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Characterization of chromium bioremediation by *Stenotrophomonas maltophilia* SRS 05 isolated from tannery effluent.

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Abstract - *Chromium is an essential micronutrient that is toxic when present in high levels. Toxicity of chromium has been also associated with cancer and as a disruption to cellular functions. The present study dealing with an approach to remediate chromium from the environment is carried out using tannery effluent. Yellow bacterial colonies were selected from the mass of colonies obtained on serial dilution of tannery effluent samples. The bacteria was identical as Stenotrophomonas maltophilia SRS05 by 16SrRNA sequencing. The sequence of Stenotrophomonas maltophilia SRS05 was submitted to the NCBI and obtained the Accession number KF558319. The hexavalent chromium remediation ability of Stenotrophomonas maltophilia SRS05 was studied and quantify the chromium (VI) in the tannery effluent using Atomic absorption spectroscopy method and was observed at the 15th day of incubation. FTIR and SEM analysis revealed some irregular fragments on the adjoining cell surfaces indicating slight morphological changes on cellular surface of the identified bacterium. The bioremediation process of the above study concluded that the Stenotrophomonas maltophilia SRS05 could be used as a promising agent for the removal of Cr(VI) in effluents.*

Key Words : 1. Hexavalent Chromium remediating *microorganisms* - *Stenotrophomonas maltophilia, 2. potassium dichromate,* 3. 1,5-diphenylcarbazide (DPC), 4. Spectrophotometer, 5. Bioadsorption studies, 6. Statistical tool - Plackett-Burman design and Box-Behnken factorial design, 7. Bioremediation, 8. Atomic absorption spectroscopy and SEM analysis.

1. INTRODUCTION

Chromium is a refractory metal and is a first element in the Group 6 of the periodic table, It's a hard and brittle metal with high melting point and many of it compounds are intensly coloured. Louis Nicolas Vauquelin first isolated chromium metal from this mineral in 1798 (Barceloux, 1999). Chromium exhibits a wide range of possible oxidation states, where the +3 state is most stable energetically; the +3 and +6 states are most commonly observed in chromium compounds. The Hexavalent chromium are more toxic than Trivalent chromium compounds in terms of its Solubility in water, Permeability through biological membrane and its interaction with cellular proteins and Nucleic acids (Sultan and Hasnain, 2005).

Chromium is an essential mineral directly involved in carbohydrate, fat, and protein metabolism, However, at high concentration it is toxic, carcinogenic and tetratogenic. It is the major pollutants being discharged from various industries (electroplating, chromate manufacturing, dyes and pigment manufacturing, wood preservation, stainless-steel production, leather tanning industry, manufacture of alloys and as corrosion inhibitor in conventional and nuclear power plants.) during mechanical operations. They mix in the water bodies and make them unusable.

Among all industrial wastes, tannery effluent are ranked as the highest pollutants and large contributors of chromium pollution. In India alone about 2000–3000 tones of chromium escapes into the environment annually from tannery industries, with chromium concentrations ranging between 2000 and 5000 mg/l in the aqueous effluent compared to the recommended permissible discharge limits of 2 mg/l (Altaf *et al*., 2008). If it exceeds the permissible discharge limit the hexavalent chromium compounds in the effluent pose health risks to humans, plants, animals and fishes (Lee and Jones, 1998; Srinath *et al*., 2002).

People are facing various health problems by being exposed to the polluted water. Due to its carcinogenicity and mutagenicity, the United States Environment Protection Agency (USEPA) has designated Cr as a "Priority pollutant" or Class A" pollutant (Srinath et al., 2002; Lee and Jones, 1998). At high levels, heavy metals like chromium damage cell membranes, alter enzyme specificity; disrupt cellular functions and damage structure of DNA (Bruins et al., 2000). Cr(VI) activates p53 by reactive oxygen species (ROS) mediated free radical reactions that occur during the oxidative reduction of hexavalent Cr within the cell. Oxidative damage is considered to be an important mechanism in the genotoxicity of Cr(VI). Hence, the need arises to remediate chromium before being discharged.

Chromium remediation is an environmental challenge. Conventional methods used for the removal of hexavalent Cr are expensive and lack specificity (Katiyar and Katiyar, 1997). Recently, bioremediation is emerging as a safe and an alternative to the traditional physicochemical methods. Bioremediation is the microbial clean up approach to remove the inorganic pollutants from the contaminated site such as water, soils, sludges, and waste streams, *in situ* or *ex situ*. Hence the present investigation primarily involves the simultaneous removal of chromium in tannery effluent using

indigenous microorganisms i.e., chromium reducing bacteria used as biosorbent for removal/reduction of heavy metals from the waste material for cleaner and healthier environment.

Stenotrophomonas maltophilia, is the member of the genus *Stenotrophomonas* - Proteobacteria group. It was first isolated in 1943 as Bacterium booker and then named *Pseudomonas maltophilia* (Hugh and Leifson, 1963) and *Xanthomonas maltophilia* (Palleroni *et al*., 1973; Swings *et al*., 1983; Palleroni and Bradbury, 1993). *Stenotrophomonas maltophilia* is an aerobic, free-living, glucose, non-fermentative, Gram negative bacterium widespread in the environment, that is frequently isolated from water sources including wells, a hypereutrophic lake, bottled water and sewage rivers, soil, animals, plant materials and hospital equipment Brooke, 2012).Growth occur at temperature ranges from 20ºC to 37ºC. Growth does not occur at temperature slower 5ºC or higher than 40ºC.

This bacterium is generally consider to be an opportunistic pathogen, and is not the part of the normal flora of health humans, but can cause severe disease in immune compromised patients (Muder et al, 1987) like cancer patients, transplant recipients, and in patients undergoing peritoneal dialysis. Colony morphology and characterization of *Stenotrophomonas maltophilia* cells are straight or slightly curved non sporulating Gram-negative bacilli that are 0.5mm to 1.5 mm long. The colonies are smooth, glistening, with entire margins and are white to pale yellow (Adjide *et al*., 2010; Brooke, 2012) Aloth Nord *et al*. (1975).

Reviews on previous researches indicated that this isolate has shown tolerance to many other heavy metals such as Ag, As, Cd, Co, Hg, Pb, Zn and selenite (Clausen 2000; Pages *et al*., 2008; Ghosh and papita 2013; Sen *et al*., 2013). Thus, *Stenotrophomonas maltophilia* is useful to recycle elements in the nature and for bioremediation purposes (Ikemoto *et al*., 1980; Binks *et al.,* 1995).

Stenotrophomonas maltophilia strains are used in biological control of plant diseases owing to their production of a number of antifungal metabolites and enzymes such as chitinase (Jacobi *et al*., 1996; Minkwitz and Berg 2001; Zhang *et al*., 2007; Robert *et al*., 2009).However, very few studies (Garg *et al.,* 2012; Verma and Singh, 2012) have been reported on the simultaneous Cr (VI) reduction and aromatics degradation using pure cultures of bacteria.

2. MATERIALS AND METHODS

2.1 Effluent sample collection and analysis

Tannery effluent was collected from leather tanneries near Erode, Tamilnadu, India. The sample was stored at 4ºC to arrest biological activity and the colour and pH of the effluent was recorded. The water sample was filtered using Whatman No.1 filter paper and given for water analysis at SITRA (South India Research Association), Coimbatore, and analyzed water quality parameters such as physical, chemical and biological characteristics of water.

2.2 Isolation and identification of chromium resistant bacteria from Tannery effluent

The tannery effluent sample was taken and diluted in sterile distilled water and the dilutions from 10^{-2} to 10^{-8} were plated on Luria Bertani (LB) medium. The plates were incubated at 37ºC for 3 days. The isolated yellow pigmented colonies were subcultured on Luria Bertani (LB) medium and the selected colonies were then grown on tryptone soy agar medium throughout the study.

The isolated colonies were then identified by routine biochemical test. A presumptive identification was performed by the following tests: Grams staining, catalase test, oxidase test, urease test, indole test, methyl red-Voges Proskauer (MR-VP) test, citrate test, starch hydrolysis, triple sugar iron (TSI) test, gelatin hydrolysis, casein hydrolysis test, decarboxylation test, nitrate reduction test, phenyl alanine deamination, DNase Test, chitin hydrolysis, lipase test and esculin hydrolysis (Bernardet and Bowman 2006).

2.3 16s rRNA Sequencing

The bacterial strain SRS05 was submitted to the Progen Biotech Pvt, Ltd, Salem for 16s rRNA sequencing.

2.4 Antibiotic Sensitivity Assay

The antibiotic sensitivity assay of the strains under the study was examined by the disc diffusion method (Bauer *et al.,* 1966). To perform the disc diffusion test, culture was grown in 5 ml of Muller Hinton broth and incubated overnight at 37ºC. The suspension was spread inoculated using sterile cotton swab on to the Muller Hinton agar plates and the antibiotic disc were placed on it. After incubating the plates at 37ºC for 24h the zone of inhibition was recorded.

The antibiotic disc includes Tetracycline, Chloramphenicol, Tobramycin, Gentamycin, Streptomycin, ciprofloxacin, Ampicillin, Rifampicin, Ofloxacin, Imipenem, Enrofloxacin, Kanamycin, Polymyxin, Piperacillin, Vancomycin, Methicillin, Trimethoprime, Amikacin, Gatifloxacin.

2.5 Minimal Inhibitory Concentration (MIC) of Chromium against *Stenotrophomonas maltophilia*

Minimal Inhibitory Concentration of chromium against *Stenotrophomonas maltophilia* is determined by tube dilution method. 600mg/ml, 400mg/ml, 200mg/ml and of chromium concentrations as stock solutions were prepared. An overnight culture of *Stenotrophomonas maltophilia* SRS05 was used as the inoculum. A set of twelve sterile test tubes were taken, nine of each were marked 1,2,3,4,5,6,7,8,9 and the rest three were assigned as T_M (Medium), T_{MC} (Medium + Compound), T_{MI} (Medium + Inoculum). 1ml of sterile TSB was poured to each of the 12 test tubes. 1ml of the chromium from the stock solution was added to the each set of 1st test

tube and mixed well and then 1ml of this content was transferred to the 2nd test tube. This process of serial dilution was continued up to the 9th test tube.10µl of inoculum was added to each of 9 test tubes and mix well. To the control test tube Tmc, 1ml of the chromium was added, mixed well and 1 ml of this mixed content was discarded to check the clarity of the medium in presence of chromium. 10µl of the inoculum was added to the control test tube T_{MI}, to observe the organism in the medium used. The control test tube T_M, containing medium only was used to confirm the sterility of the medium. All the test tubes were then incubated at 37ºC for 24 hours.

2.6 Evaluation of Chromium Tolerance

The isolated bacterium *Stenotrophomonas maltophilia* SRS05 was tested for their resistance to chromate by Agar dilution method and Broth dilution method.

2.6.1 Agar Dilution Method

The culture was inoculated into TSB broth and incubated at 37ºC for 24 h in a shaker incubator at 150 rpm. In this method, overnight culture of *Stenotrophomonas maltophilia* SRS05 was aseptically streaked into freshly prepared TSB amended with potassium dichromate at various concentrations ranging from (5 mg/ml - 400 mg/ml). Plated were then incubated at 37ºC for 72 hours.

2.6.2 Broth Dilution Method

The culture was inoculated into TSB broth and incubated at 37ºC for 24 h in a shaker incubator at 150 rpm. In this method, overnight culture of *Stenotrophomonas maltophilia* SRS05 was aseptically inoculated into freshly prepared TSB amended with potassium dichromate at various concentrations ranging from (10 mg/ml - 450 mg/ml). Tubes were then incubated at 37ºC for 72 hours in orbital shaker.

2.7 Bioadsorption studies

2.7.1 Effect of Cr(VI) on bacterial growth

24h old culture of *Stenotrophomonas maltophilia* SRS05 was prepared in TSB broth. A volume of 50 ml of TSB broth is prepared in two conical flask and sterilized. In one conical flask 50µg/ml of potassium dichromate was added and 1% overnight bacterial culture of *Stenotrophomonas maltophilia* SRS05 were inoculated aseptically into each broth. This was incubated in orbital shaker at 150rpm with 37ºC and optical density was measured at different interval of time on colorimeter at 600nm.

2.7.2 Chromium uptake

24h old culture of *Stenotrophomonas maltophilia* SRS05 was prepared in TSB broth. To 100 ml of TSB, 100µg/ml of potassium dichromate was added and sterilized. Similarly another set was prepared. To the first set, 10 ml of overnight broth culture i.e., live cells were added. To the second set, 20 ml of 24 h culture was autoclaved at 121ºC for 15 minutes i.e., killed cells was added. From above, 20 ml of samples were removed at different time intervals and centrifuged at 8000 rpm for 20 minutes. Then the pellet obtained was homogenized in 2 ml of phosphate buffer. To this 0.4µl of 1,5-diphenylcarbazide and one drop of sulphuric acid was added to the homogenate. Chromium uptake was examined using 1,5-diphenylcarbazide method at 540 nm in colorimeter (Saranraj *et al.*, 2010).

