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Optimization of As Bioleaching by *Herbaspirillum* sp. GW103 Coupled with Coconut Oil Cake

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ABSTRACT

The objective of this study was to optimize the experimental conditions for bioleaching of arsenic (As) using *Herbaspirillum* sp. GW103 and to understand the interaction between bacteria and As during bioleaching. Five variables, temperature, time, $CaCO_3$, coconut oil cake, and shaking rate, were optimized using response surface methodology (RSM) based Box-Behnken design (BBD). Maximum (73.2%) bioleaching of As was observed at 30ºC, 60 h incubation, 1.75% $CaCO_3$, 3% coconut oil cake, and 140 rpm. Sequential extraction of bioleached soil revealed that the isolate *Herbaspirillum* sp. GW103 significantly reduced 28.6% of water soluble fraction and increased 38.8% of the carbonate fraction. The results of the study indicate that the diazotrophic bacteria *Herbaspirillum* sp. could be used for bioleaching As from mine soil.

Key words : Arsenic, Bioleaching, Box-Behnken design, *Herbaspirillum* sp., Heavy metals fraction

1. Introduction

Heavy metal pollution has become a global environmental problem, with industrial activities and mining and metallurgical activities being the major contributors. South Korea has a long history of metal mining activities, which has become widespread in the last two decades (Govarthanan et al., 2013). Currently, most of the depleted metal mines have been abandoned and left unmanaged, resulting in contamination of nearby agricultural soil and aquatic environment. To address this concern, conventional physical and chemical methods have been explored, but low efficiency and high costs limit the use of these methods (Yang et al., 2008).

Hence, biological methods such as bioremediation, which uses microorganisms and plants for the remediation of contaminated soil and water, are sought after because of their simple, inexpensive, and eco-friendly nature (Govarthanan et al., 2014a, c). Bioleaching is a type of bioremediation that has been widely used for the removal of heavy metals from sludge, sediments, ores, mine tailings, and contaminated soil (Bosecker, 2001).

Microorganisms solubilize metals from solid substrates either directly or indirectly by producing organic acids and enzymes (Rulkens et al., 1995). Several bacterial isolates belonging to the genera *Acidothiobacillus*, *Acetobacter*, *Acidophilum*, *Arthrobacter*, *Pseudomonas*, and *Bacillus* have been reported as efficient bioleachers (Mulligan and Cloutier, 2003; Deng et al., 2012; Chiang et al., 2013).

This study is an extension of our previous study that reported the Cu bioleaching efficiency of the diazotrophic bacteria *Herbaspirillum* sp. GW103 isolated from mine soil (Govarthanan et al., 2014a); here we focus on the isolate's arsenic (As) bioleaching potential. It has been established that bioleaching efficiency depends on soil geochemistry and bacterial activity. The limited nutrient availability in mine soil reduces bacterial growth and activity. Hence, several organic and inorganic nutrient supplements are used to

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stimulate bacterial growth and activity in mine soil. Among the available supplements, agricultural byproducts such as oil cakes constitute cheap and good source for stimulating bacterial growth in heavy metal contaminated soil (Govarthanan et al., 2014b).

The efficiency of bioleaching is highly influenced by several physical and chemical parameters such as incubation time, temperature, agitation rate, pH, and so on (Chen and Lin, 2001; Chen and Lin, 2010), which at optimal levels help in maximizing the efficiency of leaching. Conventional methods for optimization involve changing one independent variable at a time, while the other variables remain fixed. Statistical method, on the other hand, offers several advantages over the conventional method: it is rapid and reliable, short lists significant parameters, helps understand the interactions among the parameters at various amounts, and reduces the total number of experiments (Liu et al., 2004).

The response surface methodology (RSM) is an efficient strategic experimental tool in which the optimal conditions of a multivariable system are determined (Xu and Ting, 2004; Prakash et al., 2008). Box-Behnken design (BBD), an RSM-based design, principally requires three levels (−1, 0, and +1) to optimize the coded variables (Govarthanan et al., 2015; Selvam et al., 2014). Recently, few studies have successfully used the BBD model to optimize the physical and chemical conditions for the bioleaching of heavy metals (Chen and Lin, 2010; Arshadi and Mousavi, 2014; Bajestani et al., 2014; Biswas et al., 2014).

