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Running Title: Isolation and characterization of a biosurfactant-producing heavy metal resistant *Rahnella* sp. RM

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Abstract

Objective of the study was to isolate heavy metal resistant bacteria from chromium-contaminated subsurface soil and investigate biosurfactant production and heavy metal bioremediation. Based on 16S rRNA gene sequence and phylogenetic analysis, the isolate was identified as *Rahnella* sp. RM. The biosurfactant production by heavy metal resistant *Rahnella* sp. RM was optimized using Box-Behnken design (BBD). The maximum emulsification activity was obtained 66 % at 6 % soybean meal in pH 7.0 and 33.5 °C. The biosurfactant was characterized using Field emission scanning electron microscopy (FE-SEM), Fourier transform infrared spectroscopy (FT-IR) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF). The highest metal removal rates using the biosurfactant were found 74.3, 72.5, and 70.1 %, respectively, at the 100 mg/L amended flasks at 48 h. This study indicated the biosurfactant from heavy metal resistant *Rahnella* sp. RM could be used as a potential tool to remediate the metals in contaminated environments.

Keywords: *Rahnella* sp., emulsification activity, biosurfactant, heavy metal, bioremediation.

Introduction

The release of heavy metals into the earth is one of the major environmental pollution and has become an attention of great concern in the world due to the extensive release of metal(loid)s in soil and water [1]. Elimination of heavy metals from the contaminated soil is particularly challenging as these metals are non-biodegradable. Thus, development of remediation strategies for heavy metal polluted soil is important for ecological conservation and human health. Several physico-chemical methods have been developed to control the dispersion and biomagnification of metals from contaminated soil. However, the disadvantages and ineffectiveness of these methods have been widely reported [2-4]. An alternative to the application of phyisco-chemical methods in heavy metal removal from contaminated soil/water is the use of microorganisms, capable of reduce, immobilize, transform, and leach the metal(loid)s [5-7].

Bioremediation is considered as a simple, inexpensive, and eco-friendly technology that uses biotic communities for the remediation of contaminated soils. Microorganisms are primarily used in the bioremediation to degrade or detoxify the pollutants into harmless and less toxic forms. Several studies reported the bioremediation of metal(loid)s by using microorganisms [8-11]. In addition, the use of microbial metabolic products widely analysed new tool increasing the removal of metal(loid)s from contaminated soil/water. Among the metabolic products, biosurfactants have been widely investigated because of its multiple applications including metal(loid)s removal.

Biosurfactants (BS) have been produced by microorganisms either extracellular or as part of the cell membrane from different substrates [12]. Several bacterial isolates belonging to the genus *Bacillus, Pseudomonas, Arthrobacter, Micrococcus*, and *Rahnella* have been reported as efficient BS producers [13-19]. However, the production of BS from heavy metal resistant *Rahnella* sp. is less informative. He et al. [20] reported that the endophytic *Rahnella* sp. JN6 effectively improve the efficiency of phytoremediation in soils contaminated by Cd, Pb, and Zn. Smułek et al. [21] reported that the biosurfactant production efficiency of *Rahella* sp. strain EK12 isolated from soil contaminated with crude oil. However, there are no reports about the removal of metals by BS producing heavy metal resistant *Rahnella* sp.

It has been established that the bacterial strains are very active in an optimal growth conditions and produce efficient amount of BS. Thus, the Box-Behnken design (BBD), a statistical tool employed for the media optimization leads to the enhanced production of BS. BBD employed for multiple regression analysis by using ANOVA and quantitative data obtained from the pre-designed experiments [22, 23]. Due to the potential use in environmental applications such as heavy metal removal, significant attention has been given in the past years to the production of surface active molecules from biological sources [24, 25]. In recent years the agricultural by-products have received much attention as potential substrates for the production of BS [26, 27]. The microorganisms can easily utilize the agricultural by-products as a carbon and nitrogen source, and secrete the BS in the solid state fermentation medium. Yield of BS generally depends on nature of substrate and microbial source, therefore in current study a very distinctive nutrient source, soybean meal (SM) an agro industrial by-product obtained after oil is removed from soybean seeds, has been selected for the production of BS using a selectively isolated *Rahnella* sp., RM from chromium contaminated soil. The availability, nature of renewable source and rich nutrients properties attracts the use of SM as a substrate for the production of BS by fermentation.

Hence, the objective of the study was to isolate heavy metal resistant bacteria from chromium-contaminated subsurface soil and investigate biosurfactant production and bioremediation of heavy metals using biosurfactant.

Materials and methods

Isolation of BS producing bacteria

Soil sample was collected from Ranipet, Tamil Nadu, India, a chromium contaminated site. The site is located at 12.9283° latitude and 79.3325° longitudes. Soil sample was serially diluted into saline (0.85% NaCl) up to 10^{-8} dilution and plated using the spread plate technique onto nutrient agar (Hi-Media, India) plates. The plates were incubated at 35±2 °C for 24–48 h and observed the bacterial growth. Morphologically distinct colonies were selected and tested heavy metal resistance mechanism and biosurfactants producing capacity.

Determination of minimal inhibitory concentration (MIC) of metals

The metal resistance experiments were carried out in agar dilution method [27]. The log phase cultures of the isolates were aseptically inoculated onto nutrient agar plates amended with the metals (Cr, Pb, and Cu). The inoculated plates were incubated at 33± 2 °C for 24-48 h and monitored the bacterial growth. The concentration of metal that completely inhibited the growth of the bacteria was considered as MIC.

