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Sulphate-Reducing Bacteria in Crude Oil and Procuded Water: Cell Quantification and *apsA* Gene Expression

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Abstract

The quantification of planktonic sulphate-reducing bacteria (SRB) in crude oil and produced water and their adenosine-5'-phosphosulphate reductase alpha subunit (*apsA)* gene expression were carried out using real-time polymerase chain reaction (PCR). Real-time PCR is considered a highly sensitive method for the quantification of microbial organisms and gene expression in environmental samples. This study was conducted to evaluate real-time PCR with SYBR Green detection as a quantification method for SRB and its *apsA* gene expression in crude oil and produced water. Standard curves were developed for both quantifications. The results showed that sample A (crude oil) had lesser SRB concentration (2.86 µg/ml) and *apsA* gene expression with Cycle threshold (Ct) value: 7.48than sample B (produced water) with SRB concentration of $1.27x10^6$ µg/ml and Ct-value: 6.77. The lower Ct-value of sample B showed more gene expression than sample A. This implied that sample B may have been contaminated by subterranean water.

Keywords: **Real time PCR, *apsA* genes, Produced water, Sulphate-reducing bacteria, Crude oil, Planktonic cells.**

Introduction

Sulphate-reducing bacteria (SRB) are a group of environmental anaerobic bacteria that reduce sulphate to sulphite during dissimilatory anaerobic respiration (Dunsmore*et al.*, 2002). SRB possess Adenosine-5'-phosphosulphate (APS) reductase genes and Dissimilatory sulphitereductase genes (Rabus*et al.*, 1999), which have been proposed as useful phylogenetic marker and for investigating the diversity of SRB (Ben-Dov*et al.*, 2007; Friedrich, 2002; Klein *et al*., 2001; Perez-Jimenez *et al*., 2001; Wagner *et al*., 1998).

Conventional culture-based methods for studying SRB take time as a result of slow bacterial growth rates, and do not

necessarily provide an accurate assessment of the types and numbers of bacteria present in a biological sample (Ben-Dov*et al.,* 2007). The direct methods include the use of antibodies raised against SRB (Lillebaek*et al*., 1995), an immunoassay for the enzyme APS reductase, and the use of 16S rRNA probes (Daly *et al*., 2000). Several problems are encountered when they are used *in situ* (Amann*et al*., 1995), for example, rRNA fluorescent probes are difficult to use in sediments or industrial wastewater because of high background autofluorescence of inorganic particles (Muyzer*et al.,* 1995). Furthermore, not all known types of dissimilatorysulphate reducers in environmental samples can be unequivocably identified with the RNA probes (Teske*et al*., 1996). In addition, SRB constitute a heterogenous group, thus, it is not feasible to use 16S rRNA gene as a general molecular marker (Adamczyk*et al*., 2003; Castro *et al*., 2000; Klein *et al.*, 2001; Wagner *et al.*, 1998).

Messenger RNA (mRNA), the product of gene expression, has a high turnover rate and therefore the amount of mRNA is a direct measure of the transcriptional activity in cells. After extracting total RNA from environmental samples, the amount of mRNA may be measured. This would allow to quantify the *in situ* activity of different physiological groups of microorganisms (Neretin*et al.,* 2003). Current methods to study gene expression such as Northern hybridisation, Ribonuclease protection assay and Competitive reverse transcription PCR have been found to be time-consuming and have a narrow detection range as well as low sensitivity (Ferre, 1998; Leloup*et al*., 2004; Rayemakers*et al.*, 2000). Enumeration based on culture methods and standard MPN methods appear to underestimate cell numbers of SRB (Kondo *et al*., 2004).

Analytical techniques targeting 16S rDNA or functional genes widely used for microbial quantification include hybridization-based techniques, such as membrane hybridization (Raskin*et al.,* 1995) and fluorescence in situ hybridization (FISH) (Okabe *et al*., 1999) as well as PCR-based techniques, such as denaturing gradient gel electrophoresis (Muyzer*et al.,* 1993) and cloning-sequencing (Zhang and Fang, 2001; Zhang *et al*., 2003a). PCR-based methods are capable of detecting DNA/RNA at low concentrations. However, the precision of PCR-based methods may be compromised due to reagent depletion, competition of amplicons with primers, and the loss of polymerase activity as the number of amplification cycle

increases (Schneegurt and Kulpa, 1998). To overcome such deficiency, a new technique known as real-time PCR has emerged for the detection and quantification of microorganisms at low concentrations.

