

Sustainable approach to the antifouling and corrosion inhibitive properties of Exopolysaccharide producing *Rhizobium leguminosarum* (Legume Root Nodule Associated Bacteria) on mild steel at low pH

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Rhizobium leguminosarum is isolated from legume root nodule of *Vigna mungo* and screened for exopolysaccharide production along with biofilm forming ability by bacterial attachment assay. The *Rhizobium leguminosarum* isolate is utilized for its anti-fouling potential against the fouling isolates like *Bacillus sp.* and explored as corrosion inhibitor between 293 K and 313 K temperatures at low pH. Adsorption isotherm models, electrochemical techniques (EIS and PPM), XRD, spectroscopic (UV-Vis and FT-IR) and surface morphological studies (FE-SEM - EDX and AFM) have confirmed the adherence and anticorrosive nature of *Rhizobium leguminosarum* isolate. Maximum at 298 K with the optimum concentration of 9×10^6 cfu/mL *Rhizobium leguminosarum* isolate explored its potential. Increase in concentration of *Rhizobium leguminosarum* isolate enhanced the charge transfer resistance. Polarization curves indicate the mixed type inhibiting property of *Rhizobium leguminosarum* isolate.

Keyword: Electrochemical properties, *Rhizobium leguminosarum* isolate, Nodule associated bacteria, Exopolysaccharide, Antifouling, Corrosion inhibition

1. INTRODUCTION

Mild steel (MS) is much pronouncing material in the industrial applications for fabrications and structural design because of its appreciable mechanical strength, longevity and low maintenance [1-5].

Many processing industries viz. chemical, construction, petroleum, refining etc. uses mild steel for storage purposes. Cleaning of mild steel in industry causes the corrosive attack due to the use of acid for rustling, pickling and scaling process [6-8]. In addition, mild steel has a high rate of dissolution in acid medium, which influences the corrosion on the surface of mild steel. Thus employing of corrosion inhibitors is the common and primary need for industrial process. Common methods like chemical treatments, plating, coatings, and biofilm formation on the metal are available to prevent corrosion [9, 10]. Amongst biofilm formation is the most effective due to ecofriendly, biodegradable nature and does not affect the pH of the solution [11, 12]. There are many inhibitors like organic inhibitors, green inhibitors and biological inhibitors are used to prevent the corrosion of the metals [13-17]. In the usage of organic inhibitors, the disadvantages like change in pH of the solution after adding inhibitor is observed. At the same time, finding mechanism for the interaction between surface of the metal and inhibitor is difficult due to the presence of various organic moieties present in green inhibitors [18-21]. However, biological inhibitor paves the way to prevent corrosion without loss of acidity of the solution, in addition to cost effective, ecofriendly. Prediction of mechanism is also easy due to the excretion of the single type of matter [22]. Thus, it is becoming much-interested field due to its unique property as they have high efficiency in corrosion inhibition in an acid medium [23-26].

The usage of biological bacteria for corrosion prevention is the self-renewing and ecological applicable one. Many bacteria like *Rhizobium*, *Alcaligenes*, *Pseudomonas*, *Agrobacterium* and *Rhizobium leguminosarum* isolate [27-29] are identified for secretion of exopolysaccharides. Among them *Rhizobium leguminosarum* isolate produce the exopolysaccharides in all the climatic conditions. This exopolysaccharide is also vital for the attachment and biofilm formation on the surfaces of the mild steel. In addition, the composition of rhizobial shroud is very important for the adjustment to change in environmental conditions [30]. The external surface of *Rhizobium leguminosarum* isolate cells contains a variety of exopolysaccharides (PSs), such as lipo exopolysaccharide (LPS), capsular exopolysaccharide (CPS), neutral exopolysaccharide, and gel-forming exopolysaccharide. The sustainable nature of *Rhizobium leguminosarum* isolate in low pH, salinity, drought, temperature changes, and oxidative stress insists to use the *Rhizobium leguminosarum* isolate for the corrosion inhibitor in acidic medium. To change in environmental condition and vital for attachment, biofilm formation at low pH insists to use the *Rhizobium leguminosarum* isolate as the corrosion inhibitor in acidic medium [31].

Additionally *Rhizobium leguminosarum* isolate has the great antifouling property against *Bacillus sp.* which produces the hydrogen sulphide to the environment. Hydrogen sulphide is the one of the toxic chemical for both living beings and environment. Excess inhalation of hydrogen sulfide directly affected the nervous system of human being and it binds with iron in blood and affects the respiratory system so the control of hydrogen sulphide production is very important [32]. So *Rhizobium leguminosarum* isolate is also used for the antifouling activity against *Bacillus sp.*

This work involves the culture isolation of *Rhizobium leguminosarum* root nodule associated bacteria and cultivation. The isolated culture *Rhizobium leguminosarum* is examined as corrosion inhibitor in 0.5 M H₂SO₄ on mild steel and its antifouling activity also checked against *Bacillus sp.* Both the study reveals the very good activity of *Rhizobium leguminosarum* isolate against corrosion and antifouling.

2. EXPERIMENTAL SECTION

2.1. Isolation and detection of biofilm formation of Nodule Associated Bacteria

Nodule associated bacterium was secluded from root nodules of Black gram (*Vigna mungo*) plant using Yeast Extract Mannitol Agar (YEMA) plate [33]. The biofilm forming property of the isolate was detected by bacterial attachment assay described by Niemira [34].

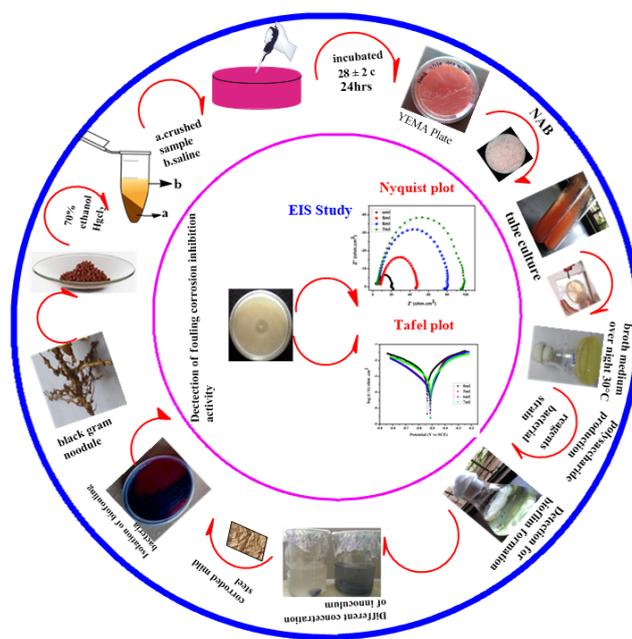
2.2. Screening and extraction of Exopolysaccharides from *Rhizobium leguminosarum*

The exopolysaccharide producing capability of NAB was detected by the formation of slime mucoidal layer of the strain on nutrient agar plates amended with 5% glucose [35]. Cultivation of EPS was performed in the production medium and exopolysaccharides was extracted according to standard method [36].

2.3. Isolation, Screening and Identification of fouling bacterium

Corrosion influencing bacteria was isolated from corroded mild steel using nutrient medium. The isolated bacterial strain was screened for fouling property and studied by the method previously adopted [37]. The fouling bacterial isolate was subjected for tentative identification using morphological and biochemical characterization.

2.4. Screening of *Rhizobium leguminosarum* for Antifouling activity



Scheme 1. The Preparation of *Rhizobium leguminosarum* root nodule associated bacterial isolate for the study of corrosion inhibition of mild steel in 0.5 M H₂SO₄.

The primary screening of antifouling activity of *Rhizobium leguminosarum* against corrosive bacteria was tested by modified agar overlay method [38]. Secondary screening of antifouling activity was detected by Schillinger and Lucke et al., for testing the antagonistic activities of *Rhizobium leguminosarum* isolate against target organisms [39].

2.5. Identification of *Rhizobium leguminosarum*

The *Rhizobium leguminosarum* isolate was identified by morphological, biochemical and molecular characterization studies. Molecular Characterization was performed using the universal eubacterial forward and reverse primer by amplifying their 16S *rRNA* gene. The fractional length 16S *rRNA* amplicon was sequenced at Optimurz Biotech Pvt. Ltd., India. The sequence of *Rhizobium leguminosarum* strain was matched up with database deposited in the NCBI GenBank using BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) and an accession number was attained. Neighbor-Joining method was employed to build phylogenetic tree in MEGA program version 6.0. Notably, the sequences studied facilitated to understand the evolutionary distances among the species.

2.6. Resistance of *Rhizobium leguminosarum* at Low pH

The acid tolerance of the isolated nodule associated bacterial isolate was studied in different pH levels from 1 to 7. Entire test solutions used were sterilized for 20 minutes at 121 °C. 10ml of each pH solution was analyzed in sterilized test tubes. The incubation of yeast extract mannitol broth inoculated with *Rhizobium leguminosarum* was carried out at 37 °C for 24 hrs. After 24 hrs of incubation, growth is determined by observing their optical density at 620 nm using sterile broth as blank.

