrate consumption declined and it could sustain the lag phase for longer time.

Recent research has revealed that growing bacterial cell suspensions entering the stationary phase owing to limitation of carbon and energy supply induce the synthesis of a whole series of new proteins that are not expressed in exponentially growing cells. Some of these proteins may be involved in switching on uptake systems for alternate substrates (Lengeler et al. 1999). Further, it has also been reported that under starvation, in stationary phase, bacteria tend to survive hunger phases without substantial losses in population in contrast to the fast growth under substrate excess (Lengeler et al. 1999). Similar growth pattern of *R. eutropha*, *P. putida* and *B. indicus* in NB media containing arsenic ions were reported by Mondal et al. (2008b).

In the absence of As(III) and As(V) the highest biomass concentration of As(III)-resistant and As(V) *C. glutamicum* MTCC 2745 was 2.1 and 2.113 g/L, respectively, and the

corresponding specific growth rate was 0.263 and 0.237 h^{-1} . With the increase in concentration of As(III) in the media from 50 to 2000 mg/L, maximum biomass concentration reduced from 2.03 to 1.864 g/L and the specific growth rate also reduced from 0.263 to 0.14 h^{-1} because of toxicity of media by As(III) ions. Increasing the concentration of As(V) from 50 to 2000 mg/L, also led to decreased maximum biomass concentration from 2.075 to 1.911 g/L and specific growth rate from 0.237 to 0.145 h^{-1} also because of toxicity of media by As(V) ions.

An analogous trend in bioaccumulation levels of RTBG dye and Cu(II) by *C. tropicalis* was also found by Gönen and Aksu (2009). Similar results were also observed by Mishra and Malik (2012), who reported that Cu(II), Pb(II) and Cr(III) exerted a substantial inhibitory effect on the biomass production.

Dönmez and Aksu (1999) reported that all the strains of nonadapted yeast showed an extended lag phase in the presence of Cu(II) in the preliminary investigations. An increase in concentration of Cu(II) too instigated a reduction in the amount of biomass and also a reduction in the bioaccumulated Cu(II). Furthermore, no growth of yeasts and uptake of Cu(II) were detected at higher concentrations of Cu(II) for some species. Thus they used the adapted yeasts for further bioremoval studies of Cu(II). Das et al. (2010) also reported that there was an increase in lag phase of the growth curve of nonadapted bacteria, *Pichia fermentans* MTCC 189, with the increasing concentrations of dyes.

Among inorganic arsenic species, As(III) is 60-fold more toxic than As(V), because of its higher cellular uptake (Kundu et al. 2004). So maximum biomass concentration and specific growth rate of *C. glutamicum* MTCC 2745 in the growth media is more in presence of As(V) than that of As(III).

Effect of contact time on bioaccumulation properties of *C. glutamicum* MTCC 2745

Data achieved on the effect of contact time on bioaccumulation % of As(III) and As(V) by the respective metal-resistant bacteria, exhibited rapid bioaccumulation up to contact time of between 4 and 8 h for both As(III) and As(V). Time for achieving equilibrium was 12 h (Fig. 7). The rate of uptake of metal is influenced by shaking or agitation (Gadd 1988), diffusion of metal via a hydrodynamic boundary layer around the biosorbent surface (Weber 1985) and biosorption of metal ions by active sites of the biomass. In this case of metal bioaccumulation by the metal-resistant bacteria, the conditions of shaking working possibly permitted a thorough mixing of adsorbate and biomass in the system and so the kinetic restrictions of bulk transport of arsenic ion [either As(III) or As(V)] across the cell membrane were inhibited (Odokuma and Akponah 2010). So the equilibrium was attained at 12 h (in less than 24 h). The results of the effect of contact time on bioaccumulation % specifies that the respective bacteria had an optimum residence time for As(III) and As(V) and when this time passed, bioaccumulation % continued either constant or diminished slightly.

This favours with the models of metal uptake, where the process can be taken as an equilibrium which includes adsorption and desorption because of saturation (Panchanadikar 1994). Su et al. (2009) reported that the bioaccumulation of As(III) in recombinant *E. coli* expressing human metallothionein increased rapidly to attain 70% of the saturated value within 1 h and attained 93% of the saturated value at 2 h. They also reported that the recombinant cells are employed in the bioremoval of

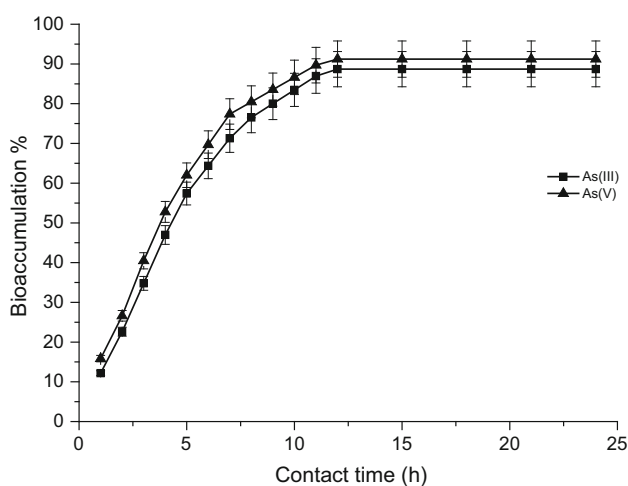


Fig. 7 Effect of contact time on bioaccumulation of As(III) and As(V) of *C. glutamicum* MTCC 2745 (S_0 : 5 g/L, pH 7.0; T : 30 °C; agitation speed: 120 rpm) (error bars represent means \pm standard errors from the mean of duplicate experiments)