The real time PCR method monitors the amount of PCR products of DNA as they are amplified in real time (Higuchi *et al.*, 1993; Heid*et al.*, 1996; Gibson *et al.*, 1996).In a conventional PCR, only the final concentration of the amplicon may be monitored using a DNA binding fluorescent dye. However, in the real time PCR, the concentration of the amplicon is monitored throughout the amplication cycles using a group of new fluorescent reagents. Fluorescent reagents used for real time PCR can be classified into three categories: (1) Dyes that bind with double-stranded DNA (Hernandez *et al.*, 2004); (2) DNA-sequence specific probes, including TaqMan Probe (Haugland*et al.*, 1999), molecular beacon (Briones and Raskin, 2003), and dual hybridization probe (Glazer and Mathies, 1997); Reina *et al.*, 2005) and (3) DNA sequence-specific primers including, Amplifuor primer (Hernandez *et al.*, 2004), scorpion primer (Taveau*et al.*, 2002), Light Upon extension primer (Donia and Pana 2005), and universal template.

Real-time PCR is considered a highly sensitive method for the quantification of microbial organisms in environmental samples (Ben-Dov*et al.*, 2007; Goerke*et al.*, 2001). It has already been applied to quantify 16S rDNA (rRNA) of different groups of microorganisms (Suzuki *et al.*, 2001; Takai and Horiko-shi, 2000; Bach *et al.*, 2002; Stubner, 2002) and functional genes like *Pseudomonas* carbazole 1,9a-dioxygenase (Widada*et al.*, 2001) in environmental samples. It has been established that real-time PCR overcomes the shortcomings of conventional PCR and has advantages over conventional methods of mRNA quantification in terms of dynamic range, sensitivity and reproducibility (Wang and Brown, 1999; Wall and Edwards, 2002), hence it is used in this current work to quantify SRB in crude oil and produced water and for the quantification of gene expression. The aim of this work therefore was to quantify the SRB and their gene expression in crude oil and produced water.

Materials and Methods

Sample Collection and Enrichment: Samples used in this research were crude oil and produced water. Five hundred millilitres each of Bonny Light Crude Oil (Sample A) and Bonny Light produced water (sample B) samples were collected aseptically from Bonny dispatch tank and oil well respectively, in sterile sample bottles from Shell Well 9, Awoba flow station in Degema Local Government Area of Rivers State, Nigeria. They were sealed for laboratory analysis. Samples were preserved in dry ice enroute the lab.

Mixed Carbon Postgate's (MCP) medium was prepared and used for culture enrichment. MCP medium is a modification of Postgate's medium (Postgate, 1984). It contained sodium lactate (60% w/v solution 1.25ml/l), sodium acetate (5.0g/l), sodium propionate (1.0g/l), sodium butyrate (0.4g/l) with sodium chloride (25g/l). The medium was prepared by dissolving the constituents in 1litre deionised water, and pH adjusted to 7.2, prior to autoclaving at 105ºC for 20 minutes to remove dissolved oxygen. The solution was cooled under nitrogen gas followed by addition of redox agents as follows: 1ml each of 10% w/v solutions of sodium thioglycollate and ascorbic acid, and the pH adjusted to 7.2 with 1M NaOH or HCL. The bulk medium was introduced into an anaerobic cabinet (Don Whitley, Yorkshire) where aliquots were dispensed as required into injection bottles (90ml aliquots in 125ml bottles, Adelphi Tubes Ltd). All vessels were closed in the anaerobic cabinet before being sterilized by autoclaving at 121ºC for 15mins. All laboratory inoculations into injection bottles were achieved by transfer with sterile hypodermic needles and syringes.