2.7.3 Chromium reduction

Reduction of chromium was determined by growing the *Stenotrophomonas maltophilia* SRS05 in TSB broth supplemented with potassium dichromate at a concentration of 50µg/ml. Cells were grown on a shaker incubator (150 rpm) for 24 hours. Supernatant obtained after centrifugation was used for chromate reduction. Chromate reduction activity was estimated by 1,5-diphenylcarbazide (DPC) method with 5% (w/v) prepared in acetone. To 1 ml of supernantant, 1 ml of phosphate buffer (pH 7.2) with chromium was added and incubated for 1 hour. After incubation, 1 drop of 0.1 M sulphuric acid and 0.4µl of 1,5-diphenylcarbazide was added. The reading were taken in colorimeter at 540nm (Saranraj *et al*., 2010).

2.8 Optimization of the physiochemical parameters for degradation of chromium using univariate and statistical tools by shake flask study.

Chromium degrading *Stenotrophomonas maltophilia* SRS05 bacteria were selected for optimization study.

2.8.1 Effect of pH on chromium remediation

The optimium pH for chromium remediation by *Stenotrophomonas maltophilia* SRS05 was examined by varying the pH of the medium (TSB broth) to 3,5,7,9 and 11 using 1N HCl and 1N NaOH, and then sterilized by autoclaving at 121ºC for 15 Minutes. *Stenotrophomonas maltophilia* SRS05 was inoculated in the above medium and incubated in shaker at 150 rpm for 24h. Chromium degradation was assessed by 1,5-diphenylcarbazide method at 540 nm by spectrophotometer.

2.8.2 Effect of temperature on chromium remediation

The effect of temperature on chromium remediation was checked. 1% overnight culture of *Stenotrophomonas maltophilia* SRS05 was inoculated in the series of five 100 ml conical flask containing 50 ml of TSB broth, The inoculated medium were incubated in shaker and kept at shaking for 24 hours at different temperatures (25ºC, 35ºC, 45ºC, 55ºC and 65ºC). Chromium degradation was assessed by 1,5-diphenylcarbazide method at 540 nm by using spectrophotometer.

2.8.3 Effect of Carbon source

The effect of carbon source on chromium remediation was checked. 1% overnight culture of *Stenotrophomonas maltophilia* SRS05 was inoculated in the series of five 100 ml conical flask containing 50 ml of TSB broth respectively with different carbon sources (Glucose, Fructose, Lactose, Galactose and Sucrose) of 0.5% concentration and incubated in shaker at 37ºC for 24 hours. Chromium degradation was assessed by 1,5-diphenylcarbazide method at 540 nm by using spectrophotometer.

2.8.4 Effect of Nitrogen source

The effect of nitrogen source on chromium remediation was checked. 1% overnight culture of *Stenotrophomonas maltophilia* SRS05 was inoculated in the series of five 100 ml conical flask containing 50 ml of TSB broth respectively with different nitrogen sources (Soy bean meal, Peptone, Ammonium oxalate, Ammonium nitrate, Ammonium carbonate) of 0.5% concentration and incubated in shaker at 37ºC for 24 hours. Chromium degradation was assessed by 1,5 - diphenylcarbazide method at 540 nm by using spectrophotometer.

2.8.5 Effect of Incubation time

The effect of Incubation time on chromium remediation was checked. 1% overnight culture of *Stenotrophomonas maltophilia* SRS05 was inoculated in the series of five 100 ml conical flask containing 50 ml of TSB broth supplemented with potassium dichromate. The inoculated medium were incubated in shaker and kept at 37ºC for various time intervals (24h, 48h, 72h, 96h and 120h). Chromium degradation was assessed by 1,5-diphenylcarbazide at 540 nm by spectrophotometer.

2.8.6 Effect of different Media

The presence of carbon source on chromium remediation was checked. 1% overnignt culture of *Stenotrophomonas maltophilia* SRS05 was inoculated into the TSB broth respectively containing Nutrient Broth, Luria Bertani Broth, Tryptic Soy Broth, Brain Heart Infusion Broth and Food Flavobacterium Broth. The different media was incubated in shaker and kept for 24 hours. Chromium degradation was assessed by 1,5-diphenylcabazide at 540 nm by spectrophotometer.

2.8.7 Effect of different Metal

The effect of different heavy metals was determined in *Stenotrophomonas maltophilia* SRS05. 1% overnight culture of *Stenotrophomonas maltophilia* SRS05 was inoculated in the series of five 100 ml conical flask containing 50 ml of TSB broth respectively with different heavy metals (Cr^{6+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and pb^{2+}) of 0.5% concentration and incubated in shaker at 37ºC for 24 h. Metal degradation was assessed at 540 nm by spectrophotometer.

2.9 Statistical methodology

2.9.1 Medium

The Cr (VI) reduction potential of *Stenotrophomonas maltophilia* SRS05 was assessed in a liquid medium containing (g/l): Lactose 10, Soy bean meal 10, NaCl 5, K_2HPO_4 2.5, $K_2Cr_2O_7$ 1000µg, pH 7, Temperature 35ºC, Time, Inoculum volume 1%. For the selection of these factors, Plackett-Burman design was used. The composition of reduction of medium varied according to the design matrix.

2.9.2 Screening of important nutrient components

Plackett–Burman design (Plackett and Burman, 1946) was used to screen and evaluate the important medium components that influence the response. In practice, all the experiments were carried out according to a design matrix, which is based on the number of variables to be studied. The matrix applied to this study is shown in Table 2.9.2 Each row represents 12 different experiments to evaluate their final effects on Cr (VI) reduction and each column represents a different variable. Each independent variable was investigated at a high (+1) and a low (-1) level. Each column should contain an equal number of positive and negative signs. Ten variables, which were expected to have an effect on Cr (VI) reduction, were identified and their concentrations are shown in Table 2.9.1 All experiments were conducted in triplicate and the averages of the results were taken as response values. Uninoculated controls were included to determine the Cr (VI) loss by the components of the culture medium.

Table: 2.9.1 Variables showing medium components and test levels used in Plackett-Burman design.

Variable	Variable code	Low level (-1)	Level (0)	High level (+1)
Temperature (^{0}C)	Temp	30	35	40
Lactose (g/l)	L	5	10	15
Soybean meal (g/l)	S	5	10	15
NaCl (g/l)	NaCl	4	5	6
K_2HPO_4 (g/l)	K_2HPO_4	1	2.5	4
$K_2Cr_2O_7$ (mg/l)	Cr	1	1.4	2
pH	pH	5	7	9
Time (h)	Time	5	20	24
Inoculum Volume (%)	In.Vol	0.75	1	1.5
Moisture (%)	M	25	50	75

Table: 2.9.2 Plackett-Burman matrix for evaluating factors influencing Cr (VI) by *Stenotrophomonas maltophilia* SRS05

Factors										
Experiment	Temp	L	S	NaCl	K_2HPO_4	Cr	pH	Time	In.Vol	M
1	1	1	-1	-1	-1	1	-1	1	1	-1
2	1	1	1	-1	-1	-1	1	-1	1	1
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
4	-1	1	-1	1	1	-1	1	1	1	-1
5	-1	-1	-1	1	-1	1	1	-1	1	1
6	-1	1	1	1	-1	-1	-1	1	-1	1
7	1	-1	1	1	1	-1	-1	-1	1	-1
8	1	-1	-1	-1	1	-1	1	1	-1	1
9	1	-1	1	1	-1	1	1	1	-1	-1
10	1	1	-1	1	1	1	-1	-1	-1	1
11	-1	-1	1	-1	1	1	-1	1	1	1
12	-1	1	1	-1	1	1	1	-1	-1	-1

2.9.3 Response Surface Methodology

The optimization study was conducted in batch mode using liquid growth media. The Box-Behnken factorial design, which is standard RSM, was established on the basis of Design Expert Factorial design (Stat Ease, 8.0 trial version). Four independent variables viz Carbon source (0.25 g - 0.75g), $K_2Cr_2O_7$ (50µg- 100µg), Time (5h-24h) and K_2HPO_4 (0.05g-0.2g) were studied at different levels to obtain the response i.e. bioremediation of Cr(VI) ions. The experiment design was obtained after the selection of four independent variables (maximum and minimum values). The present study involves four- level, three factorial Box-Behnken experimental design which constituted of 29 experiments. In each experiment 1% overnight *Stenotrophomonas maltophilia* SRS05 was inoculated and incubated at Box – Behnken experimental design time.

2.9.4 Analytical methods

Samples were aseptically drawn at regular time intervals, centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for residual Cr(VI).Chromate-reducing activity was determined as decrease of chromate over time using the Cr (VI) specific colorimetric reagent 1,5-diphenylcarbazide (APHA, 1989). Spectrophotometric measurements were made immediately at 540 nm, cell-free controls were also used for each Cr (VI) reduction assay.

2.9.5 Statistical Analysis

Statistical testing of the model was performed with F-test to obtain the mathematical relationship between response i.e., Cr(VI) biodegradation and the process variables. In order to ensure a good model, the test for significance of regression model was performed by applying the analysis of variance (ANOVA). Following quadratic equation was obtained by varying four parameters:

$$Yi = ao + \Sigma ai\ Xi + \Sigma\ aiiX2\ ii + \Sigma aijXiXj + e$$

Where, Yi (i = 4) is predicted response i.e. bioremediation of Cr (VI) ions using bacterial strain, ao is the constant coefficient, aiis ith linear coefficient or slope, aiiis the ith quadratic coefficient and aij is different interaction coefficients of the model; Xi, Xj are the independent variables and e is the residual error of the model. The independent variables are coded as A, B, C and D in the present study.The second order polynomial function was fitted to correlate the relationship between independent variables and the response for prediction of the optimum point conditions.

$$Y = ao + aiA + aiB + aiC + aiD + aiA2 + aiB2 + aiC2 + aiD2 + aijA*B + aijA*C + aijA*D + aijB*C + aijB*D + aijC*D$$

The quality of polynomial model equation was expressed statistically by the coefficient of determination (R^2) and its statistical significance was determined by using F-test. Each experimental design was carried out in triplicates. T-test was used to find the significance of the regression coefficients. The residual error, pure error and lack of fit were calculated from repeated measurements (Reddy *et al.,* 2009; Myers and Montgomery, 2002). The desirable response was selected as maximum % Cr (VI) bioremediation at optimum carbon source, time, $K_2Cr_2O_7$ and K_2HPO_4 concentration. The relationship between response and experimental levels for each of the factors could be observed as fitted polynomial equation in form of surface plots.

2.10 Bioremediation

The pH of the effluent was altered to 7.0 with NaOH and then distributed at different volume in combination with culture TSB growth medium in boiling tubes (10 ml in each). 1% sucrose and lactose was added and 1% of overnight grown culture was initiated in the medium. The tubes were kept on rotary shaker at 150 rpm at 37ºC for a period of 10 days (Table 2.10.1). Chromium was estimated at an interval of 72h to calculate the chromium depletion from the effluent by the test organisms *Stenotrophomonas maltophilia* SRS05. Chromium degradation was assessed by 1,5-diphenylcarbazide method at 540 nm by spectrophotometer.

Table: 2.10.1 Experimentation of chromium bioremediation in effluent using *Stenotrophomonas maltophilia* SRS05

S.NO	Volume of Media (ml)	Volume of Effluent (ml)	Concentration of Cr (VI) mg/ml
1	5	5	0.49 mg/ml
2	6	4	
3	7	3	
4	8	2	
5	9	1	
6	9.5	0.5	

2.11 Atomic Absorption Spectrometry (AAS)

2.11.1 Sample preparation

For the determination of hexavalent chromium in tannery effluent, 100 ml of filter sterilized tannery effluent was taken in a conical flask and pH was adjusted to 7 using NaOH. 20ml of 24 h culture was autoclaved at 121ºC for 15 min and the killed cells were added to the tannery effluent. The sample was then submitted at Chemical laboratory, SITRA (South Indian Textile Research Association) Coimbatore, for the analysis of chromium content.

Similar samples were prepared for the determination of chromium reduction in tannery effluent. Live cells were added to the tannery effluent without autoclaving the culture and incubated in orbital shaker at 150rpm with 37^0C. Chromium reduction was assessed on Atomic Absorption Spectroscopy method for 20 days at 5 days interval.

2.12 Scanning electron microscopy (SEM)

SEM analysis was carried out to study the morphology of untreated *Stenotrophomonas maltophilia* SRS05, *Stenotrophomonas maltophilia* SRS05 treated with Chromium containing tannery effluent 24.5µg/ml of TSB broth and $K_2Cr_2O_7$ 100µg/ml (Xie *et al.,* 2010; Brooke, 2012) to visualize the effect of Chromium and tannery effluent on the morphology.

