Use of marine extract as nitrogen rich growth substrate for sulfate reducing bacteria

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Abstract Marine waste extract was tested as nitrogen rich growth substrate for Sulfate Reducing Bacteria (SRB). A liquid extract was prepared from marine waste and characterized. The extract contained 14.541 g L^{-1} of nitrogen, 12.345 g L^{-1} of total organic carbon, 90.322 g L^{-1} of protein and 1.48 mg L^{-1} of magnesium. The extract supplemented medium exhibited improved performance as compared to Postgate B medium for their ability to promote the growth of SRB. It supported 95 % sulfate reduction at a rate upto 21 mg L^{-1} h^{-1} and 1.21×10^{13} cells/ml of SRB population. This indicates that the potential exists for the use of marine waste extract as growth substrate for SRB.

Keywords Nitrogen rich growth substrate, Sulfate reducing bacteria, sulfate reduction, bacterial growth

Introduction

Sulfate Reducing Bacteria (SRB) is group of prokaryotes which belongs to the several genera like *Desulfovibrio*, *Desulfotomaculum*, *Desulfosporosinus*, *Thermodesulfobium*. As a part of energy metabolism SRB utilizes the sulfate as electron acceptor and reduce it to sulfide. This characteristic made the SRB useful for the bioremediation of sulfate rich wastewater especially in acid mine drainage (AMD) environment.

Biological treatment of sulfate rich wastewater is mainly carried out using sulfidogenic bioreactor (Pol *et al.* 1998; Sarti and Zaiat 2011). Generation of adequate SRB biomass is necessary for the efficient reduction of sulfate. To produce high SRB biomass growth substrate should contain sufficient quantity of nutrient especially in the form of carbon and nitrogen (Bayoumy *et al.* 1999; Daubert and Brennan 2007, Newcombe and Brennan 2010).

Several studies are being conducted on different carbon sources and their effect on the growth of SRB (Costa *et al.* 2009; Das *et al.* 2013). Substances like trinitrotoluene (Boopathy and Kulpa 1992) taurine (Lie *et al.* 1999), urea (Neculita and Zagury 2008), nitrocellulose (Petrova *et al.* 2002), corn steep liquor (White and Gadd 1996), amino acids (Rees *et al.* 1998) are reported to be utilized as nitrogen source by SRB. Most of these substances have not been tested for their efficiency particularly as nitrogen source in terms of promoting the efficiency of sulfate reduction and SRB growth.

In most of cases the nitrogen source supplied for the growth of SRB inside sulfidogenic bioreactors include NH_4Cl (Kaksonen *et al.* 2003). Commercial growth media for SRB generally contains NH_4Cl (Postgate 1984), $(NH_4)_2HPO_4$ (Kuo and Shu 2004), NH_4HCO_3 (Mizuno *et al.* 1998) as nitrogen source. These types of nitrogen sources are expensive. Therefore, use of them is impractical during the large scale and long term treatment of sulfate rich wastewater in terms of cost effectiveness. These nitrogen sources may be substituted with the one which is available, cost effective and easily utilizable.

Wastes generated from marine fishing activities are mainly composed of the shells of crab, shrimp, mussel. These shells substances generally contain high concentration of pro-

tein, chitin and minerals (Gildberg and Stenberd 2001, Xu *et al.* 2008). The protein rich fraction has good nutritional quality and was utilized as nitrogen source in growth medium for microorganisms (Solano *et al.* 2009). As this protein rich fraction is easily obtained from the marine waste, it can be used as a nitrogen rich nutrient to grow SRB.

The objective of this study included the utilization of marine waste extract to grow SRB. To perform this, the waste was deproteinized and protein rich fraction was extracted. The growth medium supplemented with this liquid extract was compared with Postgate B medium and evaluated as an alternative to the latter one.

