Bioaccumulation of As(III)/As(V) ions by living cells of Corynebacterium glutamicum MTCC 2745

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ABSTRACT
Significant factors on simultaneous growth and bioaccumulation of arsenic ions by living cells of bacteria, Corynebacterium glutamicum MTCC 2745, were explored in growth media under experimental conditions like pH and concentrations of arsenic ions. Combined effects of the initial concentrations of peptone and arsenic (either As(III) or As(V)) ions on the specific growth rate and arsenic bioaccumulation competence of the bacteria were studied and optimized using the Response Surface Methodology. Optimum combination predicted via RSM demonstrated that the bacteria were capable of bioaccumulating As(III) and As(V) in the growth medium containing 1000 mg/L arsenic and 9 g/L peptone up to 78.4% and 77.6%, respectively.

Introduction
Arsenic is a ubiquitous element in the environment and has been established as a group 1 carcinogen. Arsenic is mobilized in natural water through a combination of natural activities such as volcanic emissions, weathering of arsenic bearing minerals, and biological activity along with a variety of anthropogenic activities such as petroleum refining, manufacturing processes, glass melting, gold mining, non-ferrous smelting, electricity generation, and the use of arsenical herbicides and pesticides and combustion of fossil fuel.

The most common arsenic species in water are inorganic forms: arsenite (As(III) as $\text{H}_3\text{AsO}_3^-$ and $\text{H}_2\text{AsO}_3^-$) and arsenate (As(V) as $\text{H}_2\text{AsO}_4^-$ and $\text{H}_2\text{AsO}_4^{2-}$). Long-term exposures to arsenic levels can result in permanent and severe damage to human health. Toxicity of arsenic causes the damage in mucous membranes, skin lesions, gastrointestinal, mutagenic, genotoxic, cardiovascular and carcinogenic effects, and finally death. So, there is a growing worldwide public concern about the pollution of arsenic in water and its health hazard on humans.

On the basis of investigation of the fatal effect of arsenic on the 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 and the European Commission in 2003. So, there are necessities of inexpensive materials and techniques for removing arsenic from water.

Common methods for removing arsenic from aqueous solution include oxidation, reverse osmosis, ion exchange, membrane filtration, oxidation/precipitation, Fe-electrocoagulation/co-precipitation, alum coagulation/precipitation, and adsorption, but most of them are not widely accepted. However, these techniques are associated with various drawbacks such as high capital and operational cost, unpredictable metal ion removal and the production of toxic residual metal sludge that is frequently harder to manage and are not appropriate for small scale industries. The sensible choice of the most suitable replacements should be on the basis of cost-benefit analysis done for each one of the probable removal methods. Bioremediation can be performed to remove metals from contaminated or wastewater and to trap metals from soil and sediments.

Bioremoval of metal ions generally falls into three varieties: biosorption of metal ions on the surface of microorganisms, chemical transformation of metal ions, and intracellular uptake of metal ions by microorganisms. The first process needs non-living, non-growing biomass or biomass products and are termed as biosorption and the latter two processes require viable and living microorganisms and are termed as bioaccumulation.
(biosorption), the microorganisms generally sequester metal ions via surface bonding only; with active biomass (bioaccumulation), metals are concentrated through a combination of surface reactions, intra and extracellular complexation reactions as well as intra and extracellular precipitation.[25]

In the second process, bioaccumulation, removed metal ions are partly bound to the cellular surface in the first passive stage, which is the same as biosorption and then are partly transferred (generally by an active transport system which requires metabolic activity and expenditures of extra cellular energy) into the cellular interior.[26] So, a part of metal ions are bound with the cellular surface and the remaining stays inside the cell. The metal accumulation phenomenon in the microorganisms will improve the toxicity of metal and sequentially decreases the microbial growth. In bioaccumulation processes, metal ions accumulate inside the cell for usage in some metabolic cycles and essential cellular activities.[27] So, more fast and effective metal uptake is achieved by growing cells than by dead cells.[28]

Since in the bioaccumulation process simultaneous growth of bioaccumulators occurs and separate biomass production can be avoided, the use of growing or living cultures in metal ion binding allows for simplifying the configuration of an installation.[29,30] So, using growing or living cultures in bioirrigation could avoid the necessity for a separate biomass production process such as cultivation, harvesting, drying, processing, and storage before use, but the applications of these processes are restricted by the requirements for maintaining growth of microbial cells.[29,31]

The efficiency of these methods usually depends on the parameters such as the oxygen level, toxicity, temperature, availability of nutrients etc.[31] However, the sensitivity of living cells to extremes of pH or high salt concentration or high metal concentration and requirement to supply metabolic energy are some of the major limitations of using growing cells for bioremediation.[25,30,32] The current investigations exhibit that the super-resistant strains (bacteria, yeast, and fungi) isolated from contaminated sites possess excellent competence of metal removal, which have, to an extent, eliminated the primary obstacle for use of growing cells. Some bacterial strains possess high tolerance to several metals and may be probable candidates for their simultaneous removal from waste. Evidently, the stage has already been established for the use of metal resistant growing microbial cells for metal removal.[32] The vital characteristics of a living biomass used in a metal ion removal process are tolerance and uptake capacities.[23,25] Genetic engineering may further improve the prospective of robust environmental strains. The living cells however have the potential for generic recombination or mutant isolation to change morphological and physiological features and also to improve the metal accumulative strain.[23,25,32,33] The efforts to meet such challenges via isolation of metal resistant bacterial/fungal strains and exploitation of organic waste as carbon substrates have already initiated.[32] However, the fact that several traditional sewage treatment processes are based on living microorganisms recommends that such restriction could not stop their uses in treatment systems regarding the bioremoval of heavy metals. If the problem of toxicity of metal to the living cells of microorganism is overcome by the use of metal resistant microorganisms, the continually self-replenishing system can be left to run repeatedly for extended periods.[25,31]

The conventional method of studying one variable at a time (OVAT) is recognized for investigating the effects of variables on the response. However this method is not only work as well as time challenging mostly for multivariable systems, but also is fully ineffective to demonstrate the effect of interaction between various factors especially while greater than one response is taken into account. So, an alternative approach including statistical method such as the response surface methodology (RSM) and factorial experimental design should be implemented for solving this difficulty tangled in wastewater treatment.[34] The RSM can be used as a stimulating scheme for implementing process parameters which lead to optimal response by carrying out the lowest number of experiments. The statistical design of experiments considers interactions among the variables and can be employed to optimize the operating parameters in multivariable systems. RSM is a combination of mathematical and statistical techniques used to develop, improve and optimize the processes and it can be employed to estimate the relative meaning of multiple influencing parameters also in the presence of complex interactions.[35,36] This methodology can be used to develop appropriate treatment technology especially to exhibit the effects of working conditions on the removal process otherwise for determining an area that fulfils the operating specifications.[37,38]

Currently, various research studies described the use of RSM to optimize parameters such as pH, concentration of contaminant and bioaccumulation of metals[35,39,40] and dyes from synthetic solutions[37]; however few investigations have described for optimizing media components to remove metal/dye[30,35,36,40,41] Very little information is reported on the use of RSM to identify and optimize the effect of concentration of substrate and toxic component on the specific growth rate and the toxic component bioaccumulation efficiency of microorganisms.[30,36]
There is the insufficiency of information on metal anions bioaccumulation by a given strain, such as, there is lack of literature studies on As(III) and As(V) ions bioaccumulation by Corynebacterium glutamicum MTCC 2745. Conversely to the best of our knowledge, no investigation has been described on use of RSM to optimize the composition media or supplementation for As(III) and As(V) removal.