arsenic, which can decrease the time length to a great extent in As(III) removing and the rapid bioaccumulation speed makes the bioremediation process more practical. Maximum bioaccumulation of As(III) at 1 h by *E. coli* expressing ArsR was reported by Kostal et al. (2004). The maximum uptake of As(III) by *M. communis* were at 24 h of exposure (Takeuchi et al. 2007) and that by sulphate-reducing bacteria was 2–3 days of exposure (Teclu et al. 2008).

Effect of temperature on growth properties of *C. glutamicum* MTCC 2745

Experiments were carried out in batch reactor to investigate the effect of temperature on the growth of *C. glutamicum* MTCC 2745 in pure media and synthetic wastewater. Various temperatures considered in the present study are 20, 25, 30, 35, 40, 45, and 50 °C. The other parameters were kept constant. Figure 8a, b represents the effect of temperature on the growth of bacteria *C. glutamicum* MTCC 2745.

The results specified that the bacterial growth of both As(III)-resistant bacteria and As(V)-resistant bacteria were optimum at 30 °C temperature. Increase in the optical density (OD_{600}) from 1.244 to 1.734 (for As(III)-resistant *C. glutamicum* MTCC 2745) and from 1.292 to 1.794 (for As(V)-resistant *C. glutamicum* MTCC 2745) were seen if the temperature was increased from 20 to at 25 °C. The maximum OD [2.189 for As(III) and 2.202 for As(V)] was found at 30 °C temperature. Drop of optical density (OD_{600}) from 1.726 to 0.943 [for As(III)] and from 1.776 to 0.968 for As(V) were observed in the bacterial growth if the temperature was increased from 35 to 50 °C. So, higher temperature does not essentially give higher growth of cell. Same trend was followed in presence of both As(III) and As(V).

The temperature has main influences on the growth of bacteria. Shivaji et al. (2005) also reported the favourable temperature for the growth of *B. arsenicus* MTCC 4380 was 30 °C. It can also be explained by the fact that when the incubation temperature was high, the temperature did not favour cell multiplication, and led towards less biomass production as compared to optimum temperature, i.e. 30 °C (Sarwat et al. 2008).

Effect of temperature on bioaccumulation of *C. glutamicum* MTCC 2745

Experiments were carried out in a batch reactor to investigate the effect of temperature on the removal of arsenic [As(III)/As(V)] from synthetic wastewater. Various values of temperature considered in the present study were 20, 25, 30, 35, 40, 45, and 50 °C. The other parameters were kept

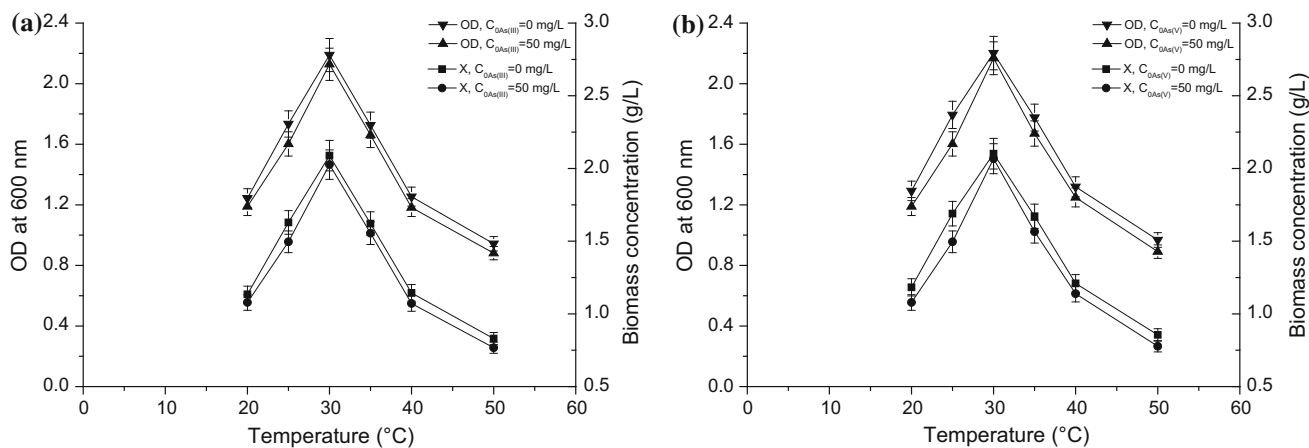


Fig. 8 Effect of temperature on growth of *C. glutamicum* MTCC 2745 in absence and presence of **a** As(III) and **b** As(V) (C_0 : 50 mg/L; S_0 : 5 g/L; agitation speed: 120 rpm; incubation time: 24 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

constant. The effect of temperature on the % removal of arsenic [As(III)/As(V)] by bioaccumulation of As(III)-resistant and As(V)-resistant *C. glutamicum* MTCC 2745 is shown in Fig. 9.