2.7. Viability of *Rhizobium leguminosarum* on the acidic environment

The *Rhizobium leguminosarum* colony was inoculated with 100 mL of YEMA broth medium overnight with appropriate conditions (30 °C, 250 rpm). Different concentration of inoculum was employed for corrosion tests. The viability of NAB in the acidic environments was determined by spreading 100 µL of acid and culture suspension from different experimental set up on to the freshly prepared yeast extract mannitol agar medium and the growth was monitored in the incubated (24 - 48 hrs at 37 °C) plates.

2.8. Strain for corrosion study

A single colony maintained in YEMA was chosen, inoculated to 100 mL of broth medium, incubated with appropriate conditions and growth was observed. The amount of cells used for the corrosion inhibition study was 10⁶ cfu/mL and various concentration of inoculum was utilized for the experimental study.

2.9. Preparation of the mild steel specimens and solutions for the study

The mild steel of the chemical composition (wt %) P- 0.39, Si – 0.18, C – 0.13, S – 0.04, Cu – 0.025 and respite being iron with an exposed area of 1 cm². A series of emery papers was used to abrade the showing area and then washed with double distilled water systematically and degreased with acetone just earlier than insertion in to the cell. All chemicals, reagents used were of critical grade and other solutions were prepared with double distilled water.

2.10. Measurement of difference in weight of mild steel in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄ at different temperatures.

Abraded mild steel specimens were immersed in different concentrations of *Rhizobium leguminosarum* isolate with 0.5 M H₂SO₄ at various temperatures between 293 K and 313 K. Later at 3 hrs, specimens were taken out to measure the difference in weights of the specimens, before and after immersion, in Mettler Toledo electronic balance having sensitivity of ± 0.1 mg. This difference in weight was used to calculate inhibition efficiency (IE%) and surface coverage (Θ) values by following equations (1) & (2):

$$IE\% = \frac{W_o - W_i}{W_o} \times 100 \quad (1)$$

$$\Theta = \frac{W_o - W_i}{W_o} \quad (2)$$

Where, W_o and W_i are weight loss (mg) of mild steel in absence and presence of inhibitor.

Corrosion rates (CR) at different concentrations of *Rhizobium leguminosarum* isolate were also computed using equation (3):

$$CR = 534 \times \frac{\Delta w}{DAT} \quad (3)$$

Where, Δw = weight loss in mg, D = density of material used (7.86 g cm⁻³), A = area of specimen in cm² exposed in acidic solution, and T = immersion time in hours.

2.11. Electrochemical measurements for the corrosion inhibition studies in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄

To recognize the electrochemical characteristics of corrosion inhibition of *Rhizobium leguminosarum* isolate on mild steel in 0.5 M H₂SO₄, an Electrochemical Work station CHI 660E (CH instrument, USA) was used with three electrode cell assembly consisting mild steel coupons (1 cm X 1 cm) as working, Ag/AgCl electrode as standard and a platinum wire as a respond to electrode. The polarization measurements was carried out from -300 mV Ag/AgCl (cathodic potential) to + 300 mV vs. Ag /AgCl (anodic potential) with reference to the open circuit potential at a scan rate of 10 mV/s and computed with the help of CHI 660E software.

2.12. Mild steel surface characterization and spectroscopic analysis in the presence and the absence of

Rhizobium leguminosarum isolate in 0.5 M H₂SO₄

To analyze the shift in wavelength during the adsorption of *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄ the UV-Visible absorption spectra were recorded using a JASCO V530 spectrophotometer. The sample scrapped from the mild steel surface were made into disk with KBr pellets and *Rhizobium leguminosarum* isolate were separately analyzed by FT-IR (SHIMADZU-FTIR-8400S spectrophotometer) to confirm the similarity of functional groups in them. The export 3-powder XRD PALYTICAL-45 KV was used to analyze the XRD pattern of corroded and inhibited mild steel. The surface morphology and elemental composition of the corroded and inhibited mild steel were measured using FE-Scanning Electron Microscope JEOL (JSM 6390), EDX (Akilan Technology UK 5500 series) and Nano Surs EASYSCAN- II Atomic Force Microscope, Zwitterchland in tapping mode to capture the image.

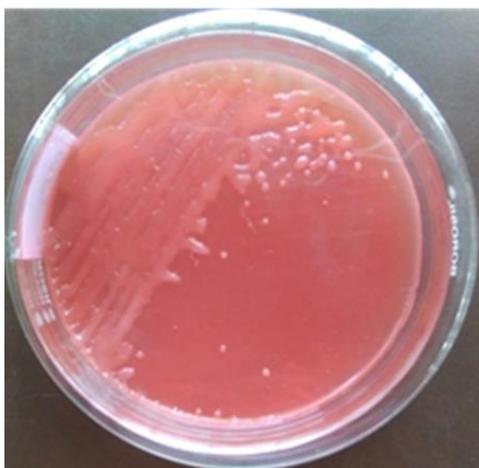


Figure 1. *Rhizobium leguminosarum* isolate identified as colorless, slimy, mucoid colonies on YEMA medium

3. RESULTS AND DISCUSSION

3.1. Characteristics of isolated *Rhizobium leguminosarum*

The root nodule associated Rhizobial isolate stand out as white, clear, shiny, lofty with mucous (slimy) colonies and rod like shape in colonial and morphological studies (Figure 1). Results of biochemical studies revealed that the culture showed positive results towards the sugar fermentation tests which differentiate *Rhizobium* from *Agrobacterium* strains.

3.2. Sequencing

The partial 16S rRNA of the potential isolate is sequenced and compared against those available sequences in the public databases. They are closely related to those of *Rhizobium leguminosarum* (100% homology) and have been deposited in the Gen-Bank database under accession number MH401128.1.

A phylogenetic tree of *Rhizobium leguminosarum* is constructed by the Neighbour-Joining method in MEGA 6.0 program (Figure 2).

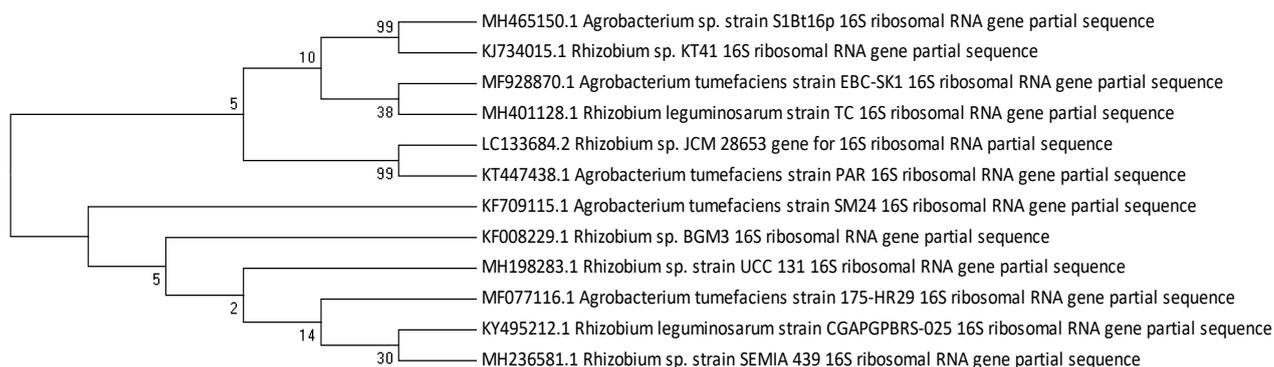


Figure 2. Phylogenetic tree of *Rhizobium leguminosarum* isolate (MH401128.1) constructed using the Neighbour-Joining method with the aid of MEGA 6.0 program.

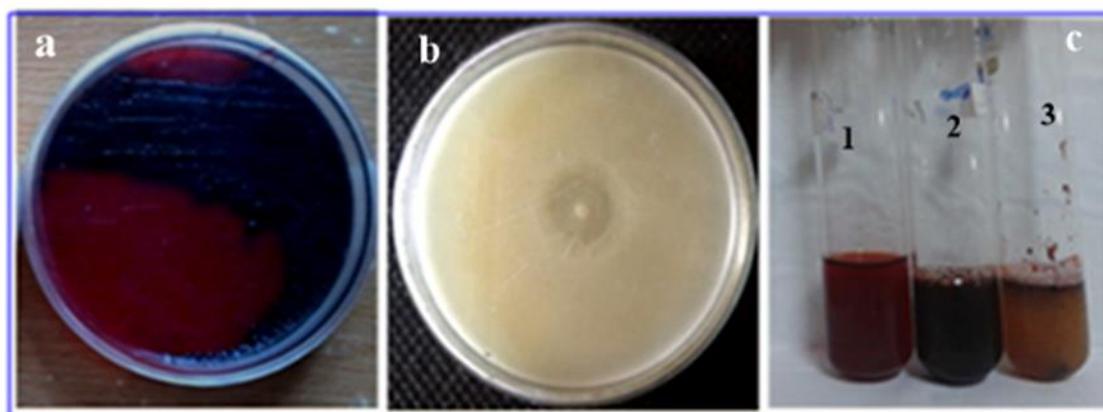


Figure 3. Antagonistic activity of *Rhizobium leguminosarum* isolate (10^6 cfu/mL) against fouling bacteria: (a) Fouling bacteria in black colour colonies, (b) Fouling bacteria with *Rhizobium leguminosarum* isolate (c) (1) Control, (2) Fouling bacteria producing black precipitate, (3) Fouling Bacteria with *Rhizobium leguminosarum* - Absence of black precipitate

3.3. Antagonistic activity of *Rhizobium leguminosarum* isolate against fouling bacteria

The potential fouling bacteria is selected based on the hydrogen sulphide production by the isolate, which resulted in the formation of black color colonies (Figure 3 (a)). The fouling bacteria is purified, subjected for morphological and biochemical characterization as per the standard methods and is identified as *Bacillus* sp. The antifouling activity of *Rhizobium leguminosarum* isolate determined using spot and tube test methods confirmed that this isolate is potential against the fouling isolates (Figure 3 (b) and (c)).

