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# Corrosion Concepts

In this forum readers will be able to present practical problems for discussion. It is envisaged that these contributions will include not only discussion of general problems and incidents of corrosion but that suggested remedies will also be presented and discussed. It is hoped that this exchange of knowledge and experience will become a permanent feature of this periodical. We are particularly anxious that both Senior Scientists and those with more practical experience will make use of this forum to exchange information, problems and potential remedies.

# Molecular characterization and corrosion behavior of thermophilic (55 °C) SRB *Desulfotomaculum kuznetsovii* isolated from cooling tower in petroleum refinery

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***Desulfotomaculum kuznetsovii* (*D. kuznetsovii*), a thermophilic sulfate-reducing bacterium (SRB), was identified in a cooling tower of a petroleum refinery by 16S rRNA gene sequencing and its functional gene encoding dissimilatory sulfite reductase (*dsr*AB). The thermophilic sulfate-reducing bacterial species have been reported for the first time in the cooling towers of an Indian petroleum refinery. The protein coded by *dsr*AB gene was cloned, expressed, and identified using recombinant DNA technology. Weight loss method, electrochemical and surface analysis showed the corrosion behavior of the isolate. In the presence of *D. kuznetsovii*, the corrosion rate was higher when compared to control at 55 °C. It suppresses the anodic reaction and enhances the cathodic reaction by the production of organic complex and iron sulfide, respectively. Numerous pitting were noticed on mild steel which is due to the presence of *D. kuznetsovii* and its role in the corrosion process has been discussed.**

## 1 Introduction

Sulfate-reducing bacteria (SRB) are strictly anerobic microorganisms responsible for the terminal mineralization of organic matter in anoxic environments. The physiology of SRB as well as their ecological and economic impact has been well described [1]. The majority of studies on SRB in oil field environments have concentrated on the ecology and physiology of mesophilic microorganisms, which grow optimally between 20 and 40 °C. Sulfate-reducing bacteria are considered to be a particular problem because they produce corrosive and toxic $H_2S$, and they have been the main focus of concern in oil field microbiology [2] and the participation of SRB in the corrosion process was identified [3]. Nuclear industry is also facing severe corrosion problem by SRB in their cooling water system where mesophilic bacteria were reported in Kalpakkam cooling water system [4]. *Abedi* et al. [5] observed and studied the influence of stress corrosion cracking (SCC) and SRB induced cracking propagation in transmission oil products pipeline. Most oil-bearing reservoirs, especially in north Sea fields and Indian Oil wells, exist in deep geological strata, with temperatures above 60 °C [6].

SRB are present in almost all types of ordinary and extreme habitats and although obligate anerobes, they can be found in a wide variety of oxygenated environments too. Generally, the temperature in condenser tubes in cooling towers is in the range between 50 and 70 °C. The incomplete description of thermo-

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philic SRB species in industrial environments has profoundly hindered the understanding of their bacterial diversity and their role in the cycling of matter or nutrients materials. The [NiFe] hydrogenase gene probe [7] was used to identify different *Desulfovibrio* spp. in oil field samples following growth on lactate–sulfate medium. The use of hydrogenase gene probes allowed the presence, appearance, or disappearance of a given species to be monitored in an oil field operation.

Sulfate reduction in SRB is catalyzed by several enzymes present in membrane and cytoplasm. Dissimilatory sulfite reductase (*dsr*), catalyzing the six electron transfer during the conversion of sulfite to sulfide, is being one of the molecular markers in the identification of SRB. So far, four different dissimilatory sulfite reductases have been distinguished as desulfoviridin in *Desulfovibrio* spp., desulfoviridin in *Desulfovibrio desulfuricans* Norway, desulfofuscidin in *Thermodesulfobacterium commune*, and P582 in *Desulfotomaculum nigrificans* [8]. All dissimilatory sulfite reductases are multisubunit enzymes containing iron–sulfur clusters and prosthetic siroheme groups. α- and β-subunits of sulfite reductases had been studied by isolation, and purification methods [9]. A third subunit γ had also been identified, expressed, and studied by *Pierik* et al. [10] and *Karkhoff-Schweizer* et al. [11].

Limited studies have been reported on the sulfite reductases of thermophilic SRB in corrosive environment and the role of thermophilic SRB in the corrosion process in industries in tropical country. Corrosion by thermophilic SRB was investigated by only few investigators [12–14]. Therefore, the identification and characterization of thermophilic SRB present in cooling towers/oil field environments is an essential requirement in order to understand its physiology and the mechanism of the corrosion process at high temperature. In the present study, sensitive and specific molecular techniques have been employed for the identification of SRB with their functional gene sequences and recombinant DNA technology for the expression and identification of dissimilatory sulfite reductase and also the corrosion behavior of the isolate was carried.