Thus, the objectives of the present study were (1) to assess the As bioleaching efficiency of *Herbaspirillum* sp. GW103 isolated from mine soil in the presence of coconut oil cake (2) to optimize the bioleaching conditions by BBD, and (3) to examine the As fraction in mine soil before and after bioleaching.

2. Materials and Methods

2.1. Samples, Materials, and Bacterium

The bacterium *Herbaspirillum* sp. strain GW103 was provided by Prof. Kui-Jae Lee, Chonbuk National University, Korea. Coconut oil cake was procured from a local market in Mallasamudram, Tamil Nadu, India. The chemical composition of the COC is presented in Table 1. All other chemicals used in this experiment were of analytical grade. The soil sample was collected from the metal contaminated mines in Yeohang-myeon, South Korea. The total concentration of As in the soil sample was 231 mg/kg. The physical and chemical characteristics of the mine soil are given in the Table 2 (Govarthanan et al., 2014b).

2.2. Bioleaching

Twenty grams of the autoclaved mine soils was treated with 5 ml (10^8 cells/ml) of bacterial suspension and 5 ml of autoclaved water. A RSM-based BBD design was used to optimize the experimental conditions for As bioleaching. The concentration of coconut oil cake (COC; 1-5%), Calcium carbonate ($CaCO_3$; 1-3%), temperature (20-40ºC), incubation time (24-60 h), and agitation rate (100-200 rpm) were considered as dependent variables, and the percentage of As in the sample was taken as the response. A total of 46

Table 1. Chemical Composition of Coconut oilcake (COC)

Chemical Components	Quantity (%)	
Dry matter	88.8	Gohl (1970)
Crude protein	25.2	
Crude fibre	10.8	
Ash	6.0	
Calcium	0.08	
Phosphorous	0.67	

Table 2. Physicochemical properties of mine soil

S.No	Parameters	Values	
1	Organic matter (%)	18.7	Govarthanan et al. (2014)
2	Ca (meg/100 g^{-1})	5.95	
3	Na (meg/100 g^{-1})	20	
4	NH_4 (meg/100 g^{-1})	54.5	
5	K (meg/100 g^{-1})	28.7	
6	pH	3.5	
7	EC (m^s/m)	0.64	

runs were performed to optimize the bioleaching parameters, and experiments were carried out according to the actual experimental design matrix. After appropriate time intervals of incubation, the contents of the flasks from bioleaching were centrifuged at 10000 rpm for 10 min and the supernatant was filtered using Whatmann No.1 filter paper. The filtrate was acidified using concentrated nitric acid and the heavy metal solubilization was analyzed using inductively coupled plasma mass spectrometry (Leemans Labs, USA). The results were analyzed by applying the coefficient of determination (R^2), analysis of variance (ANOVA), and response plots.

2.3. Sequential extraction of process optimized mine soil

Sequential removal of As was performed according to Deng et al. (2012). Seven operationally defined fractions of As were separated by this method: water-soluble (F1), ion-exchange (F2), carbonate (F3), reducible (F4), weak organic (F5), strong organic (F6), and residual fractions (F7).

F1: Two grams of soil was mixed with 25 mL of double deionised water (pH 7.0), and the flasks were incubated in a shaking incubator at 26ºC for 2 h.

F2: The soil from F1 was incubated with 25 mL of 1 mol/L $MgCl_2$ (pH 7.0), and the reaction mixture was agitated in a rotary shaker at room temperature for 2 h.

F3: The residue from F2 was extracted with 25 mL of 1 mol/L NaAc (pH 5.0), and the flasks were agitated at 26ºC for 5 h

F4: The residue from F3 was extracted with 50 mL of 0.25 mol/L $NH_2OH{\cdot}HCl$ in 0.25 mol/L HCl, and the flasks were agitated at 26ºC for 6 h.

F5: The soil from F4 was digested with 50 mL 0.1 mol/L $Na_4P_2O_7$ (pH 10), and the flasks were shaken at room temperature for 3 h.