3.4. Resistance and viability of *Rhizobium leguminosarum* isolate on Acidic Environment

The growth is observed at all pH ranges studied (Table 1). Thus, the resistance of *Rhizobium leguminosarum* isolate in different concentration of inoculum is evidenced by their significant growth on yeast extract mannitol agar medium.

Table 1. Acid Tolerance Assay of *Rhizobium leguminosarum* isolate (10^6 cfu/mL).

S. No	Name of the Isolates	OD Value in culture	Acid Tolerance Assay Different pH						
			1	2	3	4	5	6	7
1	<i>Rhizobium leguminosarum</i>	1.15 ± 0.087	-	0.10 ± 0.010	0.14 ± 0.017	0.37 ± 0.010	0.45 ± 0.010	0.91 ± 0.010	1.15 ± 0.087

3.5. Measurement of corrosion rate and inhibition efficiency from the difference in weight of mild steel in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4 at different temperatures.

The decreased corrosion rate and increased inhibition efficiency due to *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4 is investigated at various temperatures between 293 K and 313 K and it is given in Figure 4 (a) and (b). From the Figure, it is clear that the corrosion rate of the mild steel decrease with respect to increase in the concentration of *Rhizobium leguminosarum* isolate. On the other hand, inhibition increases with increasing concentration of *Rhizobium leguminosarum* isolate up to 9 mL. Efficiency increases with increasing concentration of *Rhizobium leguminosarum* isolate in the acid solution. The maximum inhibition efficiency obtained at 298 K using 9 mL of *Rhizobium leguminosarum* isolate. After adding, the high concentration of *Rhizobium leguminosarum* isolate did not give the notable increase in inhibition efficiency due to optimum secretion of exopolysaccharide by *Rhizobium leguminosarum* isolate. Inhibition efficiency of *Rhizobium leguminosarum* isolate on mild steel in various times interval at 293 K is examined which is shown in Figure 5. From that, the maximum inhibition efficiency obtained at 3 h at all the concentration of *Rhizobium leguminosarum* isolate. After that, the inhibition efficiency decreases due to the biodegradable nature of the exopolysaccharide inhibitor that is excreted by *Rhizobium leguminosarum* isolate [40]. Hence, the optimized concentration of *Rhizobium leguminosarum* isolate is 9 mL with the immersion time of 3 hrs.

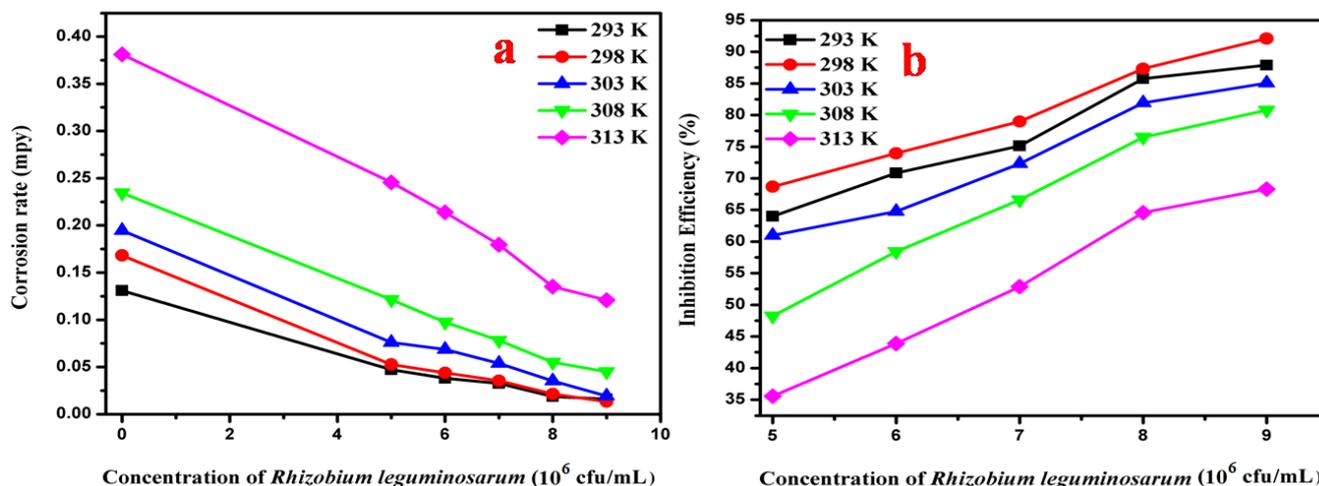


Figure 4. (a) Effect of different concentration of *Rhizobium leguminosarum* isolate (10^6 cfu/mL) on corrosion rate (mpy) and (b) Inhibition Efficiency (%) at different temperatures from 293 K to 313 K in 0.5 M H_2SO_4 .

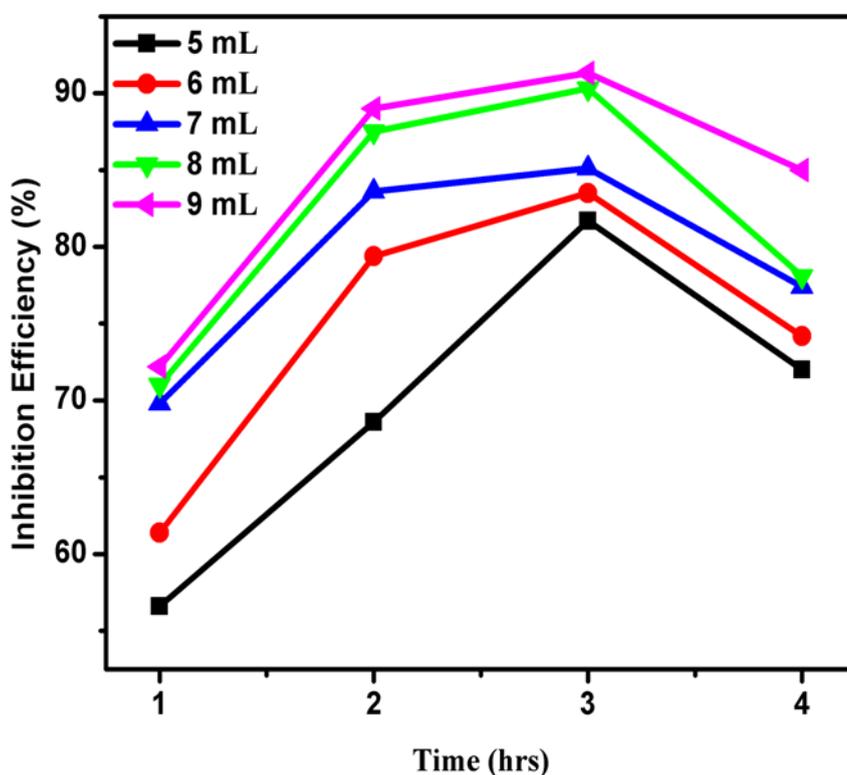


Figure 5. Effect of Time (hrs) on Inhibition Efficiency (%) in different concentration of *Rhizobium leguminosarum* isolate (10^6 cfu/mL) in 0.5 M H_2SO_4 at 298 K

3.6. Thermodynamic consideration for the corrosion inhibition process in the presence and absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4 .

Prediction of thermodynamic parameters helps to determine the nature of the corrosion process. Thermodynamic parameters like activation energy (E_a), enthalpy of activation (ΔH) and entropy of

activation (ΔS) of mild steel in 0.5 M H_2SO_4 is calculated in the absence and presence of different concentration of *Rhizobium leguminosarum* isolate from following Arrhenius and Transition equations (4) & (5) [41]:

$$\log(CR) = \log(A) - \frac{E_a}{2.303RT} \tag{4}$$

$$\log\left(\frac{CR}{T}\right) = \log\left(\frac{R}{hN_A}\right) + \left(\frac{\Delta S^*}{2.303R}\right) - \left(\frac{\Delta H^*}{2.303RT}\right) \tag{5}$$

Where A is the frequency factor, E_a is the apparent activation energy, R is the molar gas constant, T is the temperature, h is the plank's constant, N_A is the Avogadro's number, (ΔS^*) is the entropy of activation and (ΔH^*) is the enthalpy of activation.