Screening of BS producing bacteria

The biosurfactant producing bacteria was identified using oil displacement test [15] and emulsification assay [28]. The emulsification assay was performed based on the emulsification capacity of produced BS. The emulsification assay was calculated using emulsification index.

$$E24\ (\text{Emulsification index}) = \frac{\text{height of emulsification layer developed}}{\text{Total height of liquid medium}} \times 100 \qquad (1)$$

Based on the MIC and BS screening results we have chosen the isolate RM for further experiments.

16S rRNA gene identification of the isolated bacteria

Overnight grown bacterial cells from nutrient broth were harvested and chromosomal DNA was extracted according to the protocol developed by Qiagen, DNA extraction kit (QIAGEN, CA, USA). The 16S rRNA gene of the isolate was amplified using the primers, 27f (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492r (5′ TAC GGY TAC CTT GTT ACG ACT T 3′) in a thermocycler at 95 °C (5 min) followed by 35 cycles at 94 °C (1 min), 49 °C (2 min), and 72 °C (2 min) with a final extension temperature of 72 °C for 7 min. The PCR product was purified using a PCR purification kit (QIAGEN, CA, USA) and sequenced using an automated ABI PRISM 3700 sequencer (USA). The sequences were compared using the BLAST program (http://www.ncbi.nlmnih.gov/BLAST) for identification of the isolate. Phylogenetic tree was constructed using neighbour-joining distance method by software Mega 6.0.

Substrate preparation and growth kinetics

Substrate soybean meal was procured from Mallasamudram, Tamil Nadu, India. The nutrient composition of the SM is presented in Table 1 [29]. SM was reconstituted in sterile distilled water (100 mL) at 200 rpm for 5 h, filtered through Whatman no.1 filter paper then membrane filter (0.2 µm). The SM medium was used for growth of isolate and BS production. Accordingly, Log phase culture (5 mL) of the isolate RM was aseptically inoculated into the freshly prepared different concentration (1-5 %) of SM and growth was measured at the prescribed time intervals (12–72 h) in terms of increase in optical density at 600 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan).

BS production

The SM media was used for the production of BS. Briefly, the isolate RM (5 mL) was inoculated (10^8 cells mL^{-1}) into 500 mL Erlenmeyer flasks containing 100 mL SM medium and incubated at 35 °C for 48 h (200 rpm).

Optimization for BS production using BBD

The optimization experiments were carried out to seek the enhanced amount of BS production by BBD. SM concentration, pH and temperature were employed as a key optimization factors for the BS production. A total of 17 experiments were performed according to the BBD. The results were evaluated by applying the coefficient of determination (R^2), analysis of variance (ANOVA), and response plots. The second-order polynomial equation of the BBD was developed to fit the experimental results and identify the relevant model terms

$$Y = ß_0 + \sum ß_i X_i + \sum ß_i X_i^2 + \sum ß_{ij} X_i X_j \quad (2)$$

Where Y is the predicted response; $ß_0$, $ß_i$, and $ß_{ij}$ are constant regression coefficients of the model and X_i and X_j represent independent variables.

Extraction of biosurfactant

After 72 h of fermentation bacterial cells were removed from BS containing medium by centrifugation at 10,000 rpm for 30 min at 4 °C. BS was extracted according to Pereira et al [30].

Characterization of BS

The surface morphology of the produced BS was visualised under the field FESEM (AURIGA, Carl Zeiss AG, Jena, Germany) after gold coating with an accelerated voltage of 10-20 kV. To identify the major types of functional groups, FT-IR and the spectrum of BS was obtained in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets on a Perkin-Elmer FT-IR spectrophotometer (Norwalk, USA) in the region of 4000-400 cm^{-1}.

MALDI-TOF analysis was performed on a Voyager-DE STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) in a linear mode. The MALDI matrix used for the analysis was performed according to Oh et al [31].

Removal of heavy metals using BS

Removal of heavy metals was evaluated by adding BS (250 mg/L) to metal solutions containing (100-500 mg/L) of Cr, Pb and Cu, according to Dahrazma and Mulligan [32]. The effect of the contact time (48 h at 150 rpm). The pH of the culture broth was adjusted at pH 7.0 using NaOH. Samples were withdrawn at predetermined time intervals and analyzed the metal(loid)s concentration by using inductively coupled plasma mass spectrometry (ICP, Leemans Labs, USA).

Results and Discussion

Isolation and identification of heavy metal resistant bacteria for BS production

Five morphologically different heavy metal resistant strains were isolated from the chromium contaminated soil of Ranipet, Tamil Nadu, India. The total Cr(VI) content in the soil (mg/kg) was Cr(VI) 1000 ± 5.2. All of them were screened for BS producing efficiency and the strain RM was selected due to its high metal resistance mechanism and efficient BS producing activity. The strain RM showed a high degree of resistance to Cr (800 mg/L), Pb (600 mg/L) and Cu (550 mg/L). The isolate RM showed high emulsification activity (52 %) and oil displacement activity (6.7 mm). The other four isolates showed less than 25 % of emulsification activity. Based on the BS screening results the strain RM was chosen for further optimization of BS production and heavy metal removal applications. The 16S rRNA gene sequencing was carried out to identify the isolate RM. The 16S rDNA sequence of this strain showed 99% identity with *Rahnella* sp. The partial 16S rRNA (619 bp) of the isolate RM was deposited in GenBank (Accession Number: KX656894). A phylogenetic tree was

derived from the partial 16S rDNA sequences of strain RM with existing sequences in the NCBI database, and the results are shown in Fig. 1. Several studies have reported the metal tolerance mechanism and the BS producing potential of *Rahnella* sp. [21, 33]. However, the previous studies have reported the *Rahnella* sp. could tolerate metal and produce BS individually. The *Rahnella* sp. RM showed heavy metal resistance mechanism and BS producing activity has an additional advantage for the use of the strain RM in an environmental application. The metal stress allows the *Rahnella* sp. RM to thrive under the abiotic conditions with diverse biological functions and productivity of BS.