F6: (i) The soil from F5 was digested with 5 mL 30% hydrogen peroxide and 3 mL HNO_3 for 1.5 h at 83 ± 3ºC. First, 3 mL 30% hydrogen peroxide was added to the reaction mixture, and agitated for 70 min. Later, 2.5 mL of NH_4Ac (3.2 mol/L) was added to the reaction mixture, diluted to 25 ml, and incubated at room temperature for 10 h.

F7: The residue from F6 was digested with 12 mL concentrated HNO_3 for 2 h at 90ºC in a hot plate. After each extraction (F1-F7), the samples were centrifuged at 6000 rpm for 5 min, and the supernatant was acidified with concentrated HNO_3 and stored at 4ºC. Finally, 1 mL of the supernatant was filtered through 0.2 μm membrane filter and analyzed for the As concentration, after appropriate dilution.

3. Results and Discussion

3.1. Arsenic Resistance in *Herbaspirillum* sp. GW103

The present study represents an attempt to assess the As bioleaching potential of *Herbaspirillum* sp. GW103 isolated from the rhizosphere soil of *Phragmites austrails* (Lee et al., 2012). The isolate GW103 was screened repeatedly to examine its metal resistance in 1/4 strength LB agar to prevent metal precipitation. The isolate GW103 exhibited maximum resistance to As (550 mg/L), which could be due to the presence of four different arsenate reductase (*arsC*) gene present in the isolate GW103 (Govarthanan et al., 2015).

Based on the BBD design, a total of 46 experiments, including 5 replicates, were performed, and the results for each run are presented in Table 3. The results analyzed using Design Expert 8.0 (trial version) showed that maximum As bioleaching (73.2%) was achieved at the following levels of dependent variables: 30ºC, 60 h incubation, 1.75% $CaCO_3$, 3% coconut oil cake, and 140 rpm. A decrease in As leaching rate was observed for other runs, which could be due to the limited growth and reduced metabolic activity of the isolate. Numerous studies have reported on the influence of pH, temperature, and other environmental factors on the bioleaching of metals (Lee et al., 2015; Xu and Ting, 2004). Lee et al. (2015) reported that the As bioleaching efficiency was significantly lower at 40ºC than at other temperatures, indicating that As bioleaching was sensitive to the physical and chemical conditions. The optimum efficiency of metal solubilization can be achieved only when the experiments operated at optimum microbial growth conditions such as, pH, temperature, incubation time and agitation rate (Xu and Ting, 2004).

3.2. ANOVA of Aresenic Bioleaching

ANOVA of the designed matrix justified the significance

Table 3. Box-Behnken design for the selected variables and observed responses

Runs	Temperature (°C)	Time (h)	$CaCO_3$ (%)	COC (%)	Shaking rate (RPM)	As Solubilization (%)
1	30	60	1.75	3.0	140	72.8
2	35	60	1.75	3.0	180	43.1
3	25	60	1.75	1.0	140	34.3
4	35	60	3.0	3.0	140	37.2
5	30	24	0.5	3.0	140	27.4
6	30	96	1.75	5.0	140	45.5
7	25	60	3.0	3.0	140	23.7
8	30	96	3.0	3.0	140	46.9
9	35	60	1.75	3.0	100	20.5
10	30	60	1.75	1.0	100	22.6
11	35	60	0.5	3.0	140	40.2
12	30	96	0.5	3.0	140	23.5
13	25	96	1.75	3.0	140	28.8
14	35	60	1.75	5.0	140	46.8
15	30	24	3.0	3.0	140	53.4
16	30	96	1.75	1.0	140	32.7
17	30	60	1.75	5.0	180	41.5
18	30	24	1.75	5.0	140	34.7
19	25	60	0.5	3.0	140	30.5
20	30	24	1.75	3.0	180	32.8
21	25	24	1.75	3.0	140	30.7
22	35	24	1.75	3.0	140	34.8
23	35	96	1.75	3.0	140	42.6
24	30	60	1.75	3.0	140	73.2
25	30	60	3.0	3.0	180	35.6
26	30	60	1.75	1.0	180	24.8
27	25	60	1.75	5.0	140	37.4
28	30	96	1.75	3.0	100	49.7
29	35	60	1.75	1.0	140	32.4
30	30	60	0.5	3.0	100	38.6
31	30	24	1.75	3.0	100	34.6
32	30	24	1.75	1.0	140	32.5
33	30	96	1.75	3.0	180	34.7
34	30	60	1.75	3.0	140	73.1
35	30	60	0.5	5.0	140	39.8
36	30	60	1.75	3.0	140	72.9
37	25	60	1.75	3.0	100	31.7
38	30	60	1.75	3.0	140	73.5
39	25	60	1.75	3.0	180	39.8
40	30	60	3.0	3.0	100	72.9
41	30	60	3.0	5.0	140	31.7
42	30	60	1.75	5.0	100	43.7
43	30	60	1.75	3.0	140	72.6
44	30	60	0.5	3.0	180	40.6
45	30	60	0.5	1.0	140	35.4
46	30	60	3.0	1.0	140	52.3

