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TECHNICAL ARTICLE

Use of Marine Waste Extract as a Nitrogen Source for Biological Sulfate Reduction: Development of a Suitable Alternative

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Abstract Marine wastes extract (MWE), prepared from marine organic wastes, was used to develop an alternative nitrogen source for sulfate-reducing bacteria (SRB) in environments like acid mine drainage that are acidic in nature and contain high levels of sulfate and dissolved metals. The MWE contains 13.95 g L^{-1} of nitrogen, and other micronutrients like K, Na, P, S, Ca, Fe, Mg, Mn, Zn, Co, Cu and Ni, and has a C/N ratio of 0.107. A modified SRB medium (MSRB) was developed by replacing the commercial nitrogen source of standard SRB growth medium with MWE. MSRB was compared with modified Postgate B, Postgate B, and Widdel and Pfennig media, which contained bactopeptone and NH_4Cl, as nitrogen sources. Results showed that the growth media could support a total microbial population of 2.8×10^{12}–6.2×10^{12} cells mL^{-1} with 96, 80, 92.5, and 65 % SRB in MSRB, Postgate B, modified Postgate B, and Widdel and Pfennig media, respectively. The sulfate reduction efficiency was 97, 87, 72, and 68 % at reduction rates of 12.41, 11.10, 4.35, and 8.8 mg L^{-1} h^{-1}, respectively, for the same media. We conclude that MWE could be a cost-effective substitute for commercially available nitrogen sources for SRB for large-scale treatment of sulfate-rich wastewater.

Keywords Growth media · MSRB · Sulfate reducing bacteria (SRB) · Sulfide · SRB population

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Introduction

Sulfate-reducing bacteria (SRB) are anaerobic microorganisms that survive in several natural environments such as anoxic sediments, hydrocarbon seeps, oil fields, aquifers, and acid mine drainage sites (Muyzer and Stams 2008). SRB belongs to different phylogenetic lineages, like Deltaproteobacteria, Clostridia, Nitrospirae, Thermodesulfobacteria, Thermodesulfobiaceae, Euryarchaeota, and Crenarchaeota (Castro et al. 2000). As part of their energy metabolism, SRB reduce sulfate to hydrogen sulfide, and are therefore used in bioremediation of several sulfate-rich industrial wastewaters (e.g. Chang and Kim 2007; Johnson and Hallberg 2005; Sarti et al. 2009). Failure of efficient sulfate reduction in bioremediation is generally attributed to lack of nutrients especially, carbon (Cheong et al. 2010) and nitrogen (Robinson-Lora and Brennan 2009; Zagury et al. 2006). Several studies have emphasized the role of carbon-rich substances and their effect on SRB growth (e.g. Das et al. 2013), while relatively little attention has been given to the use of nitrogen-rich substrates for SRB growth. However, nitrogen-rich substrates are important to the growth, diversity, and community resilience of SRB (Bayoumy et al. 1999; Daubert and Brennan 2007).

Commercial growth media used for SRB generally contain bactopeptone (Das et al. 2013), NH_4Cl (Postgate 1984; Starkey 1938; Widdel and Pfennig 1981), NH_4HCO_3 (Mizuno et al. 1998), or $(NH_4)_2HPO_4$ (Kuo and Shu 2004) as a sole nitrogen source, which adds significantly to its cost (Aspmo et al. 2005). Therefore, using pure nitrogen sources for large-scale SRB-based bioremediation would increase the cost. Less expensive nitrogen-rich growth substrates are necessary to reduce operational costs and enhance sulfate reduction.

Several million tons of organic waste products are generated from the sea every year due to fishing activities. These waste products are generally discarded on the seashore and are poorly managed. These waste materials include items such as dead crabs, shrimps, mollusks, jelly fish, and fish scraps. Some of these wastes can be converted into value-added products like protein hydrolysates, which can be used in microbial growth media (Martone et al. 2005) and as food additives (Harnedy and FitzGerald 2012). Protein hydrolysates are rich in nutrients like protein, amino acids, and vitamins, and can serve as a nitrogen source for bacteria (Aspmo et al. 2005; Chalamaiah et al. 2012). Study of the use of protein hydrolysate as a growth substrate for SRB is limited. In this study, marine waste collected from the seashore was hydrolyzed and chemically deproteinized. The protein-rich liquid extract obtained from the hydrolyzed products was termed "marine waste extract" (MWE).

MWE was characterized in detail and used as a nitrogen-rich growth substrate in the growth medium to observe its effectiveness as a nitrogen source for SRB growth compared to conventional commercial sources of nitrogen such as bactopeptone, NH_4Cl, and yeast extract. For that purpose, an SRB growth medium (Postgate B) was modified by replacing its nitrogen source with MWE, producing a modified growth medium (MSRB). Then, the MSRB medium was compared to commercial SRB growth media, including Widdel and Pfennig (Widdel and Pfennig 1981), Postgate B (Postgate 1984), and modified Postgate B (Fortin et al. 2000), for efficiency in supporting SRB growth and sulfate reduction in sulfate-rich water. Modified Postgate B, Widdel and Pfennig, and Postgate B contained bactopeptone and NH_4Cl, respectively, as their principal nitrogen source. These growth media were selected because of their regular use to enrich and cultivate SRB.