## 2 Experimental procedure

### 2.1 Sample collection

Corrosion product from a cooling water system was collected aseptically from a cooling tower of a petroleum refinery (Chennai, India) kept in an ice bucket and transported to Sourashtra College Laboratory, Madurai for microbiological characterization. The physical and chemical characteristics of the cooling water were analyzed by standard procedures recommended [15].

### 2.2 Isolation

The sample was enriched for 7 days anerobically in sterile Modified Baar's liquid medium (1 L of medium contained 2 g of $MgSO_4$, 5 g of sodium citrate, 1 g of $CaSO_4$, 1 g of $NH_4Cl$, 0.5 g of $KH_2PO_4$, 1 g of yeast extract, and 3.5 g of sodium acetate). The enriched culture was inoculated in sterile Modified Baar's medium with carbon source acetate. The samples inoculated were kept in an anerobic jar and incubated at 55 °C for 3 days. The strain isolated was examined for its morphology and biochemical characters including Desulfoviridin test [16].

### 2.3 DNA isolation, PCR amplification, cloning, and sequencing

Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA; pH 8) buffer and Lysozyme (10 mg/mL) were added in the pelleted cells and incubated for 30 min at room temperature. SDS and Proteinase K (10 U/μL) were added and incubated at 55 °C for 2 h. DNA was extracted with phenol, chloroform, and iso-amyl alcohol and DNA was precipitated with ethanol and dissolved in TE buffer [17]. Polymerase chain reaction (PCR) was performed with a final volume of 50 μL in 0.2 mL thin walled tubes. The primers used for PCR amplification of 16S rRNA (ribosomal RNA) gene are 8F forward (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1490R reverse (5′-GGT TAC CTT GTT ACG ACT T-3′) [18] (Sigma Genosys). Each reaction mixture contained 2 μL of template DNA (100 ng), 0.5 μM of forward and reverse primers, and 25 μL of Enzyme Master Mix (Bioron). The PCR program consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of DNA denaturation at 92 °C for 30 s, primer annealing at 50 °C for 1 min, primer extension at 72 °C for 2 min, and a final extension at 72 °C for 20 min carried out in Thermal Cycler (Thermo Hybaid). The PCR products were purified by QIAquick PCR purification kit as described by the manufacturer and cloned using QIAGEN PCR cloning plus kit as described by the manufacturer. Clones were selected and plasmids with insert were isolated and sequenced with M13 sequencing primers using ABI Biosystems automated sequencer. The sequences obtained were matched with previously published sequences available in NCBI using BLAST [19].

### 2.4 Cloning, expression, and identification of dissimilatory sulfite reductase

The *dsr*AB gene was amplified with primers *DSR*1F (5′-ACG CAC TGG AAG CAC G-3′) and *DSR*4R (5′-GTG TAG CAG TTA CCG CA-3′) [20] following the PCR program used for 16S rRNA gene amplification except primer annealing that was carried out at 54 °C. The PCR products were purified by QIAquick PCR purification kit as described by the manufacturer and cloned using QIAGEN PCR cloning plus kit as described by the manufacturer. Clones were selected, plasmids with insert isolated, and sequenced with M13 sequencing primers using ABI Biosystems automated sequencer. *dsr*AB genes amplified and purified by the above method were cloned in QIAexpress UA Cloning vector (Qiagen) and transformed in competent *E. coli* M15 (pREP4) cells (Qiagen) as described by the manufacturers. The recombinant clones were selected from Luria–Bertani agar plates containing Ampicillin (100 μg/mL), and IPTG (50 mM). The clone was screened for the presence of *dsr*AB gene in the plasmid isolated from the recombinant. The screened clone was further inoculated in Luria–Bertani broth containing Ampicillin (100 μg/mL) and expression of *dsr* protein was induced with IPTG (1 mM). The induced cells were lysed in denaturing condition with lysis buffer (100 mM $NaH_2PO_4$, 10 mM Tris HCl, 8 M Urea) and centrifuged at 10 000 rpm for 10 min. The expressed proteins in the supernatant were resolved in 12% SDS–