Inoculation: Ten milliliters of sample A was dispensed into 125ml sterile bottles containing sterile 90ml MCP. Incubation was carried out at 55ºC for 28 days. The same procedure was carried out for sample B. Subcultures were later made into fresh MCP medium contained in sealed injection bottles. 10ml of the reference strain *Desulfobactercurvatus* (DSM3379) was dispensed anaerobically into 90 ml MCP in 125 ml sterile bottles, cultured under the same condition and used as positive control.

Detection of microbial hydrogen sulphide (H_2S) production: The method used was as described by Truper and Schlegel (1964) and Cline (1969).It relies on the liberation of sulphide and subsequent development of

methylene blue from *N*,*N*-dimethyl-*p*-phenylenediaminesulphate.

DNA extraction and determination of DNA concentration: DNA was prepared from a 28 days culture of samples A and B in MCP at 55ºC according to the method of Sambrook*et al.* (1989). Bacterial suspensions were centrifuged for 10 mins at 8500rpm and then resuspended in 0.6ml CTAB buffer in sterile Eppendorf tube. 0.5ml of phenol:chloroform:isoamyl alcohol (pH 8) was added and vortex briefly. This was then transferred to sterile screw cap tubes (2ml) containing 1g Zirconnia/silica beads, and bead beating was done at maximum speed of 4600rpm for 40s and later put on ice. This was followed by centrifugation at 13000rpm for 5mins and the top aqueous layer was transferred to a new sterile Eppendorf tube and 0.5ml of phenol: chloroform:isoamyl alcohol was added. This was followed by agitation (vortex) and centrifugation at 13000rpm for 5 mins. The top aqueous layer was then transferred to a new sterile Eppendorf and 1ml of 30% PEG was added, mixed and left at room temperature for two hours, after which centrifugation at 13,000rpm for 5 mins was carried out and supernatant discarded. Pellets were dried in air and resuspended in 40µl ultrapure PCR water and stored in a -20ºC freezer. Determination of the DNA concentration obtained by UV-2101PC UV-Vis spectrophotometry.

Agarose gel electrophoresis: Agarose gel electrophoresis was carried out on the DNA extraction products of samples and reference strain to separate the DNA fragments by their sizes and to visualise the fragments. 1.0g of agarose was dissolved in 100ml of 1 x TAE buffer and heated in the microwave for 30 s, swirling halfway through. It was allowed to cool and 4µl of ethidium bromide was added and poured into taped tray. Bubbles were removed with the tip of comb and the comb was inserted and allowed to set for 20mins. The tape and comb were then removed and the hyperladder and samples were loaded after mixing 5µl of each sample with 2.5µl loading dye. It was then run at 100V for 40mins and viewed under UV light.

Quantification of SRB in Samples: Quantification of SRB in samples was carried out by Real-time PCR analysis. Real-time PCR analysis was performed using the universal real-time PCR primers for apsA genes according to Ben-Dov*et al.* (2007) in a MiniOpticon Real-time PCR system. The PCR reaction consisted of 12.5ml SuperMix, 1µl forward primer, 1µl

reverse primer, 1µl DNA template and 9.5µl PCR H_20 to make a total volume of 25µl. Thermal cycling conditions were as follows: 3 mins at 95ºC, followed by 40 rounds of 15s at 95ºC and 1m in at 60ºC, 5 mins at 60ºC. To verify that the used primer pair produced only a single specific product, a dissociation protocol was added after thermocycling, to determine dissociation of the PCR products from 60ºC to 95ºC. Standard curve for quantification was prepared from the reference strain genomic DNA used in four serial dilution points. All runs included a no-template control. Detection was based on fluorescence resonance energy transfer, with a SYBR Green fluorophore. The real-time threshold cycle (Ct) was determined and standard curve was obtained by plotting the Ct-value of each dilution against the logarithm of the DNA.