Materials and Methods

Preparation of MWE

Marine waste was collected from different locations along the eastern seashore of Digha (West Bengal, India). The waste mainly contained dead crustaceans like crab, scallop, shrimp, mussel, snail, lobster, oyster, and fish scale. The waste was dried by the sun, then ground using a grinder, and sieved to pass a 40-mesh sieve (schematically represented in Fig. 1). The ground material was added at 10 % w/v to deionized water. The suspension was maintained under alkaline conditions (pH 12) by addition of 1 N NaOH, and digested at 90 °C in a closed reflux system. The digestion was carried out for 3 h to ensure complete hydrolysis. The hydrolyzed suspension was allowed to cool and the protein-rich liquid extract was collected by centrifugation (6,440 g × 20 min), followed by filtration (0.2 µm membrane filter, D121913, Whatman, GmbH, Germany).

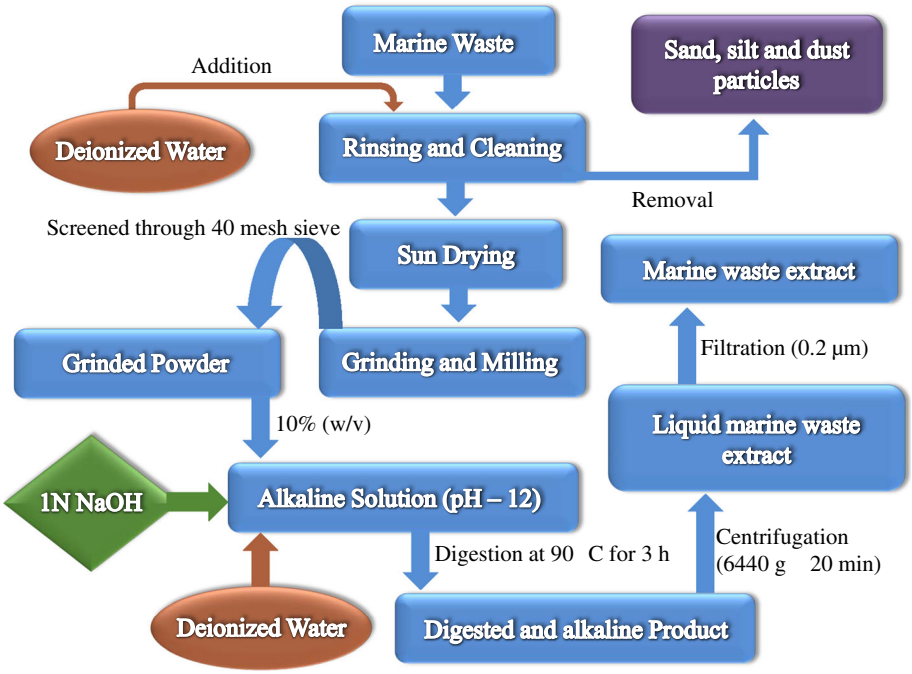

Fig. 1 Preparation of marine waste extract

Characterization of Nutrients in MWE and Growth Media

To determine nutritional quality, the MWE and MSRB media was assessed for several micro- and macro-nutrients. The nutrients present in the standard growth media were also measured and compared with those present in the MSRB medium. The selected essential nutrients included total organic carbon (TOC), total kjeldahl nitrogen (TKN), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), sodium (Na), iron (Fe), magnesium (Mg), manganese (Mn), zinc (Zn), cobalt (Co), copper (Cu), and molybdenum (Mo). To determine the actual nitrogen content present, the ground marine waste powder was analyzed for TKN before extraction.

Inoculum

A mixed bacterial culture grown in sweetmeat waste and bacto-tryptone supplemented growth medium containing 93.5 % SRB was collected from a sulfidogenic batch bioreactor (Das et al. 2013). The mix culture was repeatedly subcultured in Postgate B medium (Postgate 1984) and then used as an inoculum in the current batch study. The growth medium was prepared with deionized water, which was boiled and subsequently cooled under a continuous flow of nitrogen to remove the dissolved oxygen. The initial pH of the medium was maintained at 7.2 by the addition of 0.1 N NaOH. Then, the medium was autoclaved at 103.4 kPa (15 lbs $inch^2$) pressure, cooled, and inoculated. After the inoculation, the medium was supplemented with 10 % (v/v) reducing agent (0.7 % sodium thioglycolate +0.7 % ascorbic acid). The final mixed culture developed after the repeated subculture was analyzed using the flurescence in situ hybridization (FISH) technique (Das et al. 2013).

Batch Experiment

To produce MSRB, the NH_4Cl and yeast extract present in Postgate B medium were replaced with MWE as the only nitrogen source. The composition of the resulting MSRB medium was (in g L^{-1}): sodium lactate, 3.5; $MgSO_4{\cdot}7H_2O$, 2; $CaSO_4$, 1; Na_2SO_4, 2.58; KH_2PO_4, 0.5, and MWE, 10 % (v/v). To generate anaerobic conditions, the deionized water used to prepare the media was boiled and subsequently cooled under continuous flow of nitrogen. After the inoculation, the media were supplemented with 10 % (v/v) reducing agent (0.7 % sodium thioglycolate +0.7 % ascorbic acid).