$CaCO_3$- Calcium carbonate; COC- Coconut oilcake

Table 4. Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of Squares	Df	Mean square	*F* value	*p*-value
Model	8029.02	20	401.45	6.23	<0.0001[a]
A	90.73	1	90.73	1.41	0.2464
B	34.81	1	34.81	0.54	0.4690
C	333.25	1	333.25	5.17	0.0318
D	288.32	1	288.32	4.48	0.0445
E	1.000E.002	1	1.000E.002	1.553E.004	0.9902
AB	23.52	1	23.52	0.37	0.5510
AC	3.61	1	3.61	0.056	0.8148
AD	31.92	1	31.92	0.50	0.4879
AE	35.40	1	35.40	0.55	0.4653
BC	1.69	1	1.69	0.026	0.8726
BD	27.56	1	27.56	0.43	0.5189
BE	43.56	1	43.56	0.68	0.4186
CD	30.91	1	30.91	0.48	0.4948
CE	109.20	1	109.20	1.70	0.2047
DE	4.84	1	4.84	0.075	0.7862
A^2	3821.13	1	3821.13	59.34	< 0.0001
B^2	3013.74	1	3013.74	46.80	< 0.0001
C^2	2029.97	1	2029.97	31.52	< 0.0001
D^2	2579.75	1	2579.75	40.06	< 0.0001
E^2	2848.49	1	2848.49	44.23	< 0.0001
Residual	1609.94	25	64.40	–	–
Lack of Fit	1609.43	20	80.47	791.52	<0.0001[a]
Pure Error	0.51	5	0.10	–	–
Core Total	9638.96	45	–	–	–

[a]significant

of the model (Table 4). ANOVA of the quadratic regression model suggested that it was a highly significant model, as evident from the Fisher's F-test, which showed very low probability (6.23). Values of 'Prob > F' (0.0500) indicated that the term of the model was significant. There was only 0.01% chance that a model F-value could occur due to noise. The lack of fit value (2980.08) implies that it was also significant. There is only 0.01% chance that lack of fit F-value could occur due to noise.

The predicted R^2 (0.3320) and adjusted R^2 (0.6994) values for As solubilization were in reasonable agreement with the value of R^2 (0.7671), which is closer to 1.0, indicating the better fitness of the experimental data. The adequacy of the model was determined by evaluating the lack of fit and sequential model sum of squares. The model predicted a maximum As bioleaching 73.2%. To validate the predicted model, experiments were conducted using the optimal conditions. The predicted values were in good agreement with the values measured in these experiments, thus mitigating the validity of the As bioleaching of the response model and the necessity for optimal conditions.