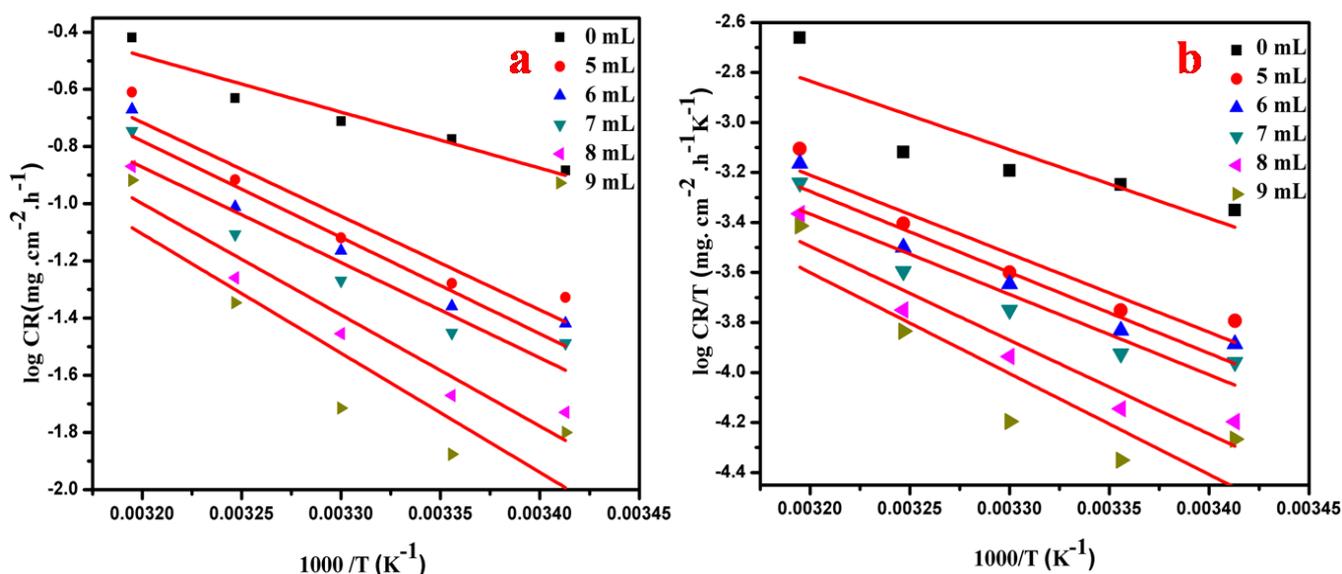


Figure 6. (a) Arrhenius plot and (b) Transition plot for corrosion inhibition of mild steel in 0.5 M H_2SO_4 in the absence and presence of different *Rhizobium leguminosarum* isolate (10^6 cfu/mL) concentration.

Table 2. Activation parameters for the corrosion inhibition process on mild steel surface in 0.5 M H_2SO_4 in the absence and presence different concentration of *Rhizobium leguminosarum* isolate (10^6 cfu/mL).

Concentration (10^6 cfu/mL)	E_a ($kJmol^{-1}$)	$-\Delta H^*$ ($kJmol^{-1}$)	$-\Delta S^*$ ($Jmol^{-1}K^{-1}$)
0	38	53	203
5	63	60	204
6	64	62	204
7	65	63	204
8	74	72	206
9	80	77	206

The calculated values are shown in Table 2. Figure 6 (a) Represents the plot between log CR vs. $1/T$ in 0.5 M H_2SO_4 at various concentrations between 5 to 9 mL of *Rhizobium leguminosarum* isolate. Activation energy (E_a) of mild steel is increased by adding the *Rhizobium leguminosarum* isolate to the 0.5 M H_2SO_4 solution that confirms the formation of productive layer on the surface of the mild steel [42]. This cause the inhibition of the mild steel in the 0.5 M H_2SO_4 solution compared to the uninhibited mild steel. The transition plot is drawn between log CR/T vs. $1/T$ in 0.5 M H_2SO_4 in the presence of *Rhizobium leguminosarum* isolate (5 to 9 mL) is shown in Figure 6 (b). From the Figure 6 (b), the straight lines of the plot also confirm the formation of exopolysaccharide film on the surface of the mild steel [43]. Additionally the enthalpy of the solution is negative in all the concentrations thereby confirmed the corrosion process in *Rhizobium leguminosarum* isolate is exothermic in nature [44]. On the other hand, the negative value of entropy (ΔS) in all the concentrations of *Rhizobium leguminosarum* isolate confirms the secreted exopolysaccharide associated on the surface of the mild steel is in higher order than the dissociation [45].

3.7. Adsorption Isotherm studies for the corrosion inhibition process in the presence and absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4

The equilibrium relationship during the contact period is described by the adsorption isotherm. A plot that relates the amount of adsorbate adsorbed at a given temperature to the concentration of the inhibitor at equilibrium. The absorptive interaction may be electrostatic or uncharged electron pair from heteroatom or π -electrons form metal or combination of two or more.

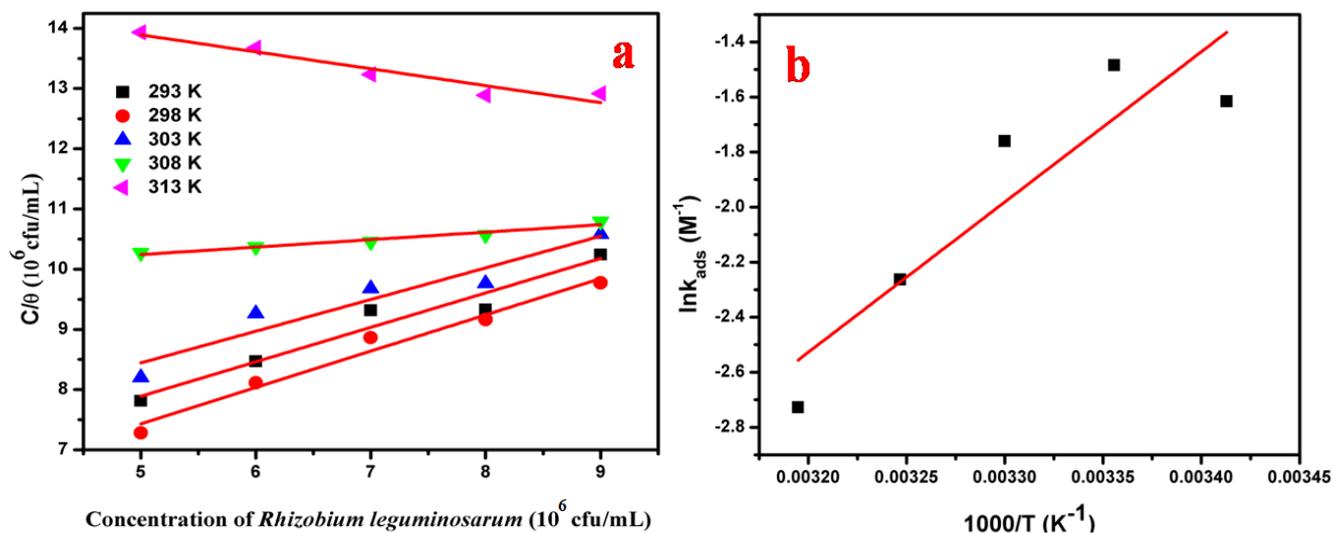
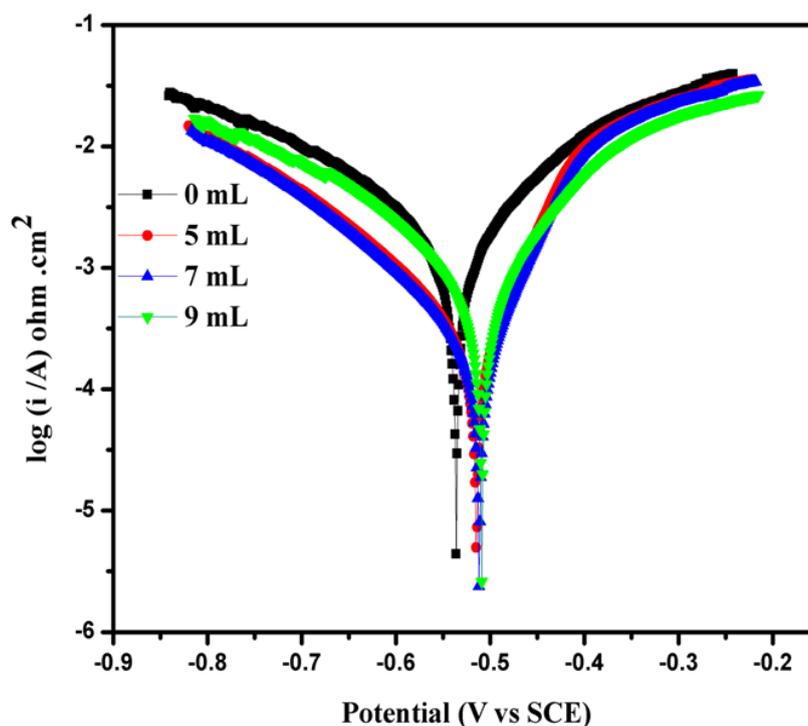


Figure 7. Adsorption isotherm: (a) Langmuir adsorption isotherm plot in different concentration of *Rhizobium leguminosarum* isolate (10^6 cfu/mL) in 0.5 M H_2SO_4 at 298 K (b) Adsorption isotherm plot of $\ln k_{ads}$ vs $1000/T$ (K^{-1}) for the adsorption of *Rhizobium leguminosarum* isolate (10^6 cfu/mL) on the surface of mild steel at 298 K.