The efficiency of the MSRB medium to support SRB growth and sulfate reduction was compared with the standard media generally used to cultivate SRB. To prepare the modified Postgate B medium (Fortin et al. 2000), bacto-tryptone was added to the Postgate B medium as a nitrogen source. Yeast extract and NH_4Cl were not added to the modified Postgate B medium; to keep the sulfate concentration high, supplementary Na_2SO_4 was added to all the media.

The experiments were carried out in four 1 L batch reactors, each with a different growth medium. All the media were prepared with deionized water, which was boiled and subsequently cooled under continuous flow of nitrogen to remove the dissolved oxygen. The initial pH of the media was maintained at 7.2. The reactors were placed inside the anaerobic system (Thermo Scientific, model 1029) and inoculated with 10 % (v/v) inocula developed after repeated subculturing in Postgate B medium. The anaerobic system was an anaerobic chamber filled with 85 % N_2, 10 % H_2, and 5 % CO_2. After the inoculation, the media was supplemented with 10 % (v/v) reducing agent (0.7 % sodium thioglycolate + 0.7 % ascorbic acid). The reactors were kept inside the incubator inside the anaerobic system and incubated at 35 °C temperature. The batch study was conducted for 14 days. Batch experiments were repeated five times to ascertain the consistency of the result.

Sulfate Reduction

The ability of different growth media to promote sulfate reduction efficiency was compared by measuring the rate and percentage of sulfate reduction.

Growth of SRB in the media was compared by measuring the total microbial cell count, the generation of alkalinity, dissolved sulfide production, and changes in the pH of the media. Total cell count was performed to count the total microbial cells present in the mixed culture. The population of SRB present in the mixed culture was measured using FISH (Das et al. 2013). The FISH was performed for the microbial samples when the maximum value in the total cell count was obtained.

Total cell count was performed using 4, 6-diamidino-2-phenylindole (DAPI) stain (Das et al. 2013). The method to perform FISH was obtained from Das et al. (2013); we used commercial (SRB385 and NON338) 16S rRNA-targeted oligonuclotide probes (Table 1). SRB385 was specific for SRB belonging to δ-proteobacteria and several gram-positive bacteria. The probe was synthesized and labeled at the 5′ end with fluorescein isothiocyanate (FITC) (Eurofins Genomic India, Bangalore, India). NON338 is a "nonsense probe" and served as a negative control. The counts were corrected by subtracting the signals observed using the NON338. Cells were counted using a Neubauer-improved counting chamber (Paul Marienfeld GmbH, Lauda-Konigshofen, Germany).

Table 1 Oligonucleotide probes used in this study targeting 16S rRNA (Das et al. 2013)

Probe	Probe sequence (5′–3′)	Target site[a]	Target bacterial group
SRB385	CGGCGTCGCTGCGTCAGG	385–402	δ-Proteobacteria and several gram-positive bacteria (e.g. Clostridium)
NON338	ACTCCTACGGGAGGCAGC	338–355	No bacterial group (negative control)

[a] Base pair position on 16 s rRNA according to *Escherichia coli*

The cells were observed using an epifluorescence microscope (Carl Zeiss, Germany, AXIO scope, A1) equipped with filter set 49 (excitation wavelength 365 nm) and filter set 09 (excitation wavelength 45–490 nm) to visualize the cells stained with DAPI and FITC, respectively. Several microscopic fields on the same slide were observed to establish the result. Microscopic fields containing 30–40 cells were considered for counting. If the fields contained very high numbers of microbial cells, the samples were diluted.

Sampling and Analysis

Samples were collected inside the anaerobic chamber at 24 h intervals. The samples were filtered using a 0.2 μm membrane and analyzed. TKN, Ca, P, dissolved sulfide, and alkalinity were measured using standard methods 4500C, 3500B, 4500E, 4500F, and 2320B, respectively (APHA 2005). Persulfate oxidation method (Das et al. 2013) was employed to measure TOC using a TOC analyzer (O.I. Analytical, Model 1030). Na, K, Mg, Fe, Co, Mn, Zn, Mo, Ni, and Cu concentrations were quantified by atomic absorption spectrophotometery (GBC Scientific Equipment, model N814). Sulfate concentration was measured using a spectrophotometric method (Roy et al. 2011). To remove interfering agents, the sample was initially treated with ammonium molybdate and passed through a Dowex-50W-X8 ionic form-H^+ column. Barium chloranilate was added to the treated sample and the absorbance was measured at 350 nm. The pH was measured with using a pH probe (ROSS sure-flow pH electrode, Orion, 8172BNWP) attached to a multi-parameter water analyzer (Orion VERSASTAR, Thermo Scientific, USA).