The interaction of two factors (2FI) was not significant using the RSM. Fig. 1 represents the three-dimensional pictures of regression equation and helped in understanding the influence variables on As bioleaching. The coefficients of the regression equation were calculated, and the following regression equation was obtained (Eq. 1).

$$Y = 73.02 + 2.38A + 1.47B + 4.56C + 4.25D - 0.025E + 2.43AB + 0.95AC + 2.82AD + 2.97AE - 0.65BC + 2.62BD - 3.30BE - 2.78CD - 5.22CE - 1.10DE - 20.92A^2 - 18.58B^2 - 15.25C^2 - 17.19D^2 - 18.07E^2 \quad \text{(Eq. 1)}$$

where Y represents As concentration, A-temperature, B-

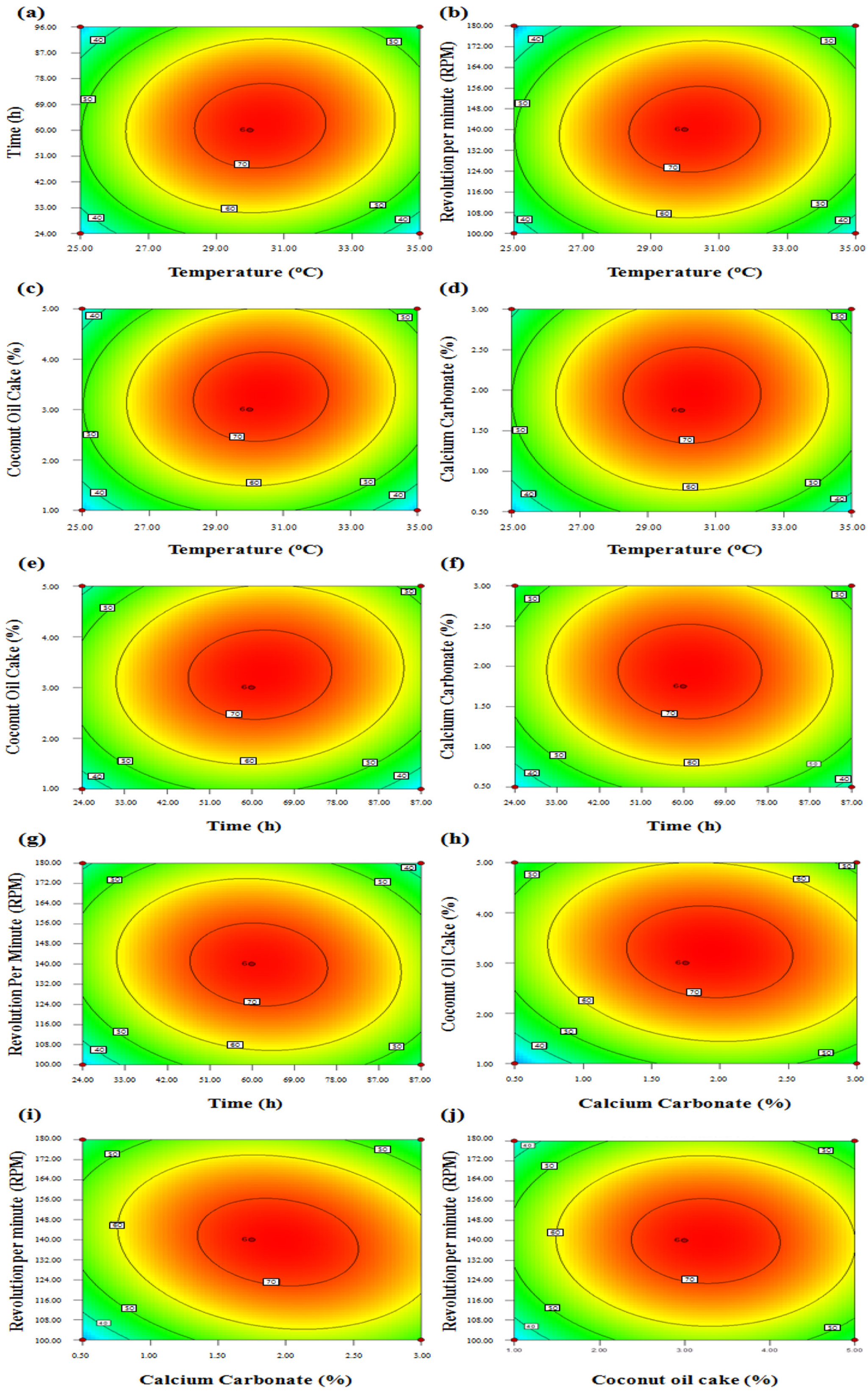

Fig. 1. Response surface contour plots of the combined effects of two variables on As bioleaching by GW103 (a-j).