Table 3. Thermodynamic parameters for adsorption of *Rhizobium leguminosarum* isolate (10^6 cfu/mL) on the mild steel surface in 0.5 M H_2SO_4 solution.

Temperature (K)	K_{ads} $10^4 (M^{-1})$	$-\Delta G_{ads}$ (kJ mol $^{-1}$)	$-\Delta H_{ads}$ (kJ mol $^{-1}$)	$-\Delta S_{ads}$ (J mol $^{-1}$ K $^{-1}$)	R^2 (Langmuir)
293	1.9	5.8	45	135	0.95
298	2.3	6.3	45	131	0.98
303	1.7	5.7	45	131	0.92
308	1.0	4.5	45	133	0.96
313	0.7	3.4	45	134	0.93

**Figure 8.** Potentiodynamic polarization curves for mild steel in 0.5 M H_2SO_4 in the presence and absence of *Rhizobium leguminosarum* isolate (10^6 cfu/mL) at 298 K

The different adsorption isotherm models like Langmuir, Freundlich, Frumkin and Temkin are used to test the adsorption characteristics of biofilm on the mild steel surface. The analysis indicates that the all the adsorption isotherm model describes the best fit for the defense of mild steel by biofilm in their view. That is, the Langmuir shows monolayer adsorption, Freundlich describes the ease of adsorption of corrosion inhibitors on mild steel whereas Temkin and Frumkin predict the attractive nature of adsorbed biofilm layer. Adsorption isotherm of *Rhizobium leguminosarum* isolate is investigated by fitting various isotherms such as Langmuir, Temkin, Frumkin, Freundlich, Florry-Huggins and Bockris-Swinkels adsorption isotherms.

Table 4. Tafel plots of mild steel immersed in 0.5 M H₂SO₄ with and without *Rhizobium leguminosarum* isolate (10⁶ cfu/mL).

Concentration (mL)	-E _{corr} (mV)	I _{corr} (μA.cm ⁻²)	-bc (mV.dec ⁻¹)	-ba (mV.dec ⁻¹)	R _p	IE%
0	536	1522	170	132	21	-
5	515	593	156	116	31	61
7	515	258	168	130	24	83
9	512	217	144	107	45	85

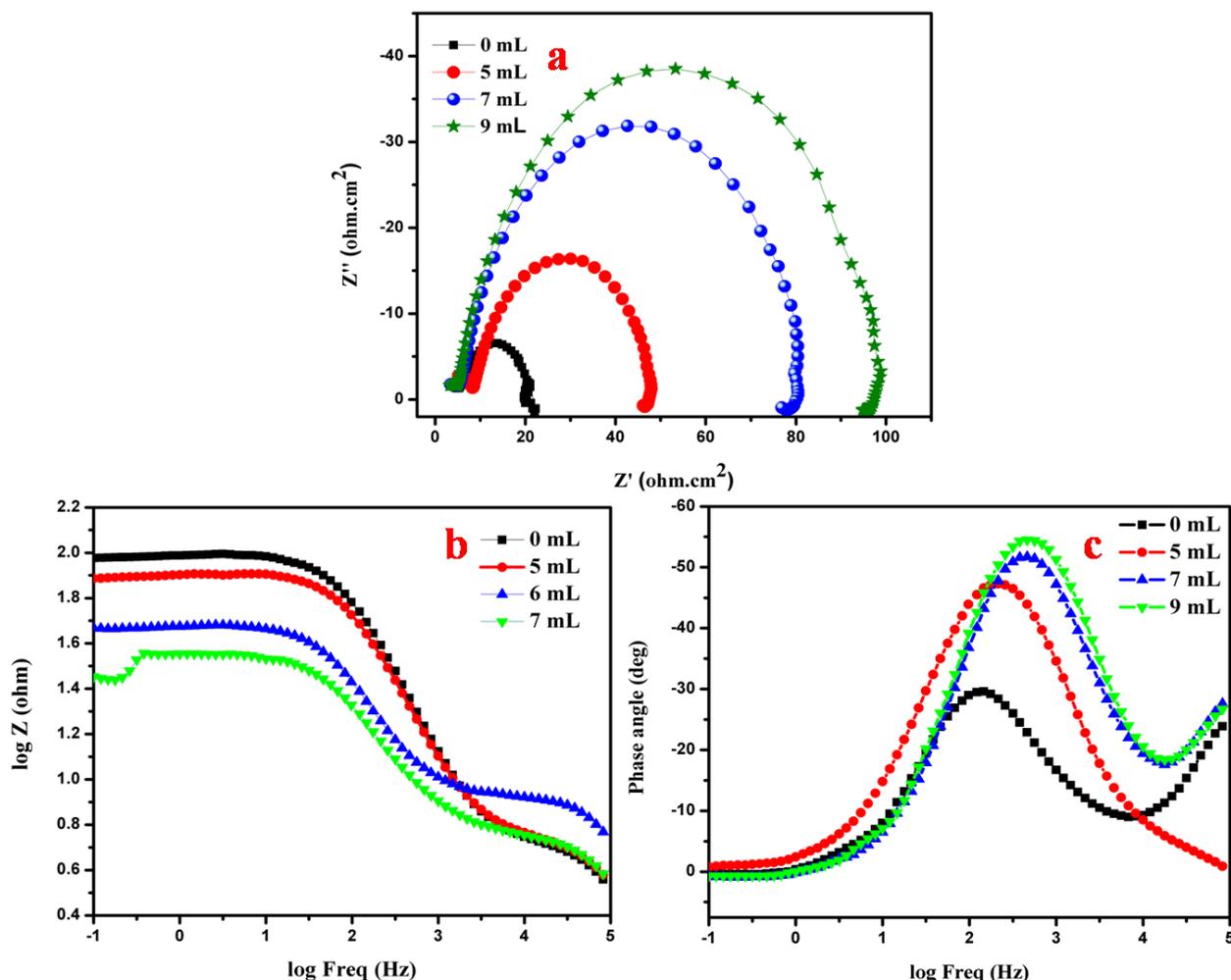


Figure 9. (a) Nyquist, (b) Bode total impedance and (c) Phase angle plots against frequency (Hz) for mild steel immersed in 0.5 M H₂SO₄ in the presence and absence of *Rhizobium leguminosarum* isolate (10⁶ cfu/mL) at 298 K.

Among them studied *Rhizobium leguminosarum* isolate as an inhibitor on mild steel in 0.5 M H₂SO₄ is best fitted to Langmuir adsorption isotherm than other adsorption isotherm. Langmuir adsorption isotherm is expressed as follows [46]:

$$C / \varnothing = 1 / K_{ads} + C \tag{6}$$

Where C/θ is the concentration of inhibitor (10^6 cfu/mL) and K_{ads} is the equilibrium constant of an adsorption process. Figure 7(a) and (b) shows the Langmuir adsorption isotherm plots of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4 . The adsorption parameters such as adsorption coefficient (K_{ads}) and free energy (ΔG) are studied for all the temperatures (298 K - 313 K) and tabulated in Table 3. From obtained results, R^2 values for the plots are nearby unity, which indicates the adsorption of *Rhizobium leguminosarum* isolate inhibitor best fitted for Langmuir adsorption isotherm [47].

Adsorption coefficient is used to predict the free energy of adsorption of the inhibitor *Rhizobium leguminosarum* isolate by the following equation (7) [48]:

$$\Delta G_{ads} = -RT \ln(55.5 \times K_{ads}) \quad (7)$$

Where T is the temperature, R is the gas constant and 55.5 is the molar concentration of water in solution. The adsorption free energy of *Rhizobium leguminosarum* isolate is calculated for all the temperatures ranging between 298 K and 313 K. The free energy values are obtained in negative, which confirms the formation of biofilm by exopolysaccharides on the surface of the mild steel is simultaneous process. Addition to that the obtained ΔG values are around -5 KJ Mol^{-1} confirms the physisorption type of adsorption [49].

3.8. Potentiodynamic polarization study for the corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4

Potentiodynamic polarization study is useful to identify the inhibitor as anodic or cathodic type. Tafel plot of the mild steel in 0.5 M H_2SO_4 with and without *Rhizobium leguminosarum* isolate is shown in Figure 8. The corrosion parameters like corrosion potential (E_{corr}), corrosion current (i_{corr}), cathodic slope (bc), anodic slope (ba), and linear polarization resistance (LPR) are calculated by Tafel plot (Table 4). The inhibition efficiency is calculated by the following formula in Tafel slope (8) [50].

$$IE\% = \frac{i_{corr(blank)} - i_{corr(inhib)}}{i_{corr(blank)}} \times 100 \quad (8)$$

Where $i_{corr(blank)}$, $i_{corr(inhib)}$ are uninhibited and inhibited corrosion current density, respectively. From Tafel plot the i_{corr} value decreases (i_{corr} value decreases from 1522 to 217 $\mu\text{A cm}^2$) when *Rhizobium leguminosarum* isolate concentration increases which indicates the construction of biofilm on the surface of the mild steel with restrict the electron transfer from mild steel to solution. From the Table 4, ba and bc both values are decreased after adding the *Rhizobium leguminosarum* isolate to the solution which indicates that *Rhizobium leguminosarum* isolate is a mixed type inhibitor it resists both anodic and cathodic electron transfer [51]. The maximum inhibition efficiency obtained 85% in 9 mL of *Rhizobium leguminosarum* isolate. This observation is supported with the weight loss measurement.