Cost Analysis

Cost analysis of MWE was performed based on its sourcing, transportation, and processing. The MWE was then compared with nitrogen sources used in other commercial growth media in terms of cost effectiveness. In Postagte B, NH_4Cl (Sigma Aldrich, V800041-500G) and yeast extract (Sigma Aldrich, 92144-500G-F) were used as nitrogen sources. In modified Postgate B, bacto-tryptone was used as the nitrogen source (Sigma Aldrich, 95039-50G-F). In the Widdel and Pfennig medium, NH_4Cl was used as the nitrogen source.

Statistical Analysis

During the characterization, all of the parameters were measured 5 times. Mean and standard deviations of the data, including characterization and batch experiments, were determined.

Results

Characterization

The detailed characterization of MWE and the growth media is shown in Table 2. The MWE contained different macro- and micro nutrients like C, N, P, K, Ca, Na, K, S, Fe, Mg, Zn, Cu, Ni, and Co. The TKN value of the MWE was 13,951 mg L^{-1}. The ratio of TOC and TKN was 0.107, indicating that the MWE contained a high nitrogen content compared to the total carbon content. The nitrogen sources generally used in microbial growth media, like bactotryptone, bactosoytone, malt extract, and yeast extract, contain total nitrogen contents of 13.3, 9.4, 1.1, and 11.4 %, respectively (Aspmo et al. 2005). In contrast, the total nitrogen content of the ground marine waste from which the MWE was extracted was 55.80 % nitrogen. The MWE might also serve as a source of carbon as it contained 1,500 mg L^{-1} of TOC.

To prepare the MSRB medium, the original MWE was diluted by a factor of 10. The extract with supplemented salts provided essential nutrients for the MSRB medium. The TKN value of the MSRB medium was 1,405 mg L^{-1}. The TKN value in the MSRB medium was highest among those measured in the remaining growth media. The lowest TKN value, 80.08 mg L^{-1}, was found in the Widdel and Pfennig medium. The TOC value of the MSRB medium was 1,124 mg L^{-1}. Postgate B medium had the highest TOC value, followed by modified Postgate B, MSRB, and Widdel and Pfennig growth media. The content of P, K, S, Ca, and Na in the MSRB compared favorably to Postgate B and Widdel and Pfennig growth media. Trace elements such as Mn, Zn, Cu, Co, and Ni were higher in the MSRB than the Widdel and Pfennig and these elements were not observed in either the Postgate B or modified Postgate B medium. The presence of these micro- and macro nutrients made the MSRB medium comparable to the other SRB growth media in terms of nutritional quality.

Table 2 Comparison of micro and macronutrients present in MWE and the SRB growth media; all concentrations in mg/L

Elements	MWE	Growth media			
		MSRB	Postgate B	Modified Postgate B	Widdel and Pfennig
TOC[a]	1,500	1,124.397	2,509	1,807	20
TKN	13,951 ± 0.61	1,405 ± 0.61	340 ± 0.61	1,200 ± 0.61	80.08 ± 0.61
P	75 ± 0.2	125 ± 0.2	115 ± 0.2	ND	46 ± 0.2
S	300 ± 0.7	585 ± 0.7	550 ± 0.7	450 ± 0.7	680 ± 0.7
K	120 ± 0.5	157 ± 0.5	140.6 ± 0.5	ND	320 ± 0.5
Ca	2,090 ± 3.5	510 ± 3.5	300 ± 3.5	ND	41 ± 3.5
Na	401 ± 2	132 ± 2	92 ± 2	92 ± 2	880 ± 2
Fe[a]	2.09	10.5	10.04	10.04	0.421
Mg[a]	1.48	198	197.3	197.3	47.820
Mn[a]	0.29	0.027	ND	ND	0.027
Zn[a]	1.91	0.190	ND	ND	0.033
Co[a]	1.04	0.101	ND	ND	0.047
Cu[a]	0.91	0.089	ND	ND	0.00633
Ni[a]	1.15	0.109	ND	ND	0.00592
Mo[a]	ND	ND	ND	ND	0.014

ND not determined

± Standard error of the mean (s.e.m.), n = 5

[a] % of RSD calculated by instrument was <5 %

Growth of SRB

The pattern of microbial growth throughout the course of the experiment is represented in Fig. 2a. A lag phase of 48 h was observed in both modified Postgate B and Widdel and Pfennig growth media. In comparison, a very short lag phase was found in the MSRB and Postgate B media. The exponential phase of microbial growth continued up to 144–168 h for all the growth media. The highest microbial count and SRB population observed in the growth media is represented in Table 3. MSRB was able to support both the SRB and microbial population. The medium was found superior in its ability to support the growth of the mixed microbial culture than the other growth media. The microbial cell counts in MSRB and modified Postgate B media were comparable from 96 h to the end of the batch study, possibly due to total nitrogen content, which did not differ much between these two media. Similarly, the microbial population grown in Postgate B was less than that in modified Postgate B, but better than in the Widdel and Pfennig media, apparently reflecting their nitrogen contents.