Methods

Mixed SRB culture was previously acclimatized for 6 months in Postgate B medium (Postgate 1984) with the following composition: KH_2PO_4 0.5 g L^{-1}, NH_4Cl 1 g L^{-1}, $CaSO_4$ 1 g L^{-1}, $MgSO_4.7H_2O$ 2 g L^{-1}, sodium lactate 3.5 g L^{-1}, ascorbic acid 0.1 g L^{-1}, thioglycolic acid 0.1 g L^{-1} and $FeSO_4.7H_2O$ 0.5 g L^{-1}. pH of the medium was kept to 7.0-7.2 using.

Marine waste was collected from the seashore area of Digha (West Bengal, India). The waste was manly composed of shells of crabs, mussel, prawn, shrimp, tails and exoskeletons of fishes. The waste was sun dried and milled. The powdered form was added as 10 % (w/v) to 1000 mL of deionized water. The suspension was kept to alkaline condition by adjusting pH to 11.5 using 2(N) NaOH and boiled at 70 °C for 2h with constant stirring. Protein rich liquid extract was separated by centrifugation at 6440×g for 15 minutes followed by filtration through 0.2 µm polycarbonate membrane filter (Millipore).

The extract was analyzed for total kjeldahl nitorogen (TKN), total organic carbon (TOC), protein content and magnesium.

Batch experiment was carried out to compare growth in medium supplemented with marine waste extract as nitrogen source, with that of the commercial growth medium like Postgate B containing NH_4Cl as nitrogen source.

Growth of study SRB mixed culture was carried out in a batch mode using 250 mL of conical flasks. Growth medium supplemented with the liquid extract was termed as marine waste extract (MWE) medium. The MWE medium had the following composition: sodium lactate, 3.5 g L^{-1}, KH_2PO_4, 0.5 g L^{-1}, $CaSO_4$, 1 g L^{-1}, $MgSO_4.7H_2O$, 2 g L^{-1}, marine waste extract, 10.322 % (v/v). To compare MWE with Postgate B, the media were added individually to different conical flasks. The Media were prepared with deionized water, boiled and cooled under continuous flow of nitrogen gas to remove dissolved oxygen and autoclaved at 121 °C temperature under 15 lbs cm^{-2} pressures for 15 minutes. Subsequently the growth media were inoculated with 10 % (v/v) SRB inoculum and incubated at 35 °C. MWE medium without added inoculum was considered as control. The growth study was carried out for 192 hours and samples were collected during every 24 hours intervals. Efficiency of SRB growth was analyzed in terms of SRB population, efficiency and rate of sulfate reduction, increase in pH and the growth media were compared accordingly. Inoculation, incubation, periodic transfer of inoculum and sampling were done inside anaerobic system (Thermo Scientific, Model 1029).

Result and Discussion

Marine waste extract exhibited TKN value of 14.541 g L^{-1} and C:N ratio of 0.854 which indicated it as a nitrogen rich substrate. TOC value of the extract was 12.345 g L^{-1}. It also contained 90.322 g L^{-1} of protein and 1.48 mg L^{-1} of magnesium. The value of protein, nitrogen, carbon and magnesium indicated the extract as substrate with balanced nutritional quality.

In the growth study the residual sulfate concentration in MWE medium was 130 mg L^{-1} and exhibited 95 % sulfate reduction. In Postgate B medium the values were 570 mg L^{-1} with 79 %. The highest rate of sulfate reduction observed in MWE and Postgate B medium

were 21 mg L^{-1} h^{-1} and 18 mg L^{-1} h^{-1} respectively. The efficiency and rate of sulfate reduction in MWE medium was always higher than that of Postgate B medium (Fig. 1). Higher efficiency and rate of sulfate reduction were mainly due to the supposedly higher activity of SRB in MWE compared to the Postgate B medium.

Number of bacterial cells in MWE medium increased from approximately 7.43×10^{11} cells/mL to about 1.21×10^{13} cells/mL. Similarly, in Posgate B medium the number increased from 3.17×10^{11} cells/mL to approximately 9.02×10^{12} cells/mL.

The population of SRB in MWE medium was always higher than Postgate B medium throughout the experiment (Fig.2). The lag phase of SRB growth in MWE medium was 24 hours whereas in Postgate B medium it was 72 hours. Short lag phase in MWE medium may be due to the rapid supply of nutrient at the initial stage of the experiment (Robinson-Lora and Brennan 2009). This result was much similar to that observed in the experiment of Robinson-Lora and Brennan (2009) who used crab shell chitin as nitrogen source for SRB.