3.9. Electrochemical impedance study and Bode plot analysis for the corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4

Electrochemical impedance studies of mild steel in 0.5 M H_2SO_4 in the absence and the presence of *Rhizobium leguminosarum* isolate is shown in Figure 9 (a). Impedance study reveals that charge

transfer resistant (R_{ct}) increase with increasing concentration of *Rhizobium leguminosarum* isolate identical to weight loss measurement. The maximum inhibition efficiency obtained for 9 mL of *Rhizobium leguminosarum* isolate is 80%. The impedance parameters R_s , R_{ct} , C_{dl} values are calculated from Nyquist plot which is shown in Table 5. The inhibition efficiency is calculated by the following equation (9).

$$IE\% = \frac{R_{ct(inhib)} - R_{ct(blank)}}{R_{ct(inhib)}} \times 100 \tag{9}$$

Where $R_{ct(blank)}$, $R_{ct(inhib)}$ are uninhibited and inhibited charge transfer resistance values respectively. Diameter of the semicircle increasing and double layer capacitance value decreasing along with respect to rise in concentration of *Rhizobium leguminosarum* isolate indicates the formation of biofilm on the surface of the mild steel in 0.5 M H_2SO_4 [52].

Table 5. Electrochemical impedance parameter for corrosion inhibition process of mild steel in 0.5 M H_2SO_4 in the absence and the presence of *Rhizobium leguminosarum* isolate (10^6 cfu/mL).

Concentration (10^6 cfu/mL)	R_s ($\Omega.cm^2$)	R_{ct} ($\Omega.cm^2$)	C_{dl} (F.cm ²)	IE(%)
0	3.4	20	1.23×10^{-2}	-
5	3.5	42	3.90×10^{-3}	52
7	7.1	78	4.17×10^{-4}	74
9	4.1	99	6.39×10^{-4}	80

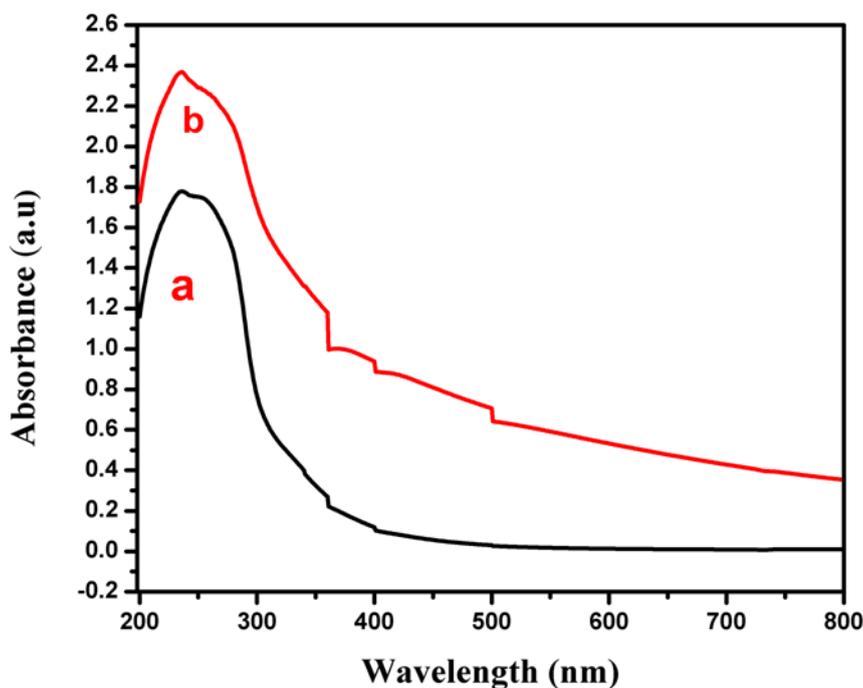


Figure 10. UV-Visible spectrum of solution containing 0.5 M H_2SO_4 before (a), and after (b) the mild steel immersed in (9×10^6 cfu/mL) of *Rhizobium leguminosarum* isolate.

Classic Bode modulus and phase angle plots with and without *Rhizobium leguminosarum* isolate is displayed in Figure 9 (b) and (c). In the phase angle, the low frequency region (29.50° , 47.05° , 52.01° and 54.48°) is close to -70° shows the existence of passivation film on the mild steel surface immersed in 0.5 M H_2SO_4 . Figure 9 (c) presents a shift towards higher phase angle at low frequencies (1.99 Hz, 1.91 Hz, 1.69 Hz and 1.55 Hz) in the presence of *Rhizobium leguminosarum* isolate is a sign of biofilm formation and indicative of lower electrochemical corrosion rate on the mild steel surface. Further, the reduced phase angle of depressed semicircles in Figure 9 (b) and (c) shows the roughness caused by the corrosion of electrode surface. Thus, the phase angle increases with reduction in roughness of the surface with increase in inhibitor concentration [53].

3.10. UV-Visible spectroscopic study for the corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4

UV-Visible spectrum of the solution of mild steel immersed in 0.5 M H_2SO_4 (Figure 10(a)) and the same solution with optimum concentration of *Rhizobium Leguminosarum* isolate (Figure 10(b)) shows the identical absorption band at 200 nm – 237 nm due to the $n - \sigma^*$ and $\pi - \pi^*$ which confirms the adsorption of carboxyl, ester, exopolysaccharide layer of *Rhizobium leguminosarum* isolate on mild steel surface in 0.5 M H_2SO_4 . Figure 10 (b) additionally shows the absorption band around 236 nm to 273 nm which is attributed to the $\pi - \pi^*$ electronic transition of the exopolysaccharide compound found in most conjugated molecules [54].

3.11. FTIR Studies for the corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4

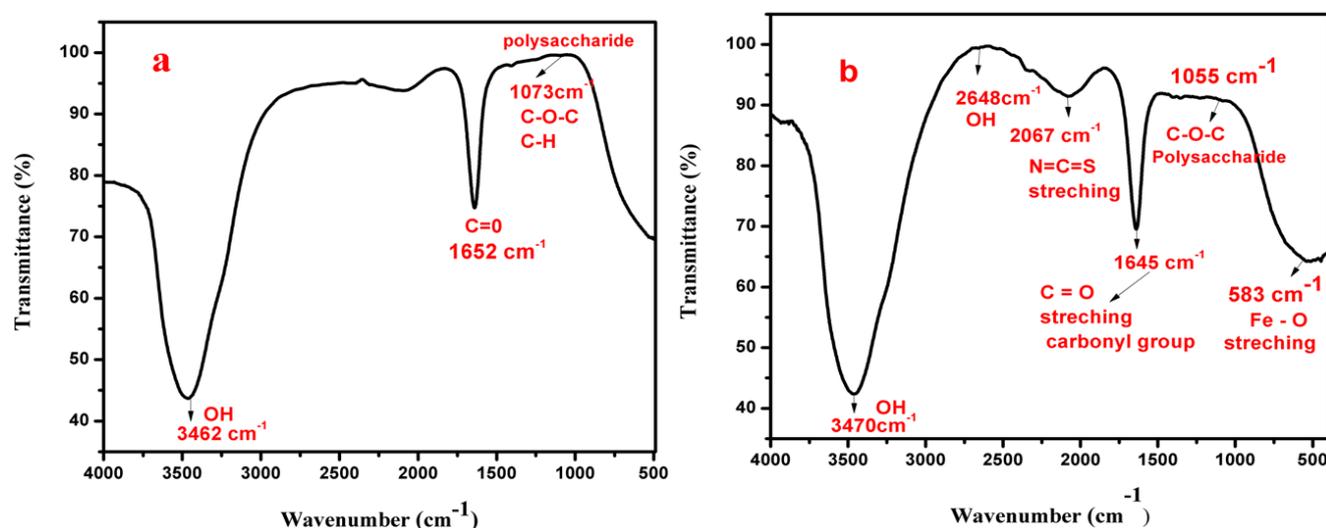


Figure 11. IR spectrum of (a) (9×10^6 cfu/mL) of *Rhizobium leguminosarum* isolate and (b) *Rhizobium leguminosarum* isolate scratched from mild steel surface after immersion in 0.5 M H_2SO_4 with (9×10^6 cfu/mL) of *Rhizobium leguminosarum* isolate.