The temperature has important effects on bioaccumulation process. It is found in the above study that the biomass concentration increased when the temperature increased from 20 to 30 °C. For 20 °C temperature, As(III) bioaccumulation % was 85.217, which was increased to 88.696 at a temperature of 30 °C for As(III)-resistant bacteria and then reduced to 67.826 at a temperature of 50 °C. It was supported by the change in the biomass concentration from temperature 20 to 50 °C. The highest biomass concentration of 5.73858 g/L was found at 30 °C temperature.

Increasing the concentration of As(V), also followed the same trend. For 20 °C temperature, the As(V) bioaccumulation % was 86.615, which was increased to 91.231 at a

temperature of 30 °C for As(V)-resistant bacteria and then decreased to 71.231 at a temperature of 50 °C. It was also supported by the change in biomass concentration. The highest biomass concentration 5.739 g/L was found at 30 °C temperature.

It can also explained by the cellular metabolic activity. Over the range investigated (20–50 °C), temperature-related influences did not appear to be particularly noticeable. Accumulation processes that depend on cellular metabolism, like active uptake, would be those that are the most expected to be inhibited by low temperatures, while high temperatures could disturb the integrity of the cell membranes and obstruct the compartmentalization of arsenic ions, also resulting in the reduced uptake levels (Brady and Duncan 1994).

Effect of initial As(III) and As(V) concentrations on bioaccumulation properties of *C. glutamicum* MTCC 2745

The effect of initial metal ion concentration on bioaccumulation of *C. glutamicum* MTCC 2745 was performed by changing arsenic [either As(III) or As(V)] concentrations (50, 100, 200, 500, 800, 1000, 1200, 1500, 1800 and 2000 mg/L) in the growth media, initial peptone concentration was kept constant at 5 g/L at an initial optimized pH value of 7.0.

Bioaccumulation % and bioaccumulation level of the As(III) and As(V) by the bacterial species at different initial arsenic [either As(III) or As(V)] concentrations were determined as given in Table 2. For all the two arsenic species [As(III) and As(V)], bioaccumulation % in *C. glutamicum* MTCC 2745 reduced as the arsenic species concentration increased from 50 to 2000 mg/L, presented in Fig. 10.

It is observed in the above research that the biomass concentration decreased while the amount of

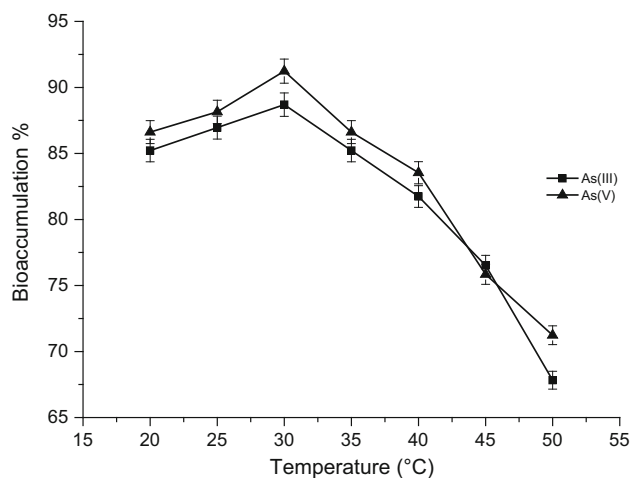


Fig. 9 Effect of temperature on bioaccumulation of As(III) and As(V) of *C. glutamicum* MTCC 2745 (S_0 : 5 g/L, pH 7.0; t : 12 h; agitation speed: 120 rpm) (error bars represent means \pm standard errors from the mean of duplicate experiments)

bioaccumulated arsenic species [either As(III) or As(V)] increased from 50 to 2000 mg/L. In the presence of 50 mg/L of As(III), the bioaccumulation % was 88.7, which was reduced to 53.25 at 2000 mg/L of As(III). It was supported by the reduction in biomass concentration from 2.1 to 1.864 g/L and the specific growth rate from 0.263 to 0.14 h⁻¹. The reduction in bioaccumulation % was also caused because of the toxicity of arsenic at higher concentrations which decreased the biomass concentration. Altering the concentration from 50 to 2000 mg/L resulted in increased bioaccumulation levels from 44.35 to 1065 mg/L. An analogous trend in bioaccumulation levels of RTBG dye and Cu(II) by *Candida tropicalis* was also found by Gönen and Aksu (2009). It was also reported by Das et al. (2011) for bioaccumulation of the synthetic dye Basic Violet 3 and heavy metals in single and binary systems by *C. tropicalis*.

Increasing the concentration of As(V), also led to decreased bioaccumulation %. The bioaccumulation % reduced from 91.23 to 55.01 while the concentration of As(V) also increased from 50 to 2000 mg/L. This consequence was supported by a decrease in the biomass concentration from 2.113 to 1.911 g/L and specific growth rate from 0.237 to 0.145 h⁻¹. Varying the concentration of As(V) from 50 to 2000 mg/L also resulted in increased bioaccumulation levels from 45.62 to 1100.35 mg/L.