Growth kinetics of *Rahnella* sp. RM

Till now, pure substrates such as glucose and hydrocarbonslike hexadecane, palm oil, olive oil etc have beenused for biosurfactant production [8]. In this study, SM was investigated for effective cell growth. Growth profile of the *Rahnella* sp. RM in the presence of different concentrations (1-5 %) of SM is presented in Fig.2. The cell growths were increased with increase of culture time by 42 h irrespective of SM concentration. However, they were decreased from 42 h of culture. Also, when SM concentration was increased, the cell growth was increased. These results indicate that cell growth of *Rahnella* sp. RM was strongly affected by SM concentration. The rich nutrients present in the SM had extended the log phase of growth kinetics. Our previous studies reported the nutrient properties of oil cakes and the growth profile of microorganisms [5-7]. The growth kinetics showed that the SM could be used as an alternative to the synthetic medium for the cultivation of bacteria. The presence of abundant nutrients of SM enhances the growth of bacteria as well as metabolic products synthesis. Thus, the SM was used as a substrate medium for the production of BS by fermentation.

Optimization of SM, temperature, and pH for BS production using BBD

SM plays an important role in the production of BS by *Rahnella* sp. In order to optimize physicochemical conditions for BS production, various parameters such as SM concentration, pH and temperature were used. The isolate RM showed high emulsification activity (52%) and oil displacement activity (6.7 mm) in SM medium. To obtain the maximum BS production, effect of the variables interaction on the BS production using levels of SM, temperature, and pH were determined by BBD. The experimental results obtained from three factorial design matrix, and the model was fitted well into the second order polynomial equation of the BBD. The observed results were assessed to test the significance of regression model and the results of ANOVA are presented in Table 2 and 3. The ANOVA analysis showed that the second order polynomial equation and the relationship between designed response and the significant variables. The significance of the regression model was further confirmed by the Fishers F-test with a very low probability value (F value = 32.27). The value of $P< 0.0001$ indicated the BBD model term was significant. The fitness of the model showed with the coefficient determination value (R^2). The predicted R^2 (0.8404) and adjusted R^2 (0.9462) values for BS emulsification were in reasonable agreement with the value of R^2 (0.9765), which is closer to 1.0, indicating the better fitness of the model in the experimental data. The influence of significant variables on BS production was depicted using 3D plots (Fig. 3). The observed results illustrated that the optimization of fermentation medium enhanced the BS production with the combination of variables. The results clearly indicated that the SM could be effectively used as an alternative media for the production of BS in fermentation. The coefficients of the regression equation were calculated and the following regression equation was obtained

$$Y = 63.80 + 0.38A + 0.50B + 0.12C - 1.50AB - 0.75AC - 1.00BC - 10.78A^2 - 4.52B^2 - 6.28C^2 \quad 3)$$

Where, Y stands for emulsification index, A is SM, B is temperature, and C is pH.

According to the BBD model, the maximum BS emulsification was achieved at 6.0 % SM, 33.5 °C and pH 7.0. To verify the accuracy of the model, the BS emulsification activity (66 %) was performed according to the predicted optimum fermentation condition. The results illustrated that the BS emulsification was 65.91±0.2 %, which is closely related with the predicted value of 66 %. The results from this study indicated that SM concentration, pH and temperature play a major role in the BS production from *Rahnella* sp. RM.

Characterization of BS

The structural morphology of the BS is shown in Fig.4. The results showed that the structural porous webs of BS. To identify the preliminary chemical compounds of BS, FT-IR was carried out to analyze the functional groups present in the BS (Fig.5). The results clearly indicated that the BS exhibited an absorption band at 3490 cm^{-1} of N—H stretching. The band at 2940 cm^{-1} represents the aliphatic chains (—CH_3—CH_2) of BS. A strong absorption band at 1660 cm^{-1} indicating CO—N stretching and band at 1550 cm^{-1} indicating the presence of N—H bond combined with C—N stretching pattern. The FT-IR results confirmed the presence of various functional groups of the BS [31, 34, 35]. The molecular mass of the BS was measured using MALDI TOF mass spectrometry (Fig.6). The MALDI results showed the resolved groups of peaks at 1000-1575 m/z. The extensive peaks at 1062.31, 1048.26, 1468.30; 1483.19 m/z indicated the structural analogs of BS. The results clearly indicated the presence of O-linked and N-linked polysaccharide moieties of BS obtained from *Bacillus pumilus* DSVP18 grown on potato peels [34].

Removal of heavy metals using BS

The potential of the BS obtained from *Rahnella* sp. RM in the removal of heavy metals, such as Cr, Pb and Cu was investigated in 500 mL flasks containing working volume 100 mL of metal solution (100-500 mg/L) of Cr, Pb and Cu and 250 mg/L of BS at 37 °C for 48 hr at 150 rpm. The bioremediation results of heavy metals using BS were presented in Fig.7 (a-c).