2.12.1 Sample preparation

TSB broth was sterilized and the effluent was filter-sterilized prior to the experiment. To each volume of 10ml TSB broth, 100µg/ml of sterilized potassium dichromate; 0.5ml of filter sterilized effluent was added separately along with 0.5% overnight bacterial culture. A separate tube containing 10ml of TSB broth and 0.5% overnight bacterial culture was maintained as control. All the tubes were incubated in shaker at 150rpm with 37ºC for 18 h and further processed for analysis.

The cells were harvested by centrifugation at 6,500 g for 15 min and fixed using 2.5% (v/v) aqueous glutaraldehyde for 2h. These cells were dehydrated using a gradient of ethyl alcohol (10–100%) and a final wash was done with absolute ethyl alcohol. The dried cells were lyophilized and subjected to Scanning Electron Microscopy (HITACHI S - 4500).

3. RESULT AND DISCUSSION

3.1 Physiochemical analysis

Industrial effluent was collected and its physiochemical parameters analyzed (Table 3.1.1). The effluent contain 490 mg/l chromium.

Table 3.1.1: Physicochemical analysis of tannery effluent

S. No	Property	Sample Analysis
1	Color	Hazen (17075)
2	pH at (30°C)	5.88
3	Dissolved Oxygen	4.56
4	Biological Oxygen Demand (BOD) (mg/l)	992
5	Chemical Oxygen Demand (COD) (mg/l)	9869
6	Total Dissolved Solid (TDS) (mg/l)	10448
7	Temperature °C	27°C
8	Electrical Conductivity	14.18
9	Total Carbon (mg/l)	3700.88
10	Chromium with effluent control (recommended permissible discharge limit) is 2 mg/l	490 mg/l

3.2Charaterization of *Stenotrophomonas maltophilia*

From tannery effluent samples, the isolated bacteria were Gram negative rods. The colony morphology and biochemical properties of the isolated strains are given in Tables 3.2.1. 10 strains showing yellow pigmentation were isolated and maintained on Tryptic Soy Agar medium. Among all the strains, on the basis yellow pigmented colonies and other biochemical test showing positive for Citrate, Catalase, KOH Assay, DNase, Decarboxylation, Esculin and Chitin hydrolysis and Chromium tolerance capability, one of the isolate SRS05 was selected for further study as it showed high degrading potential. From these biochemical properties of the isolated strains, it was identified as *Stenotrophomonas spp*.

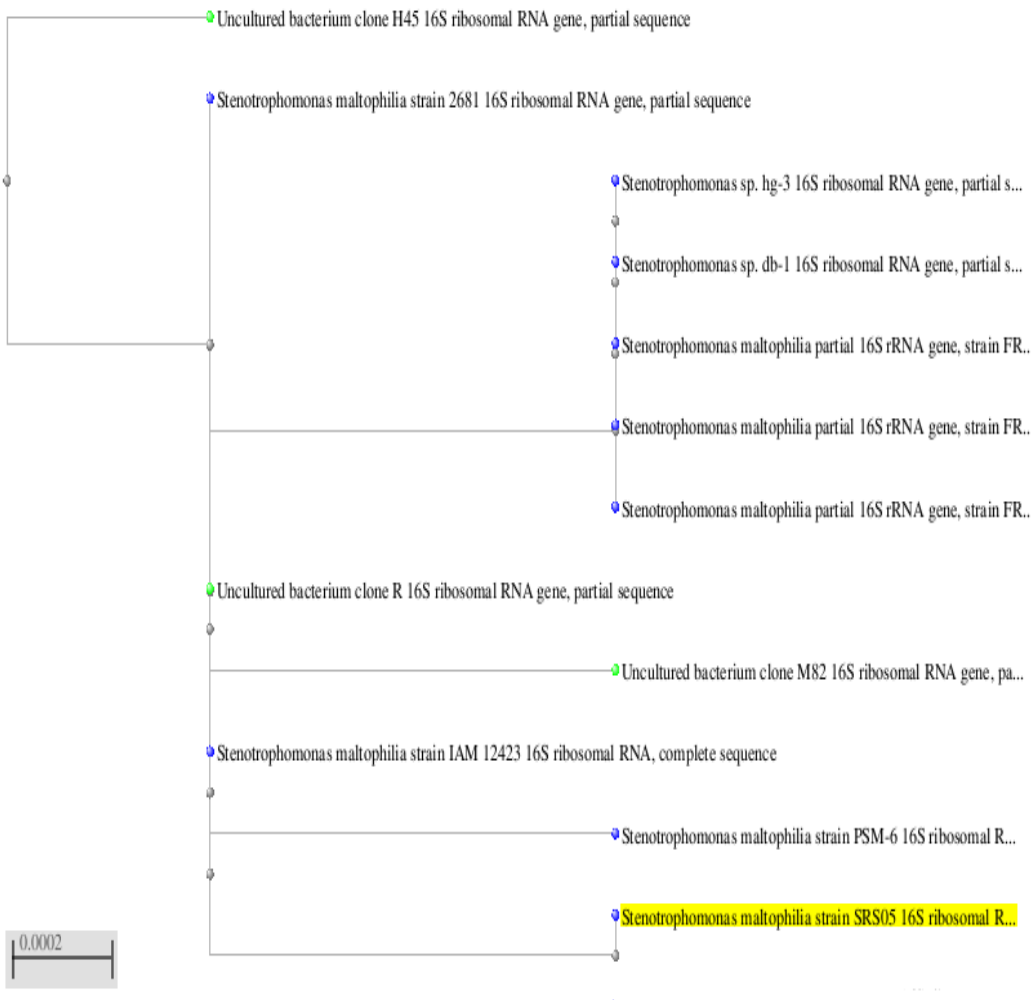
Table: 3.2.1 Biochemical Characterization of Bacterial strains isolated from Tannery Effluent

Bacterial Strain	Morphology	Gram Staining	Motility	Indole Test	MR Test	VP Test	Citrate Test	Catalase Test	Urease Test	KOH Assay	Oxidase Test	DNase Test	Nitrite Reduction	Nitrate Reduction	Lipase Test	TSI &H2S Production	Esculin Test	Chitin Hydrolysis	Deaminase Test	DecarboxylationTest (lysine)	Casein Hydrolysis	Starch Hydrolysis
SRS01	Pale Yellow, smooth with mucoid growth	Gram Negative Rods	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	+	-	+	-	-
SRS02	Pale Yellow, smooth with mucoid growth	Gram Negative Rods	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	+	-	-
SRS03	Pale Yellow, smooth with mucoid growth	Gram Negative Rods	+	-	-	-	-	+	+	+	-	-	-	-	-	+	+	-	-	-	+	+
SRS04	Pale Yellow, smooth with mucoid growth	Gram Negative Rods	+	-	-	-	-	+	+	+	-	-	-	-	-	+	+	-	-	+	+	+
SRS05	Pale Yellow, smooth with mucoid growth	Gram Negative Rods	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	+	-	+	+	-
SRS06	Yellow, spreading edges with rhizoidal growth	Gram Negative Rods	+	-	-	-	-	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-
SRS07	Yellow, Rhizoidal growth with spreading edges	Gram Negative Rods	+	-	-	-	-	+	+	-	-	-	+	-	+	+	+	-	-	+	+	-
SRS08	Pale yellow, Mucoid with smoothing edges	Gram Negative Rods	+	-	-	-	+	+	+	+	-	+	-	-	+	+	-	-	-	+	+	-
SRS09	Yellow, Rhizoid growth with spreading edges	Gram Negative Rods	+	-	-	-	+	+	+	+	-	+	+	-	+	+	+	-	-	-	+	-
SRS10	Yellow, Rhizoid growth with spreading edges	Gram Negative Rods	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	-	-	+	-	-

3.3 16srRNA Sequencing

Phylogenetic analysis of the strain SRS05 was found to be most similar to *Stenotrophomonas maltophilia* strain (IAM 12423). The next closest homologue was found to be *Stenotrophomonas maltophilia* R551-3 strain.The sequenced strain SRS05 was found to be *Stenotrophomonas maltophilia* and was submitted to Gen bank and obtained Accession no: KF558319.

Figure: 3.3.1 Phylogenetic Tree of SRS05 Strain



3.4 Antibiotic sensitivity assay

The strain of *Stenotrophomonas maltophilia* SRS05 analyzed in this study were resistant to antibiotics such as Tetracycline, Polymyxin, Vancomycin, Methicillin, Amikacin, Gatifloxacin, Gentamycin, Tobramycin, Ampicillin, Rifampicin, Streptomycin, Kanamycin, Ofloxacin, Enrofloxacin. However, Stenotrophomonas maltophilia SRS05 was sensitive to chloramphenicol, ciprofloxacin, trimethoprime and imipenem (Table 3.4.1). The breakpoints of each antibiotic was taken and confirmed with respect to the resistance and susceptibility values of the zone of inhibition (a clear halo) by the HiMedia, India.

3.5 Minimum Inhibitory Concentration of the strains

Resistance of *Stenotrophomonas maltophilia* SRS05 to chromium was determined by tube dilution method with three different concentration of chromium. (200 mg/ml, 400 mg/ml and 600 mg/ml). Good growth was seen in concentration up to 400 mg/ml beyond which growth was inhibited. The MIC of *Stenotrophomonas maltophilia* SRS05 against chromium was determined to be 400 mg/ml (Table 3.5.1).

Table: 3.4.1 Antibiotic Sensitivity Assay of *Stenotrophomonas maltophilia* SRS05

S.No	Anitibiotics	Disc Concentration (mcg)	*Stenotrophomonas maltophilia* SRS05-Antibiotic susceptibility
1	Tetracycline	30	R
2	Chloramphenicol	10	S
3	Tobramycin	10	R
4	Gentamycin	10	R
5	Streptomycin	10	R
6	Ciprofloxacin	5	S
7	Ampicillin	2	R
8	Rifampicin	5	R
9	Ofloxacin	5	R
10	Imipenem	10	R
11	Enrofloxacin	5	R
12	Penicillin	2 units	R
13	Kanamycin	30	R
14	Polymyxin	50	R
15	Piperacillin	100	R
16	Vancomycin	30	R
17	Methicillin	5	R
18	Trimethoprime	10	S
19	Amikacin	30	R
20	Gatifloxacin	5	R

R-resistance S-sensitive

3.6 Evaluation of chromium tolerance

In agar dilution method, the bacterial isolate *Stenotrophomonas maltophilia* SRS05 was resistant to chromium at 200 mg/ml. Growth was found to be inhibited at concentration higher than 200mg/ml. In broth dilution method, the bacterial isolate *Stenotrophomonas maltophilia* SRS05 was resistant to chromium at 400 mg/ml. Growth was found to be inhibited at concentration higher than the 400mg/ml. The results indicated that *S.maltophilia* SRS05 had a higher degrading capacity of chromium. The wide difference in these results might be attributed to the fact that the growth conditions are more favorable in the broth due to better aeration and agitation conditions when compared to an agar plate.

3.7 Bioadsorption study

3.7.1 Effect of Cr(VI) on bacterial growth

The growth of the isolates were studied in the presence and the absence of Cr (VI). The optical density of *Stenotrophomonas maltophilia* SRS05 were determined at 600 nm.The growth of the isolate is much better in presence of Cr (VI) in the medium which is clearly shown in the Figure:3.7.1.1.

Table: 3.5.1 MIC of the *Stenotrophomonas maltophilia* SRS05 against different concentration of chromium

S. No	Inoculum added in 1 ml of nutrient broth (µl)	Concentration of Chromium (600 mg/ ml)		Concentration of Chromium (400 mg/ ml)		Concentration of Chromium (200 mg/ ml)	
		Dilution of Chromium (mg/ml)	Growth observed against Cr	Dilution of Chromium (mg/ml)	Growth observed against Cr	Dilution of Chromium (mg/ml)	Growth observed against Cr
1	10	600	-	400	+	200	+
2	10	300	+	200	+	100	+
3	10	150	+	100	+	50	+
4	10	75	+	50	+	25	+
5	10	37.5	+	25	+	12.5	+
6	10	18.5	+	12.5	+	6.25	+
7	10	9.325	+	6.25	+	3.175	+
8	10	4.687	+	3.175	+	1.562	+
9	10	2.34	+	1.562	+	0.781	+
TMC	0	600	-	400	-	200	-
TMI	10	-	+	-	+	-	+
TM	0	-	-	-	-	-	-

+ indicates growth - indicates no growth

3.7.2 Chromium uptake

Bioadsorption studies were done to test the ability of *Stenotrophomonas maltophilia* SRS05 to accumulate chromium at different time intervals 30minutes, 1h and 5h. The rate of chromium accumulation was rapid. It showed that the cells exhibited better chromium uptake in short period of time. The results were shown in Figure: 3.7.2.1

3.7.3 Chromium reduction

The ability of *Stenotrophomonas maltophilia* SRS05 to reduce chromium at various time intervals from 30 min, 1h and 5h was 5, 7.8, 10.9 µg/ml of chromium and the chromium reduction is shown in Figure: 3.7.3.1 It showed that the chromium reduction is increased in increasing time intervals.