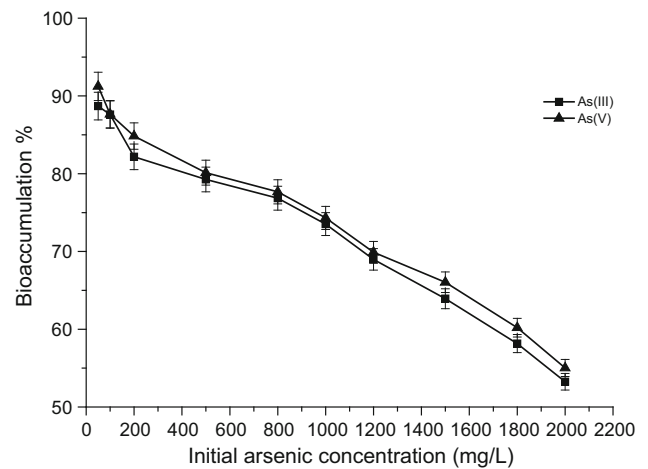


Fig. 10 Effect of initial concentration on bioaccumulation of As(III) and As(V) of *C. glutamicum* MTCC 2745 (S_0 : 5 g/L, pH 7.0; T : 30 °C; agitation speed: 120 rpm; t : 12 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

Effect of initial peptone concentration on growth of *C. glutamicum* MTCC 2745

Peptone comprises carbon, nitrogen and other growth factors which are vital for bacterial growth. Conversely peptone, a mixture of protein degradation products, is a preferred carbon source for fungal growth (Yan et al.

Table 2 Comparison of As(III) and As(V) bioaccumulation, at different levels of As(III) and As(V) concentration

Arsenic species	C_0 (mg/L)	Bioaccumulation (%)	Arsenic conc. (mg/L)
As(III)	0	–	–
	50	88.70	44.35
	100	87.61	87.61
	200	82.17	164.35
	500	79.26	396.30
	800	76.85	614.78
	1000	73.52	735.22
	1200	68.99	827.83
	1500	63.93	958.91
	1800	58.15	1046.74
	2000	53.25	1065.00
As(V)	0	–	–
	50	91.23	45.62
	100	87.65	87.65
	200	84.85	169.69
	500	80.13	400.65
	800	77.67	621.38
	1000	74.32	743.15
	1200	69.9	838.77
	1500	66.04	990.65
	1800	60.20	1083.58
	2000	55.017	1100.35

2012). It was reported that organic nitrogen peptone usually have stimulating effects (Hewitt and Solomons 1996; Hamilton et al. 1999).

In this study, peptone was utilized in higher amount compared to other media components (yeast extract and beef extract). Thus the effect of initial peptone concentration on growth rate of *C. glutamicum* MTCC 2745 in arsenic-free media was examined in the peptone concentration range of 1.0–9.0 g/L, at pH 7.0 and at a temperature of 30 °C. As given in Fig. 11a, b, the relationship of specific growth rate to peptone concentration for As(III)-resistant *C. glutamicum* MTCC 2745 and As(V)-resistant *C. glutamicum* MTCC 2745, respectively, often adopts the saturation kinetics form. It was observed that both biomass concentration and specific growth rate increased with increasing initial peptone concentration from 1.0 to 9.0 g/L. Any inhibition of substrate was not acquired in the concentration interval inspected. In the absence of either As(III) or As(V) ions, the specific growth rate improved from 0.197 to 0.371 h⁻¹ and 0.16 to 0.371, respectively, with an increase initial peptone concentration from 1 to 9 g/L. Biomass concentration of As(III) and As(V) resistant *C. glutamicum* MTCC 2745 also increased from 2.068 to 2.078 g/L and from 6.202 to 2.121 g/L, respectively.

Combined effect of initial peptone concentration and initial arsenic concentration on growth of *C. glutamicum* MTCC 2745

This time the growth behaviour of *C. glutamicum* MTCC 2745 was examined at an initial pH value of 7.0 and at 30 °C temperature at rising initial peptone concentration both in the absence and presence of a constant either As(III) or As(V) level. When initial peptone concentration was varied from 1 to 9 g/L, initial arsenic [either As(III) or

As(V)] concentration was kept constant between 50 and 2000 mg/L for each experiment set. Comparison of biomass concentration and specific growth rate at different concentrations of arsenic [either As(III) or As(V)] ions with rising peptone concentration is shown in Table 3. Figure 12a, b shows the effect of initial peptone concentration on specific growth rate of *C. glutamicum* MTCC 2745 at different levels of As(III) and As(V) ions, respectively.

When peptone concentration was held constant and initial arsenic [As(III)/As(V)] concentration was varied from 50 to 2000 mg/L, growth rate of bacteria was decreased. It was found from the study that the maximum biomass concentration and the specific growth rate increased while the concentration of peptone increased from 1 to 9 mg/L at a constant initial arsenic concentration [As(III)/As(V)].