Preparation of samples and reference strain for RNA extraction:

Samples and reference strain were prepared for mechanical disruption using RNAprotect bacterial reagent. One millilitre of RNAprotect was added to 0.5ml of culture, mixed by vortexing and left at room temperature for 5mins. Centrifugation was carried out at 5000rpm for 10mins, after which supernatant was removed and it was allowed to dry for about 5-10mins. Samples were stored at -20˚C until ready for RNA extraction.

RNA extraction: RNA extraction was carried out so as to get mRNA of APS reductase genes. This was done using the RNeasy Mini kit according to manufacturer's instructions. Three hundred and fifty microlitres of buffer RLT was added to each sample mixed by vortexing strongly for 5 – 10s to ensure that the pellets are thoroughly resuspended in buffer RLT. The suspension was transferred into sterile 2ml safe-lock tubes containing acid-washed beads and disrupted in a TissueLyser for 5mins at maximum speed. Centrifugation was carried out for 10s at maximum speed and supernatant was then transferred into new sterile tubes. Three hundred and fifty microlitres of 70% ethanol was added and mixed well by pipetting.

Purification of RNA: With reference to Gibson*et al*.,1996; Hermandez*et al*., 2004;Ben-Dov *et al*., 2007, a 700µl of the lysate including any precipitate that may have formed was transferred into an RNeasy Mini-spin column placed in a 2ml collection tube. Centrifugation was carried out at >10,000 rpm for 10s and flow through

discarded. The collection tube was reused. Seven hundred microlitres of buffer RW1 was added to the spin column, lid closed gently and centrifugation was carried out at >10,000 rpm for 15s. Flow-through was discarded with the collection tube. The RNeasy column was placed in a new 2ml collection tube and 500µl of buffer RPE was added to mini spin column, lid closed gently and centrifuged for 15s at >10,000 rpm. Flow-through was discarded and collection tube reused. Five hundred microlitres of buffer RPE was added to the column and centrifuged for 2mins at >10,000 rpm. The spin column was gently removed so that the spin column did not touch the flow-through. The spin column was placed in a new 1.5ml collection tube and 50µl of RNase-free water added directly to the spin column membrane. Centrifugation was carried out for 1 min at >10,000 rpm. The eluted RNA was then collected in RNA tubes and stored at -80˚C until needed for DNase treatment.

DNase treatment of purified RNA: This was carried out prior to Reverse Transcription-Polymerase Chain Reaction (RT-PCR) carried out to synthesize cDNA. The digestion reaction was set with 8µl of RNA, 1µl of RQ1 RNase-free DNase 10X reaction buffer, 0.5µl of RQ1 RNase-free DNase and 0.5µl of nuclease-free water to make a volume of 10µl. Incubation was carried out at 37˚C for 30mins. One microlitre of RQ1 DNase Stop solution was added to terminate the reaction and incubated at 65˚C for 10mins to inactivate the DNase and RT-PCR carried out to synthesize cDNA.

Reverse Transcription-Polymerase Chain Reaction: cDNA of mRNA for APS reductase genes was generated with *apsA*-specific primers with 5µl of extracted DNase-treated RNA serving as template. For RT reactions, Thermoscript Reverse Transcriptase, invitrogen kit (Invitrogen Technologies) was used according to manufacturer's instructions. The reaction mixture consisted of 2µl of 10mM dNTP mix, 3µl of DEPC-treated water and 5µl of DNase-treated RNA to make a total volume of 12µl. Incubation was carried out at 65˚C for 5 mins and 7µlmixture of 5x cDNA synthesis buffer (4µl), DTT (1µl), RNaseOUT (1µl), and DEPC-treated water (1µl) added. One hundred microlitres RT enzyme later was added and incubation at 25˚C for 10mins was carried out, followed by gentle mixing and brief centrifugation. This was followed by incubation at 50˚C for 60 mins and at 85˚C for 5mins. The cDNA samples were stored at -80˚C before Q-PCR analysis was carried out. Control

experiments to check DNA contamination was performed by preparing reactions in which no Reverse Transcriptase was added to the extracted RNA.

DNA contamination was checked with agarose gel electrophoresis following RT-PCR by performing control experiments in which no reverse transcriptase was added to extract RNA before the PCR step. RNA concentration was determined by absorption at 260 nm with a Biophotometer (Eppendorf, Hamburg, Germany). Purified RNA was stored at −80 °C.