C. glutamicum MTCC 2745 species is of specific attention due to its high capability for abatement biologically. C. glutamicum could construct a corynbacterial strain with special capabilities to resist and accumulate arsenic, which can be utilized for bioremediation. C. glutamicum genome exposed the presence of two complete ars operons (ars1 and ars2) comprising the typical three gene structure arsRBC, with an extra arsC1 located downstream from arsC1 (ars1 operon), and two orphan genes (arsB3 and arsC4). The involvement of both ars operons in arsenic resistance in C. glutamicum helps in the bioaccumulation of arsenic. So, the bacteria C. glutamicum can depollute arsenic wastewater, by accumulation outside the cells and/or biosorption of the ion on their surface as was defined earlier for E. coli and R. eutropha. 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.

The objective of the current research were (1) to examine the effect of pH and the initial arsenic (either As(III) or As(V)) concentrations on the growth of C. glutamicum MTCC 2745, (2) to explore the effect of the initial arsenic (either As(III) or As(V)) concentration on the growth of C. glutamicum MTCC 2745, (3) to find the combined effects of the initial concentrations of peptone and arsenic (either As(III) or As(V)) ions on the growth and bioaccumulation properties of the bacteria C. glutamicum MTCC 2745 in a batch system, and (4) to observe whether the specific growth rate plus bioaccumulation % of both As(III) or As(V) by the bacteria C. glutamicum MTCC 2745 can be modelled by employing RSM regarding peptone and the initial arsenic (either As(III) or As(V)) concentrations.

**Theory of the RSM**

RSM generally comprises three stages: (1) design and experiments, (2) response surface modelling via regression and (3) optimization. The purpose of RSM is for determining an appropriate approximation for the true functional relationship between the set of independent variables (factors) \( X_1, X_2, \ldots \) and the dependent variable (response) \( Y \). If information regarding the true response surface shape is inadequate, primary efforts usually attempt for approximating the shape by fitting a first order model to the response values. Conversely, the first order model is advanced by addition of higher order terms to it if the first order model suffers from the lack of fit rising from the presence of surface curvature. The second order model is the next higher order model which is expressed as follows:

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon
\]

(1)

The test variables were coded to develop this regression equation as follows:

\[
x_i = X_i - \left( \frac{X_i}{\Delta X_i} \right) \text{ where } i = 1, 2, 3, \ldots
\]

(2)

Central composite design (CCD), a famous second order experimental design, is used for designing the experiment. The CCD is a proficient design which is used for sequential experimentation and offers a realistic amount of data to test the goodness of fit (GOF) and does not require significantly large number of design points thus decreasing the total cost accompanying with the experiments. CCD has following three set of experimental runs: (1) Fractional factorial runs in which factors are investigated at \( +1 \) and \( -1 \) levels, (2) centre points with all factors at their centre points that give assistance to understand the curvature and duplication gives assistance for calculating pure error and (3) Axial points analogous to centre points, however one factor takes values higher and lower than the median of two factorial levels usually both outside their range. Axial points make the design rotatable.

A 2^2 full factorial Central Composite design (CCD) for two independent variables is adopted for fitting a second order polynomial model, which specifies that 13 data were compulsory for this method. The statistical software package Design Expert, Stat-Ease Inc., Minneapolis, USA can be utilized for regression analysis of experimental data and for plotting response surface.

**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 by deionized double distilled water. The stock solutions of As(III) (1000 mg/L) and As(V) (1000 mg/L) were prepared by dissolving 1.734 g of sodium arsenite (NaAsO_2) and 4.16 g of sodium...
arsenate (Na$_2$HAsO$_4$, 7H$_2$O), purchased from Himedia Laboratories Pvt. Ltd. Mumbai India, in 1 L of double distilled water, respectively. All other necessary chemicals used in the experiments, were purchased from Himedia Laboratories Pvt. Ltd. Mumbai India. The working solutions of As(III) or As(V) were prepared by diluting the stock solution to the desired concentration.

**Microorganisms 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 consists of the following components: Beef Extract 1 g/L, Yeast extract 2 g/L, Peptone 5 g/L, NaCl 5 g/L and agar 15 g/L. The pH of the growth medium was adjusted to 7.0 by adding 1 N NaOH and 1 N HCl and Cultivation condition is maintained at 30°C temperature.

**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 in the current investigation.

The revived culture was initially grown in MTCC prescribed growth media in a 250 ml round bottom flask tightly closed with cotton plug as follows: *C. glutamicum* MTCC 2745 was cultivated in 250 mL flask containing 100 mL of the growth media with As (III) and As(V). The cultures were acclimatized to As (III) and As(V) individually exposing the culture in a series of shake flasks.

The bacterial inoculum was prepared by transferring a loop full of bacterial culture from the nutrient agar tubes to the flask containing sterilized growth media, incubated at 30°C for 24 h with moderate agitation (120 rpm) in an incubator cum orbital shaker. Then the acclimatization of *C. glutamicum* MTCC 2745 in arsenic environment was carried out as follows [47]:

After 24 h the synthetic medium in the flask had turned milky specifying significant bacterial growth in the flask. An appropriate amount of arsenic (As(III) or As(V)) was added into the flask having 100 mL sterilized growth media to acquire a concentration of 50 mg/L of arsenic. Firstly growth of *C. glutamicum* MTCC 2745 was inhibited and the growth started after 2 h. After 24 h of incubation at 30°C with moderate agitation (120 rpm), 5 mL of the arsenic resistant bacterial inoculum was periodically added in a series of 250 ml flasks containing 100 mL of arsenic containing sterilized growth media (As(III) or As(V) concentration, 100, 200, 500, 800, 1000, 1200, 1500 and 1800 mg/L) under sterile conditions in a laminar hood chamber. After 24 h later, another fresh growth media containing arsenic (As(III) or As(V) concentration, 2000 mg/L) was also inoculated with 5 mL of the last culture (As(III) or As(V) concentration, 1800 mg/L) to ensure that the bacteria was already adapted to both As (III) or As(V). For inoculum, a further sub culturing was performed and all the inoculum transfers were done in exponential phase (OD value ~1 at 600 nm).

**Preparation of bioaccumulation medium containing metal ions**

A 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 5 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**

An aliquot of 5 mL of pregrown culture aseptically harvested from exponential phase was transferred to 100 mL of synthetic wastewater. The influences of pH on growth of bacteria were investigated by varying the pH of the pure media and synthetic wastewater containing 50 mg/L of arsenic (either As(III) or As(V)) from 1.0 to 12.0. The effects of the 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.