PAGE with protein molecular weight marker (MBI Fermentas). The proteins were stained using Coomassie-R250 staining solution, destained and the bands were visualized [21]. The proteins obtained were quantified using Bradford's method [22]. 0.1 and 0.5 mL of sample were withdrawn and made up to 1 mL using 0.15 M NaCl and to that 1 mL of Bradford's reagent (50 mg of Coomassie Brilliant Blue G-250 in 25 mL ethanol) was added, kept at 37 °C for 2 min and reading was taken at 595 nm (Shimadzu UV1700) against NaCl that was used as a blank. Bovine Serum Albumin standard solution was used for the calibration. The proteins were purified using Ni-NTA spin kit (Qiagen) for His tagged protein [23] as described by the manufacturers. A Maldi-TOF MS (Kratos Analytical) was used for mass analysis. The matrix 3,5-dimethoxy-4-hydroxycinnamic acid was made up to a saturated solution with 0.1% trifluoroacetic acid/50% acetonitrile. 0.5 μL of the sample and 0.5 μL of matrix were applied to a sample plate and vacuum dried. A pulsed UV nitrogen laser was used to desorb ions from the sample. The instrument was operated at an accelerating voltage of 24 kV. The spectra were generated by 50–60 laser shots. The data were acquired from the instruments operating in the positive reflectron mode.

### 2.5 Weight loss method

Mild steel coupons of size 1 cm × 5 cm were mechanically polished to mirror finish and then degreased using trichloro ethylene. Modified Baar's medium (250 mL) has been taken as the system and polished coupons were immersed in the medium inoculated with SRB isolate and incubated at 55 °C. The control system without bacteria by employing Baar's medium was also maintained for 7 days. After 7 days, the coupons were removed and pickled in pickling solutions, washed in water, and dried with air drier. Final weights of the six coupons in each system were taken and the average corrosion rates were calculated. The standard deviation for each system is also presented.

### 2.6 Polarization study

Mild steel coupons were embedded in araldite with an exposed area of 1.0 $cm^2$ as a working electrode. The specimens were immersed for 7 days at 55 °C in sterile as well as SRB isolate inoculated in Modified Baar's medium. Polarization study with conventional three-electrode cell assembly was done by employing potentiostat (PGP 201, potentiostat with voltammeter-1 software). A three-electrode setup was used consisting of test coupon as the working electrode, saturated calomel electrode (SCE) as the reference, and a platinum electrode as the auxillary. The test coupon was first immersed in the corrosion cell for 10 min to allow equilibrium with the electrolyte. The Tafel polarization curves were obtained by scanning from open circuit potential towards 200 mV anodically and cathodically with the scan rate of 120 mV/min. IR drop composition was not needed since this was a high conductivity electrolyte.

### 2.7 X-ray diffraction (XRD), Fourier transform infra red (FTIR) spectroscopy, and Scanning electron microscopy (SEM) analysis

A computer controlled XRD (JEOL Model JDX-8030) was used to scan the 2θ of corrosion products between 10 and 85° with copper Kα radiation (Ni filter) at a rate of 40 kV, 20 mA. The dried corrosion products were collected and crushed into a fine powder and used for XRD analysis for determining the nature of oxides present in the corrosion product in the presence of bacteria. To verify the adsorption of corrosion products on the metal surface in control and inoculated system, the film formed on the metal surface was carefully removed and dried, mixed thoroughly with potassium bromide (KBr), and made as pellets. These pellets are subjected to FT-IR spectra (Perkin–Elmer, Nicolet Nexus −470) to find out the nature of film formed on the surface of the metal coupons. The surface morphological characteristics of the bacteria were observed under scanning electron microscope (SEM) (Hitachi model S-3000 H) at magnification ranging from 50 to 200× operated at an accelerating voltage of 25 kV.

### 2.8 Confocal microscopy

The mild steel specimen exposed to culture was air-dried, stained using acridine orange and observed under confocal laser scanning microscope (CLSM) (Leica TCS-SP2-RS, Germany) equipped with argon ion laser light source with an excitation wavelength of 488 nm was used to image the corrosion pits. The images were obtained in a fluorescence mode and images were generated with an objective magnification of 20× and numerical aperture set at 0.5 (20× magnification and 0.5 NA), selecting a scan format of 512 × 512 pixels and scan speed of 4000 Hz and pinhole set at 0.63. The stage was moved vertically (z-direction) between the first and last detectable light reflex and a z series consisting of optical sections (~1.5 μm) as per numbers optimized by the software was generated.

## 3 Results and discussion

### 3.1 Isolation and identification

The physical and chemical characteristics of the cooling water are presented in Table 1. The thermophilic strain isolated was analyzed for its morphology and biochemistry and the observations are tabulated in Table 2.