Primer design: Primers were designed for apsA genes using the Genscript software. To determine whether the primers were suitable, gene-specific qualitative PCR was performed and primers were evaluated for real time PCR (Chin *et al.*, 2004).

Preparation of standard curve for quantification of gene expression: Standard curve for the real time PCR quantification was prepared from dilution series of purified RT-PCR products of the reference strain. The cDNA was amplified with *apsA* gene-specific primers and the resulting amplicon was purified. PCR conditions were: 95˚C (5mins); 40 cycles of 95˚C (40s); 58˚C (1min); 72˚C (1min), followed by a final extension at 72˚C for 7mins. The purified RT-PCR product was quantified and five serial dilutions made. Q-PCR analysis was then carried out with the dilutions. Standard curve of the *apsA* RNA copies was obtained by plotting the Ct-values of each dilution against the log copy number. The copy number was calculated on the basis of the measured *apsA* RNA concentration (ng/µL), an average molecular mass of 330 for a base in a single-stranded RNA and the *apsA* RNA size which was 115 bases. The lower detection limit was about 3-8 copies of target mRNA.

Quantification of Gene expression: Quantification of *apsA*expression was performed in an MJ Mini Thermal Cycler and detected by a Mini Opticon system for Real-time PCR detection (Biorad Laboratories, Inc.), using iQ SYBR Green Supermix (Biorad). Analysis was undertaken using designed *apsA* specific primers shown in Table 1 and obtained from MWG Biotech (Germany). Quantification of samples was carried out by determining the threshold cycle value and by comparing results to a standard curve. The PCR reaction consisted of 12.5µl of SYBR Green Supermix, 0.5µl of each 10 pM/µL forward and reverse primers, and 1.0 µl of each cDNA template, in a total volume of 25µl. Thermal cycling conditions were started at

95˚C for 3mins, followed by 40 cycles of denaturation (95˚C for 20s) and annealing (60˚C for 1 min) and a final extension (60˚C for 5min). No template-negative controls were included in each Real-time PCR.

Results and Discussion

The results of this work are shown in Tables 1–4 and in Figures 1 –3. Growth and production of hydrogen sulphide were observed in sample enrichments, even after subculturingas shown in Table 1.DNA extraction from growth cultures of samples A and B was successful as confirmed by agarose gel electrophoresis (Figures 1 and 2). Quantification of SRB (Table 2) in samples using real-time PCR analysis as obtained from Figure 3 showed, concentrationof SRB in sample A, 2.86µg/ml and sample B, 1.27 x 10^6µg/ml.Q-PCR analysis for quantification of gene expression was successful (Figure 4). This showed a higher Ct-value for sample A(7.48) than sample B (6.77) as seen in Table 3, with the successful design of primers for *aps*A genes shown in Table 4.

Table 1: Growth of SRB in Enrichment Medium

SAMPLE	SAMPLE TREATMENT	INCUBATION TEMPERATURE (ºC)	ENRICHMENT RESULTS	SUBCULTURE
A (Bonny light crude oil)	Direct mix in MCP	55	Slight blackening and H_2S detected	Slow blackening and H_2S production
B (Bonny light produced water)	Direct mix in MCP	55	Blackening and H_2S detected	Rapid blackening and H_2S production

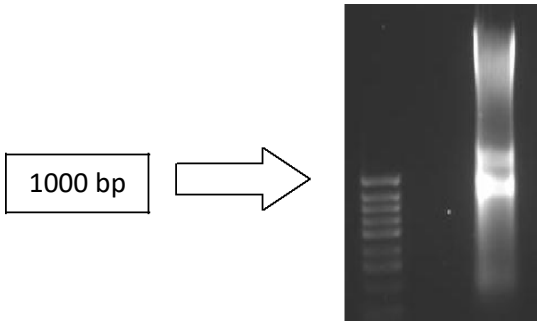

Figure 1: Agarose gel electrophoresis of sample DNA. Lane 1: Hyperladder IV; lane 2: DNA fragment of sample A.