The presence of arsenic in the growth media suppressed the growth of the bacteria irreversibly and the effect of inhibition increased with the arsenic concentrations for all initial peptone concentration. The increase in growth rate of *C. glutamicum* MTCC 2745 with rising initial concentration of peptone at constant arsenic concentration could be because of cell defence mechanisms, for example acclimation to toxicity. Analogous results of noteworthy reduction in specific growth rate with rising Remazol blue concentration were too described in case of *C. tropicalis* (Aksu and Dönmez 2005). Gönen and Aksu (2009) also described that the increase in concentration of sugar caused an increase in concentration of cell and as well in specific growth rate. It is obvious from the results of the current research that the initial peptone concentration played a main role in the bacterial growth and reduced the inhibitory effects of arsenic on the bacterial growth. Furthermore, the residual peptone concentration in the media was observed

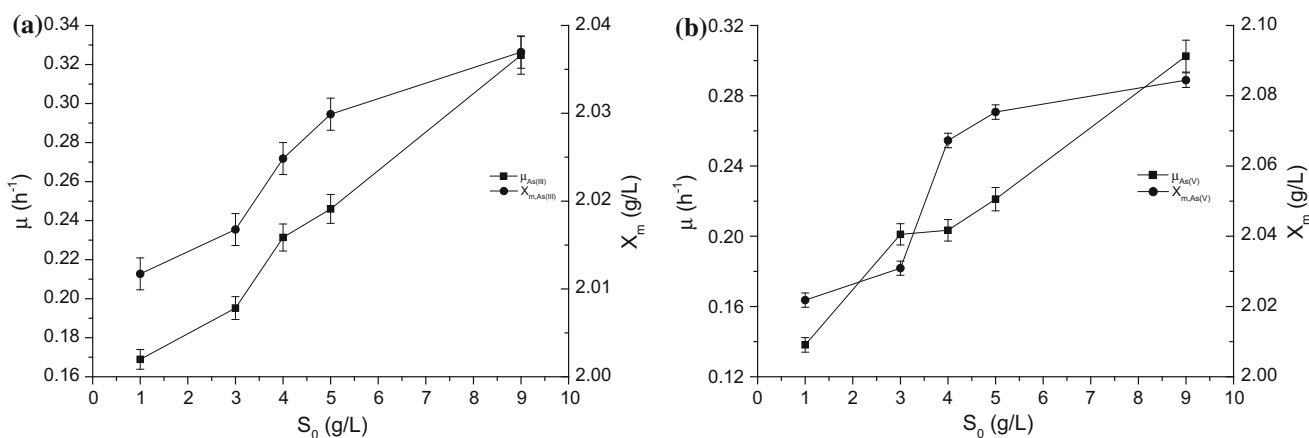


Fig. 11 Effect of initial peptone concentration on the specific growth rate and biomass concentration of **a** As(III)-resistant *C. glutamicum* MTCC 2745 and **b** As(V)-resistant *C. glutamicum* MTCC 2745 (pH

7.0; T : 30 °C; agitation speed: 120 rpm; incubation time: 102 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

Table 3 Comparison of specific growth rate (μ) and maximum biomass concentration (X_m) at different levels of initial peptone concentration (S_0) in absence and presence of either As(III) or As(V) concentration

	As(III)				As(V)			
	S_0 (g/L)	$C_{0As(III)}$ (mg/L)	μ (h^{-1})	X_m (g/L)	S_0 (g/L)	$C_{0As(V)}$ (mg/L)	μ (h^{-1})	X_m (g/L)
1	0	0	0.197	2.068	1	0	0.16	2.078
3	0	0	0.221	2.077	3	0	0.218	2.086
4	0	0	0.256	2.095	4	0	0.228	2.102
5	0	0	0.263	2.10	5	0	0.237	2.113
9	0	0	0.371	2.115	9	0	0.371	2.121
1	50	50	0.169	2.012	1	50	0.138	2.022
3	50	50	0.195	2.017	3	50	0.201	2.031
4	50	50	0.231	2.025	4	50	0.203	2.067
5	50	50	0.246	2.03	5	50	0.221	2.075
9	50	50	0.325	2.037	9	50	0.302	2.084
1	500	500	0.102	1.966	1	500	0.107	2
3	500	500	0.182	1.972	3	500	0.169	2.013
4	500	500	0.184	1.981	4	500	0.183	2.023
5	500	500	0.2	1.994	5	500	0.215	2.029
9	500	500	0.264	2.005	9	500	0.299	2.038
1	1000	1000	0.074	1.913	1	1000	0.086	1.95
3	1000	1000	0.155	1.926	3	1000	0.17	1.957
4	1000	1000	0.165	1.935	4	1000	0.175	1.963
5	1000	1000	0.165	1.944	5	1000	0.179	1.977
9	1000	1000	0.247	1.954	9	1000	0.294	1.985
1	1500	1500	0.058	1.871	1	1500	0.065	1.915
3	1500	1500	0.131	1.878	3	1500	0.143	1.919
4	1500	1500	0.142	1.884	4	1500	0.157	1.925
5	1500	1500	0.15	1.891	5	1500	0.16	1.932
9	1500	1500	0.234	1.954	9	1500	0.282	1.94
1	2000	2000	0.046	1.841	1	2000	0.047	1.872
3	2000	2000	0.107	1.851	3	2000	0.1	1.88
4	2000	2000	0.116	1.856	4	2000	0.109	1.895
5	2000	2000	0.14	1.864	5	2000	0.145	1.911
9	2000	2000	0.226	1.872	9	2000	0.278	1.92

to be very low which would not cause any discarding problem.