The ability of MWE medium to promote higher biomass and sulfate reduction efficiency was found superior to Postgate B. The presence of marine waste extract in MWE made it comparatively improved growth medium to support SRB growth than Postgate B. Besides providing efficient source of nitrogen the liquid extract was also the source of high organic carbon, protein and trace quantity of magnesium. The protein rich fraction could also be degraded to short chain organic electron donor and NH_4^+ (Neculita *et al.* 2007, Robinson-Lora and Brennan 2010). NH_4^+ was thought to be utilized directly. Therefore the presence of several electron donors in addition to nitrogen, carbon, magnesium promoted improved growth and sulfate reduction in MWE than Postgate B.

In MWE and Postgate B medium, pH was increased gradually throughout the course of the experiment (Fig. 3). The final pH in MWE and Postgate B medium was pH 7.35 and pH 7.32 respectively. After 24 hour there was a reduction of pH value. This was mainly due to the generation of several volatile fatty acids which lowered the pH. The change in pH with respect to time was similar in both the growth medium. As a part metabolism SRB generated alkalinity which increased pH during the course of incubation (Bilek and Wagner 2012).

The experiment showed that marine waste extract acted primarily as nitrogen source which developed both SRB population and thereby sulfate reduction efficiency. Similar behavior was observed on the effect of corn steep liquor as nitrogen source for SRB (White

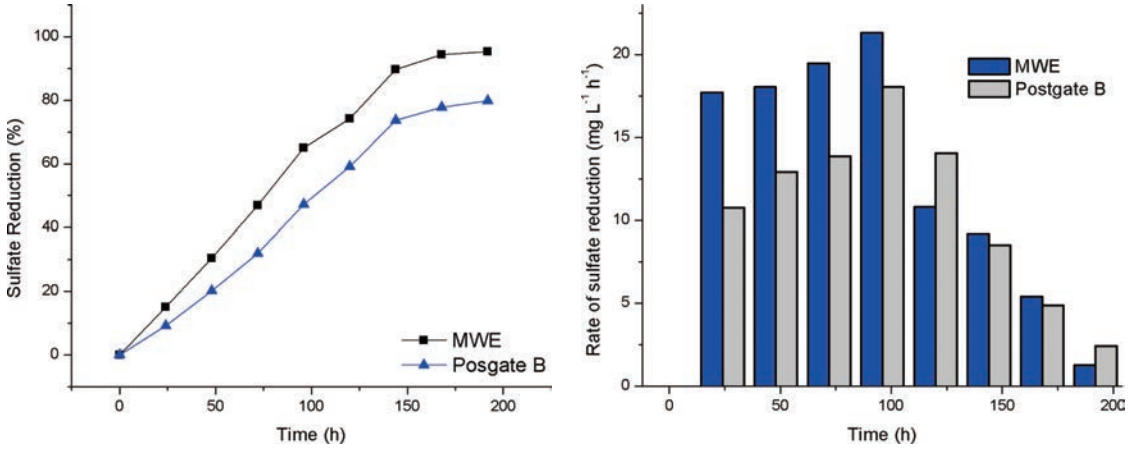

Fig. 1 *Comparison of (left) Sulfate reduction (%), (right) Sulfate reduction rate (mg L^{-1} h^{-1})*