**Table 1.** Physical and chemical characteristics of the cooling water

| S. No | Factors | Observations |
|---|---|---|
| 1 | Temperature | 60 °C |
| 2 | pH | 8.2 |
| 3 | Total solids | 285 ppm |
| 4 | Total dissolved solids | 225 ppm |
| 5 | Total suspended solids | 60 ppm |
| 6 | Dissolved oxygen | 6.3 ppm |
| 7 | Chloride | 280 ppm |
| 8 | Total hardness | 150 ppm |
| 9 | Sulfate | 78 ppm |
| 10 | Calcium | 68 ppm |
| 11 | Magnesium | 82 ppm |

 

**Table 2.** Morphological and biochemical characters of isolates

| S. No. | Characters | Isolate |
|---|---|---|
| 1 | Shape | Rod |
| 2 | Gram reaction | Positive |
| 3 | Motility | +[a] |
| 4 | Spore formation | Subterminal |
| 5 | Optimal growth temperature | 55 °C |
| 6 | Oxidation of organic substrates | Acetate |
| 7 | $H_2S$ production | +[b] |
| 8 | Desulfoviridin test | –[c] |

[a]motile, [b]$H_2S$ produced, [c]no Desulfoviridin.

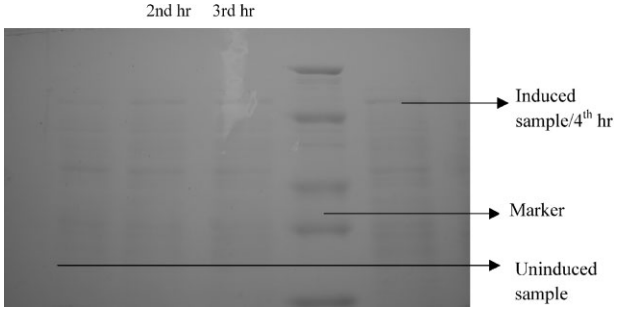


**Figure 1.** Resolved proteins expressed in SDS–PAGE

### 3.2 Molecular identification

Nucleotide database was searched with the sequences obtained using NCBI BLAST (Blastn) tool (http://www.ncbi.nlm.nih.gov/BLAST) and showed 99% similarity with 16S rRNA genes of *D. kuznetsovii* of the database sequences.

### 3.3 Cloning, sequencing, and expression of dissimilatory sulfite reductase protein

The sequences searched in the NCBI database showed 99% similarity with the *dsr*AB gene sequences of *D. kuznetsovii* species in the database sequences. Bands of expressed proteins observed in induced cell lysates inferred a molecular weight of ~96 kDa in the SDS–PAGE electrophoretograph given in Fig. 1. Quantification of proteins in the crude lysate showed higher protein concentration in induced cells than in uninduced cells (Table 3). Mass peaks (96.1 kDA) given in Figs. 2 and 3 in the unpurified and His Tag purified samples of expressed proteins identified in MALDI MS supported the protein size analysis in the SDS–PAGE. It is expected that sulfite from sulfate is further reduced to sulfide through six electron transfer catalyzed by dissimilatory sulfite reductase having the size of ~44 kDa for α-subunit and

**Table 3.** Concentration of proteins expressed and His tag purified

| S. No. | Samples | Concentration of protein μg/mL of *D. kuznetsovii* |
|---|---|---|
| 1 | Uninduced samples | 87 |
| 2 | Induced sample after 2 h | 92 |
| 3 | Induced sample after 3 h | 114 |
| 4 | His-tag purified sample | 11 |

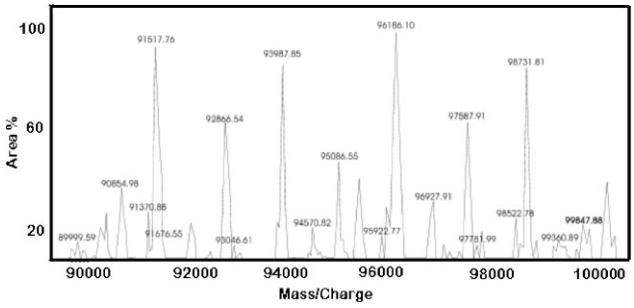


**Figure 2.** MALDI MS analysis of unpurified protein extract

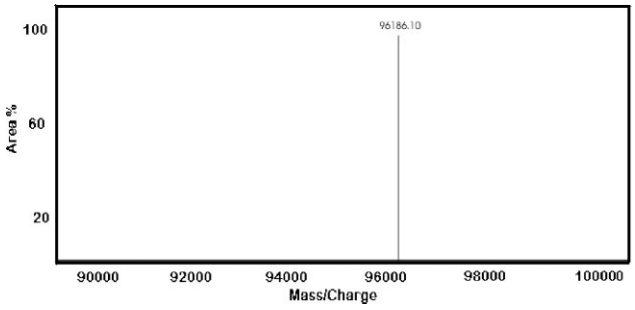


**Figure 3.** MALDI MS analysis of purified His tagged protein extract

**Table 4.** Corrosion rate of mild steel

| S. No. | System | Weight loss (mg) | Corrosion rate (mm/year) |
|---|---|---|---|
| 1 | Control uninoculated medium | 45 ± 2 | 0.0026 |
| 2 | Experimental system *D. kuznetsovii* | 118 ± 1 | 0.0687 |