Figure: 3.7.1.1 Growth of *Stenotrophomonas maltophilia* SRS05 in chromium containing medium

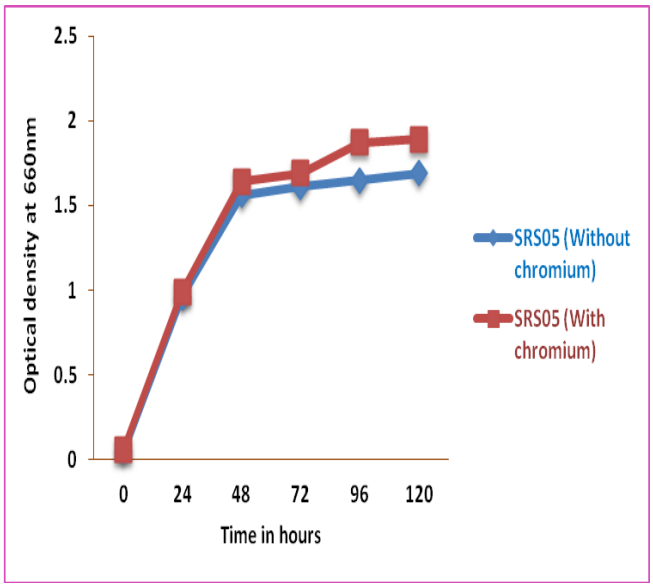


Figure: 3.7.2.1 Chromium uptake by *Stenotrophomonas maltophilia* SRS05 at Different time intervals

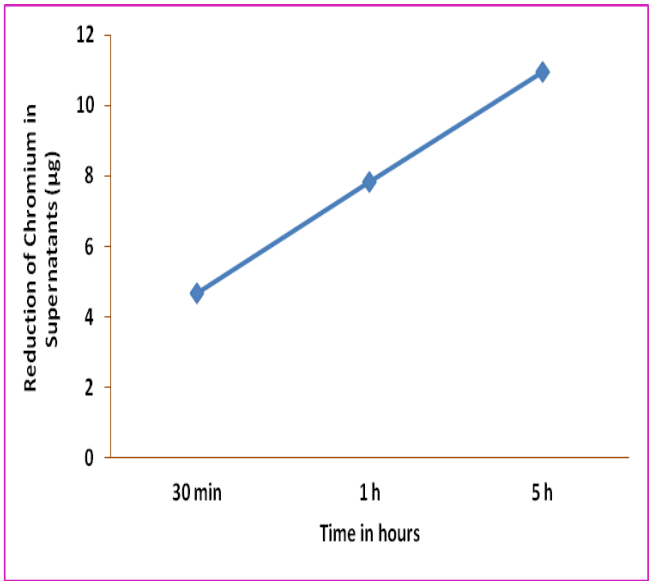
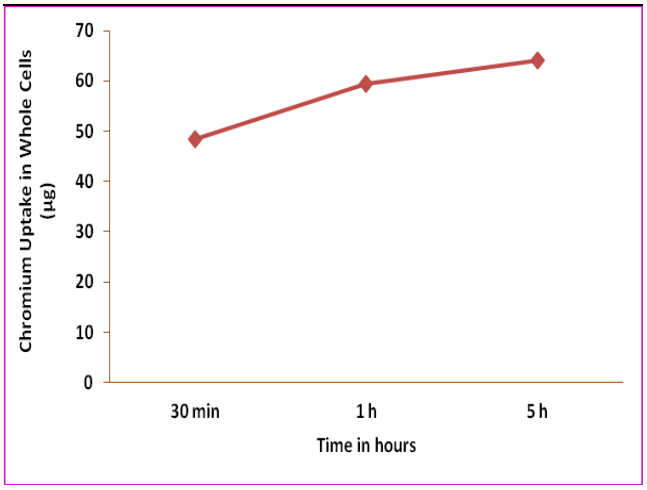


Figure: 3.7.3.1 Chromium reduction by *Stenotrophomonas maltophilia* SRS05 at different time intervals



The bioadsorption studies were done to determine the chromium uptake and reduction by *Stenotrophomonas maltophilia* SRS05 using 1,5-Diphenylcarbazide method. The chromium levels were determined in the cell biomass and media supernatant after incubation for overnight. The chromium levels were found to be higher in the cell biomass when compared to media supernatant. These results indicated that the uptake of chromium was higher than its reduction. The higher presence of chromium in cell biomass indicates that the organism utilizes the chromium for its metabolic activities and hence it is present in higher quantities in the cell pellet. Kader *et al*. (2007) showed that the rate of chromium accumulation by active cells was also faster compared to chromium reduction. Shahida and Thakur. (2007) found that *Brevibacterium* sp. showed higher chromium uptake than the pellet of killed cells when time intervals increased. The chromium remediation might be due to excellent potential of metal biosorption. This potential in metal recovery and remediation can be due to binding of other metals along with chromium (Butt and Jecker, 1987). Of the different consortia of bacteria and fungi (Katiyar and Katiyar, 1997) *Saccharomyces* and *Pseudomonas* was found to be most efficient. The high efficiency might be contributed to the chromium reductase genes in *P.aeroginosa* and the Mt genes in *S.cerevisiae. Stenotrophomonas maltophilia* SRS05 showed good degradation capacity.

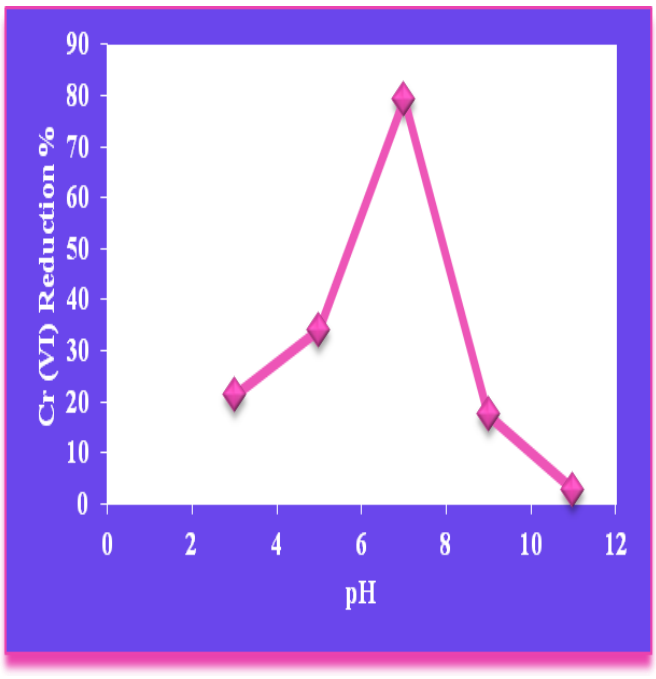
The bioremediation process of the above study concluded that the *S.maltophilia* could be used as a promising agent for the removal of Cr(VI) in effluents.

3.8 Screening of variables for Plackett-Burmann design

3.8.1 Effect of different pH on chromium reduction

The percentage of Cr (VI) reduction by *Stenotrophomonas maltophilia* SRS05 was evaluated at various pH ranging from 3-11. Figure: 3.8.1 shows that, *Stenotrophomonas maltophilia* SRS05 could reduce Cr (VI) in a wide range of pH (5-7) with maximum Cr(VI) reduction at pH 7.0 and therefore pH 7 was selected for the rest of the experiments. The growth of *Stenotrophomonas maltophilia* SRS05 was also found to be maximum at pH 7. These results are in accordance with the findings of other reports (Wang and Xiao, 1995; Liu *et al*., 2004; Mona and Mabrouk, 2008; Sukumar, 2010; Damaris *et al*., 2013; Gunasundari and Muthukumar, 2013). However, since Cr (VI) reduction is enzyme-mediated, pH changes affects the enzyme ionization rate, changes the protein's conformation and consequently affects the enzyme activity (Farrell and Ranallo, 2000). The difference in optimum pH value suggests that pH modification is important for different cultures to achieve the maximum Cr(VI) reduction in the bioremediation of chromate.

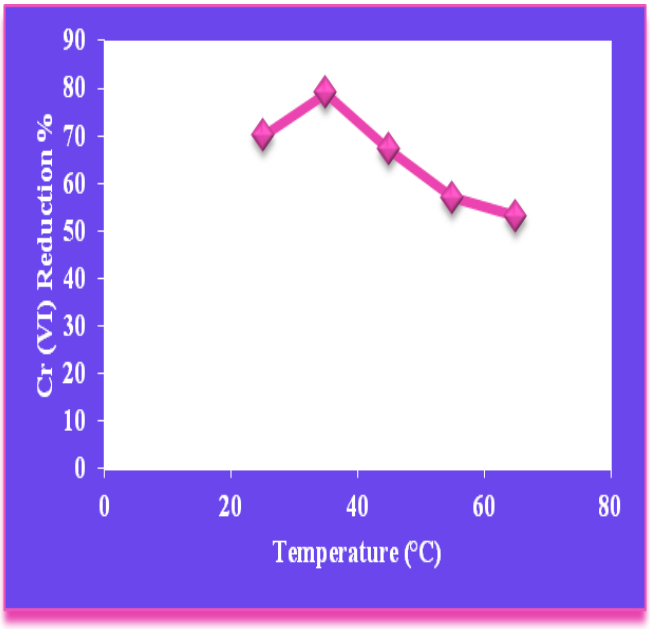
Figure: 3.8.1 Effect of different pH on reduction of Cr(VI) by *Stenotrophomonas maltophilia* SRS05



3.8.2. Effect of different Temperature on chromium reduction

The percentage of Cr (VI) reduction by *Stenotrophomonas maltophilia* SRS05 was evaluated at various temperatures ranging from 25°C - 65°C. Reduction was exhibited over the temperature range 30 – 45°C with maximum at 35°C, after that the Cr(VI) reduction percentage is gradually decreased with increased temperature and therefore temperature 35°C was selected for the rest of the experiments (Figure: 3.8.2). The growth of *Stenotrophomonas maltophilia* SRS05 was also found to be maximum at 35°C Losi *et al.* (1994), Camargo *et al.* (2003), Sukumar (2010), Mohammad llias *et al.* (2011), Damaris *et al*. (2013), Gunasundari and Muthukumar (2013) Rachna bhateria and Rajesh dhankhar (2013) reported an optimal temperature of 30 – 37°C for Cr(VI) reduction.

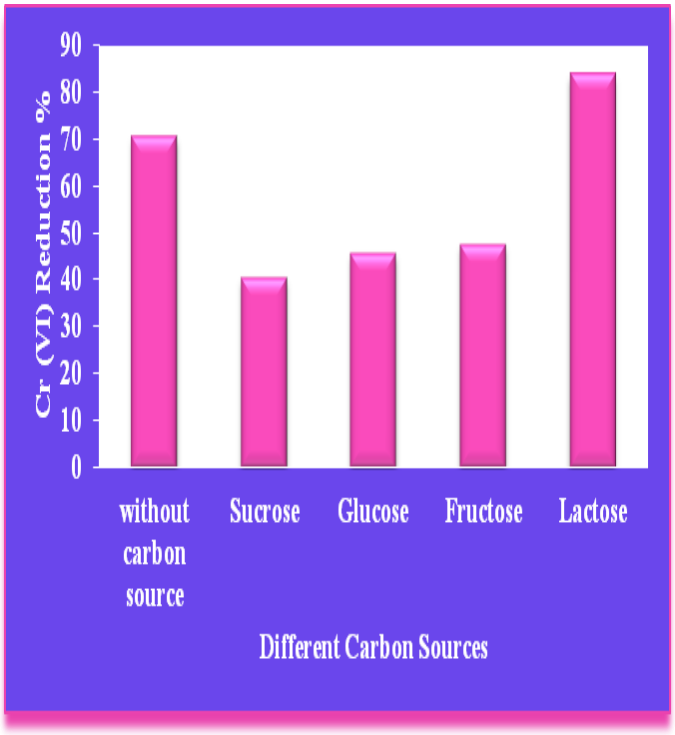
Figure: 3.8.2 Effect of different Temperature on reduction of Cr (VI) by *Stenotrophomonas maltophilia* SRS05



3.8.3. Effect of different Carbon Sources on growth and chromium reduction

The carbon source was found to be essential for the growth of *Stenotrophomonas maltophilia* SRS05. The percentage of Cr (VI) reduction by *Stenotrophomonas maltophilia* SRS05 was evaluated at different carbon sources (Glucose, Fructose, Lactose, Galactose and Sucrose). Among these, the percentage of Cr (VI) reduction was maximum in lactose containing medium and therefore lactose was selected for the rest of the experiments. It was also observed that Cr (VI) reduction was growth related. An addition of lactose was found to enhace the growth of the bacterium (Figure:3.8.3). Growth-related chromate reduction was also reported previously (Sultan and Hasnain, 2007; Preetha *et al.,* 2007; Magdi *et al.*, 2010; Sukumar, 2010).