The combined effect of peptone and arsenic (either As(III) or As(V)) concentration on the specific growth rate was investigated by varying the arsenic concentration from (0, 50, 500, 1000, 1500 and 2000 mg/L at constant peptone concentration (1, 3, 4, 5 and 9 g/L). The combined effect of peptone and arsenic (either As (III) or As(V)) concentration on bioaccumulation was
studied by varying the arsenic concentration (1000, 1500 and 2000 mg/L) at constant peptone concentration (1, 5 and 9 g/L). The pH of the medium was adjusted by dropwise addition of sterile 1 N HCl and 1 N NaOH. The inoculated flasks were incubated in an incubator cum orbital shaker at temperature 30°C for 24 h shaking at 120 rpm. 

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 analyzed for residual concentration of either As(III) or As(V) ion. Arithmetic mean of results of two similar experiments was utilized to estimate data.

**Arsenic analysis**

Samples were preserved using 10% v/v trace metal grade nitric acid. Arsenic was analyzed using a ThermoFisher Scientific iCE 3000 Series AA graphite furnace atomic absorption (GFAA) spectrometer, with Zeeman background correction (GF-AAS) equipped with GTA 100-graphite tube atomizer and programmable sample dispenser. A general guide for the application of the graphite furnace is given in (ASTM D2972, 2003). Practice pyrolytic graphite coated tubes (notched partition, Varian Canada Inc., Toronto) with forked pyrolytic platforms were used in the experiment and argon gas (ultrahigh purity 99.995%) was used to sheath the atomizer and to purge internally. An arsenic high-intensity hollow-cathode lamp (Varian Canada Inc., Ontario) was used at a wave length of 193.7 nm with a slit width of 0.5 nm for determining As <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 Pokhrel and Viraraghavan\[48\] and Michon et al.\[49\] The arsenic measurement in all the cases corresponds to total arsenic. No speciation of arsenic was carried out in the study.

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

\[
\text{Bioaccumulation} \% = \frac{(C_0 - C_f)}{C_0} \times 100
\]

**Biomass analysis**

The centrifuged cells were washed with 1 mL of double distilled water and resuspended in 2 mL of double distilled water and then the OD was measured. A correlation for converting OD600 values to bacterial dry weight was established from the calibration curve.\[50,51\]

**Statistical analysis**

RSM was utilized for designing experiment, modelling and optimizing two response variables viz. the specific growth rate and metal bioaccumulation %.

The current investigation was performed not only for investigating the combined effects of peptone and either As(III) or As(V) concentrations on the specific growth rate and bioaccumulation % of *C. glutamicum* MTCC 2745, but also for determining the model equations demonstrating the specific growth rate and bioaccumulation %. The optimization procedure engaged investigating the response of statistically designed combinations, evaluating the coefficients by fitting the experimental data to response functions, thus forecasting the response of the model and verifying the competence of the model in terms of $R^2$ values. The initial peptone concentration (g/L) ($S_0$) and the initial concentrations of either As(III) or As(V) (mg/L) ($C_0$) in the bioaccumulation medium were selected as independent variables ($X_1$ and $X_2$). The levels of each variable were varied in the range $-1$ to $+1$, respectively, as revealed in Table S1 of supplementary materials. The first independent variable initial peptone concentration was changed over two main levels designated by $-1$ and $+1$ (1 and 9 g/L) relative to the centre point designated by 0 (5 g/L). The second independent variable initial arsenic (either As(III) or As(V)) concentrations were changed over two levels designated by $-1$ and $+1$ (1000 and 2000 mg/L) with respect to the centre point designated by 0 (1500 mg/L) (Table S1 of supplementary materials). The critical ranges of designated parameters were set by the initial experiments on the basis of the literature survey. With the purpose of studying the combined effects of these variables on the responses, 13 sets of experiments with proper combinations of concentrations of peptone and arsenic (either As(III) or As(V)) ions were performed using Box Wilson statistical method which included the concentrations of both peptone and arsenic (either As(III) or As(V)) coded by the a value as presented in Table S2 (As (III)) and Table S3 (As(V)). These 13 sets of experiments were performed with five replications at the design center to estimate the pure error and were
carried in randomized order as essential in several design processes.

Data were analysed using 2² full factorial central composite design with respect to the coded and uncoded values as listed in Table S2 (As(III)) and Table S3 (As (V)). Numerical analysis to evaluate the responses of the specific growth rate and arsenic (either As(III) or As(V)) bioaccumulation % and the graphical analysis of the data were performed by using Design Expert® Package (Version 6.0.8 Stat-ease Inc., Minneapolis, MN, USA). The goodness of fit of the model was estimated by the coefficient of determination $R^2$ and by the $F$-test analysis of variance (ANOVA). In relation to ANOVA, a high $F$-value specifies that maximum variation in response can be clarified by regression equation. The associated $p$-value is utilized to evaluate whether $F$-value is huge enough for holding a statistical significance. A $p$-value lower than 0.05 specifies the model is statistically significant.\[^{30,35,38,53}\] The lack of fit $F$-test defines the variation of data around the fitted model. Lack of fit will be non-significant if the model fits the data well. Adequate precision compares the range of predicted values at the design points to the average prediction error. A ratio greater than 4 is desirable and specifies model discrimination.

**Results and discussion**

**Calibration curve**

Correlations for converting OD600 values to bacterial dry weight were established from the calibration curves. The correlations used for both As(III) resistant C. glutamicum MTCC 2745 and As(V) resistant C. glutamicum MTCC 2745 can be expressed by Eq. (4) with $R^2$ value 0.99, as follows:

$$
\text{Cell concentration (g/L)} = 1.01 \times \text{OD600} - 0.122 \quad (4)
$$

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

pH plays a main role for the growth properties of C. glutamicum MTCC 2745. Influence of the 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 bacteria could not grow at extreme low pH 1.0 and at extreme high pH 12.0 for both As(III) and As(V) resistant bacteria (Fig. 1(a)–(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 were determined as 2.19 and 2.09 g/L and 2.2 and 2.1 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 were also determined at pH 7.0 as 2.13 and 2.03 g/L and 2.17 and 2.07 g/L, respectively, in the media containing 50 mg/L As(III) and As(V) ions, respectively. Shivaji et al.\[^{53}\] reported that the bacterial growth is very sensitive to pH and the encouraging pH for the growth of Bacillus arsenicus MTCC 4380 is 5.5–8.0. 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.\[^{54}\]

**Effect of the 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)) from 50 to 2000 mg/L in the growth media are shown in Fig. 2(a)–(b).

In the present case, the bacteria was previously acclimatized up to 2000 mg/L arsenic (As(III) and As(V) separately) comprising growth media. Therefore the lag phase did not increase while arsenic was supplemented in the growth media. Presence of arsenic (either As(III) or 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 increases the cell numbers while nitrogen source increases the cell mass of bacteria.\[^{51}\] In the current investigation, yeast extract acts as carbon source and peptone acts as nitrogen source for all the cases.