In the FISH study, the cells stained with both DAPI and SRB358 were counted as SRB (Fig. 3). The FISH study revealed that 95 % of the microorganisms in the inoculums were SRB. The FISH study of the microbial samples with the largest populations during the batch study revealed the difference between SRB populations grown in the different media (Table 3).

Formation of sulfide as a result of biological sulfate reduction during the growth of SRB in the media is represented in Fig. 2b. The interrelated behavior between microbial growth and hydrogen sulfide generation is shown in Fig. 2a, b. The generation of the sulfide was very similar in MSRB and modified Postgate B media. Similarly, sulfide generated in both Postgate B and Widdel and Pfennig growth media was comparable, but differed greatly from that generated in MSRB. The sulfide generated in MSRB was always greater than the other media. The maximum concentration of sulfide generated was between 192 and 216 h in all growth media (Table 3). After 192 h, the sulfide concentration started decreasing in all the media.

The pattern of alkalinity generated due to the SRB growth in the media throughout the course of the experiment is represented in Fig. 2c. Similarly, an increase in pH due to SRB activity was observed in all the media from 24 h until the end of the experiment (Fig. 2d). Table 3 shows the final alkalinity generated and the final pH attained in all the media at the end of the batch experiment. The alkalinity generated in MSRB was much higher than that generated in the other growth media. The initial alkalinity in MSRB medium was 5,250 mg L^{-1} $CaCO_3$, which was much higher than the values found in the other media. Initial alkalinities were measured as 3,500, 3,670, and 3,458 mg L^{-1} $CaCO_3$ in Postgate B, modified Postgate B and Widdel and Pfennig media, respectively.

Sulfate Reduction

The efficiency of sulfate reduction and the overall sulfate reduction rate in different growth media is compared in Table 3. The rise in sulfate reduction efficiency throughout the experiment is presented in Fig. 4. In MSRB medium, 58 % sulfate reduction was obtained at 72 h. Equivalent