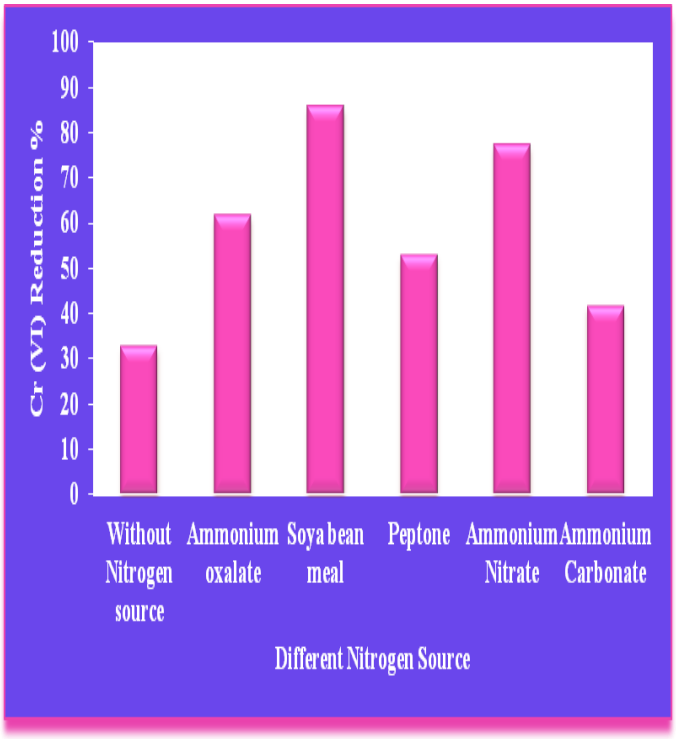
Figure: 3.8.3 Effect of different carbon source on reduction of Cr (VI) by *Stenotrophomonas maltophilia* SRS05



3.8.4. Effect of different Nitrogen Sources on growth and chromium reduction

The nitrogen source was found to be essential for the growth of *Stenotrophomonas maltophilia* SRS05. The percentage of Cr (VI) reduction by *Stenotrophomonas maltophilia* SRS05 was evaluated at different nitrogen sources (Soy bean meal, Peptone, Ammonium oxalate, Ammonium nitrate, Ammonium carbonate). Among these, the percentage of Cr (VI) reduction was maximum in Soy bean meal containing medium and therefore Soy bean meal was selected for the rest of the experiments. It was also observed that Cr (VI) reduction was growth related. An addition of Soy bean meal was found to enhance the growth of the bacterium. (Figure: 3.8.4). Growth-related chromate reduction was also reported previously (Sultan and Hasnain, 2007; Preetha *et al.*, 2006; Magdi *et al.,* 2010).

Figure: 3.8.4 Effect of different Nitrogen Sources on reduction of Cr (VI) by *Stenotrophomonas maltophilia* SRS05



3.8.5 Effect of incubation time on chromium reduction

The incubation time was found to be an important parameter for the growth and reduction of Cr (VI) by *Stenotrophomonas maltophilia* SRS05. The growth pattern of the Chromium resistant bacterial culture *Stenotrophomonas maltophilia* SRS05 was observed and to accumulate chromium at various time interval from 24 h to 120 h. Over the period of time the percentage of Cr (VI) reduction by *Stenotrophomonas maltophilia* SRS05 was maximum in120 h. It was also observed that Cr (VI) reduction was growth related. Growth-related chromate reduction was also reported previously (Sultan and Hasnain, 2007). The results were shown in the following graph (Figure: 3.8.5).

3.8.6. Effect of different Media on growth and chromium reduction

The culture *Stenotrophomonas maltophilia* SRS05 was inoculated in different media which included Nutrient Broth, Luria Bertani Broth, Tryptic Soy Broth, Brain heart infusion Broth and Food Flavobacterium Broth to determine the growth and chromium reduction percentage. The growth pattern of *Stenotrophomonas maltophilia* SRS05 and its reduction of chromium percent were found to be highest in TSB medium (Figure: 3.8.6) and therefore Tryptic Soy Broth was selected for the rest of the experiment.

3.8.7. Determination of Heavy metal degradation by *Stenotrophomonas maltophilia* SRS05

The resistance of chromium degrading cultures was determined against with other heavy metals (Figure: 3.8.7). Result as follows,

SRS05 degrades Lead > Chromium > Cobalt > Mercury > Copper >Cadmium >Zinc.

When compared to growth conditions without the presence of metals it can be deduced that the organism is sensitive to mercury, copper and cadmium.

Figure: 3.8.5 Effect of incubationtime on chromium reduction of *Stenotrophomonas maltophilia* SRS05 on its uptake of chromium

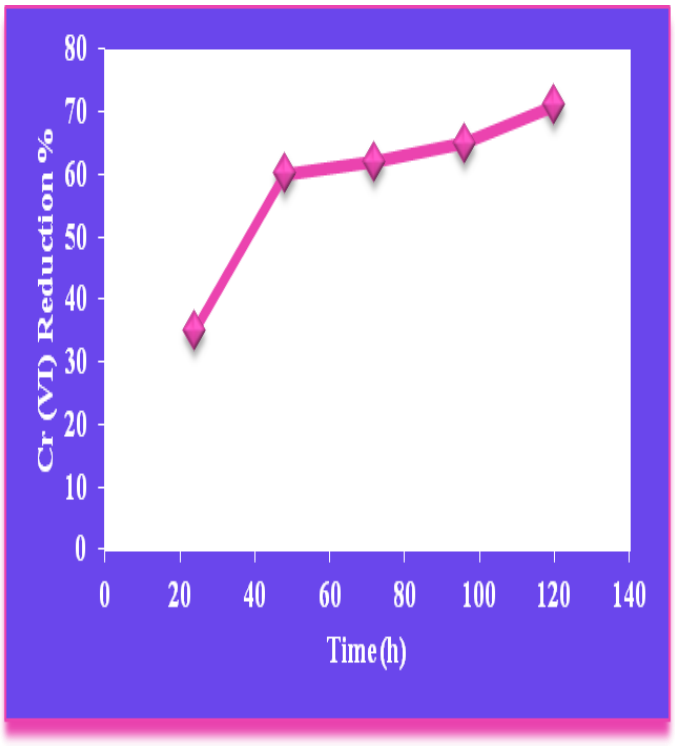


Figure: 3.8.6 Chromium reduction by *Stenotrophomonas maltophilia* SRS05 on different media

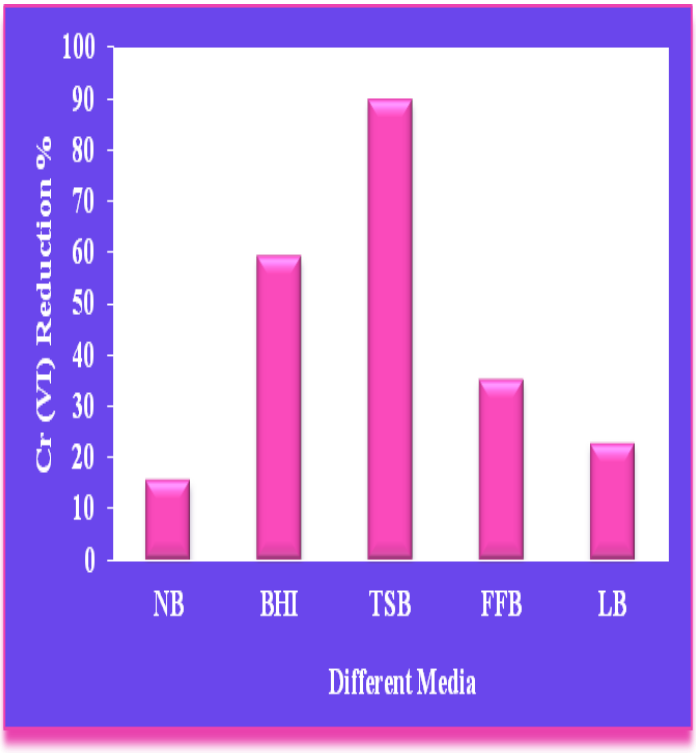
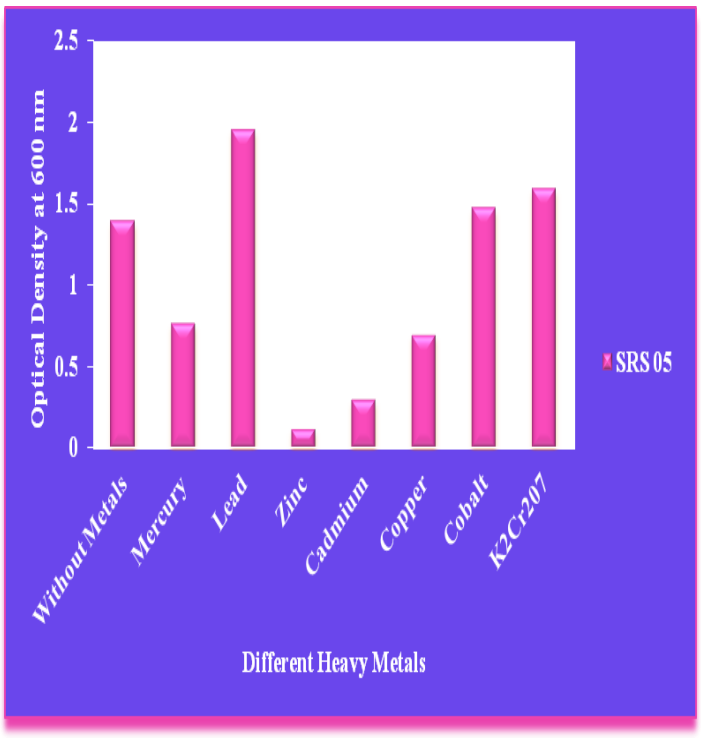


Figure: 3.8.7 Effect of different Heavy metals on growth of *Stenotrophomonas maltophilia* SRS05



3.8.8 Evaluation of culture conditions affecting Cr (VI) reduction by *Stenotrophomonas maltophilia* SRS05

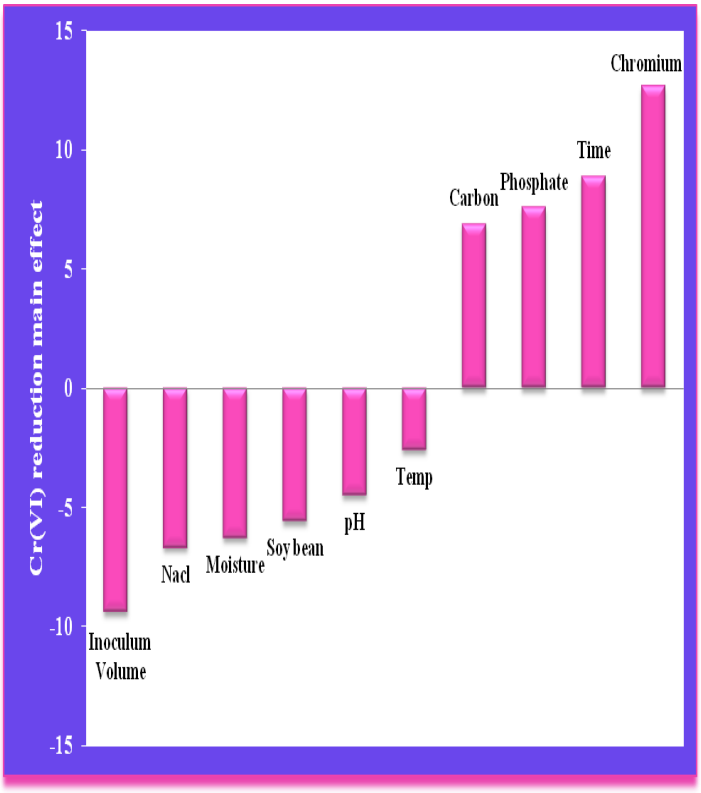
The use of Plackett–Burman design aided in ranking factors from different categories to enable better understanding of the medium effect. A wide variation in Cr(VI) reduction was clearly observed, which reflects the importance of medium optimization to attain high reduction. The main effects of the examined factors on Cr(VI) reduction were calculated and are presented graphically in Figure 3.8.8 The data showed that carbon source (lactose), $K_2Cr_2O_7$, Time and K_2HPO_4 within the test range had a positive effect on Cr (VI) reduction, whereas Nitrogen source (soy bean meal), NaCl, and Temperature, contributed negatively. Some researchers thought that the variables with confidence level above 80% (Pujari and Chandra, 2000) or 85% (Xiong *et al*., 2004) were significant. The components were screened at the confidence level of 80% on the basis of their effects (either positive or negative). Table 3.3 represents the results of Plackett–Burman experiment with respect to the *t*-value, *p*-value and confidence level of each component. A significance at or above the 80% confidence level, indicates that the component was effective in Cr(VI) reduction.