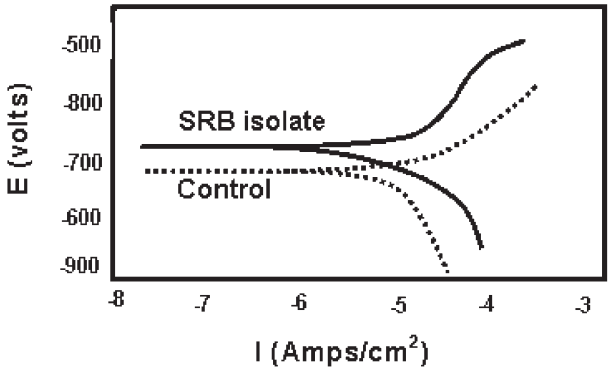


**Figure 4.** Polarization study with SRB isolate

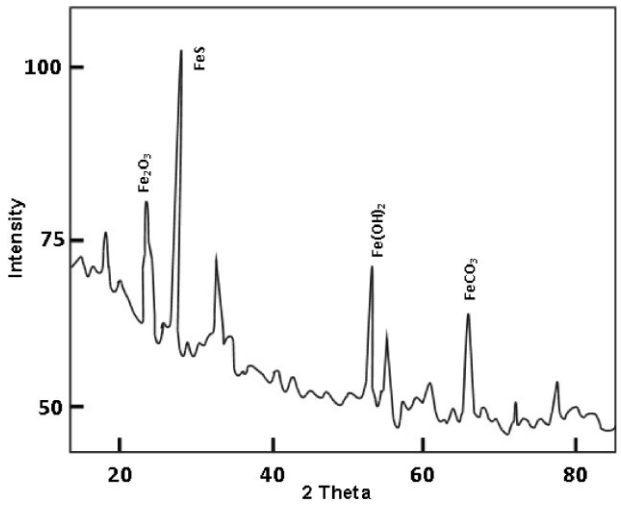


**Figure 5.** XRD pattern of the corrosion product

~41 kDa for β-subunit correlated with the report of *Molitor* et al. [24].

### 3.4 Weight loss method

In the control system (uninoculated), the weight loss was 45 mg whereas in the presence of *D. kuznetsovii*, the weight loss was 118 mg (Table 4). It indicates that the corrosion rate was higher (0.069 mm/year) in the presence of *Desulfotomaculum kuznetsovii* than the control.

### 3.5 Polarization study

Figure 4 shows the polarization study for mild steel in the presence and absence of *D. kuznetsovii*. The OCP of steel in the control system was −690 mV SCE while inoculating bacteria, potential shifted to negative side about −745 mV SCE. The corrosion current for the control system was $8 \times 10^{-4}$ A/cm$^2$ and the presence of bacteria was $2 \times 10^{-5}$ A/cm$^2$. It indicates that bacteria enhance the corrosion by shifting of potential to negative side. However, the $i_{corr}$ was lower in the presence of bacteria. The nature of the curve indicates that the bacteria enhance cathodic current and suppress anodic current.

### 3.6 XRD, FTIR, and SEM analysis

Figure 5 shows the details of XRD peaks corresponding to the phases present in the corrosion product collected from the mild steel in the presence of bacteria. The presence of a broad peak with 2θ between 0 and 20° indicates the organic complex produced by bacteria. Besides peaks of higher intensity were noticed with $Fe(OH)_2$, FeS, $Fe_2O_3$, and $FeCO_3$ in the presence of bacteria. A peak at 2θ below 20° indicates the presence of iron and organic complex. The FTIR spectrum of the biofilm collected from steel is presented in Fig. 6. The presence of $CH_3$ and $CH_2$ stretching vibrations was noticed between 2000 and 2500 cm$^{-1}$ which attributed to fatty acid. The presence of amide I and II bands was noticed at the wavelength range of 1700–1500 cm$^{-1}$ which indicates the peptide of polypeptides (proteins) relevant relationships between the various secondary protein substructures. A broadband was noted in the range of 3000–3500 cm$^{-1}$, which was assigned to the presence of adsorbed water molecule and OH/NH groups. The band at 450–700 cm$^{-1}$ probably originated mainly from $\gamma$-$Fe_2O_3$ (610 cm$^{-1}$). The other peaks were noticed at 1685 and 1640 cm$^{-1}$ which was due to free acidic group and iron phosphate, respectively. It suggests that the extracellular polymeric substances (EPS) produced by *D. kuznetsovii* contained acidic groups. It can be concluded that the formation of fatty acids, polypeptides, and nitrogen group is due to the attachment of *D. kuznetsovii* on the mild steel surface. SEM analysis (Fig. 7) showed the presence of rod-shaped to ovoid-shaped bacterial cells and adsorbed corrosion products on mild steel coupon and the morphology of the bacteria supports with the observations made by *Nazina* et al. [25].