Furthermore, in the current study, the arsenic concentration was the 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. However, the uptake rate of carbon is slower in presence of arsenic than in absence of arsenic. This prolongs not only the log phase but also the stationary phase. In the current study, since the bacteria was previously acclimatized in 2000 mg/L arsenic (either As(III) or As(V)) containing media, the lag phase did not increase while arsenic was added in the growth media.
It is possible that the rate of substrate consumption in absence of arsenic (either As(III) or As(V)) was high; while as the arsenic (either As(III) or As(V)) concentration was increased, the rate of substrate consumption declined and it could sustain the lag phase for longer time.

Recent research revealed that the growing bacterial cell suspensions entering the stationery 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 alternative substrates.\(^5\) Furthermore, it was also reported that under starvation, in stationary phase, bacteria tend to survive the starvation phases without substantial losses in population in contrast to the fast growth under excess substrate.\(^5\)

Similar growth pattern of *R. eutropha*, *P. putida* and *B. indicus* in NB media containing arsenic ions were reported by Mondal *et al.*\(^4\)

In the absence of As(III) and As(V) the highest biomass concentrations of As(III) resistant and As(V) *C. glutamicum* MTCC 2745 were 2.1 g/L and 2.11 g/L, respectively, and the corresponding specific growth rates were 0.263 and 0.237 h\(^{-1}\). With the increase in concentration of As(III)) in the media from 50 to 2000

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**Figure 1.** 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 ± standard errors from the mean of duplicate experiments).
A.1. Introduction

In the study, the researchers investigated the bioaccumulation of As(III) and As(V) by the yeast C. tropicalis. They found that increasing the concentration of As(III) from 50 to 2000 mg/L led to a decrease in the maximum biomass concentration from 2.03 to 1.86 g/L and a decrease in the specific growth rate from 0.246 to 0.14 h^{-1} because of the toxicity of As(III) ions. Increasing the concentration of As(V) from 50 to 2000 mg/L also led to a decrease in the maximum biomass concentration from 2.08 to 1.91 g/L and a decrease in the specific growth rate from 0.221 to 0.145 h^{-1} because of the toxicity of As(V) ions.

An analogous trend was observed in the bioaccumulation levels of RTBG dye and Cu(II) by C. tropicalis. Gönen and Aksu [39] reported that all the strains of non-adapted yeast showed an extended lag phase in the presence of Cu(II) in the preliminary investigations. An increase in concentration of Cu(II) also 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 found 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. [30] also reported that there

Figure 2. 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_o 5 g/L; pH: 7; T: 30°C; Agitation speed: 120 rpm; Incubation time: 102 h) (Error bars represent means ± standard errors from the mean of duplicate experiments).
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 much more poisonous than As(V), because of its higher cellular uptake. [57] So, the maximum biomass concentration and the specific growth rate of *C. glutamicum* MTCC 2745 in the growth media were more in presence of As(V) than that of As(III).

**Combined effect of the initial peptone concentration and initial arsenic 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. [58] It was reported that organic nitrogen peptone usually have stimulating effect. [59–61] In the present study, peptone was used in a higher amount compared to other media components (yeast extract and beef extract).

In the present study, the growth behaviour of *C. glutamicum* MTCC 2745 was explored at an initial pH value of 7.0 and at 30°C temperature when the initial peptone concentration was varied from 1 to 9 g/L, the initial arsenic (either As(III) or As(V)) concentration was kept constant between 50 and 2000 mg/L for each experiment set. Figure 3(a)–(b) exhibits the effect of the initial peptone concentration on the specific growth rate of *C. glutamicum* MTCC 2745 at different levels of As(III) and As(V) ions, respectively. Comparison of the corresponding biomass concentration and the specific growth rate at different concentration of arsenic ions with increasing peptone concentration are presented in Table 1. When peptone concentration was held constant and initial arsenic (either As(III) or As(V)) concentration was varied from 50 to 2000 mg/L, the growth rate of bacteria decreased. It was found from the study that the maximum biomass concentration and the specific growth rate increased while the concentration of peptone was increased from 1 to 9 g/L at a constant initial arsenic concentration (either As(III) or As(V)) (Table 1).

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. An increase in the growth rate of *C. glutamicum* MTCC 2745 with increasing the initial peptone concentration at constant arsenic concentration could be owing to cell defence mechanisms such as acclimation to toxicity. Analogous results of significant reduction in the specific growth rate with increasing Remazol blue concentration were also described in case of *Candida tropicalis*. [62] Gönen and Aksu [39] also described that the increase in concentration of sugar resulted in an increase in cell concentration as well as the specific growth rate. It is clear from the results of the present investigation that the initial peptone concentration played a major role in the bacterial growth and decreased the inhibitory effects of arsenic on the bacterial growth. Moreover, the residual peptone concentration in the media was found to be very low which will not cause any discarding problem.

**Combined effects of peptone and the 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 studied separately at pH 7.0 and 30°C temperature in the growth media for 10 h. The initial concentration of peptone was varied from 1 to 9 g/L keeping the initial arsenic concentration constant between 1000 and 2000 mg/L. Comparison of the bioaccumulation % and corresponding biomass concentration and the specific growth rate at different concentration of arsenic ions with increasing peptone concentration are presented in Table 1. Combined effects of the initial concentrations of peptone and arsenic (either As(III) or As(V)) ions on bioaccumulation properties of *C. glutamicum* MTCC 2745 are shown in Fig. 4(a)–(b).

The bioaccumulation % of arsenic (either As(III) or As(V)) decreased from 1000 to 2000 mg/L, when the biomass concentration and the specific growth rate showed a declining trend, signifying that the media toxicity increased with increasing arsenic concentration. However, increased arsenic concentrations at constant peptone concentration reduced the biomass concentration, specific growth rate and bioaccumulation %.

Since the bioaccumulation is dependent on the bacterial growth, the increase in peptone concentration at constant arsenic concentration increased the biomass concentration, specific growth rate and bioaccumulation %. The bioaccumulation % increased while concentration of peptone was increased from 1 to 9 mg/L at an initial arsenic concentration of 1000 mg/L (either As(III) or As(V)). Furthermore, 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.
Response surface evaluation for the combined effects of the initial concentration of peptone and arsenic ions on the specific growth rate and bioaccumulation properties of C. glutamicum MTCC 2745

The requirement for confirming the efficiency of the biological treatment of wastewater with living cells has increased the attention in the use of mathematical models to the behaviour of microbes.

The main problem in the maximum predictive models is the effort to obtain adequate reproducible data appropriate to the model. The goal of this study is not only to investigate the combined effects of the initial concentrations of peptone and arsenic (either As(III) or As(V)) ions on the growth and bioaccumulation properties of As(III) resistant and As(V) resistant C. glutamicum MTCC 2745, but also to determine the best model indicating the growth and bioaccumulation process.