Of the ten culture factors tested, only Carbon source (lactose), K_2HPO_4, $K_2Cr_2O_7$, and Time of the medium had a significant effect on Cr(VI) reduction at a confidence level above 80% and are thus regarded to be the most significant variables (Table 3.8.8). From the results obtained in this experiment, it has observed that the confidence level of Carbon source (lactose 85%), K_2HPO_4 (89%), $K_2Cr_2O_7$ (90%), and Time (83%) are favored for Cr(VI) reduction.

Table: 3.8.8 Statistical analysis of the explicative factors on Cr (VI) reduction from the results of Plackett-Burman design.

Variables	p-Value	Confidence level (%)	Main effect	t-Value
Carbon Source	0.17	85	6.9	1.4
Soy bean meal	0.82	81	-5.6	2.5
Time	0.04	83	8.9	1.5
Chromium(VI)	0.1	90	12.67	1.99
pH	0.61	76	-4.5	2.7
Phosphate	0.01	89	7.6	1.89
NaCl	0.53	65	-6.7	3.83
Inoculum Volume	0.45	74	-9.4	3.53
Moisture	0.13	69	-6.3	3.88
Temperature	0.22	72	-2.6	3.45

Figure: 3.8.8 Effect of different factors on Cr (VI) reduction by *Stenotrophomonas maltophilia* SRS05 based on the result of Plackett-Burman Design



The above results indicate that the Plackett–Burman design is a powerful tool for determination of relevant variables, which had a significant influence on Cr (VI) reduction and shows a much higher level of Cr(VI) reduction. Based on the *t*-test and *P* values as well as the % confidence level obtained from Plackett-Burman experiments (Table 3.8.8), Carbon source (lactose), K_2HPO_4, $K_2Cr_2O_7$ and Time have positive effect and being statistically significant, and were selected for further optimization The present study provides evidence indicating that *Stenotrophomonas maltophilia* SRS05 may be used in developing a bioremedial process for chromate-contaminated industrial waste discharge.

3.9 Response Surface Methodology and Statistical Analysis

Box-behnken design matrix and RSM experiments were used to optimize the process of bioremediation of Cr (VI) ions using *Stenotrophomonas maltophilia* SRS05. Four significant parameters Carbon source, Time, $K_2Cr_2O_7$ and K_2HPO_4, were studied. The domain factor and level selected for designing the Box-Behnken design is presented in Table 3.9.1

Table: 3.9.1 The experimental domain factor and level for the Box-Behnken design

Code	Name of factor	Range and Level (Coded)		
		-1	0	1
A	Carbon Source (g/l)	5	10	15
B	Time (h)	5	14.5	24
C	$K_2Cr_2O_7$ (mg/l)	1	1.4	2
D	K_2HPO_4 (g/l)	1	2.6	4

Experiments were performed according to Box-Behnken design and observed response for biodegradation of Cr (VI) by *Stenotrophomonas maltophilia* SRS05 is summarized in Table 3.9.2.

The relationship between independent variables and response was drawn by second-order polynomial equations. The regression equation coefficients were calculated and the result revealed that the response i.e., Cr(VI) bioremediation fitted to the second-order polynomial equation. Significance of each coefficient was determined by Student's *t*-test and *P* values. Table 3.9.3 shows the result of ANOVA for bioremediation of Cr (VI) ions by the studied bacterial strains.

Table 3.9.3 shows the result of ANOVA for bioremediation of Cr (VI) by the studied bacterial strains. The model *F*-value of 3.10 implies the model is significant. There is only a 1.78% chance that a "Model F-value" this large could be due to noise. Values of "Prob> F" less than 0.05 indicate model terms are significant. In this case D, BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms. The "Lack of Fit F-value" of 2.92 implies the Lack of Fit is not significant relative to pure error. There is a 15.5 % chance that a "Lack of Fit F- value" this large could occur due to noise. Non-significant lack of fit is good. The "Pred R-Squared" of 0.0278 is not as close to the "Adj R-Squared" of 0.4284 as one might normally expect. This may indicate a large block effect or a possible problem with this model. Things to consider model reduction, response transformation, outlires, etc. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. This model indicates ratio of 6.681, which is an adequate signal that this model can be used to navigate the design space.

Table: 3.9.2 The Box-Behnken design matrix for experimental design and observed response for Cr(VI) biodegradation using *Stenotrophomonas maltophilia* SRS05.

Experimental Run	Carbon Source (g/l)	Time(h)	$K_2Cr_2O_7$ (mg/l)	K_2HPO_4 (g/l)	Yield
1	10	5	2	2.6	0.057
2	10	5	1.4	1	0.038
3	10	14.5	1.4	2.6	0.056
4	15	14.5	1.4	4	0.068
5	15	14.5	1	2.6	0.069
6	10	24	1.4	1	0.044
7	10	14.5	1.4	2.6	0.057
8	10	14.5	2	1	0.049
9	5	24	1.4	2.6	0.059
10	5	14.5	1	2.6	0.046

11	10	5	1.4	4	0.056
Experimental Run	**Carbon Source (g/l)**	**Time(h)**	**$K_2Cr_2O_7$ (mg/l)**	**K_2HPO_4 (g/l)**	**Yield**
12	10	14.5	1.4	2.6	0.06
13	5	5	1.4	2.6	0.126
14	10	14.5	2	4	0.032
15	15	14.5	2	2.6	0.064
16	15	14.5	1.4	1	0.054
17	5	14.5	2	2.6	0.068
18	10	24	1.4	4	0.058
19	10	24	2	2.6	0.69
20	10	14.5	1.4	2.6	0.057
21	10	14.5	1	1	0.043
22	5	14.5	1.4	1	0.039
23	15	24	1.4	2.6	0.072
24	10	14.5	1	4	0.068
25	5	14.5	1.4	4	0.036
26	15	5	1.4	2.6	0.063
27	10	24	1	2.6	0.059
28	10	14.5	1.4	2.6	0.045
29	10	5	1	2.6	0.112

Table: 3.9.3 Analysis of Variance for RSM variables fitted to quadratic model

Bacterial strain	Source	Sum of Squares	d.f.	Mean square	F-Value	P-Value	Probe>F
SRS 05	Model	0.008481	10	0.000848	3.098125	0.0178	Significant
	Residual	0.004928	18	0.000274			
	Lack-of-fit	0.004489	14	0.000321	2.922762	0.155	not Significant
	Pure error	0.000439	4	0.00011			
	residual square	0.632512					
	Adj -R square	0.428353					

3.9.1 Effect of $K_2Cr_2O_7$ and Time

Figure 3.9.1 shows the interactive effect of two variables $K_2Cr_2O_7$ (C: 50 µg -100 µg) and Time (B: 5-24h) on biodegradation of Cr (VI) ions using *Stenotrophomonas maltophilia* SRS05, and to determine their ability of bacteria at different concentration of $K_2Cr_2O_7$. From the contour plot, it was observed that, the biodegradation of Cr(VI) ions were increased in the midpoint of the variables $K_2Cr_2O_7$ and time, following which biodegradation of Cr(VI) ion decreased with increase in concentration of $K_2Cr_2O_7$ and time. As the bacterial population in the culture media is not increased with increasing $K_2Cr_2O_7$ concentration, bacterial metabolic uptake of $K_2Cr_2O_7$ is less. In the midpoint $K_2Cr_2O_7$ and time the bacterial growth in the culture media is increased so bacterial metabolic uptake of $K_2Cr_2O_7$ as well as binding of $K_2Cr_2O_7$ to the cell surface also increased. After a certain time period the depletion of an essential nutrient in the medium the bacteria attained stationary phase, indicated with decreased yield of $K_2Cr_2O_7$.

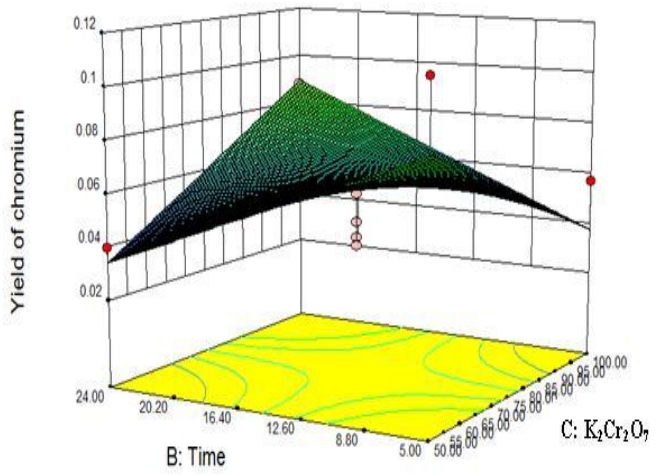


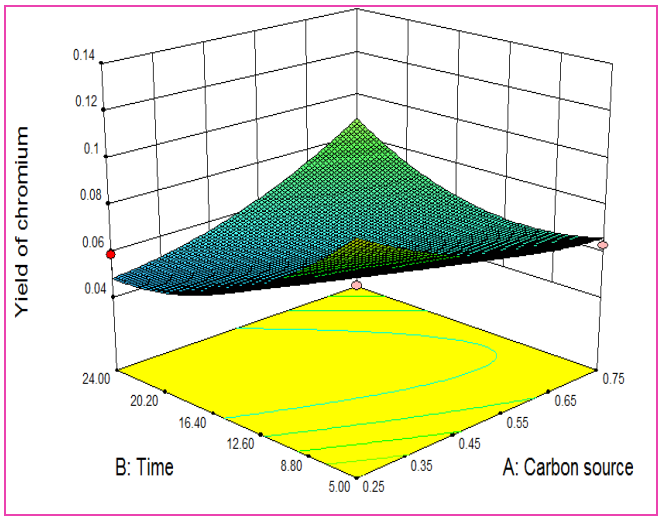
Figure: 3.9.1 The 3D-Contour plot shows the interactive effect of $K_2Cr_2O_7$ and Time on Cr(VI) biodegradation by *Stenotrophomonas maltophilia* SRS05

3.9.2 Effect of Carbon source and Time

Figure 3.9.2 shows the interactive effect of two variables Carbon source (A: 0.25g-0.75g) and Time (B: 5-24h) on biodegradation of Cr (VI) using *Stenotrophomonas maltophilia* SRS05. It could be analyzed from the figure that

in the midpoint of the variables Carbon source and time, the biodegradation of Cr (VI) is decreased, following which biodegradation of Cr (VI) ion increased with increase in concentration of Carbon source and Time. It was observed that as the carbon source was increased, the biodegradation of the Cr(VI) also increased, this is due to the increase in bacterial cell density in the culture media; as a result Cr(VI) was attached with the bacterial cell surface and metabolically could be uptaken by the bacterial cells.

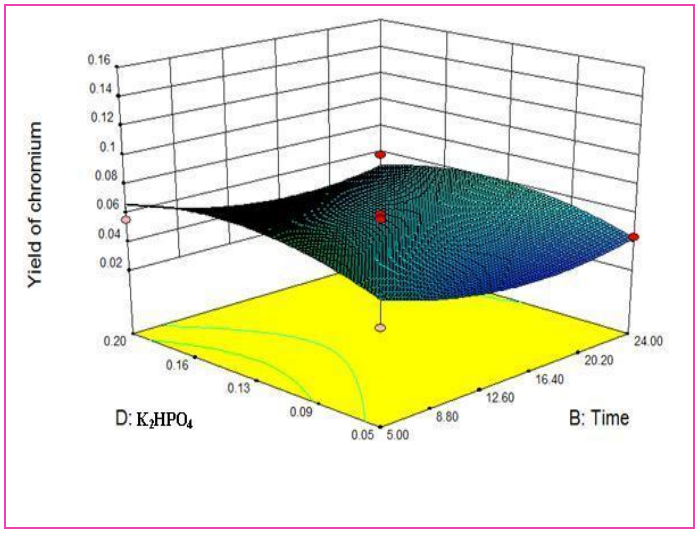
Figure: 3.9.2 The 3D-Contour plot shows the interactive effect of Carbon source and Time on Cr(VI) biodegradation by *Stenotrophomonas maltophilia* SRS05



3.9.3 Effect of Time and K_2HPO_4

Figure 3.9.3 shows interactive effects of Time (B: 5-24h) and K_2HPO_4 (D: 0.05g-0.20g) on biodegradation of Cr(VI) using *Stenotrophomonas maltophilia* SRS05. From the contour plot, it was observed that, the biodegradation of Cr(VI) ions was increased in the initial point of the variables following which the biodegradation of Cr(VI) is stable. There is no variation in increased or decreased level of biodegradation of Cr(VI) with increased K_2HPO_4 concentration and time.