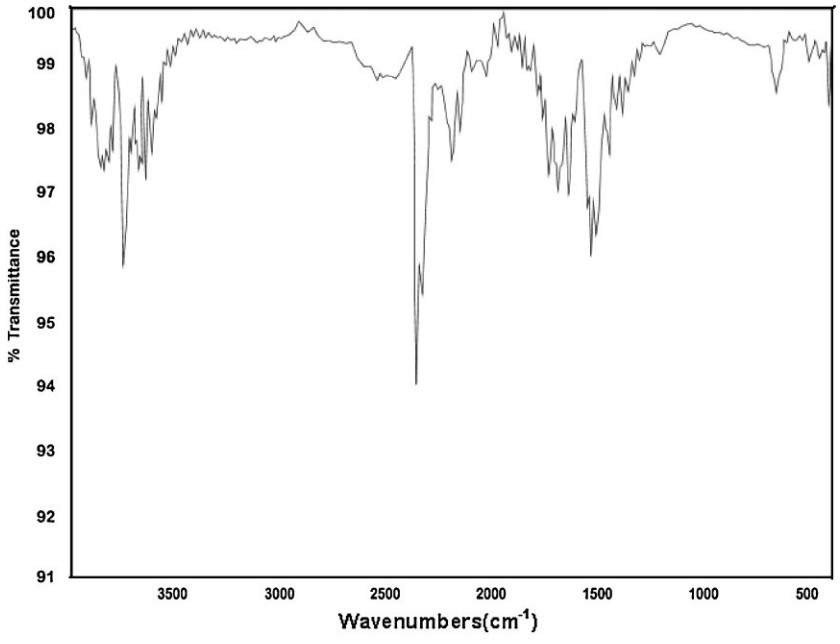


**Figure 6.** FTIR analysis of the corrosion product

### 3.7 CLSM analysis

Figure 8 shows the presence of micropits throughout the surface. Several pits of size up to 150 μm diameter and depth were observed.

Corrosion damage associated with bacteria in petroleum chemical industry and nuclear power plants was frequently noted and the amount of damage was estimated to be in excess of several billion US dollars [26]. Many observations made by investigators on mesophilic SRB corrosion in various industries namely cargo oil tank and fuel oil tank of an aircraft, but limited literatures are available on thermophilic SRB corrosion. SRB are a complex physiological bacterial group and various properties have been used in traditional classification schemes. The most impor-

 

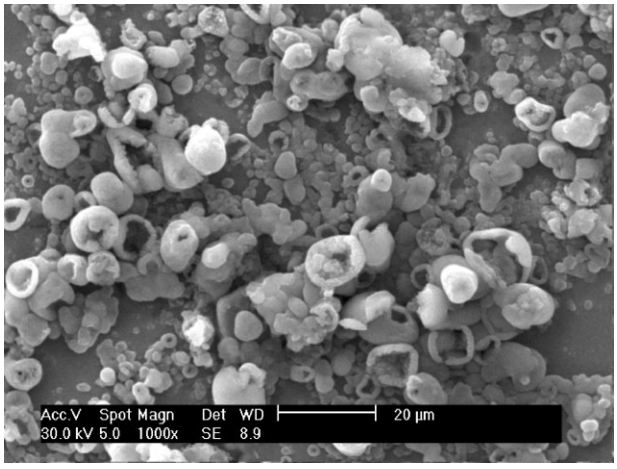


**Figure 7.** Scanning electron micrograph of mild steel coupon exposed to thermophilic SRB isolate

tant of these properties were cell shape, motility, GC content of DNA, presence of desulfoviridin and cytochromes, optimal temperature, and complete and incomplete oxidation of acetate. The temperature, pH, salinity, in situ pressure, and degraded hydrocarbon products encourage the growth of thermophilic and mesophilic SRB in hydrocarbon-rich environments [27]. In the present study, anerobic growth at high temperature, sporulation, and oxidation of carbon source acetate, $H_2S$ production, and Desulfoviridin production were observed (Table 1) in the isolate confirming the presence of thermophilic Gram positive SRB. Nutritionally similar new mesophilic and thermophilic rod-shaped sulfate reducers were included in the genus *Desulfovibrio* and all these *Desulfotomaculum* and *Desulfovibrio* species oxidize their substrates, such as lactate, ethanol, or malate, incompletely to acetate [28, 29]. The oxidation of organic substrates in sulfate-reducing bacteria may