Binary effects of the initial concentrations of peptone and arsenic (either As(III) or As(V)) ions on the specific growth rate and arsenic (either As(III) or As(V)) bioaccumulation % by C. glutamicum MTCC 2745 were
Table 1. Comparison of bioaccumulation %, maximum biomass concentration and specific growth rate at different concentration of arsenic species (As(III) or As(V)) with increasing peptone concentration.

| Arsenic species | C₀ (mg/L) | S₀ (g/L) | Bioaccumulation % | Xₙ (g/L) | µ (h⁻¹) |
|-----------------|-----------|----------|-------------------|----------|---------|
| As(III)         | 1000      | 1        | 69.9              | 1.91     | 0.074   |
|                 | 1000      | 5        | 73.5              | 1.94     | 0.165   |
|                 | 1000      | 9        | 78.4              | 1.95     | 0.247   |
|                 | 1500      | 1        | 59.5              | 1.87     | 0.058   |
|                 | 1500      | 5        | 63.9              | 1.89     | 0.15    |
|                 | 1500      | 9        | 68.9              | 1.95     | 0.234   |
|                 | 2000      | 1        | 49.3              | 1.84     | 0.046   |
|                 | 2000      | 5        | 53.3              | 1.86     | 0.14    |
|                 | 2000      | 9        | 58.8              | 1.87     | 0.226   |
| As(V)           | 1000      | 1        | 70.6              | 1.95     | 0.086   |
|                 | 1000      | 5        | 74.3              | 1.98     | 0.179   |
|                 | 1000      | 9        | 77.6              | 1.99     | 0.294   |
|                 | 1500      | 1        | 62.2              | 1.92     | 0.065   |
|                 | 1500      | 5        | 66                | 0.16     | 1.93    |
|                 | 1500      | 9        | 70                | 0.282    | 1.94    |
|                 | 2000      | 1        | 50.6              | 1.87     | 0.047   |
|                 | 2000      | 5        | 55                | 0.145    | 1.91    |
|                 | 2000      | 9        | 59                | 1.92     | 0.278   |

studied and data acquired by experiments were analyzed by RSM.

The experimental results of the specific growth rate and bioaccumulation % of arsenic (either As(III) or As(V)) were fitted to a second order quadratic equation, yielding two numerical correlations (for both As(III) and As(V)) to evaluate the responses of the specific growth rate (Y₁) and arsenic (either As(III) or As(V)) Bioaccumulation % (Y₂).

The responses Y₁ and Y₂ for As(III) resistant bacteria are obtained as follows:

\[
Y_1 \text{ (Specific growth rate)} = 0.15 + 0.088X_1 - 0.013X_2 - 0.004X_1^2 + 0.003X_2^2 + 0.001X_1X_2 \quad (5)
\]

\[
Y_2 \text{ (As(III) Bioaccumulation %)} = 63.9 + 4.56X_1 - 10.1X_2 + 0.47X_1^2 - 0.347X_2^2 + 0.245X_1X_2 \quad (6)
\]

The responses Y₁ and Y₂ for As(V) resistant bacteria are obtained as follows:

\[
Y_1 \text{ (Specific growth rate)} = 0.16 + 0.109X_1 - 0.015X_2 + 0.014X_1^2 + 0.002X_2^2 + 0.006X_1X_2 \quad (7)
\]

\[
Y_2 \text{ (As(V) Bioaccumulation %)} = 66.1 + 3.87X_1 - 9.63X_2 - 0.118X_1^2 - 1.5X_2^2 + 0.351X_1X_2 \quad (8)
\]

It can be supposed from the response function coefficients that the initial arsenic (both As(III) and As(V)) concentration unfavourably affected both the specific growth rate as well as arsenic (either As(III) or As(V)) Bioaccumulation %. Effect of the initial arsenic concentration was robust compared with the initial peptone concentration. The values of µ and arsenic (either As(III) or As(V)) Bioaccumulation % agreed very well with the forecasted values of µ and arsenic (either As(III) or As(V)) Bioaccumulation % at all concentration combinations investigated.

Using analysis of variance (ANOVA), the statistical meaning of the ratio of mean square variation because of regression and mean square residual error were basically verified. The statistical technique ANOVA subsections the total variation in a data set into parts of component linked with definite sources of variation to test the hypotheses on the parameters of the model. The data achieved from the above model equations were significant, as tested by the F-test ANOVA (Tables S4 and S5 for As(III) and Tables S6 and S7 for As(V)). The statistical significance of the model equation was evaluated by this analysis. The ANOVA results of these quadratic models indicated that these quadratic models could be used for navigating the design space.

The meaning of each coefficient of model equations for µ and Bioaccumulation % of either As(III) or As(V) were determined by calculating p-values, as listed in Tables S4 and S5 of supplementary materials (for As(III)) and Tables S6 and S7 of supplementary materials (for As(V)). The associated Prob > F-values for each model (µ and bioaccumulation % of arsenic (either As(III) or As(V))) were lower than 0.05 (i.e. a = 0.05) indicating that quadratic models were significant. This outcome specified that it was significant statistically at 99.95% confidence level for both µ and bioaccumulation % of arsenic (either As(III) or As(V)) values. The ANOVA for the response surface model gave an F-value of 1.8E+05 for µ (Table S4 of supplementary materials) and 6.43E+04 for As(III) Bioaccumulation %.
Table S5 of supplementary materials) and 6.42E+04 for \( \mu \) (Table S6 of supplementary materials) and 2.03E+05 for As(V) Bioaccumulation % (Table S7 of supplementary materials).

The \( R^2 \) values 0.9999 for \( \mu \) of As(III) resistant bacteria and 0.9998 for As(III) bioaccumulation % and 0.9998 for \( \mu \) of As(V) resistant bacteria and 0.9999 for As(V) Bioaccumulation % (Table 4) acquired to be near to 1.0 similarly supported a high correlation between the experiential and predicted values. This specifies that the regression model offers an outstanding explanation of the relationship between the independent variables (peptone and arsenic (either As(III) or As(V)) concentrations) and the responses (\( \mu \) and arsenic (either As (III) or As (V)) Bioaccumulation %). This recommends that 99.99 and 99.98% of the sample variation for the specific growth rate of the As(III) resistant bacteria and As(III) bioaccumulation % and 99.98% and 99.99% of the sample variation for the specific growth rate of the As(V) resistant bacteria and As(V) Bioaccumulation % (Table 4) are clarified by the independent variables and this too specifies that the model did not clarify only about 0.01% and 0.02% of sample variation for \( \mu \) and As(III) Bioaccumulation % and 0.02% and 0.01% of

Figure 4. 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 ± standard errors from the mean of duplicate experiments).
The coefficient of variation was 0.155% for μ of As (III) resistant bacteria and 0.237% for As(III) Bioaccumulation % and 0.905% for μ of As(V) resistant bacteria and 0.123% for As(V) Bioaccumulation % (Table 4), signifying that the experiments performed in duplicates were extremely precise and consistent and that the model was very significant. The lack of fit tests compares the residual error to the Pure Error from the duplicated experimental design points. Experimental and predicted values for μ and As(III) bioaccumulation % were within 3.73E-07% (Table S4 of supplementary materials) and 0.16% (Table S5 of supplementary materials), respectively, and those for μ and As(V) Bioaccumulation % were within 1.61E-05% (Table S6 of supplementary materials) and 0.045% (Table S7 of supplementary materials), respectively. The adequate precision for the initial peptone concentration and the initial As(III) concentration were 1.29E+03 and 285, respectively, and for the initial peptone concentration and the initial As(V) concentration were 241 and 495, respectively (Table 4). The high adequate precision values (greater than 4 for both As(III) and As