The maximum removal rate of Cu was observed 74.3 % at the 100 mg/L. However, when the Cu concentration was increased from 100 mg/L to 500 mg/L, the total removal amount was increased from 7.43 mg to 26.93 mg, which was about 3.62 times higher than that of 100 mg/L of Cu. In the case of Cr removal, the maximum removal rate of Cr was observed at 72.5 % at 48 h at the 100 mg/L. When the Cr concentration was increased from 100 mg/L to 500 mg/L, the total removal amount was increased from 7.25 mg to 24.35 mg, which was about 3.28 times higher than that of 100 mg/L of Cu. whereas, the maximum removal rate of Pb was observed at 70.1 % at 48 h at the 100 mg/L. When the Pb concentration was increased from 100 mg/L to 400 mg/L, the total removal amount was increased from 7.01 mg to 25.36 mg, which was about 3.61 times higher than that of 100 mg/L. However, in the case of over 500 mg/L, it was decreased to 24.60 mg. To conclude**,** the biosurfactant obtained from *Rahnella* sp. RM increased the removal of metals in the order of Cu>Cr>Pb at 100 mg/L of each metal concentration. On the other, in the case of 500 mg/mL, it was increased in the order of Cu>Pb>Cr. Asha A. et al. [36] reported that rhamnolipid biosurfactant by *Pseudomonas aeruginosa* BS2 selectively favours mobilization of metals in the order of Cr>Pb>Cu [36]. Apart from differential removal of metals to soil, the extraction and mobilization of metals depends on various factor like stability constant of the metals of concern with the rhamnolipid [37]. The bioremediation rate of isolate *Rahnella* sp. RM should be strongly dependent on the population of cells at optimal growth conditions. The results also suggest that the isolate *Rahnella* sp. RM can survive under the high concentration of heavy metals and has been identified as a potential candidate for application in bioremediation of heavy metals in contaminated environments. De Franca et al. [3] reported that maximum removal rate of Cu was at 48 h. It is suggested that the presence of functional groups of BS may promote the chelation of the metal(loid)s. Mulligan et al. [12, 38] reported that the carboxylic groups of amino acids significantly contribute to metal chelation by

surfactant. However, further work will address the interactions between the metal ions, and the BS in metal removal mechanism. The application of BS in heavy metal removal is potential alternative for chemical surfactants.

Conclusion

A new strain, heavy metal resistant *Rahnella* sp. RM is isolated from chromium-contaminated subsurface soil and is found to produce large quantities of BS and remove heavy metals using biosurfactant. To our knowledge, this might be the first study to screen heavy metal resistant bacteria and its ability to remediate heavy metal using biosurfactant. This study demonstrated the efficient BS emulsification was achieved using SM as an alternative substrate in fermentation. Utilization of these by-products in biotechnological production processes has importance in economical and ecological fields. For that reason, the feasibility of usage of these wastes as substrates to produce valuable biotechnological products has gained importance, recently. The BS produced from *Rahnella* sp. RM showed the maximum emulsification activity (66 %) at 6 % SM and 33.5 °C culture in pH 7.0. The bioremediation rates using the biosurfactant increased in the following order: Cu (74.3 %)> Cr (72.5 %) >Pb (70.1 %). The morphological studies, structural and functional analysis revealed that the nature of BS. Furthermore, the BS has high potential to be used in bioremediation of metal(loid)s.

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Figure legends

Fig. 1A neighbour-joining tree constructed using Mega 6.0 showing the phylogenetic relationship of 16S rDNA sequence of *Rahnella* sp. RM from closely related sequences from GenBank. Accession numbers at the GenBank of National Centre for Biotechnology Information (NCBI) are shown in parenthesis.

Fig. 2 Growth kinetics of *Rahnella* sp. RM at various concentration of soybean meal (SM). Error bars indicate standard deviation of means.

Fig. 3 Response surface 3-D plots of emulsification activity of biosurfactants produced by *Rahnella* sp. RM.

Fig. 4 FE-SEM micrographs of BS produced by *Rahnella* sp. RM.

Fig. 5 FTIR spectra of BS produced by *Rahnella* sp. RM.

Fig. 6 MALDI-TOF MS spectra of BS produced by *Rahnella* sp. RM.

Fig. 7 (a) Removal of Cr, **(b)** Removal of Cu, **(c)** Removal of Pb by using BS in batch cultures. Error bars indicate standard deviation of means.

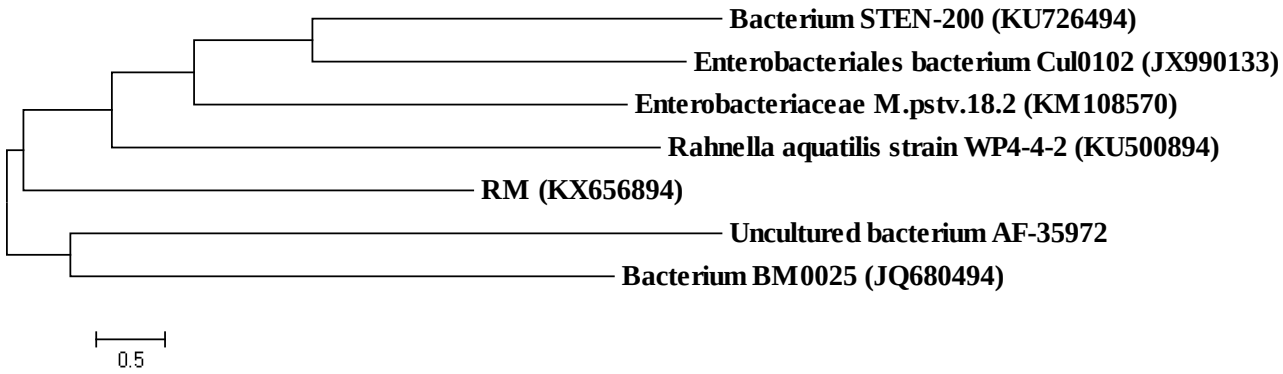
Bacterium STEN-200 (KU726494)
Enterobacteriales bacterium Cul0102 (JX990133)
Enterobacteriaceae M.pstv.18.2 (KM108570)
Rahnella aquatilis strain WP4-4-2 (KU500894)
RM (KX656894)
Uncultured bacterium AF-35972
Bacterium BM0025 (JQ680494)
0.5