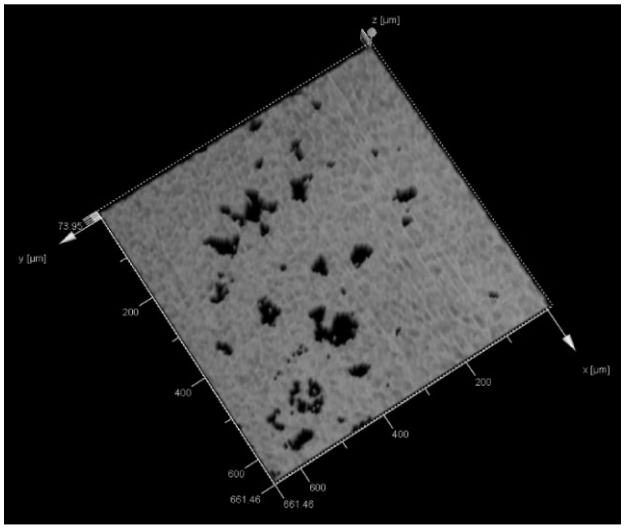


**Figure 8.** Confocal microscopy showing pits on mild steel exposed to thermophilic SRB isolate

be complete, leading to $CO_2$ or incomplete with acetate usually being the end product. In the present study, the isolate given in Table 2 infers the complete oxidation of acetate into $CO_2$ and the presence of a pathway for acetyl-CoA oxidation that is usually associated with the ability to use free acetate as a growth substrate [30].

Because of the critical role played by the SRB in the functioning of the ecosystem, environmental remediation and their involvement in corrosion processes has resulted in an increased interest in SRB over the last decade. 16S rRNA gene-targeted PCR primer sequences specific for SRB subgroups have been designed and used to detect phylogenetic subgroups of SRB [31]. The isolate collected from cooling water system of a refinery had similar characters and exhibited sequence similarity (99%) to *D. kuznetsovii* sequence (Y11569) in the Genbank database which was also identified [32] by 16S rRNA genes and comparative analysis done with the closest representatives of the genus *Desulfotomaculum*. However, 16S rRNA-based analysis does not provide an unambiguous link to the physiology or metabolic capacities of a bacterium, particularly in newly discovered phylogenetic lineages without cultured isolates and known phenotypes [33]. Hence, in the present study, the functional gene approach (*dsr*AB, a key enzyme present only in SRB groups) has been used to identify the bacteria responsible for biogeochemical processes in the environment [34]. A molecular approach based on the *dsr*AB genes had been used to characterize SRB in a variety of environmental settings [35, 36]. The key gene encoding *dsr*AB (EC 1.8.99.3) was found in all SRBs. The 1.9 kb DNA fragment encoding most of the α- and β-subunits of *dsr*AB have been amplified and analyzed by PCR from all recognized lineages of SRBs [36]. The present work shows the 99% similarity of gene sequences with *dsr*AB genes of database sequences and confirms the presence of *D. kuznetsovii*. As *dsr* is a highly conserved enzyme in all SRBs and phylogenetic analysis of gene sequenced in the present study confirms the isolate as *D. kuznetovii* and suggests a paralogous origin of α- and β-subunits, *dsr* from sulfate reducers formed a separate cluster with the genes from *Desulfotomaculum thermocisternum* and *Desulfovibrio vulgaris* branching together, next to *dsr* from *Archaeoglobus profundus* and *Archaeoglobus fulgidus* [37].

Hydrogenase, a key enzyme of hydrogen metabolism, which plays a central role in the energy yielding mechanisms of SRB and three different classes of hydrogenases have been found as (Fe)-only, (NiFe), and (NiSeFe) [38] and well documented. Hydrogenase consumes the hydrogen produced and so enhances the rate of sulfate reduction to form $H_2S$ and mechanism exactly corresponds to the so-called theory of cathodic depolarization [39]. The gene coding hydrogenase was used as a probe for the identification of SRB in addition to 16S rRNA sequencing [40]. The *dsr*AB gene, as not only a convincing marker for the identification of SRB in the environment, but also involves in the dissimilatory sulfate reduction to convert sulfite to sulfide which is then converted to $H_2S$ with membrane bound hydrogenase makes this current study an important one to find a way to knock out the gene or the enzyme dissimilatory sulfite reductase to control the corrosion process. Hence, recombinant DNA studies and expression profile on dissimilatory sulfite reductase carried out like the present study may disclose many factors behind its

structure, function, and involvement in corrosion. In this study, the dissimilatory sulfite reductase protein was expressed and identified with MALDI MS peaks which correlate with the following findings. *Steuber* et al. [41] analyzed the expressed sulfite reductase of *D. desulfuricans* with the enzyme of *D. vulgaris* by immunodetection with antibodies raised against α, β, and γ-subunits of *D. vulgaris* enzyme. They observed significant differences in their subunit composition, especially with regard to the γ-subunit between the enzymes of the two species. α- and β-subunits of dissimilatory sulfite reductases in hyperthermophilic archaeon *Pyrobaculum islandicum* were expressed and purified by recombinant DNA methods and immunoblotting methods [24].