Combined effects of peptone and initial arsenic concentration on bioaccumulation properties of *C. glutamicum* MTCC 2745

The bioaccumulation of both As(III) and As(V) by *C. glutamicum* MTCC 2745 was examined separately at the an optimal pH of 7.0 at 30 °C in the growth media at an optimized contact time of 12 h. Initial concentration of peptone was varied from 1 to 9 g/L keeping the initial arsenic concentration constant between 1000 and 2000 mg/L. Combined effects of initial concentrations of peptone and arsenic [either As(III) or As(V)] ions on bioaccumulation properties of *C. glutamicum* MTCC 2745 are revealed in Fig. 13.

The amount of bioaccumulated arsenic [either As(III) or As(V)] increased from 1000 to 2000 mg/L, while the biomass concentration and specific growth rate exhibited a declining trend, indicating that media toxicity increased with increasing arsenic concentration. However, the increased arsenic concentrations at constant peptone concentration decreased the biomass concentration, specific growth rate and bioaccumulation %.

Since the bioaccumulation is dependent on bacterial growth, the increase in peptone concentration at constant arsenic concentration increased the biomass concentration, specific growth rate and bioaccumulation %. The bioaccumulation % increased from 69.87 to 78.39 while the concentration of peptone increased from 1 to 9 mg/L at an initial As(III) concentration of 1000 mg/L. Furthermore, it was found from the above study that the maximum biomass concentration increased from 1.913 to

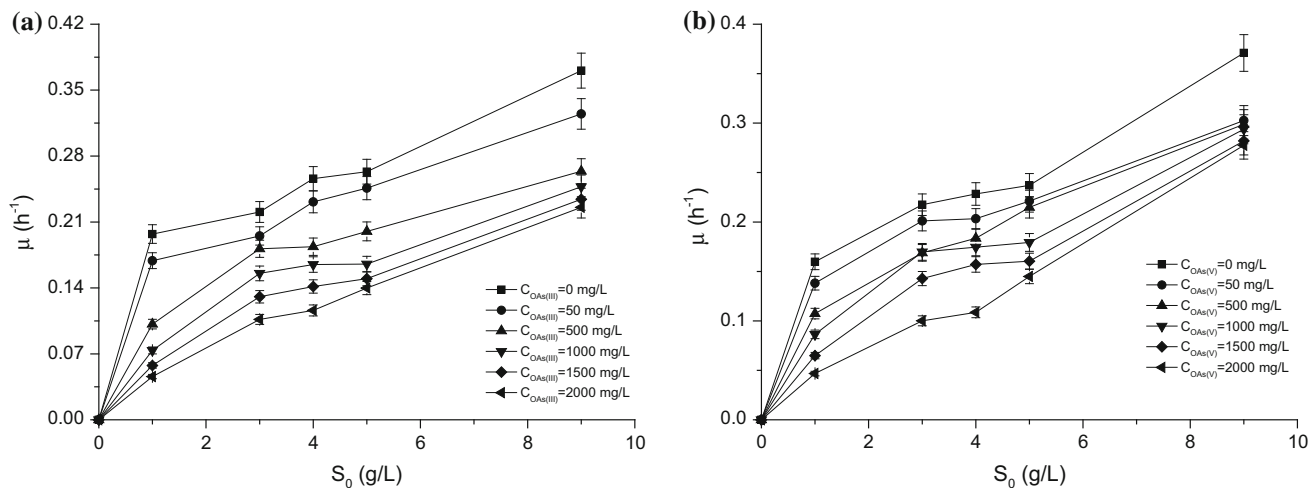


Fig. 12 Effect of initial peptone concentration on the specific growth rate at different levels of initial concentration of **a** As(III) and **b** As(V) (pH: 7.0; T : 30 °C; agitation speed: 120 rpm; incubation

time: 102 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

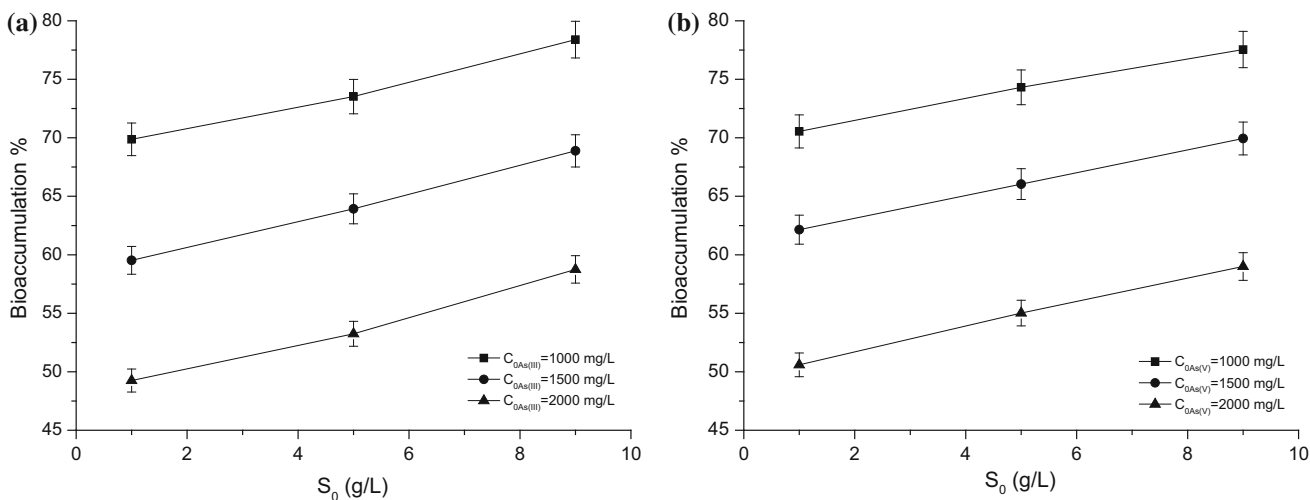


Fig. 13 Effect of initial peptone concentration on bioaccumulation % at different levels of initial concentration of **a** As(III) and **b** As(V) (pH: 7.0; T : 30 °C; agitation speed: 120 rpm; incubation