Fig.1

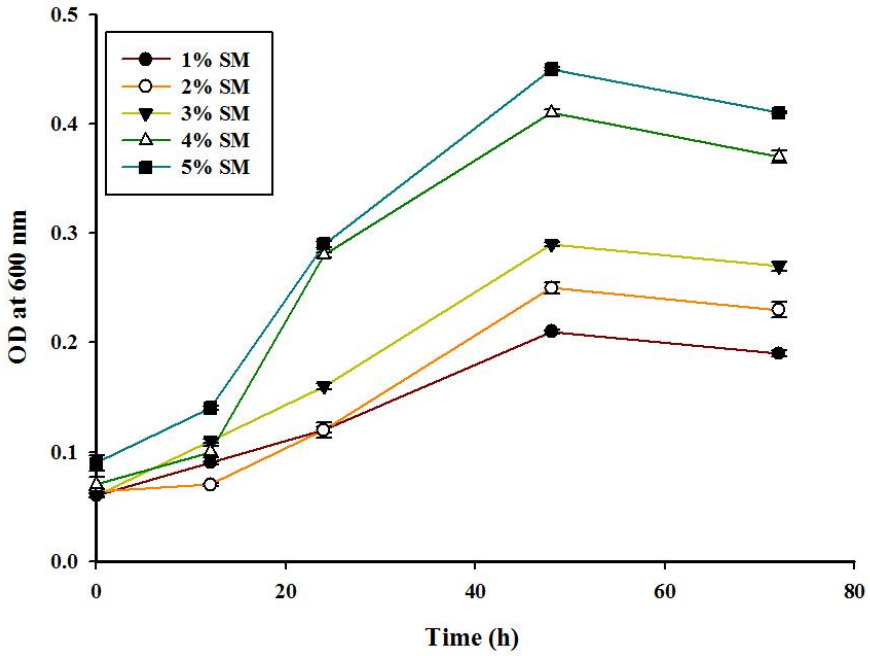
1% SM
2% SM
3% SM
4% SM
5% SM
OD at 600 nm
Time (h)
0.0
0.1
0.2
0.3
0.4
0.5
0
20
40
60
80

Fig.2

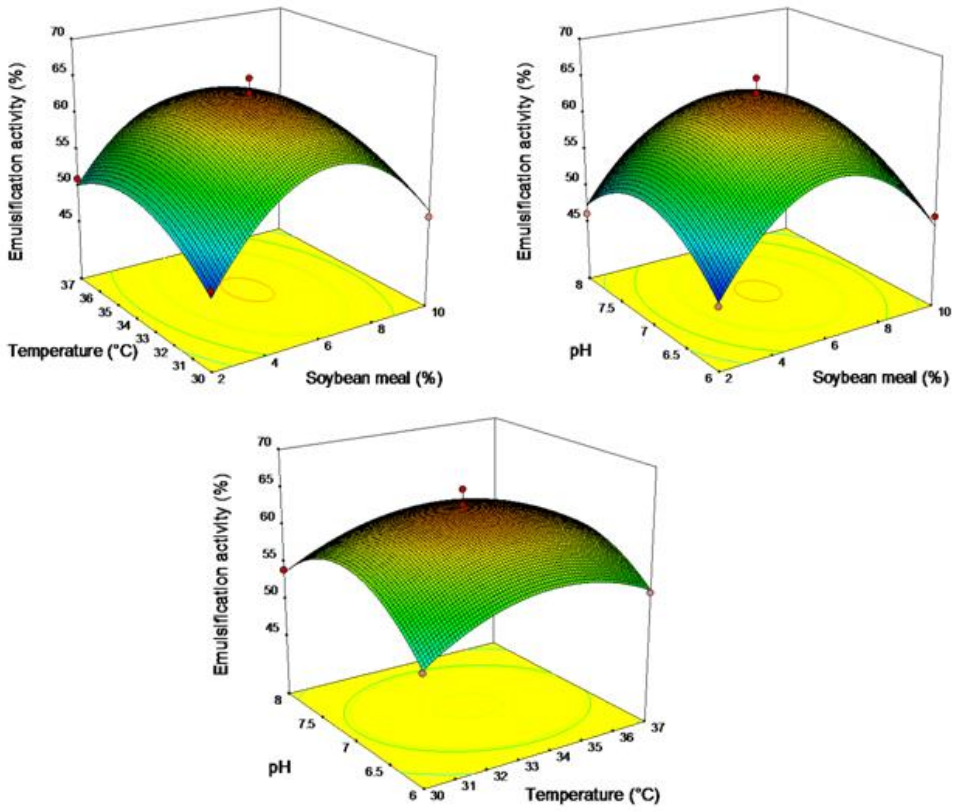
Emulsification activity (%)
70
65
60
55
50
45
Temperature (°C)
37
36
35
34
33
32
31
30
Soybean meal (%)
2
4
6
8
10
pH
8
7.5
7
6.5
6