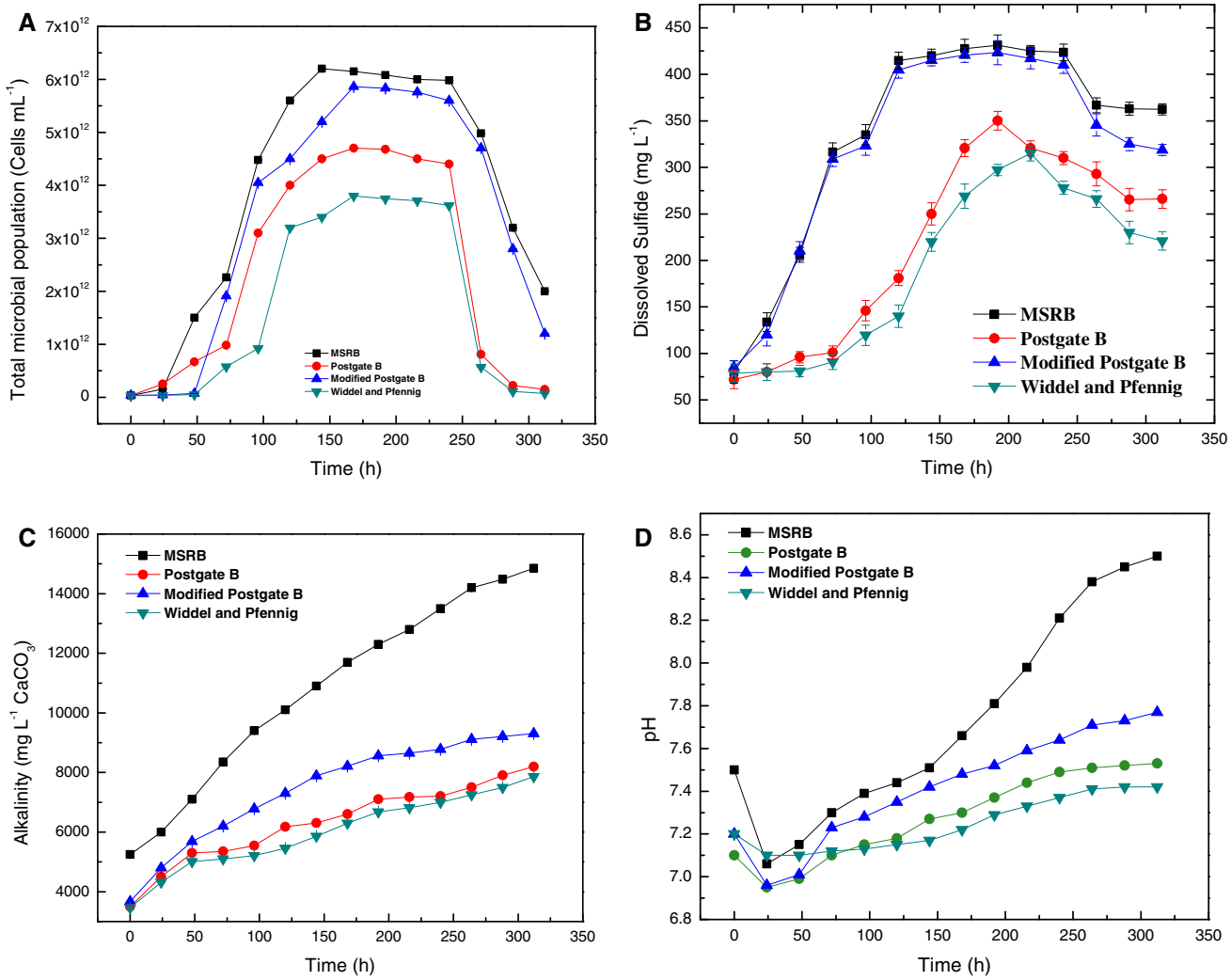

Fig. 2 Comparison of parameters related to SRB growth in different media: **a** total microbial population, **b** generation of sulfide, **c** progressive increase in alkalinity, and **d** change in pH; measurements were replicated 5 times (n = 5) for each parameter

sulfate reduction was obtained in modified Postgate B, Postgate B, and Widdel and Pfennig growth media at 96, 192, and 216 h, respectively. At 312 h, sulfate reduction in all the media reached the stationary phase and remained constant.

The initial sulfate concentration measured was 4,000 mg L^{-1} in all the media. This concentration was withstood by the SRB mixed culture, which exhibited varying sulfate reduction capacity in different growth media. SRB could reduce the sulfate concentration to 119 mg L^{-1} in MSRB, below the 250 mg L^{-1} recommended by US Environmental Protection Agency (2009) as the maximum sulfate concentration in drinking water. Table 3 presents the residual sulfate concentration measured at the end of the batch experiment. The sulfate reduction capacity observed in MSRB was comparable to that of modified Postgate B medium throughout the study. The sulfate reduction efficiency, on the other hand, was similar in Postgate B and Widdel and Pfennig growth media (Table 3).

Cost Analysis

The complete cost for the preparation of 1 L of MWE was USD 0.021, which includes sourcing, transportation, and processing. Since 103.322 mL of MWE was required to prepare 1 L of MSRB medium, the MWE used to prepare 1 L of MSRB cost around USD 0.002. Table 4 compares the MWE with other nitrogen sources in terms of cost effectiveness.

Discussion

The importance of both micro- and macro-nutrients to microbial growth and the necessity of their presence in

Table 3 Comparison of the growth media in terms of their ability to support biological sulfate reduction

Parameters	MSRB	Postgate B	Modified Postgate B	Widdel and Pfennig
Initial sulfate concentration[a]	4,000 ± 6.75	4,000 ± 7.21	4,000 ± 5.8	4,000 ± 6.2
Final sulfate concentration[a]	119 ± 4.9	1,081 ± 6.6	506 ± 5.5	1,251 ± 4.1
Sulfate reduction efficiency[b]	97 ± 0.27	72 ± 0.11	87 ± 0.11	68 ± 0.06
Overall sulfate reduction rate[c]	12.41 ± 0.01	9.35 ± 0.01	11.1 ± 0.01	8.8 ± 0.01
Highest total microbial count[d]	$(6.2 \pm 7) \times 10^{12}$	$(4.7 \pm 7.1) \times 10^{12}$	$(5.8 \pm 9.4) \times 10^{12}$	$(3.8 \pm 8.5) \times 10^{12}$
SRB population in mixed culture[b]	96 ± 0.05	80 ± 0.05	92.5 ± 0.07	65 ± 0.4
Maximum dissolved sulfide generated[a] (mg L^{-1})	431.2 ± 11	350.12 ± 10	423.27 ± 13	297.23 ± 6
Final alkalinity[a]	14,850 ± 18	8,200 ± 10	9,305.2 ± 20	7,862.3 ± 15
Final pH	8.5	7.53	7.77	7.42

± Standard error of the mean (s.e.m), n = 5

[a] mg L^{-1}; [b] %; [c] mg L^{-1} h^{-1}; [d] cells mL^{-1}

growth substrates has been discussed (Kayhanian and Rich 1995). In similar situations, MWE could be used as a nutrient-rich growth substrate. The TOC/sulfate ratios of MSRB, Postgate B, modified Postgate B, and Widdel and Pfennig media were 0.281, 0.627, 0.451, and 0.005, respectively. A chemical oxygen demand (COD): sulfate (COD/SO_4^{2-}) ratio of 0.5 was reported to be suitable for SRB activity (Neculita et al. 2007). Significant sulfate reduction was reported at COD/SO_4^{2-} ratios of 0.67 and 0.34 (Mockaitis et al. 2010). At low COD/SO_4^{2-} ratios, electron flow is directed more favorably towards sulfate reduction and SRB out-competes other bacterial communities (McCartney and Oleszkiewicz 1993). Treating the TOC as COD, the COD/SO_4^{2-} ratio ranged between 0.281 and 0.451 in MSRB, Postgate B, and modified Postgate B; it was therefore assumed that these ratios did not significantly impact SRB growth. The lowest SRB growth and sulfate reduction was observed in Widdel and Pfennig media, where the COD/SO_4^{2-} ratio was only 0.005, which might have inhibited SRB activity.