### Table 2. Comparison of the values of μ and As(III) Bioaccumulation % experimentally acquired and forecasted from RSM.

| Run | S₀ (g/L) | C₀(As(III)) (mg/L) | μ (h⁻¹) (–exp) | μ (h⁻¹) (–pred) | As(III) Bioaccumulation % (–exp) | As(III) Bioaccumulation % (–pred) |
|-----|---------|---------------------|----------------|----------------|----------------------------------|----------------------------------|
| 1   | 9       | 2000                | 0.226          | 0.226          | 58.8                             | 58.7                             |
| 2   | 5       | 1500                | 0.15           | 0.15           | 63.9                             | 63.9                             |
| 3   | 5       | 1500                | 0.15           | 0.15           | 63.9                             | 63.9                             |
| 4   | 9       | 1000                | 0.247          | 0.248          | 78.4                             | 78.4                             |
| 5   | 1       | 2000                | 0.046          | 0.046          | 49.3                             | 49.1                             |
| 6   | 5       | 1500                | 0.15           | 0.15           | 63.9                             | 63.9                             |
| 7   | 1       | 1000                | 0.074          | 0.074          | 70                               | 69.8                             |
| 8   | 5       | 2000                | 0.14           | 0.141          | 53.3                             | 53.4                             |
| 9   | 5       | 1000                | 0.165          | 0.165          | 73.5                             | 73.6                             |
| 10  | 9       | 1500                | 0.234          | 0.234          | 68.9                             | 68.9                             |
| 11  | 5       | 1500                | 0.15           | 0.15           | 63.9                             | 63.9                             |
| 12  | 1       | 1500                | 0.058          | 0.057          | 59.5                             | 59.8                             |
| 13  | 5       | 1500                | 0.15           | 0.15           | 63.9                             | 63.9                             |

### Table 3. Comparison of the values of μ and As(V) Bioaccumulation % experimentally acquired and forecasted from RSM.

| Run | S₀ (g/L) | C₀(As(V)) (mg/L) | μ (h⁻¹) (–exp) | μ (h⁻¹) (–pred) | As(V) Bioaccumulation % (–exp) | As(V) Bioaccumulation % (–pred) |
|-----|---------|------------------|----------------|----------------|---------------------------------|---------------------------------|
| 1   | 9       | 2000             | 0.278          | 0.276          | 58.1                            | 59.1                            |
| 2   | 5       | 1500             | 0.16           | 0.16           | 66                              | 66.1                            |
| 3   | 5       | 1500             | 0.162          | 0.162          | 66                              | 66.1                            |
| 4   | 9       | 1000             | 0.294          | 0.294          | 77.6                            | 77.6                            |
| 5   | 1       | 2000             | 0.047          | 0.046          | 50.6                            | 50.6                            |
| 6   | 5       | 1500             | 0.16           | 0.16           | 66                              | 66.1                            |
| 7   | 1       | 1000             | 0.086          | 0.088          | 70.6                            | 70.6                            |
| 8   | 5       | 2000             | 0.145          | 0.147          | 55                              | 55.1                            |
| 9   | 5       | 1000             | 0.179          | 0.178          | 74.3                            | 74.2                            |
| 10  | 9       | 1500             | 0.282          | 0.283          | 69.9                            | 69.9                            |
| 11  | 5       | 1500             | 0.162          | 0.167          | 66                              | 66.1                            |
| 12  | 1       | 1500             | 0.065          | 0.065          | 62.2                            | 62.1                            |
| 13  | 5       | 1500             | 0.16           | 0.16           | 66                              | 66.1                            |

### Table 4. Summary of fit for As(III) and As(V) resistant bacteria.

**As(III) Bioaccumulation %**

| Specific growth rate (μ) | Standard deviation | Mean of response | CV | PRESS | Adeq precision |
|--------------------------|--------------------|------------------|----|-------|----------------|
| As(III) Bioaccumulation % | 0.151              | 63.9             | 0.15 | 1.25  | 285            |

**As(V) Bioaccumulation %**

| Specific growth rate (μ) | Standard deviation | Mean of response | CV | PRESS | Adeq precision |
|--------------------------|--------------------|------------------|----|-------|----------------|
| As(V) Bioaccumulation %  | 0.08               | 65.332           | 0.08 | 1.61E-04 | 495            |

### sample variation for μ and As(V) Bioaccumulation %, respectively.

The coefficient of variation was 0.155% for μ of As (III) resistant bacteria and 0.237% for As(III) Bioaccumulation % and 0.905% for μ of As(V) resistant bacteria and 0.123% for As(V) Bioaccumulation % (Table 4), signifying that the experiments performed in duplicates were extremely precise and consistent and that the model was very significant. The lack of fit tests compares the residual error to the Pure Error from the duplicated experimental design points. Experimental and predicted values for μ and As(III) bioaccumulation % were within 3.73E-07% (Table S4 of supplementary materials) and 0.16% (Table S5 of supplementary materials), respectively, and those for μ and As(V) Bioaccumulation % were within 1.61E-05% (Table S6 of supplementary materials) and 0.045% (Table S7 of supplementary materials), respectively. The adequate precision for the initial peptone concentration and the initial As(III) concentration were 1.29E+03 and 285, respectively, and for the initial peptone concentration and the initial As(V) concentration were 241 and 495, respectively (Table 4). The high adequate precision values (greater than 4 for both As(III) and As
confirmed that models are significant for the process. This model can be used to navigate the design space.

Studies of the output of fit summaries exhibited that the quadratic models are important statistically for the responses. So these equations may be used for advance analysis. Usually it is important for confirming that the nominated model is offering a satisfactory approximation to the real system. By applying the diagnostic plots for example the predicted versus actual value plot, the model competence can be tested. The correlation coefficient between actual and predicted values for $\mu$ ($Y_1$) and As(III) Bioaccumulation % ($Y_2$) were 0.9973 and 0.9988, respectively, and for $\mu$ ($Y_1$) and As(V) Bioaccumulation % ($Y_2$) were 0.9998 and 0.9999. These $R^2$ values elucidate good agreement between the predicted and experiential outcomes within the range of experiments.

The three dimensional (3-D) response surface graphs (Fig. 5(a)–(b) for As(III) and Fig. 5(c)–(d) for As(V)), two dimensional (2-D) contour plots (Fig. 6(a)–(b) for As(III) and Fig. 6(c)–(d) for As(V)) and perturbation plots (Fig. S1(a)–(b) of supplementary materials for As(III) and Fig. S1(c)–(d) of supplementary materials for As(V)) of the quadratic models are showed for signifying the combined effects of concentration of peptone and arsenic (either As(III) or As(V)) ions on the specific growth rate of C. glutamicum MTCC 2745 and bioaccumulation %.