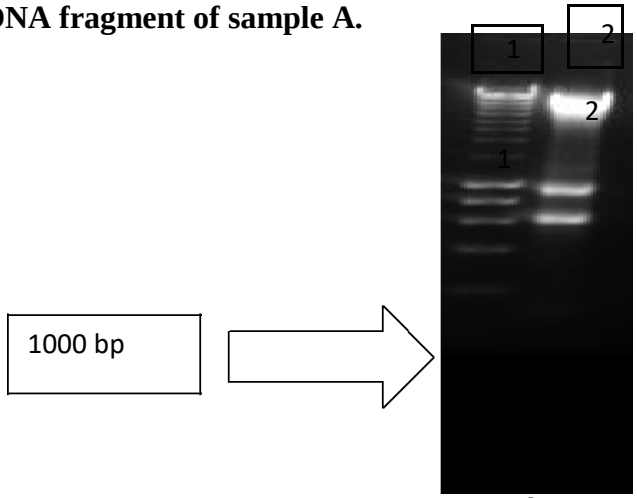

Figure: 2: Agarose gel electrophoresis of sample DNA. Lane 1: Hyperladder I; lane 2: DNA fragment of sample B.

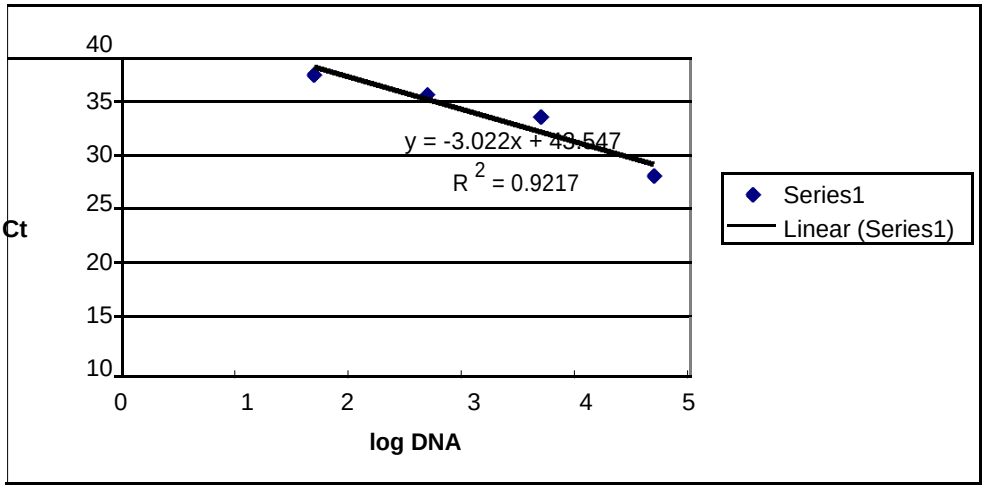

Figure 3: Standard curve for SRB concentrations

Table 2: Concentration of SRB cells in samples

Sample	Concentration of SRB (μg/ml)
A (BLCO)	2.86
B (BLPW)	$1.27X10^6$

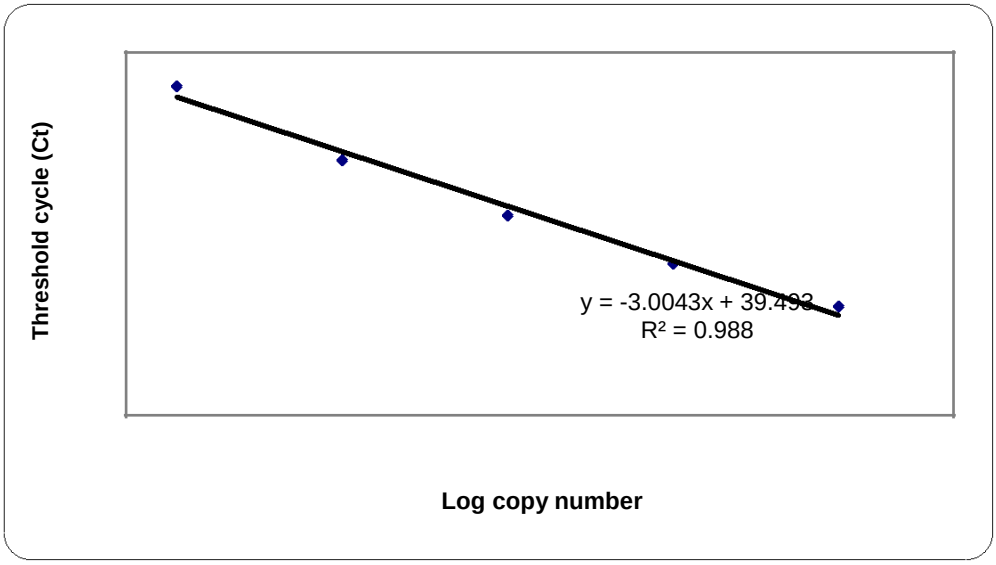

Figure4: Standard curve for *aps*A genes.

Table 3: Quantification of Gene Expression

Sample	Ct-value
A	7.48
B	6.77

- The lower Ct-values of sample B showed more gene expression than sample A.

Table 4: Primers designed for real-time PCR

Primer	Sequence
Forward Primer	5′-GAG CCT TAC CTG CTT GGT TC-3′
Reverse Primer	5′- TAT AGA CCT TGC CGT TCT TG-3′

Discussion of Results

SRB growth, Sulphide production and DNA extraction: Distinctive changes in the appearance of the growth medium were observed after inoculation. Prior to inoculation, MCP medium typically contained mild grey precipitate but after addition of the inoculum (10ml), the medium darkened rapidly with time. The precipitate flocculated and took on a clumped appearance. The clumps aggregated and blackened as insoluble ferrous sulphide concentration increased. After three days, all the clumps were consolidated into a mass of sticky black material at the bottom of the growth vessel, the overlying fluid being clear. Sample A showed slight blackening in culture enrichment as compared to sample B which showed rapid blackening. This possibly implies low level of SRB contamination and H_2S production by sample A. Growth of SRB in the MCP medium took about 28 days and there was evidence of growth and presence of sulphidogenic activity in the enrichment medium seen as culture turbidity, blackened particulate material, and sulphide production in the medium (Table 1). Subculturing from the initial enrichments into further fresh medium was successful. This observation