time: 12 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

1.954 g/L, and the specific growth rate increased from 0.074 to 0.247 h^{-1} .

In the presence of 1 g/L of peptone, the bioaccumulation % was 70.55, which increased to 77.55 at 9 g/L of peptone while initial As(V) concentration was kept constant at 1000 mg/L. Also it was observed from the above study that the maximum biomass concentration increased from 1.95 to 1.985 g/L and the specific growth rate increased from 0.086 to 0.294 h^{-1} .

An analogous trend was also found for bioaccumulation of both As(III) and As(V) at a constant initial concentration of 1500 and 2000 mg/L.

Inhibition models

Determination of kinetic constants

Monod equation can be linearized in double reciprocal form. A plot of $1/\mu$ versus $1/S$ yields a linear line with a y-axis intercept of $1/\mu_m$ and a slope of K_S/μ_m and. The highest specific growth rate (μ_m) and the saturation constant (K_S) of As(III) resistant bacteria and As(V) resistant bacteria in arsenic [either As(III) or As(V)] free media were determined as 0.309 h^{-1} and 0.633 g/L and 0.312 h^{-1} and 1.004 g/L, respectively, from the double

reciprocal form of Monod equation by linear regression method. As the Monod expression for the growth kinetics did not indicate the inhibitory effects of toxic metal ions, competitive inhibition model, noncompetitive model and uncompetitive model were verified for characterizing the arsenic inhibition kinetics of both As(III)-resistant and As(V)-resistant *C. glutamicum* MTCC 2745. Thus main objective of this research was: (1) to find the inhibition type of As(III) ions on the growth of As(III) *C. glutamicum* MTCC 2745 when the initial concentrations of As(III) ions were increased from 50 to 2000 mg/L (Fig. 14a) and (2) to find the inhibition type of As(V) ions on the growth of As(V) *C. glutamicum* MTCC 2745 when the initial concentrations of the As(V) ions were also varied between 50 and 2000 mg/L (Fig. 14b). The μ_m values could be estimated from the intercept of linear Lineweaver–Burk plots of $1/\mu$ versus $1/S$ at different initial arsenic concentrations [either As(III) or As(V)] (Fig. 14a, b). The maximum specific growth rates (μ_m) and the saturation constants (K_S) in the presence of increasing concentrations of either As(III) or As(V) ions are compared with those acquired in the arsenic-free media in Table 4. The net effect of competitive inhibition is an increased value of K'_S ,app and so reduced reaction rate. K'_S ,app values were evaluated from the K_S values (Eq. (5)) and they exposed an increasing trend at higher concentration levels, indicating that the competitive inhibitory model was more encouraging compared to the noncompetitive and uncompetitive inhibitory models (Açikel and Alp 2009). In the noncompetitive and uncompetitive inhibition models, the K_S values should have reduced (Açikel and Alp 2009; Das et al. 2010, 2011), which was not preferred in the current investigation.

The maximum specific growth rate μ_m appeared to remain constant approximately at 0.298 h^{-1} for As(III) and 0.308 h^{-1} for As(V) and this value was almost similar with the maximum specific growth rate acquired in arsenic-free growth medium, when the Monod constants (K_S) increased in the media containing the arsenic ions at increasing concentrations. The existence of As(III) and As(V) ions separately seems to have a competitive inhibition effect on the growth of As(III)-resistant and As(V)-resistant *C. glutamicum* MTCC 2745. Figure 14a, b also confirms competitive metal inhibition in the form of double reciprocal plots of Monod equation.

Determination of inhibition constants

The inhibition effect of As(III) and As(V) ions on the specific growth rate of *C. glutamicum* MTCC 2745 was inspected by the Eqs. (4–14) derived for competitive, noncompetitive and uncompetitive inhibition. The inhibition constants K_I for As(III) and As(V) ions from experimental specific growth rate data were evaluated as shown in Table 5. The competitive inhibition model defined the experimental specific growth rate data of As(III)-resistant and As(V)-resistant *C. glutamicum* MTCC 2745 in the presence of As(III) and As(V) ions separately better than the noncompetitive and uncompetitive inhibition models. Consistent with the competitive inhibition model, the apparent saturation constant (K'_S ,app) value in the presence of arsenic ions increases with regard to intrinsic Monod constant; however, the maximum specific growth rate (μ_m) remains constant (Shuler and Kargi 1992; Berg et al. 2006; Nelson and Cox 2008). In the current research, the almost