During microbial growth, the length of the lag phase depends on medium pH, temperature, inoculum size, and available nutrients (Swinnen et al. 2004). Incubation temperature and inoculum size for all the media were the same. The initial pH of the growth media ranged between 7.1 and 7.5. Therefore, it can be postulated that the probable causes of the difference between these lag phases among microbial population grown in different media were the nutrient composition, the nitrogen content in it, and its availability to SRB. The short lag phase of microbial growth in MSRB and Postgate B was possibly due to the ability of the media to provide available nutrients during the initial stages of microbial growth.

Stimulation of SRB growth due to nitrogen addition has been reported (White and Gadd 1996; Azabou et al. 2005; Chockalingam and Subramanian 2006; Martins et al. 2011). Similarly, the growth of a SRB mixed culture decreased when the concentration of bacto-tryptone (a nitrogen source) in growth media was cut by half (Das et al. 2013). Thus, nitrogen is clearly important to the growth of SRB. The major difference between the media was the nitrogen source. Comparable sulfate reduction efficiency in MSRB and modified Postgate B media (97 and 87 %, respectively) occurred because there was little difference in their nitrogen content (Table 2). Due to low nitrogen content (Table 2), the Widdel and Pfennig growth medium had the lowest sulfate reduction efficiency (68 %). However, the MSRB medium also supplies essential macronutrients for the growth of bacteria and synthesis of several structural and functional components of living cells.

In a sulfate-rich environment, the growth of SRB leads to sulfide generation. Part of the sulfide escapes to the gas phase; the remainder either reacts with dissolved metals to form metal sulfide precipitates or remains as dissolved sulfide (Velasco et al. 2008). Therefore, sulfide generation was considered to be indicative of SRB activity (Bayoumy et al. 1999). There was not much difference in the SRB population in MSRB and modified Postgate B media, and therefore, their sulfide generation was similar (Fig. 3b; Table 3). Less sulfide generation in Postgate B and Widdel and Pfennig media indicates less SRB activity compared to MSRB. In the growth media, the decrease in sulfide generation after 192 h of incubation was possibly due to progressive increases in pH and retardation of microbial growth. Figure 2a indicates that microbial population