Microbial growth and precipitation of sulfides in the oil reservoir may also reduce the permeability of the oil formation [42]. Some common species of mesophilic SRB grow best at temperatures from 25 to 40 °C and that for thermophilic species is 45–65 °C or higher and the effect of some thermophilic SRB on pitting behavior of SS 304 has been studied [43]. Salinity changes in the range of 2–50 g of NaCl per liter had a large effect on the resident SRB population in oil fields. Injection waters of the highest available salinity were recommended for the effective control of SRB [44]. In the present study, the thermophilic strain isolated is able to survive at a temperature range of 55 °C by forming spores. Their vegetative forms might develop and multiply when the optimum temperature of growth is encountered. It converts the available acetate to $CO_2$ in the carbon metabolism for biomass production, besides the bacteria converts sulfate to sulfide at 55 °C as noticed. It is also well known that the metabolism carried by the isolate suppresses the anodic reaction by accumulating organic complex on steel surface which were noticed by XRD and FTIR analysis. It is expected that the presence of iron sulfide determines the cathodic reaction which acts cathodic to parent metal. Besides iron hydroxides were also noticed in the corrosion product. *Lee* et al. [45] suggested that iron hydroxide might be formed due to dissolved oxygen in the medium reacting with FeS, pyrite a non-acid volatile sulfide may be formed in the aerobic condition, and mackinawite found in XRD patterns may be formed in anerobic condition as acid volatile sulfide and the impact of these sulfides. The pyrite and troilite formed at low $H_2S$ concentration were relatively protective whereas mackinawite at high $H_2S$ concentration was not protective. It is also well known that the formation of marcasite and pyrite (FeS) were protective in nature [46]. The sulfate reduction and substrate oxidation found at 55 °C supports the observation made by *Mueller* and *Nielsen* [47]. The anodic reaction was suppressed by the organic end products of acetate metabolism. Since iron sulfide acts as cathodic to parent metal, it shifts the potential to negative side. The authors assume that since biofilm is heterogenous, the formation of iron sulfide is also heterogeneous. It may create electrochemical cell on the metal surface and enhances corrosion at 55 °C. *Villanueva* et al. [48] also noticed anodic corrosion process by thermophilic and mesophilic SRB influenced by passivation due to corrosion products film and biofilm formed on the steel. They also argued that the corrosion activation process was due to decrease in pH by the increased concentration of $H_2S$ that made solutions acidic and corrosive. On the basis of the influence of dissimilatory sulfite reductase on SRB and acetate metabolism, a mechanism proposed for the physiology of SRB isolate is given in Fig. 9.

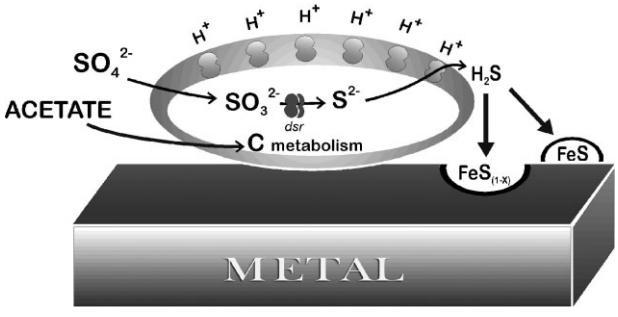


**Figure 9.** Sulfate reduction and corrosive mechanism of SRB on metal surface

## 4 Conclusions

Thermophilic SRB *D. kuznetsovii* was isolated and identified by molecular techniques from cooling tower of a petroleum refinery.

1. The enzyme responsible for sulfite reduction to sulfide was expressed from its *dsr*AB gene by employing rDNA technology and identified as having ~96 kDa with Maldi-TOF analysis.
2. The protective and corrosive nature of metabolic corrosion products were noticed on the mild steel surface. It may be assumed that the proliferation of thermophilic SRB may be encouraged by higher temperature about 60 °C in heat exchanger tubes and it is also possible for the involvement of SRB in the corrosion process in cooling towers and heat exchanger tubes of petroleum refineries.

Sequences submitted in GenBank (NCBI) under accession numbers: DQ155286 and DQ455689.

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