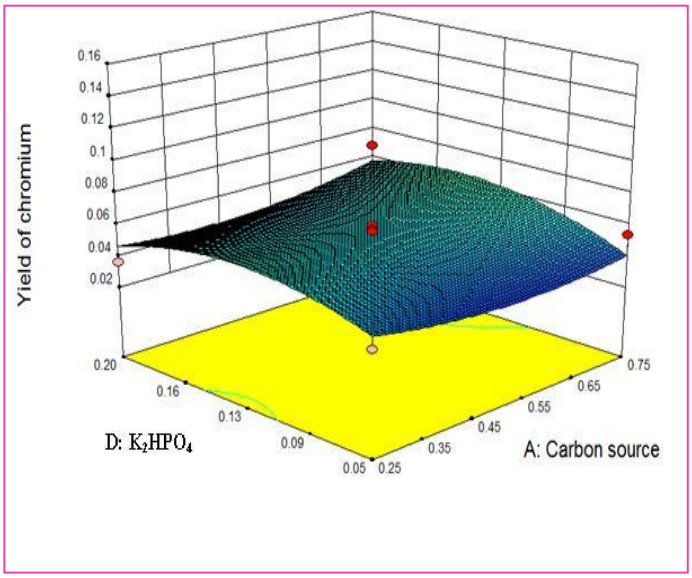
Figure: 3.9.3 The 3D-Contour plot shows the interactive effect of Time and K_2HPO_4 on Cr(VI) biodegradation by *Stenotrophomonas maltophilia* SRS05



3.9.4 Effect of Carbon Source and K_2HPO_4

Figure 3.9.4 shows interactive effects of Carbon Source (A: 0.25g-0.75g) and K_2HPO_4 (D: 0.05g-0.20g) on biodegradation of Cr(VI) using *Stenotrophomonas maltophilia* SRS05. From the contour plot, it was observed that, the biodegradation of Cr(VI) ions was increased in the initial point to the midpoint of the variables Carbon and K_2HPO_4 and after that biodegradation of Cr (VI) is maintained in stable condition, with no variation in increased or decreased Cr (VI) biodegradation level with increased K_2HPO_4 concentration and time.

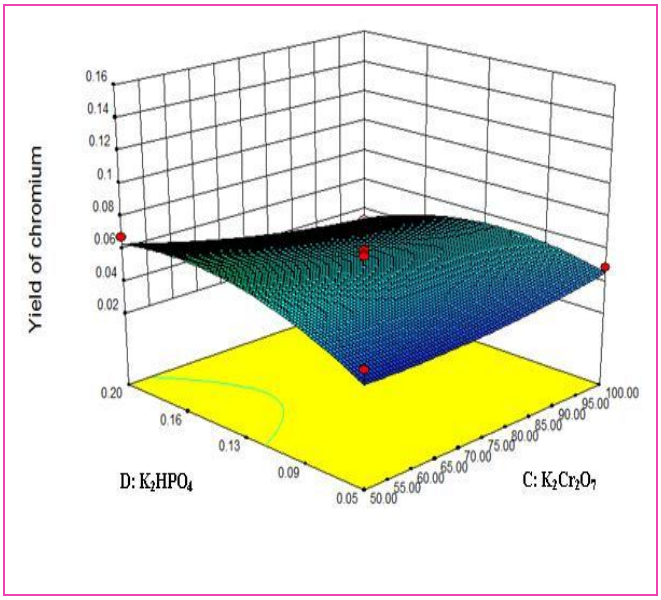
Figure: 3.9.4 The 3D-Contour plot shows the interactive effect of Carbon Source and K_2HPO_4 on Cr (VI) biodegradation by *Stenotrophomonas maltophilia SRS05*



3.9.5 Effect of $K_2Cr_2O_7$ and K_2HPO_4

Figure 3.9.5 shows interactive effects of $K_2Cr_2O_7$ (C: 50µg-100µg) and K_2HPO_4 (D: 0.05g-0.20g) on biodegradation of Cr (VI) using *Stenotrophomonas maltophilia SRS05.* From the contour plot, it was observed that, the biodegradation of Cr (VI) ions was increased in the initial point of the variables $K_2Cr_2O_7$ and K_2HPO_4 and it attained slight Cr (VI) yield variation in their mid point of the variables after which biodegradation of Cr (VI) is decreased with increased $K_2Cr_2O_7$ concentration and K_2HPO_4. It was observed that, the bacterial growth in the culture media was increased in the decreased concentration of $K_2Cr_2O_7$ and K_2HPO_4 so bacterial metabolic uptake of $K_2Cr_2O_7$ as well as binding of $K_2Cr_2O_7$ to the cell surface also increased. The bacterial growth was decreased with increasing concentration of $K_2Cr_2O_7$ and K_2HPO_4, therefore bacterial metabolic uptake of $K_2Cr_2O_7$ is less resulting in decreased yield or biodegradation.

Figure: 3.9.5 The 3D-Contour plot shows the interactive effect of $K_2Cr_2O_7$ and K_2HPO_4 on Cr (VI) biodegradation by *Stenotrophomonas maltophilia* SRS05

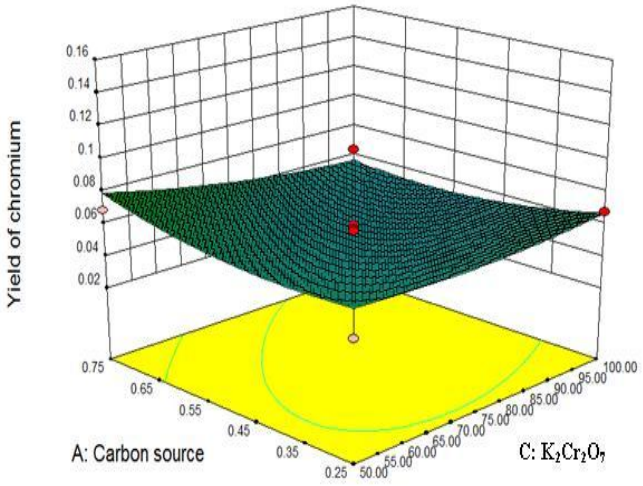


3.9.6 Effect of $K_2Cr_2O_7$ and Carbon Source

Figure 3.9.6 shows interactive effects of $K_2Cr_2O_7$ (C: 50µg-100µg) and Carbon Source (A: 0.25g-0.75g) on biodegradation of Cr (VI) using *Stenotrophomonas maltophilia* SRS05. From the contour plot, it was observed that, increased concentration of $K_2Cr_2O_7$ and Carbon source yield decreased biodegradation of chromium as the bacterial growth in the culture medium was decreased with increasing concentration of $K_2Cr_2O_7$ and Carbon Source. Therefore the bacterial metabolic uptake of $K_2Cr_2O_7$ is less.

It was also observed that Cr (VI) reduction was growth related. Growth- related chromate reduction was also reported previously (Sultan and Hasnain, 2007).

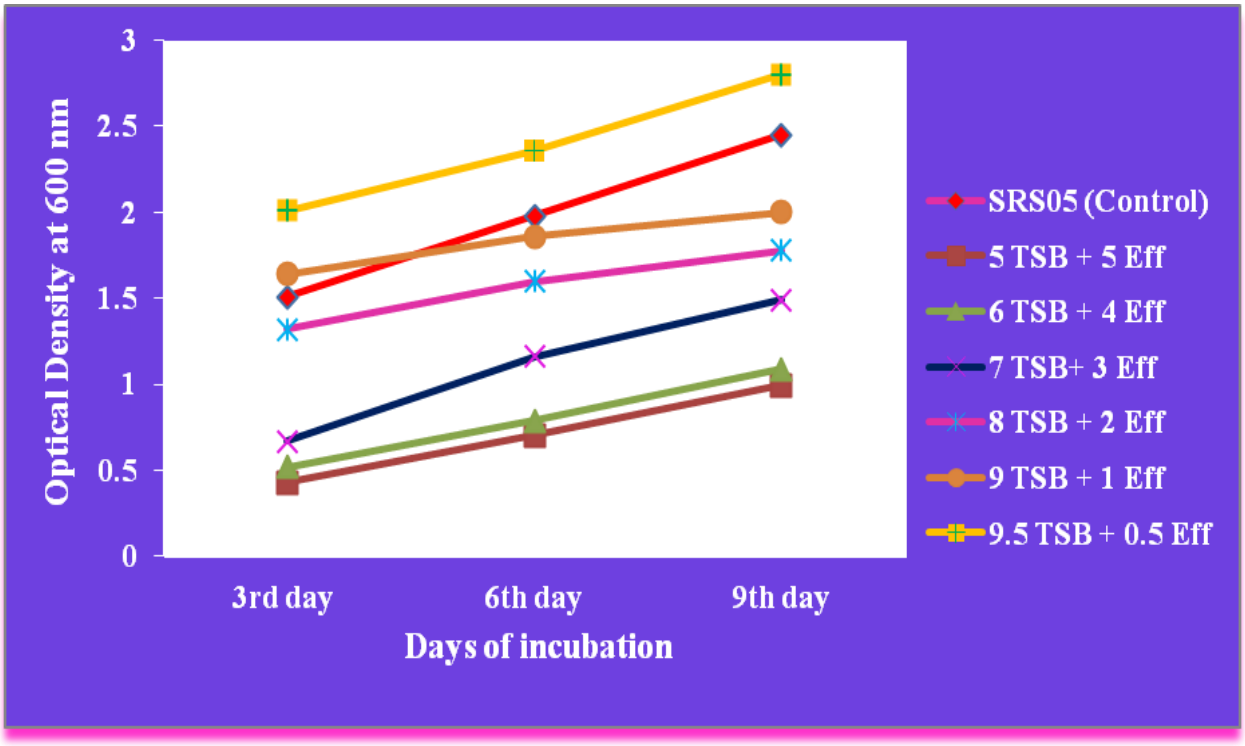
Figure: 3.9.6 The 3D-Contour plot shows the interactive effect of $K_2Cr_2O_7$ and Carbon Source on Cr (VI) biodegradation by *Stenotrophomonas maltophilia* SRS05



3.10 Bioremediation of Tannery effluent

Bioremediation is a popular field of environmental microbiology, used to degrade toxic organic pollutants, heavy metals using growing cells of bacteria, fungi, algae etc. (Malik A 2004; Andrezza *et al*., 2010; Ghosh and Saha 2012). The bioremediation studies were carried out with the effluent collected from tannery industry (Figure: 3.10.1). The incubation time was found to be an important parameter for the growth and reduction of Cr (VI) by *Stenotrophomonas maltophilia* SRS05. The growth pattern of the Chromium resistant bacterial culture *Stenotrophomonas maltophilia* SRS05 and the degradation of Cr(VI) in tannery effluent was analyzed by comparing the degradation in the test sample at different dilution (ml) of TSB medium and effluent (5+5, 6+4, 7+3, 8+2, 9+1, 9.5+0.5) against the control at 3-day interval (ie., 3rd, 6th and 9th day). Over the period of time the bacterial growth and bioremediation of Cr(VI) by *Stenotrophomonas maltophilia* SRS05 was maximum in 9th day in the dilution of 9.5 ml TSB medium + 0.5 ml Effluent, because the organism utilized the minimum volume of effluent as a nutrient supplement (ie., 0.5 ml Effluent in 9.5 ml TSB medium). The 3rd day sample showed bacterial growth and reduction in the concentration of Cr(VI) as compared with control. There was difference in the bacterial growth and chromium (VI) concentration in the 6th day sample when compared to the 9th day sample. The 3rd day to 9th day sample showed bacterial growth and reduction in Cr(VI) concentration with a high growth and reduction of concentration in the 9th day sample when compared with the control.

Figure: 3.10.1 Growth of *Stenotrophomonas maltophilia* SRS05 in different effluent concentration at different incubation time.



The results indicated that *S.maltophilia* SRS05 had a higher degrading capacity of chromium. The level of chromium tolerance and bioadsorption studies of the strain SRS05 using 1,5-Diphenylcarbazide method were studied in previous chapter. The chromium remediation might be due to excellent potential of metal biosorption. This potential in metal recovery and remediation can be due to binding of other metals along with chromium (Butt and Jecker, 1987). Of the different consortia of bacteria and fungi (Katiyar and Katiyar, 1997) *Saccharomyces* and *Pseudomonas* was found to be most efficient. The high efficiency might be contributed to the chromium reductase genes in *P.aeroginosa* and the MT genes in *S.cerevisiae (*Stanberg *et al., 1981). Stenotrophomonas maltophilia* SRS05 showed good degradation capacity. The growth and degradation potential of *Stenotrophomonas maltophilia* SRS05 was high after incubation for 9 days in tannery effluent.