As exposed in the figures, both the bacterial growth and Bioaccumulation % of either As(III) or As(V) improved with increasing initial peptone concentration from 1 to 9 g/L and reduced with the increase in the initial arsenic (either As(III) or As(V)) concentrations from 1000 to 2000 mg/L. It is also quite easy as well as valuable for locating optimum levels of two variables through these 3-D plots and their corresponding contour and perturbation plots. Furthermore, the best value of growth and arsenic (either As(III) or As(V)) bioaccumulation happened near to the upper point suggesting the values of 1000 mg/L either As(III) or As(V) and 9 g/L peptone.

The influence of interaction between peptone and either As(III) or As(V) ($X_1X_2$) on the specific growth rate (Fig. 5(a) for As(III) and Fig. 5(c) for As(V)) exhibited was a steady increase in the specific growth rate with increasing initial peptone concentration and after that stabilized growth was found. The 3-D plot too signified that there was a steady decrease in the specific growth rate with the increase in concentration of arsenic (either As(III) or As(V)) ions along the initial arsenic concentration ($C_0$) axis. The effect of interaction between peptone and arsenic (either As(III) or As (V)) ($X_1X_2$) on bioaccumulation showed that there was
an increase in Bioaccumulation % with increase in the initial peptone concentration and reduction in arsenic (either As(III) or As(V)) concentrations which was exhibited by the upward increase of the 3-D plot (Fig. 5(b) for As(III) and Fig. 5(d) for As(V)) along the initial peptone concentration axis and a reducing tendency was found along the axis of the initial arsenic (either As(III) or As(V)) concentration ($S_0$).

So it is understood that peptone acted a constructive part for bacterial growth as a source of carbon and nitrogen and directly affected the process of bioaccumulation. Similarly the 2-D contour plots (Fig. 6(a)–(b) for As(III) and Fig. 6(c)–(d) for As(V)) gave a clear idea that C. glutamicum MTCC 2745 showed a high specific growth rate and high bioaccumulation % with peptone concentrations varying in the range of 5–9 g/L indicated by the response flags having the highest value in this region.

Perturbation plots (Fig. S1(a)–(b) of supplementary materials for As(III) and Fig. S1(c)–(d) of supplementary materials for As(V)) exhibited a steep uprising curve for the initial peptone concentration and a down falling curve for the initial arsenic (either As(III) or As(V)) concentration so confirming a positive response of C. glutamicum MTCC 2745 towards the specific growth rate and Bioaccumulation % recognized by inversely connecting these two parameters. The terms A, i.e., $X_1$, and B, i.e., $X_2$ denotes the initial peptone concentration and arsenic (either As(III) or As(V)) concentrations.

Based on the present examination it can be understood that RSM was successfully employed as a fast and error free method to optimize As(III) or As(V) bioaccumulation by C. glutamicum MTCC 2745 with respect to parameters of arsenic (either As(III) or As(V)) and the initial peptone concentration as medium components. In addition, the interactive study between these two components yielded further benefit of using RSM. Furthermore, the system performance at any experimental points with different combinations of variables.

Table 5. Optimized medium composition for bioaccumulation of As(III) and As(V) by RSM.

| Component   | Concentration | Specific growth rate (h⁻¹) | Bioaccumulation % |
|-------------|---------------|----------------------------|-------------------|
|             |               | Predicted | Experimental | Predicted | Experimental |
| Peptone (g/L) | 9             | 0.248     | 0.247       | 78.4      | 78.4         |
| As(III) (mg/L) | 1000          |            |             |           |              |
| Peptone (g/L) | 9             | 0.294     | 0.294       | 77.6      | 77.5         |
| As(V) (mg/L)  | 1000          |            |             |           |              |

Figure 6. 2–D contour plots presenting (a) combined impacts of peptone and As(III) concentrations on the specific growth rate of C. glutamicum MTCC 2745 (b) combined impacts of peptone and As(III) concentrations on the As(III) Bioaccumulation % of C. glutamicum MTCC 2745 (c) combined impacts of peptone and As(V) concentrations on the specific growth rate of C. glutamicum MTCC 2745 (d) combined impacts of peptone and As(V) concentrations on the As(V) Bioaccumulation % of C. glutamicum MTCC 2745.
as well as arsenic (either As(III) or As(V)) bioaccumulation at intermediate levels which were not studied experimentally may be assessed by employing this technique.

**Optimization**

The comparison of the specific growth rate and bioaccumulation % of As(III) and As(V) for optimized media using RSM is given in Table 5. RSM has predicted the specific growth rate and bioaccumulation % was 0.248 h\(^{-1}\) and 78.4 for As(III), respectively, and 0.294 h\(^{-1}\) and 77.6, respectively, for As(V). The maximum experimental specific growth rate and bioaccumulation % obtained in both case were almost the same for RSM optimized inputs. The difference between the predicted and experimental results can be contributed to the extent deviation in predictive capacity of the

![Figure 7](image)

**Figure 7.** Scanning electron micrographs (SEM) (10,000×) and EDX of (a) native living cells of *C. glutamicum* MTCC 2745, (b) As(III) acclimatized living cells of *C. glutamicum* MTCC 2745 at loaded stage and (c) As(V) acclimatized living cells of *C. glutamicum* MTCC 2745 at loaded stage.
model. So, RSM can be most widely used method in growth medium optimization. It is one of the effective approaches for non-linear optimization.

**Characterization**

**SEM–EDX analysis**
The surface morphology of living *C. glutamicum* MTCC 2745 biomass without and with bioaccumulation of arsenic (either As(III) or As(V)) ions during the bioaccumulation process was measured with the help of SEM–EDX. Figure 7(a) exhibited that, without bioaccumulation of arsenic ions, *C. glutamicum* MTCC 2745 cells were of spherical shape and with a smooth surface. The morphological changes with respect to the size of the bacteria after bioaccumulation of arsenic (either As(III) or As(V)) ions with *C. glutamicum* MTCC 2745 cells are presented in Fig. 7(b)–(c). It can be clearly found that the size of biomass has become short after arsenic (either As(III) or As(V)) ions bioaccumulation. It is found that in native bacterial biomass the surface is smooth while after arsenic (either As(III) or As(V)) treatment the surface becomes rough in both the bacterial structures. Such roughness of the surface may be because of the bioaccumulation of arsenic (either As(III) or As(V)) over the surface that makes the surface coarser than its original form.

It recommends that the presence of arsenic (either As(III) or As(V)) does not make the medium selective towards any of the strains and the biological nature of the bacteria 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 bacteria 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 EDX spectra of arsenic (III) ion unloaded and loaded biomass obtained are shown in Fig. 7(a)–(c), respectively. So, it can be concluded that arsenic (either As(III) or As(V)) ions were bioaccumulated on the bacteria.