was supported by Postgate (1984) who found that mixed carbon Postgate's medium encourages growth of SRB than any other media for anaerobic sulphidogens. Preliminary indication of microbial H_2S production from the samples was identified by characteristic black ferrous precipitate (Iron sulphide) which remained visible after agitation of the sample. Both samples were observed to produce H_2S as there wasliberation of sulphide and subsequent development of

methylene blue from *N,N*-dimethyl-*p*-phenylenediamine sulphateas described by Truper and Schlegel (1964) and Cline (1969). DNA extraction from growth cultures of samples A and B was successful as confirmed by agarose gel electrophoresis (Figures 1 and 2).

Quantification of SRB (Table 2) in samples using real-time PCR analysis as obtained from figure 3 showed, concentration of SRB in sample A, 2.86µg/ml and sample B, 1.27 x 10^6µg/ml. This showed less SRB concentration in sample A than sample B.Successfulmolecularanalysisof functional genes was observed. This was similar to that observed by Ben-Dov*et al*. (2007) who found APS genes responsible for H_2S production in SRB. Q-PCR analysis for quantification of gene expression obtained from figure 4 revealed a higher Ct-value for sample A than sample B (Table 3). The lower Ct value of sample B showed more gene expression than sample A. These findings were consistent with those of Singh *et al.* (2006) who discovered that planktonic cells possess differentially expressed genes.Primers designed for apsA genes are shown in Table 4.

Conclusion

Data presented confirms presence of genes expressed by SRB cells as well as showing the concentration of SRB in both samples. The lower Ct value of sample B showed more gene expression than sample A. Higher concentration of SRB in sample B than sample A was revealed, indicating a possible contamination from subterranean water.

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