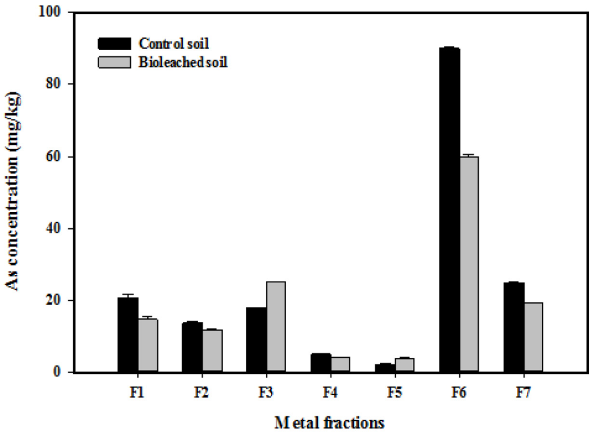

Fig. 2. Distribution of As fractions in control and bioleached soils (Three replicates). Error bars indicate standard deviation of mean, where absent, bars fall within symbols (F1- water soluble, F2- Ion-exchangeable, F3- Carbonate, F4- Reducible, F5- Weak organic, F6- Strong organic, F7-Residual fractions).

incubation time, C-$CaCO_3$ concentration, D-coconut oil cake concentration, and E-the shaking rate (rpm).

3.3. Sequential Extraction of Arsenic

To provide a comprehensive picture of GW103 and As interaction, the As concentration in bioleached soil was determined by sequential extraction methods, and the results are shown in Fig. 2. Seven different fractions, water soluble (F1), ion exchange (F2), carbonate (F3), reducible (F4), weak organic (F5), strong organic (F6), and residual (F7), were determined by this method. The order of As distribution in the control soil was found to be strong organic > residual > water soluble > carbonate > ion-exchangeable > reducible > weak organic fraction, whereas in bioleached soil the order was strong organic > carbonate > residual > water soluble > ion exchangeable > reducible > weak organic fraction.

The results indicate that the bacteria leached As from F1, F2, F6, and F7 fractions. The maximum leaching (33.3%) in strong organic fractions could be due to the secondary metabolites of the isolate GW103. Similarly, leaching in residual fraction indicates that isolate GW103 had direct interaction with metal particles. The results are consistent with previous study by Govarthanan et al.(2014a), who reported a decrease in different fraction of metals after bioleaching. Deng et al.(2012) reported the potential activity of microbial metabolites on enhanced solubilization of heavy metals. The metabolites of the microorganisms indirectly act as reactive species and solubilize metal sulfides and oxides during the bioleaching process (Mishra et al., 2008). However, the biogenically produced organic acids play a major and direct role in the bioleaching process. Aung and Ting(2005) reported that the bioleaching efficiency is directly proportional to the organic acids present in the leaching medium.

The isolate GW103 had significantly increased (38.8%) carbonate fraction in bioleached soil, and it could be due to calcite induced carbonate precipitation of As. The crystal structure of the calcite might have adsorbed As on their surface and incorporated As by substituting them for Ca^{2+} in the lattice. Thus, the carbonate fraction was not easily leached by the isolate GW103. The results are in agreement with previous studies reporting significant increase in the carbonate fraction of metals after bioaugmentation with bacterial isolates (Achal et al., 2012; Govarthanan et al., 2013).

4. Conclusions

The results of the study indicate the potential of *Herbaspirillum sp.* GW103 in the bioleaching of As from contaminated mine soil. The isolate effectively solubilize the As at optimal bioleaching conditions. Maximum As bioleaching (73.2%) was observed at 30ºC, 60 h of incubation with 1.75% of $CaCO_3$ and 3% COC at 140 rpm by applying BBD. Soil fraction studies indicated that the isolate had effectively decreased the ion-exchangeable fraction of As. These findings indicate the potential role of the isolate GW103 in bioleaching of As from mine soil.

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