Fig.3

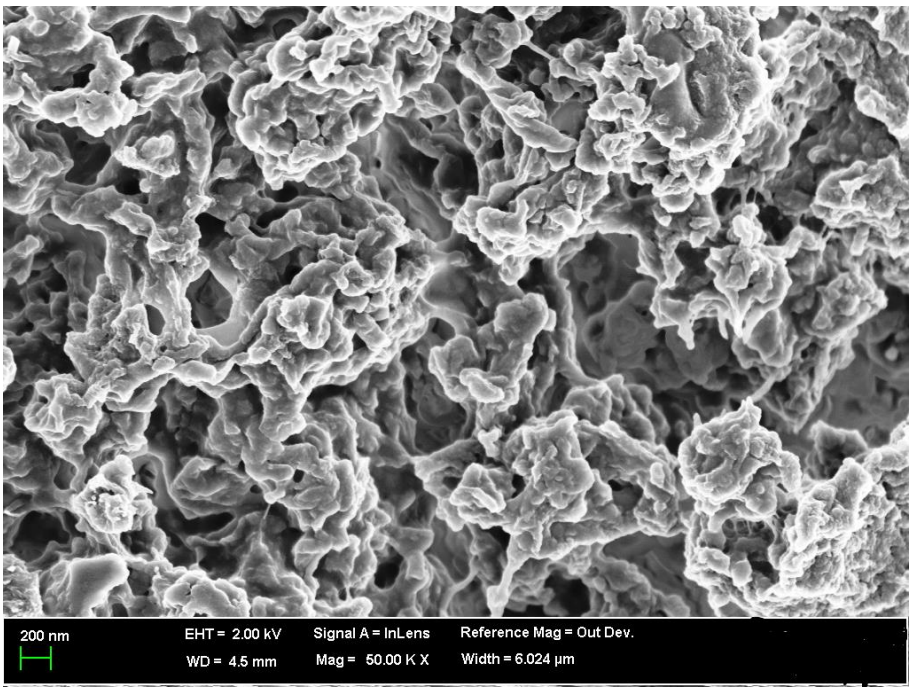
200 nm
EHT = 2.00 kV
WD = 4.5 mm
Signal A = InLens
Mag = 50.00 K X
Reference Mag = Out Dev.
Width = 6.024 µm

Fig.4

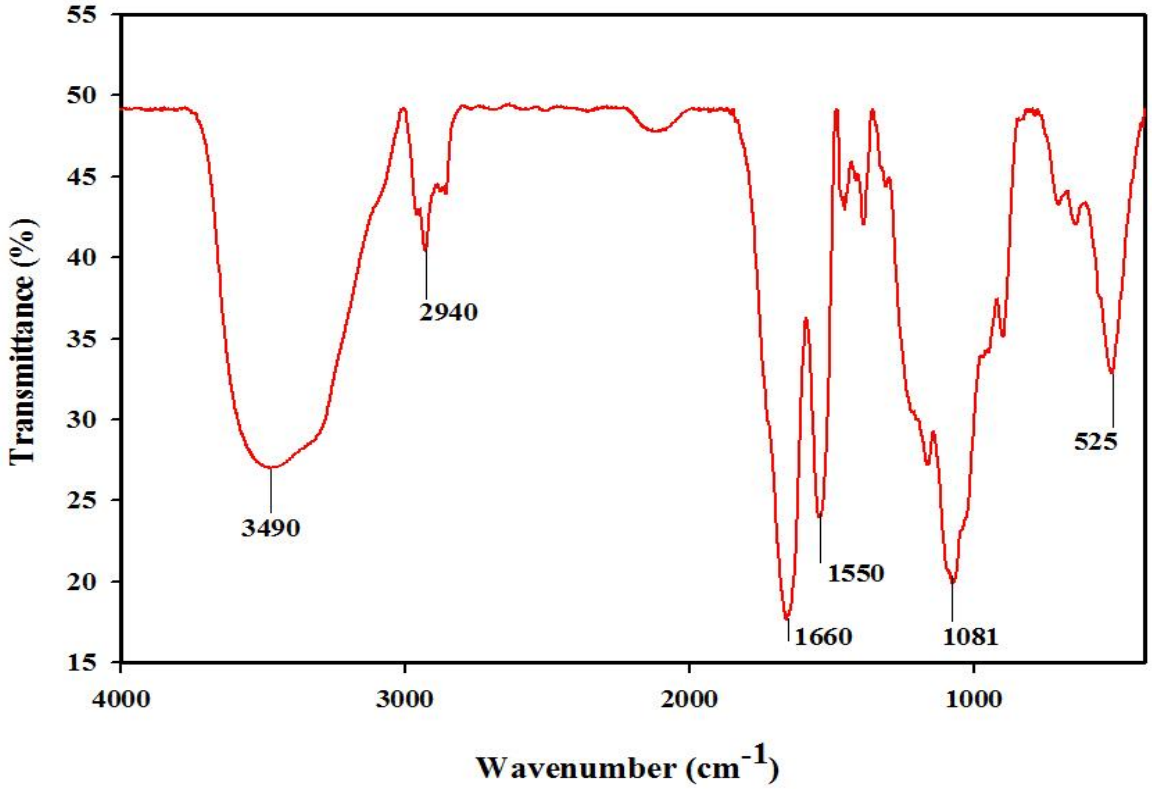
Transmittance (%)
55
50
45
40
35
30
25
20
15
4000
3000
2000
1000
Wavenumber (cm-1)
3490
2940
1660
1550
1081
525