3.11 Atomic Absorption Spectrometry (AAS)

Atomic absorption spectroscopy (AAS) is a spectro analytical procedure for the quantitative determination of chemical elements employing the absorption of optical radiation (light) by free atoms in the gaseous state. In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 62 different elements in solution or directly in solid samples employed in pharmacology, biophysics and toxicology research (Walsh, 1955; L'vov, 2005).

Chromium is the most toxic and common among the heavy metal pollutants of industrial effluents. Bioremediation is the microbial clean up approach. It is very efficient and eco-friendly techniques to remove the inorganic pollutants from contaminated site *in situ* or *ex situ* for cleaner and healthier environment (Lloyd *et al.*, 2001; Lovely and Coates, 1997; Ilias *et al.*, 2011; Damaris *et al.*, 2013). The hexavalent chromium remediation ability of *Stenotrophomonas maltophilia* SRS05 was studied and quantify the chromium (VI) in the tannery effluent using Atomic absorption spectroscopy method.

Processed Tannery effluent was given to SITRA (South Indian Textile Research Association) CBE, for the determination of chromium reduction in Atomic Absorption Spectrophotometer.

Stenotrophomonas maltophilia SRS05 reduced chromium (VI) under aerobic conditions. This may be due to the presence of chromium reductase. The degradation of Cr(VI) in tannery effluent was analyzed by comparing the degradation in the test sample (Tannery effluent + live cells) against the control (Tannery effluent + killed cells) at 5-day intervals. The chromium (VI) content in the effluent was around 490 mg/l before remediation, after which it was reduced to 260 mg/l. The best activity was observed by *S.maltophilia* SRS05 at 15th day of incubation. The bioremediation of Cr(VI) was analyzed by Atomic absorption spectroscopy comparing the degradation in the test sample (Tannery effluent + live cells) against the control (Tannery effluent + killed cells) at regular intervals. The 5th day sample showed reduction in the concentration of Cr(VI) as compared with control.

Table 3.11.1: The Bioremediation of Cr (VI) in tannery effluent was analyzed by Atomic absorption spectroscopy

S.NO	Initial Cr(VI) Concentration mg/l in tannery effluent	Days of incubation	Hexavalent Cr(VI) Concentration after remediation mg/l
1	490 mg/l	-	-
2	490 mg/l	5	396 mg/l
3	490 mg/l	10	317 mg/l
4	490 mg/l	15	260 mg/l
5	490 mg/l	20	260 mg/l

There was difference in the chromium (VI) concentration in the 10th day sample when compared to the 15th day sample. The 5th day to 15th day sample showed reduction in Cr(VI) concentration with a high reduction of concentration in the 15th day sample when compared with the control. There was no difference in the Cr(VI) concentration when compared to the 20th day sample because the bacteria attained stationary phase (Table 3.11.1).

The Stationary phase is often due to a growth-limiting factor such as the depletion of an essential nutrient, and/or the formation of an inhibitory product such as an organic acid. Stationary phase results from a situation in which growth rate and death rate are equal. The number of new cells created is limited by the growth factor and as a result the rate of cell growth matches the rate of cell death. The result is a smooth, horizontal linear part of the curve during the stationary phase. Poornima *et al*. (2010), Damodara *et al*. (2011), Raja Rao *et al*. (2014), also evaluated the other heavy metals and chromium before and after treatment of indeginious microorganisms using Atomic Absorption Spectrophotometer.

3.12 Scanning electron microscopy (SEM)

Scanning electron microscopes are frequently equipped with an energy dispersive X-ray analyser. This equipment permits elemental analysis with a high horizontal resolution of the inspected specimens. Scanning electron microscopy is a key tool to study the effect of physicochemical properties on adhesion phenomena (pH, roughness, topography, temperature, etc). SEM plays also a paramount role for assessing the microbial populations, three - dimensional structure, physiology, thickness, etc (Akernan *et al*., 1993).

SEM is a powerful technique for revealing the fine structure of living systems and has been applied to biofilms (Eighmy *et al*., 1983; Richards and Turner, 1984; Weber *et al*., 1978) and is one of the many methods available for the visual effect of antibacterial or antifungal on biofilm development (Camargo *et al*., 2005; McDowell *et al*., 2004; Sasidharan *et al*., 2010; Sevinc and Hanley, 2010; Zameer and Gopal, 2010; Zeraik and Nitschke, 2010). Sasidharan *et al*., (2010) used SEM to study the effects of potential antifungal extracts from natural sources in *Candida albicans* biofilm.

SEM presents many advantages: (i) higher resolution of visualization of microbial biofilms (Walker *et al*., 2001) than other imaging techniques, typically 3.5nm, (ii) able to measure and quantify data in three dimensions. It has also been of specific importance in elucidating biofilm structure for understanding the physiology and ecology of these microbial systems (Blenkinsopp and Costerton, 1991). For example, electron - microscopic studies proved that the biofilm is composed of bacterial cells wrapped in a dense glycocalyx, i.e. exopolysaccharide matrix (Eighmy *et al*., 1983; Blenkinsopp and Costerton, 1991).

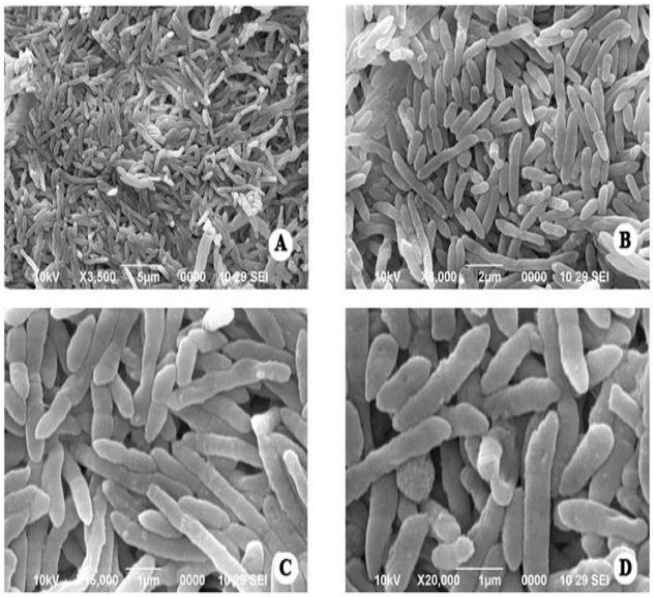
Chromium has been indicated to be a pollutant in various sites and heavy water including tannery effluents. These waters when mixed with the ground seem to cause various problems (Oliver *et al.,* 2003). The biological approach to degradation of heavy metals has been an important case study for bioremediation via biosorption of these metals using indeginious microorganisms (Diestra *et al.,* 2007; Xie *et al.,* 2010). The application of resistant bacteria for this purpose has been involved with its growth and morphological parameters. In this study, we used scanning electron microscopy for analyzing the changes in morphology of *Stenotrophomonas maltophilia* SRS05. This study was mainly undertaken to adjudge if there was any effect on the growth of the bacteria which may affect its biosorption potential.

The growth characteristics of *S.maltophilia* indicates rod or slightly curved rod like features with smooth walled cells (Brooke, 2012). In this chapter, untreated *Stenotrophomonas maltophilia* SRS05 grown on TSB for 24 h (control); *Stenotrophomonas maltophilia* SRS05 treated with 100µg/ml of chromium and effluent containing 24.5µg/ml of chromium treated *Stenotrophomonas maltophilia* SRS05 were observed by SEM. The SEM analysis conducted at a magnification of X3500, X8000, X15000 and X20,000 respectively revealed intense layering of biofilm.

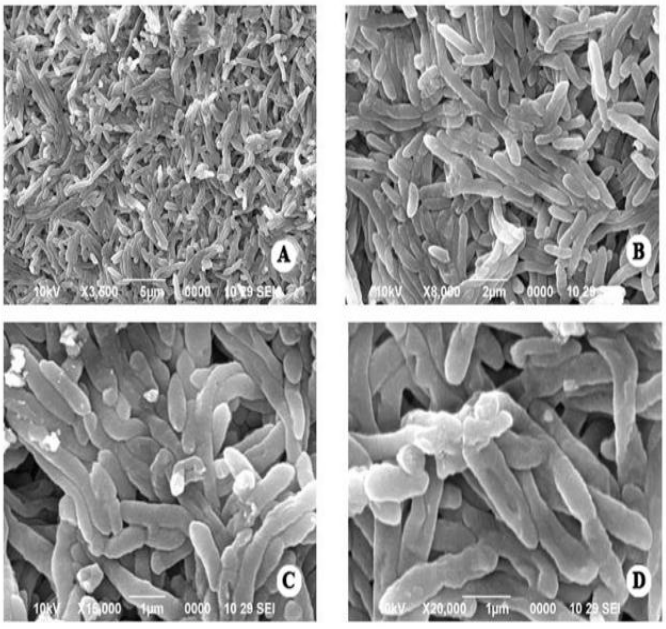
After 18 h, the cells treated as control is observed as rod shaped, smooth intact cell surface with no damages. However, in the bacteria treated with chromium, there are some irregular fragments on the adjoining cell surfaces indicating slight morphological changes on cellular surface. The treatment of culture with effluent as effect on its degradation was further confirmed using scanning electron microscope visually by rough cell surface although the rod shaped characteristic of the cells remained intact. These results are in accordance with the findings of other reports (Sethuraman *et al.,* 2010; Thakur and Srivastava, 2011) and slightly vary with the previous studies of Feng *et al.* (2000) and Kim *et al.* (2005) where morphological changes were observed on the bacterial cell. David *et al*. (1973), Beveridge (1988), Baldi *et al*. (1990) and Radehaus & Schmidt (1992) stated that, the microorganisms have excellent nucleation sites for grained mineral formation, due to their high surface area and volume ratio and the presence of electronegative charges on the cell wall. Surface functional groups (e.g. carboxyl, phosphoryl and hydroxyl) play major role in bioaccumulation of metals and significantly removed chromate which is toxic (David *et al*., 1973; Beveridge, 1988; Radehaus and Schmidt, 1992; Leusch *et al*., 1995). The biosorbed chromate was assumed to be Cr(III), as Cr(VI) is reduced to Cr (III) in the living cells due to reducing environment and enzymes present inside the cell (Thakur and Srivastava, 2011).

Therefore, scanning electron microscopy helps us to conclude that *Stenotrophomonas maltophilia* SRS05 can be used for biosorption of chromium owing to its excellent growth but further studies have to be performed with other heavy metals.

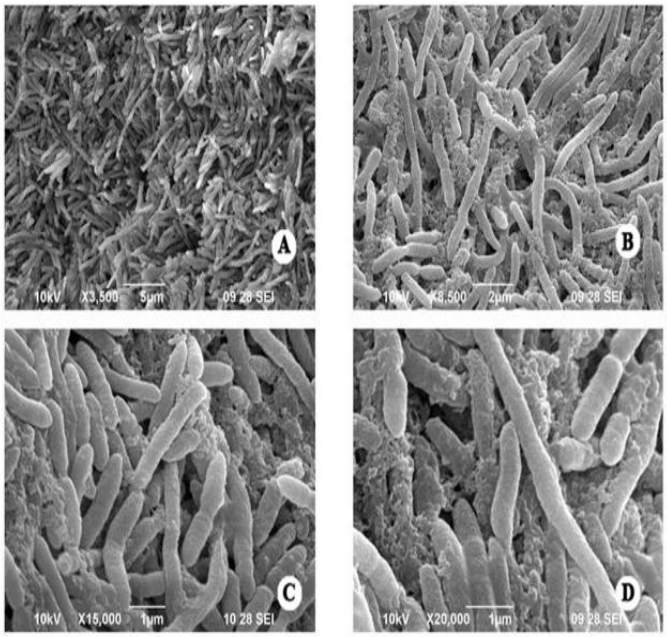
A. SEM Micrograph of S.maltophilia SRS 05 in TSB



B. SEM Micrograph of S.maltophilia SRS05 in TSB with 100µg/ml of Potassium dichromate for 18h



C. SEM Micrograph of S.maltophilia SRS05 in TSB with effluent (24.5µg/ml of cr for 18h



CONCLUSION

This study helps us to conclude that *Stenotrophomonas maltophilia* SRS05 can be used for biosorption of chromium owing to its excellent growth but further studies have to be performed with other heavy metals. The bioremediation process of the above study concluded that the *S.maltophilia* SRS05 could be used as a promising agent for the removal of Cr (VI) in effluents.

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