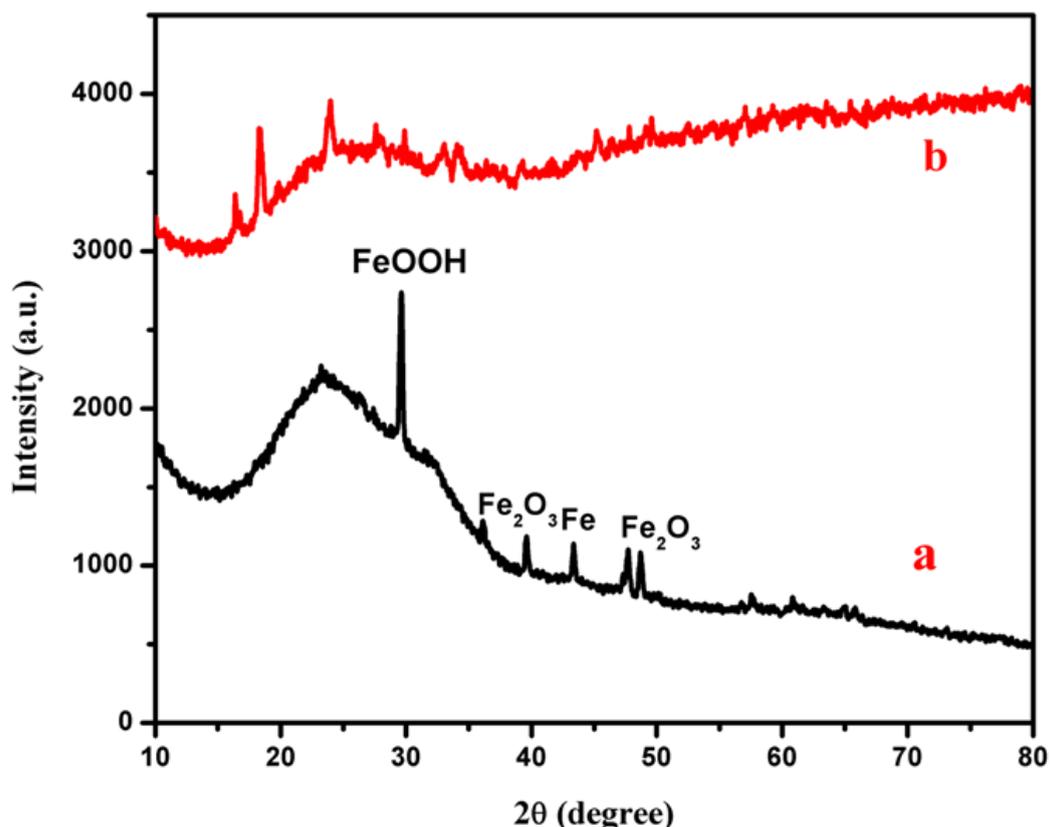


Figure 12. XRD Pattern of (a) Corroded mild steel in 0.5 M H₂SO₄ (b) Mild steel immersed in 0.5 M H₂SO₄ with (9×10^6 cfu/mL) of *Rhizobium leguminosarum* isolate.

Figure. 11(a) and (b) shows the FT-IR spectrum of *Rhizobium leguminosarum* isolate and scratched sample of *Rhizobium leguminosarum* isolate from mild steel surface immersed in 0.5 M H₂SO₄ after 3h, respectively. Both the spectrum is nearly identical with the additional iron peak at 583 cm⁻¹ in Figure 11(b). In both figures, the stretching vibration of O-H causes the peak centered at 3462 cm⁻¹ and 3470 cm⁻¹ and the peaks at 1645 cm⁻¹ and 1652 cm⁻¹ indicates C=O stretching. The further peaks at 1073 and 1055 cm⁻¹ are due to the presence of C-O-C exopolysaccharide moiety which prevents the corrosion of mild steel [55].

3.12. XRD analysis for the corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄

Figure 12 (a) and (b) shows the XRD spectrum of scratched powder from mild steel surface after immersion at 3h in the presence and absence of *Rhizobium leguminosarum* isolate. There are small peaks in the region at 29.8°, 39.5°, 43.4°, 47.8° due to the presence of the FeOOH and Fe₂O₃ molecules. On the other hand, oxides of iron (FeOOH and Fe₂O₃) peaks are absent in the XRD analysis of scratched powder from inhibited mild steel. Additionally, the broad shoulder appeared for exopolysaccharide at 24.2°. These results confirmed the formation of protective exopolysaccharide layer on the surface of mild steel, which is secreted by *Rhizobium leguminosarum* isolate [56].

3.13. Field Emission Scanning electron microscope and EDX analysis for the corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄

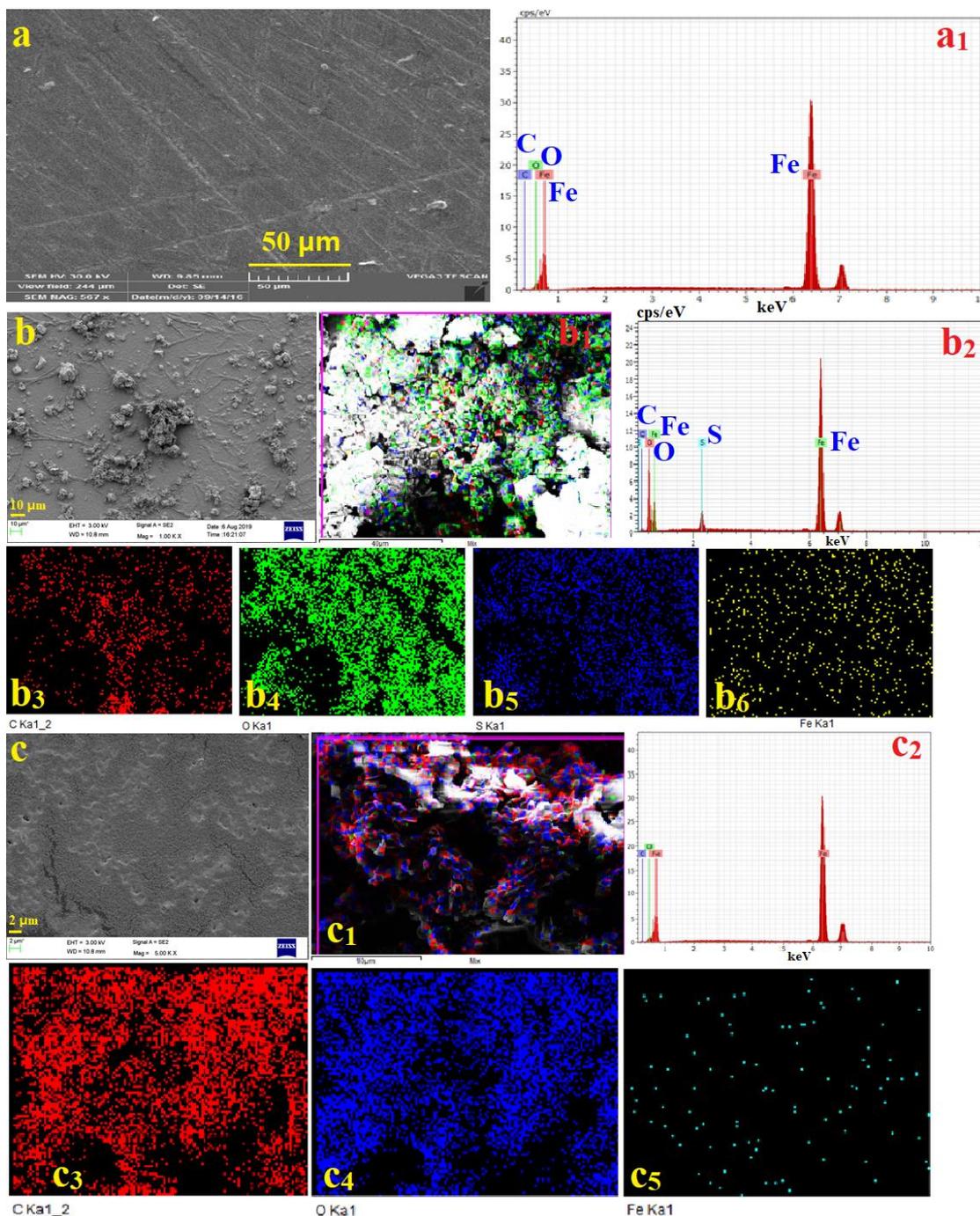


Figure 13. (a) FE-SEM of polished mild steel (a₁) EDX micrograph of polished mild steel, (b) FE-SEM of corroded mild steel (b₁). Overall mapping elements on the same spot: corresponding to (b₂) EDX micrograph, (b₃) carbon, (b₄) oxygen, (b₅) sulphur, (b₆) Fe mapping analysis of mild steel immersed in 0.5M H₂SO₄, and (c) FE-SEM of mild steel (c₁). Overall mapping elements on the same spot: corresponding to (c₂) EDX micrograph, (c₃) carbon, (c₄) oxygen, (c₅) Fe mapping analysis of mild steel immersed in 0.5M H₂SO₄ containing (9 × 10⁶ cfu/mL) *Rhizobium leguminosarum* isolate.