Fig.5

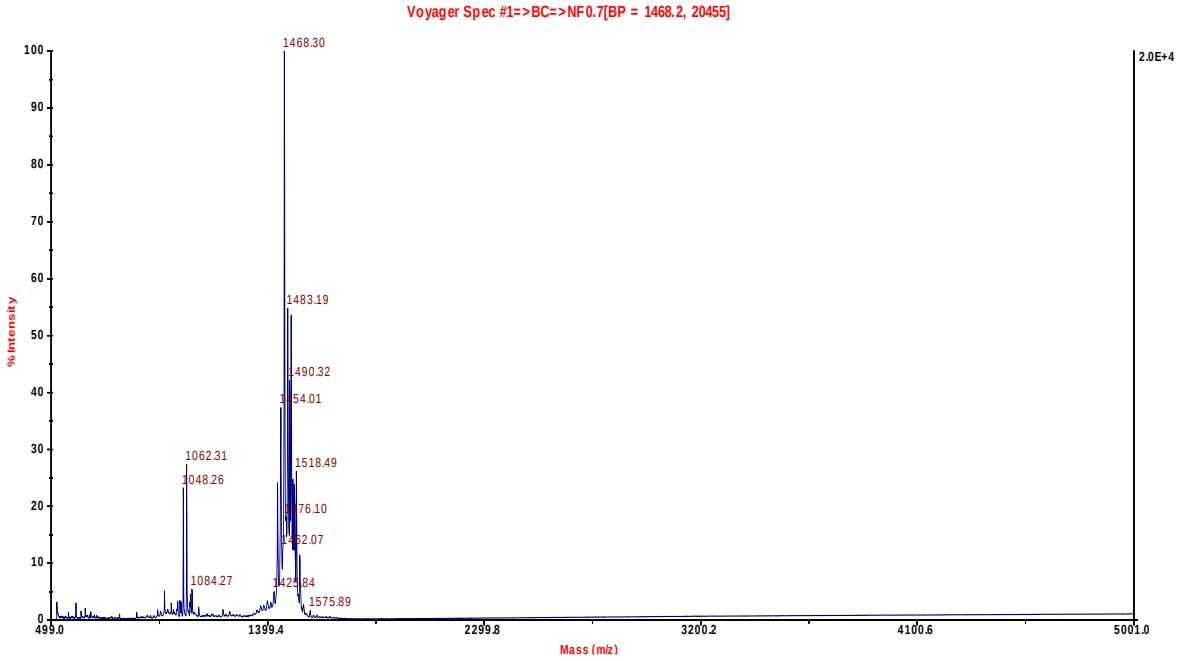
Voyager Spec #1=>BC=>NF0.7[BP = 1468.2, 20455]
% Intensity
Mass (m/z)
2.0E+4
1468.30
1483.19
1490.32
1062.31
1048.26
1518.49
1084.27
1575.89
499.0
1399.4
2299.8
3200.2
4100.6
5001.0

Fig.6

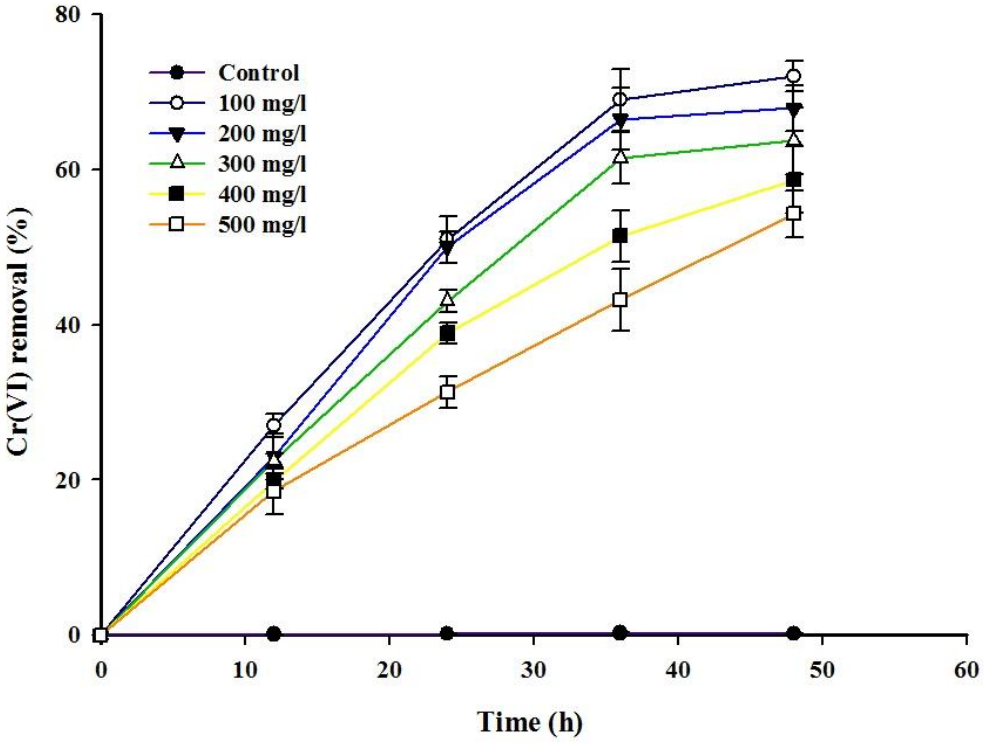
Control
100 mg/l
200 mg/l
300 mg/l
400 mg/l
500 mg/l
Cr(VI) removal (%)
Time (h)
0
20
40
60
80
0
10
20
30
40
50
60

Fig.7(a)