**FT–IR analysis**
The FTIR spectra of the *C. glutamicum* MTCC 2745 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 bioaccumulation of As (III) and As(V) ions, are presented in Fig. 8. The FTIR spectra of the *C. glutamicum* MTCC 2745 biomass without As(III) and As(V) ions loaded exhibited a number of absorption peaks, indicating the complex nature of the bacterial biomass (Fig. 8). The spectra of unloaded and loaded with either As(III) or As(V) ions are compared and observed the following shifting (Fig. 8, Table 6). The spectra of living bacterial cells exhibited an absorption band at 3421.154 cm$^{-1}$ because of bonded $–$OH and $–$NH

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![Figure 8. FT–IR spectra of *C. glutamicum* MTCC 2745 control and loaded As(III) and As(V) ions.](image-url)
stretching vibration which was shifted to 3302.326 cm\(^{-1}\) (As(III)) and 3399.94 cm\(^{-1}\) (As(V)) which may be possibly because of the complexation of –OH and –NH groups with As(III) or As(V) ions.\(^{64-67}\) Aliphatic C–H stretching may be responsible for biosorption of As(III) and As(V) on the bacterial biomass as wavenumber shifted from 2960.243 to 2929.387 cm\(^{-1}\) for both As(III) and As(V), possibly because of the complexation of secondary amine group with As(III) and As(V) ions.\(^{65}\) The next absorption peaks at 2355.969 cm\(^{-1}\) shifted at a lower frequency to 2350.388 cm\(^{-1}\) for both As(III) and As(V), probably because of the complexation of –CH stretching vibration of alkyl chains.\(^{68}\) The next biosorption peaks at 1677.792 shifted to 1652.722 cm\(^{-1}\) for both As(III) and 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.\(^{65,66,69}\) Wavenumber shifted from 1538.941 to 1532.403 cm\(^{-1}\) for both As(III) and As(V), probably because of the complexation of secondary amine group with As(III) and As(V) ions.\(^{65}\) Another shift was found from 1454.087 to 1449.922 cm\(^{-1}\) for both As(III) and As(V), possibly due to the complexation of nitrogen with As(III) and As(V) ions of the N–H group\(^{70,71}\) and also due to the complexation with carboxyl groups.\(^{65}\) Wavenumber shifted from 1386.589 to 1382.326 cm\(^{-1}\) for both As(III) and As(V) assigned the reactivity of carboxylate anion C=O stretching for the biosorption process.\(^{72}\) Wavenumber shifted from 1304.186 to 1294.264 cm\(^{-1}\) (As(III)) and 1247.752 cm\(^{-1}\) (As(V)) assigned the symmetric bending of CH\(_3\) group.\(^{65}\) Wavenumber 1234.238 shifted to 1234.238 cm\(^{-1}\) for both As(III) and As(V) assigned for –SO\(_3\) stretching for the biosorption process.\(^{65}\) The peaks at 1078.03 cm\(^{-1}\) may be attributed to the C–N stretching vibrations of amino groups which was shifted and appeared at 1071.628 cm\(^{-1}\) for both As(III) and As(V), due to the interaction of nitrogen from the amino group with As(III) and As(V) ions.\(^{68,73}\) The other weak adsorption peak shifted from 875.039 to 870.078 cm\(^{-1}\) for both As(III) and As(V), corresponding to the O–C–O scissoring vibration of polysaccharide.\(^{74,75}\) The presence of As(III) and As(V) on the bacterial biomass can be assured from the bands appeared at 719.38 and 848.992 cm\(^{-1}\), respectively.\(^{76-78}\) 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) biosorption. It should be distinguished that the As–O band after biosorption of arsenic was not clearly observed because of the broad overlapping peaks in this region.\(^{79}\)

**Conclusions**

The results of present study recognize that the living *C. glutamicum* MTCC 2745 was proficient of bioaccumulating As(III) and As(V) using peptone as the main source of carbon and nitrogen in the growth medium in a batch system. This study also confirmed that RSM can deliver statistically consistent outcomes to analyze the effect of numerous parameters on the specific growth rate of *C. glutamicum* MTCC 4380 and the Bioaccumulation % of both As(III) and As(V). The results obtained in the present study indicated that:

- The highest biomass concentration achieved at pH 7.0 in the absence of As(III) and As(V) ions was determined as 2.09 and 2.1 g/L, respectively.
- There is also a risk that the metal would pose toxicity to the cells. So, the growth of arsenic resistant bacteria could also be decreased with increasing concentration of arsenic ions (either As(III) or As(V)).
- The statistical analysis based on a 2\(^2\) full factorial central composite design showed that the growth medium containing 1000 mg/L arsenic (either As(III) or As(V)) and 9 g/L peptone were the best combination to bioaccumulate As(III) and As(V) using *C. glutamicum* MTCC 2745 up to 78.4% and 77.6%, respectively. The maximum experimental bioaccumulation % obtained in this case is almost the same for RSM optimized inputs (78.4% for As(III) and 77.5% for As(V)). The difference between the predicted and experimental results can be contributed to the extent deviation in predictive capacity of the model.
- FT–IR and EDX analysis established the fact that both As(III) and As(V) was bioaccumulated on the living cells of *C. glutamicum* MTCC 2745.

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**Table 6. Wavenumber (cm\(^{-1}\)) for the dominant peak from FT–IR for bioaccumulation 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                         | 3421.154       | 3302.326               | 3399.940             |
| Aliphatic C–H stretching                                | 2960.243       | 2929.387               | 2929.387             |
| –CH stretching vibration of alkyl chains                | 2355.969       | 2350.388               | 2350.388             |
| Amide group (N–H stretching and C=O stretching vibration) | 1677.792       | 1652.722               | 1652.722             |
| Secondary amine group                                   | 1538.941       | 1532.403               | 1532.403             |
| N–H group and Carboxylate anion                         | 1454.087       | 1449.922               | 1449.922             |
| Carboxylate anion                                       | 1386.589       | 1382.326               | 1382.326             |
| Symmetric bending of CH\(_3\) group                    | 1304.186       | 1294.264               | 1247.752             |
| –SO\(_3\) stretching                                    | 1234.238       | 1232.248               | 1232.248             |
| C–N stretching vibrations of amino groups               | 1078.030       | 1071.628               | 1071.628             |
| O–C–O scissoring vibration of polysaccharide            | 875.039        | 870.078                | 870.078              |
| As(III)–O                                               | ×              | 719.380                | ×                    |
| As(V)–O                                                 | ×              | ×                      | 848.992              |
Nomenclature

- $C_0$: initial metal concentration (mg/L)
- $C_f$: final metal concentration (mg/L)
- $S_0$: initial peptone concentration (g/L)
- $X$: concentrations of dried cell mass (g/L)
- $X_i$: the uncoded real value of the $i$th independent variable
- $X_i^*$: the coded value of the $i$th independent variable
- $X_k$: input variables
- $X_m$: maximum biomass concentration (g/L)
- $X_1$: initial concentration of peptone (g/L)
- $X_2$: initial concentration of peptone (g/L)
- $Y$: predicted response

Greek symbols

- $\alpha$: alpha
- $\beta_0$: independent term of regression equation
- $\beta_i$: linear term of regression equation
- $\beta_{ij}$: second-order term of regression equation
- $\beta_{ij}$: interactive term of regression equation
- $\mu$: specific growth rate of the microorganisms (h$^{-1}$)
- $\Delta X_i$: the step change value
- $\epsilon$: random error

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