Figure 13 shows the FE-SEM, EDX and mapping analysis of surface of the polished mild steel (a & a₁), mild steel after immersion in 0.5 M H₂SO₄ (b to b₆) and mild steel in 0.5 M H₂SO₄ with *Rhizobium leguminosarum* isolate (c to c₅). The smooth surface image of FESEM and increased elemental composition of EDX and mapping analysis of inhibited mild steel than uninhibited mild steel confirms the formation of exopolysaccharide on the surface of mild steel in 0.5 M H₂SO₄ with *Rhizobium leguminosarum* isolate [57].

3.14. Atomic force microscope studies analysis for the corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄

The protective film formation on the surface of the inhibited mild steel is confirmed by AFM analysis. AFM images of polished mild steel, inhibited and corroded mild steel are shown in Figure 14 (a) and (b) and (c). A critical attack on the surface by the corroding medium is observed in the uninhibited metal surface. The average roughness (Ra) value calculated are shown in Table 6 polished mild steel for 8 nm, 69 nm for blank and 23 nm for *Rhizobium leguminosarum* isolate. The reduction in Ra values from the blank values indicates the decrease in surface roughness, among them inhibited mild steel surface is covered by the exopolysaccharide secreted by *Rhizobium leguminosarum* isolate. On the other hand uninhibited mild steel surface is fully corroded and it seems like punctured due to acid attack [58].

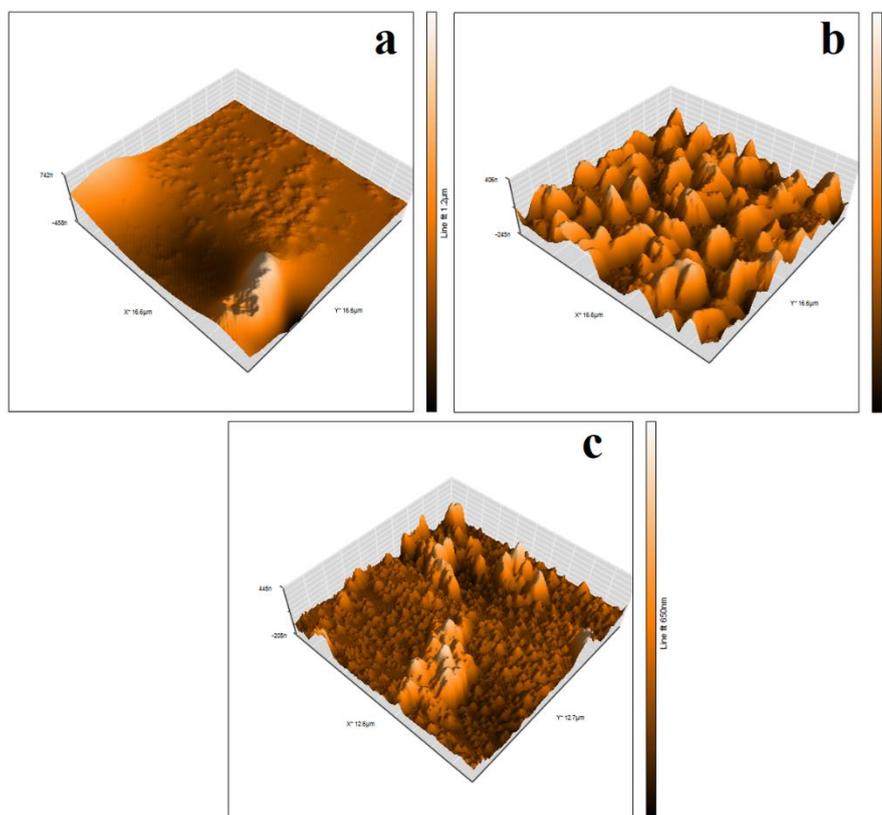
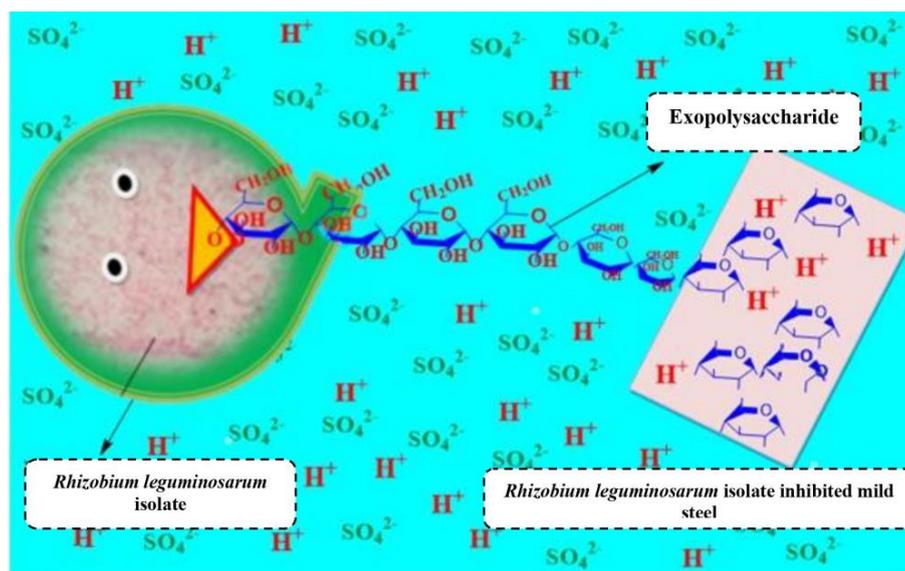


Figure 14. AFM images of (a) polished mild steel surface (b) Mild steel immersed in 0.5M H₂SO₄ (c) Mild steel immersed in 0.5 M H₂SO₄ containing 9 x 10⁶ cfu/mL of *Rhizobium leguminosarum* isolate.

Table 6. AFM data for mild steel surface dipped in *Rhizobium leguminosarum* (10^6 cfu/mL) and 0.5M H_2SO_4 medium.

Samples	R_q (nm)	R_a (nm)	R_{p-v} (nm)
Polished mild steel	7	8	36
Mild steel in 0.5M H_2SO_4	89	69	336
Mild steel in 0.5 M H_2SO_4 + 9 mL <i>Rhizobium leguminosarum</i>	29	23	136

3.15. Mechanism of corrosion inhibition process in the presence and the absence of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4

**Scheme 2.** Mechanistic view of corrosion inhibition using *Rhizobium leguminosarum* isolate on mild steel surface in 0.5 M H_2SO_4

Formation of protective layer of exopolysaccharide secreted by *Rhizobium leguminosarum* isolate on the surface of mild steel in 0.5 M H_2SO_4 is confirmed by spectroscopic and surface studies. FTIR spectrum and isotherm data confirms the type of adsorption as physisorption. The above factors leads to the two confirmations (i) *Rhizobium leguminosarum* isolate excreted the exopolysaccharides in 0.5 M H_2SO_4 concomitantly (ii) The electrostatic interaction has been formed between the activated surface of mild steel and the π electrons of oxygen in the exopolysaccharide [59]. The plausible mechanism for *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4 is shown in Scheme 2.

4. CONCLUSION

In summary, the *Rhizobium leguminosarum* isolate perform as anti-fouling and biological inhibitor for the corrosion of mild steel in 0.5 M H_2SO_4 . The corrosion of mild steel and formation of exopolysaccharide matrix of *Rhizobium leguminosarum* isolate in 0.5 M H_2SO_4 is confirmed by weight

loss measurement, Potentiodynamic polarization, Electrochemical impedance study, UV, FTIR, XRD, FE-SEM, EDX and AFM. The inhibition efficiency of *Rhizobium leguminosarum* isolate on mild steel in 0.5 M H₂SO₄ is increased with increasing concentration of *Rhizobium leguminosarum* isolate. The mixed type inhibiting nature of *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄ is confirmed by potentiodynamic polarization studies. Formation of protective layer of biofilm on the surface of mild steel is studied by EIS. The obtained ΔG values for adsorption at all the temperatures are around 5 KJ mol⁻¹ reveals that *Rhizobium leguminosarum* isolate in 0.5 M H₂SO₄ is physisorption. Langmuir adsorption isotherm is well fitted for the adsorption of *Rhizobium leguminosarum* isolate on mild steel in 0.5 M H₂SO₄. Characterization and surface techniques like UV, FTIR, FE-SEM, EDX and AFM studies confirm the formation of exopolysaccharide matrix on the surface of mild steel in 0.5 M H₂SO₄.

The current exploration endorses the upshot that polysaccharides made by microorganisms display anti-corrosive belongings. First and foremost, exopolysaccharides showed interesting results for the protection of steel. Measurements specify that it take some time to form layers of biopolymers on the metal to figure a complete protective layer. Our facts disclosed that *Rhizobium leguminosarum* harvest exopolysaccharide, which oblige as corrosion inhibitor for mild steel. Supplementary studies are desired to appraise the potential of the exopolysaccharides as anticorrosive agents. Corrosion inhibition by microbes is not constantly curbed to a single mechanism or a single species of microorganism. Mostly, the inhibition activity is at the metal-bulk solution interface owing to the neutralizing action of the microorganisms through the production of exopolysaccharide with metal binding abilities and by consuming cathodic electron or by the production of antimicrobials.

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