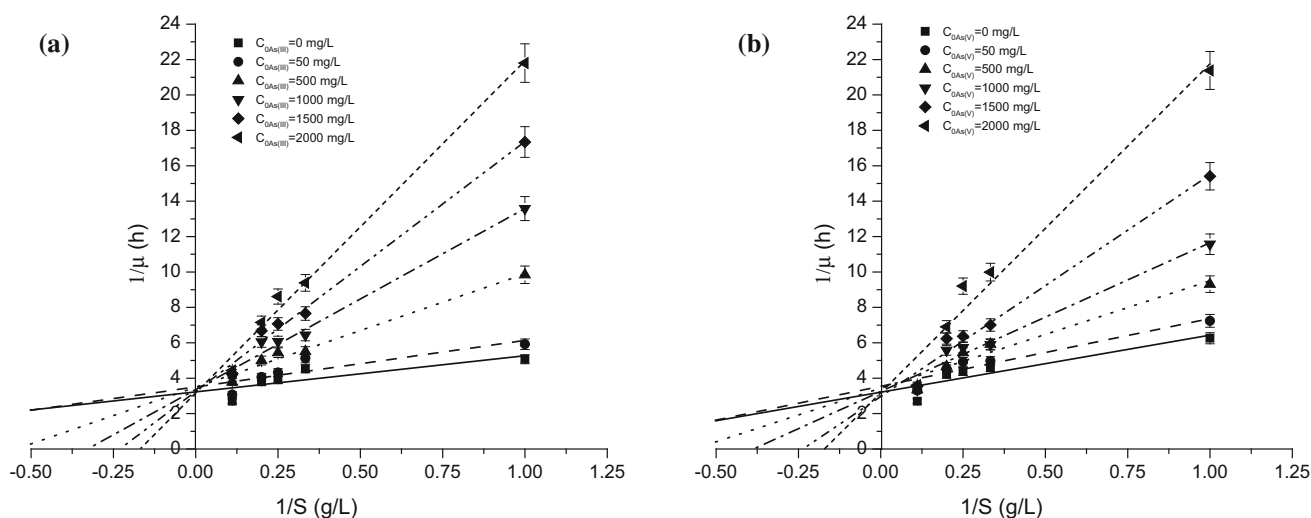


Fig. 14 Lineweaver–Burk plots for the growth of *C. glutamicum* MTCC 2745 obtained in the metal-free media and in the presence of increasing concentrations of **a** As(III) and **b** As(V) ions in the range of

1000–2000 mg/L (pH: 7.0; T : 30 °C; agitation speed: 120 rpm; incubation time: 102 h) (error bars represent means \pm standard errors from the mean of duplicate experiments)

Table 4 Comparison of the maximum specific growth rates and the saturation constants acquired in the arsenic-free growth media and in the presence of increasing concentrations of either arsenic [either As(III) or As(V)] ions

Arsenic species	Concentration of the metal ions, C_0 (mg/L)	Competitive inhibition model parameters	
		Maximum specific growth rate, μ_m (h^{-1})	Monod constants, $K_{S,\text{app}}$ (g/L)
As(III)	50	0.285	0.749
	500	0.286	1.834
	1000	0.298	3.042
	1500	0.307	4.339
	2000	0.316	5.926
As(V)	50	0.282	1.079
	500	0.291	1.7633
	1000	0.308	2.586
	1500	0.337	4.223
	2000	0.315	5.846

Table 5 Comparison of the inhibition constants obtained in the presence of increasing concentrations of arsenic [either As(III) or As(V)] ions

Arsenic species	Concentration of the metal ions, C_0 (mg/L)	Competitive inhibition model parameter Inhibition constants, K_I (mg/L)
As(III)	0	0
	50	272.856
	500	263.623
	1000	262.713
	1500	256.199
As(V)	2000	239.167
	0	0
	50	672.937
	500	661.474
	1000	634.7
	1500	467.712
	2000	414.693

constant value of the maximum specific growth rate (μ_m) and the increasing values of the saturation constant ($K'_{S,\text{app}}$) were also confirmed with the linearization of the Monod model in the absence and presence of inhibitors. Consistent with competitive inhibition model, the smaller the value of K_I , the potent is the inhibition (Berg et al. 2006). At higher concentration levels, the inhibition constants K_I reduced which specified higher toxicity. The inhibition constant (K_I) value was also evaluated from assessed $K'_{S,\text{app}}$ and known initial arsenic ion concentrations values. The inhibition constants for As(III) and As(V) ions decreased from

272.856 to 239.167 mg As(III)/L and from 672.937 to 414.693 mg As(V)/L with increasing arsenic ion concentrations from 50 to 2000 mg/L in both cases. So from the experimental data, it is agreed that As(III) is more toxic than As(V).

Conclusions

The acquired results identify that the living *C. glutamicum* MTCC 2745 was proficient in bioaccumulating As(III) and As(V) using peptone as the main source of carbon and nitrogen in the growth medium in a batch system. Also, bioaccumulation % of arsenic species [both As(III) and As(V)] could also be increased with increasing concentration of peptone which reduced the inhibitory effects of arsenic on growth of bacteria. Inhibition kinetics showed that both As(III) is more toxic than As(V). Experiments related to the optimization show that the bacteria *C. glutamicum* MTCC 2745 can be grown easily in lab and at industry level due to survival in broad range of pH and temperatures.

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