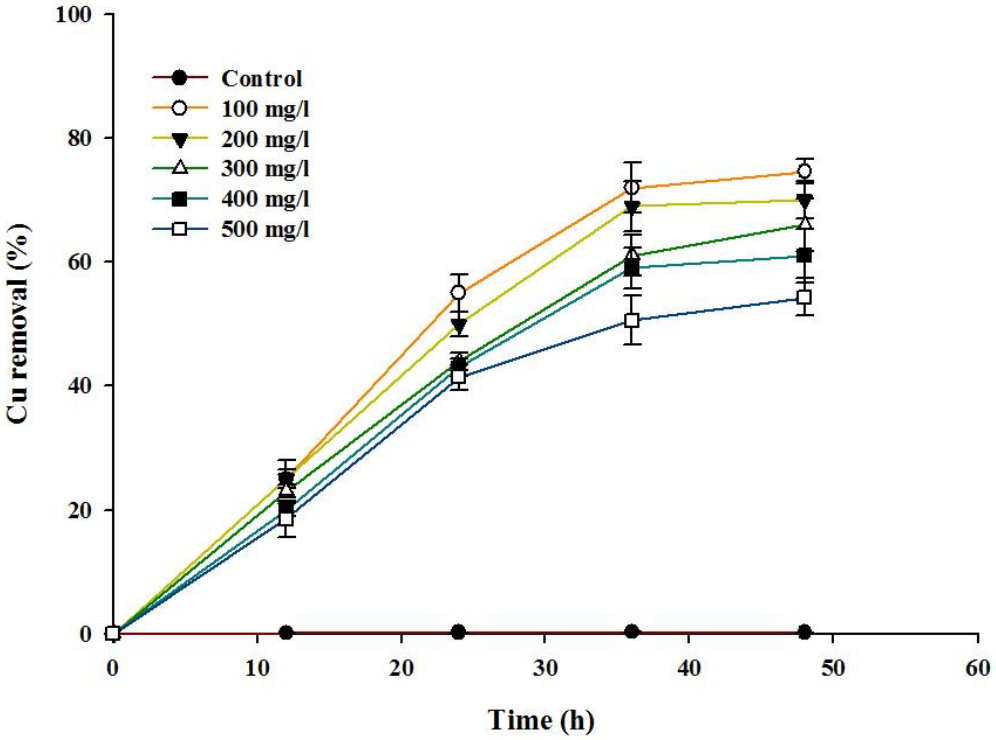
Control
100 mg/l
200 mg/l
300 mg/l
400 mg/l
500 mg/l
Cu removal (%)
Time (h)
0
20
40
60
80
100
0
10
20
30
40
50
60

Fig.7 (b)

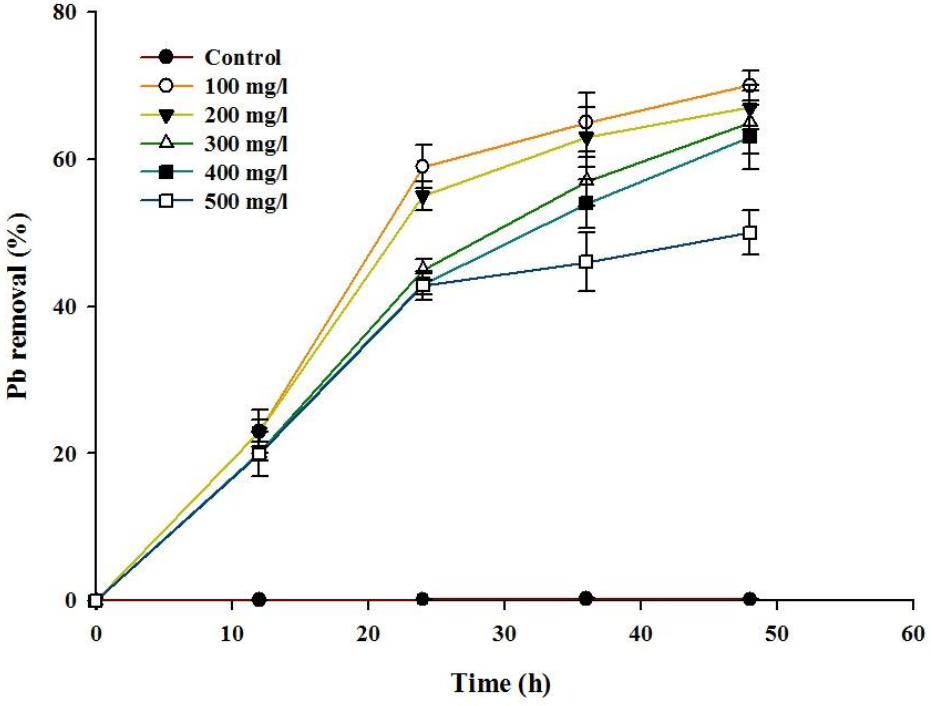
Control
100 mg/l
200 mg/l
300 mg/l
400 mg/l
500 mg/l
Pb removal (%)
Time (h)
0
20
40
60
80
0
10
20
30
40
50
60

Fig. 7 (c)

Table legends

Table 1. Nutrient Composition of SM

Table 2. Box–Behnken design for the variables and the experimental observed responses

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model

Table 1.

Chemical Components	Quantity (%)	
N-free extractive	31.82	Banaszkiewicz (2000)
Crude protein	44.40	
Crude fibre	6.75	
Ash	6.65	
Neutral detergent fiber	15.51	
Acid detergent fiber	9.5	
Crude fat	2.18	
Starch	6.3	

Table 2

S.NO	Soybean meal concentration (%)	Temperature (°C)	pH	Emulsification (%)
1	2	37	7	51
2	6	33.5	7	64
3	6	30	6	51
4	10	30	7	49
5	6	37	6	54
6	10	33.5	8	47
7	6	33.5	7	64
8	2	30	7	47
9	6	33.5	7	66
10	6	33.5	7	64
11	10	37	7	47
12	10	33.5	6	49
13	6	30	8	54
14	6	37	8	53

15	6	33.5	7	61
16	2	33.5	8	46
17	2	33.5	6	45

Table 3.

Source	Sum of Squares	Df	Mean square	*F* value	*p*-value
Model	831.83	9	92.43	32.27	<0.0001[a]
A	1.13	1	1.13	0.39	0.5507
B	2.00	1	2.00	0.70	0.4310
C	0.13	1	0.13	0.044	0.8405
AB	9.00	1	9.00	3.14	0.1196
AC	2.25	1	2.25	0.79	0.4049
BC	4.00	1	4.00	1.40	0.2759
A^2	488.84	1	488.84	170.67	<0.0001
B^2	86.21	1	86.21	30.10	0.0009
C^2	165.79	1	165.79	57.88	0.0001
Residual	20.05	7	2.86	-	-
Lack of Fit	7.25	3	2.42	0.76	0.5743
Pure Error	12.80	4	3.20	-	-

Core Total	851.88	16	-	-	-

a- significant