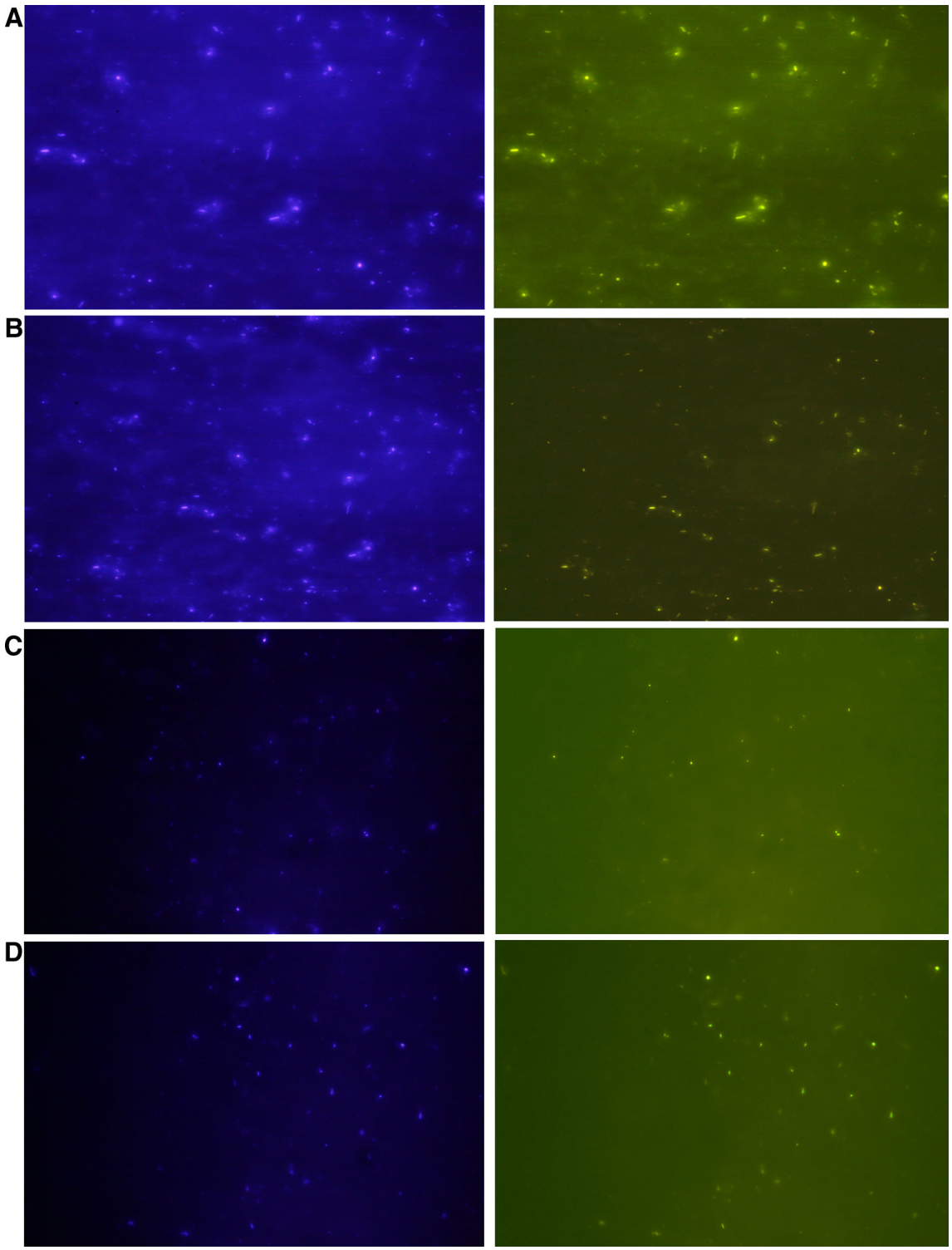

Fig. 3 Epifluorescence micrograph of microbial cells stained with both DAPI and SRB385 grown in: **a** modified Postgate B, **b** Postgate B, **c** Widdel and Pfennig, and **d** MSRB media. Each horizontally paired image represents the same microscopic field observed using the two different filter sets

moved towards a stationary phase after 192 h, with the death phase following after 240 h. Therefore, less microbial activity resulted in a continuous decrease in sulfide concentrations after 192 h onwards. Figure 4d shows the continuous increase in pH from 24 h until the end of the experiment. At neutral pH, sulfide is generally present as

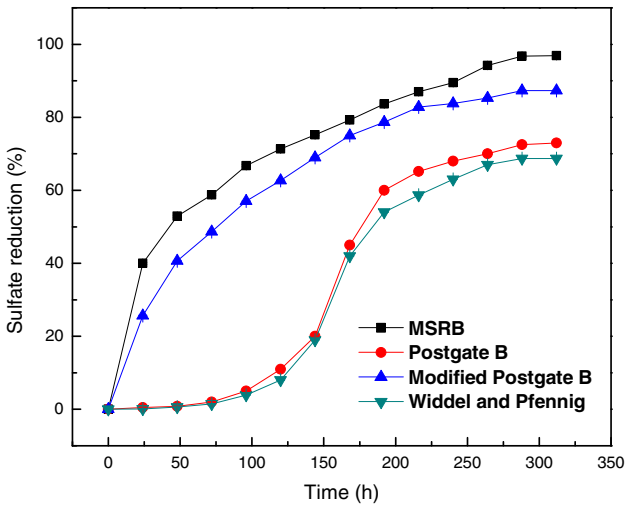

Fig. 4 Sulfate reduction efficiency of SRB mixed culture during growth in different media

Table 4 Comparison of the nitrogen sources with MWE in terms of cost

Growth media	Nitrogen source	Concentration used in the media	Equivalent price ($)
Postgate B	NH_4Cl	1 g L^{-1}	0.0099
	Yeast Extract	1 g L^{-1}	0.230
Modified Postgate B	Bacto-tryptone	10 g L^{-1}	4.65
Widdel and Pfennig	NH_4Cl	0.3 g L^{-1}	0.0029
MSRB	MWE	103.32 mL L^{-1}	0.0022

undissociated H_2S; with increasing pH, it begins to convert into the ionized form, HS^- (Colleran et al. 1995; Kaksonen and Puhakka 2007). Therefore, the progressive increase in pH might also have contributed to decreased sulfide generation.

Growth of SRB is also associated with the generation of alkalinity (Johnson and Hallberg 2005). SRB can generate alkalinity due to both the production of bicarbonate ions and ammonia from nitrogen-containing substrates (Zagury et al. 2006). However, the initial high alkalinity in MSRB was attributed to the fact that the MWE contained a high $CaCO_3$ content from the crustacean shells in the marine waste. The decrease in pH in all the growth media at 24 h could be due to the formation of organic acids (Neculita et al. 2011). On the other hand, the high pH in MSRB possibly aided SRB activity.

Our cost analysis of the nitrogen sources indicates that MWE was cost effective as a nitrogen source (Table 4) and that MSRB is a cost competitive alternative to other commercially-available bacterial growth substrates. MWE can also be used as a substrate to grow various bacterial cultures where other, more expensive commercially-available substrates are used.

Conclusion

The MWE contains high concentrations of nitrogen as well as other essential nutrients, and as a result, the MWE-supplemented MSRB medium supported better SRB growth and the highest sulfate reduction efficiency compared to other conventional SRB growth media that contained different commercial nitrogen sources. Preparation of MSRB is cost effective as it substitutes MWE for the use of more expensive conventional nitrogen sources, such as NH_4Cl, NH_4HCO_3, $(NH_4)_2HPO_4$, and bactopeptone. Therefore, conventional SRB growth media, like Postgate B, can be replaced with MSRB. Similarly, using MSRB to grow SRB in large-scale operational process should enhance cost effectiveness and improve performance. Such effective alternative uses of abundant marine waste can lead to the development of sustainable biotechnological processes.

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