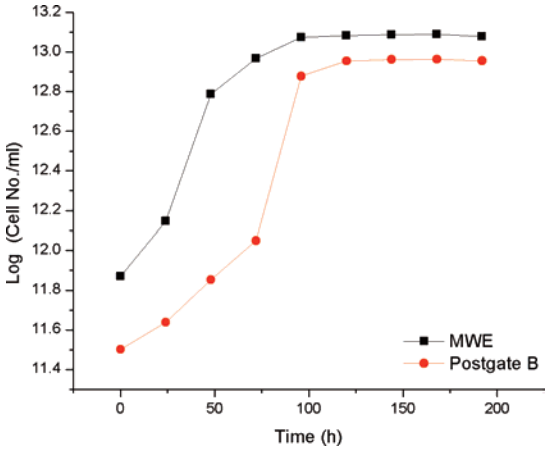

Fig. 2 *Log (Cell number/ml) as a function of time following the SRB population*

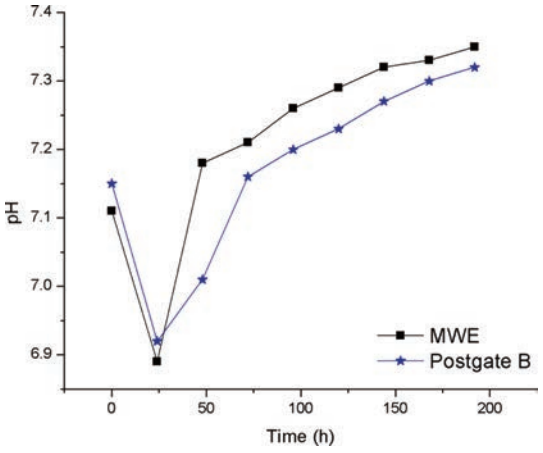

Fig. 3 *Changes in pH as a function time during the growth of SRB in MWE and Postgate's B medium.*

and Gadd 1999). The authors reported that corn steep liquor enhanced both the SRB population as well as sulfate reduction performance.

The rate of sulfate reduction and SRB growth was much better than that reported by Neculita and Zagury (2008) who used urea as nitrogen source for the growth of SRB. (Boopathy and Kulpa 1992) used trinitrotoluene as nitrogen source for SRB. In our experiment MWE medium exhibited much lower lag phase than that reported by the authors. SRB population grew in MWE medium was much higher than that reported by Chockalingam *et al.* (2005) who studied husk filtrate as nutrient source for SRB. Along with rice husk the authors added ferrous ammonium sulfate which could be used as nitrogen source.

In our experiment the liquid extract exhibited 14.541 g L^{-1} of nitrogen and C:N ratio of 0.854 which indicated very high concentration of nitrogen. However, successful sulfate reduction of 95 % with the growth of SRB population upto 1.21×10^{13} cells/mL was obtained using the extract. Substrate with C:N ratio more than 45 is reported to inhibit the growth of SRB due to unavailable nitrogen source (Gibert *et al.* 2004; Robinson-Lora and Brennan 2009). El Bayoumy *et al.* (1999) mentioned about the toxicity effect of nitrogen on SRB growth above the value of 500 mg L^{-1}. Several complex substrates have been studied for their efficiency to promote sulfate reduction activity in both batch and continuous scale reactors. These substrates have not been reported to have effect on SRB growth as nitrogen source. But these substrates contained different quantities of nitrogen content and vary in C:N ratio (Tab. 1).

Marine waste extract was proved to be a nitrogen rich growth substrate which can promote the growth of SRB in an efficient manner. MWE medium used in this study can be effectively used as a substitute for commercial growth medium like Postgate B to cultivate SRB. Moreover, the expenditure of operational process to treat sulfate rich wastewater can be reduced by substituting the expensive nitrogen sources like NH_4Cl, $(NH_4)_2HPO_4$, NH_4HCO_3. with marine waste extract. Thus the extract prepared from marine waste serves as available, cost effective and suitable source of nitrogen for SRB.

Conclusions

- Slow rate and inefficient sulfate reduction during the long term treatment of sulfate rich wastewater is mainly due the absence

Sl No.	*Name of the Substrate*	*Nitrogen (%)*	*C:N ratio*	*References*
1	Sheep manure	1.98 ± 0.07	19.52 ± 2.66	Gibert et al. 2004
2	Compost	1.26 ± 0.05	21.58 ± 2.91	
3	Poultry Manure	1.61 ± 0.07	16.94 ± 1.58	
4	Mushroom compost	2.47	10	Neculita et al. 2011
5	Cow Manure	2.15	18	
6	Rice straw	0.71	54	
7	Poultry manure	5.80 ± 0.30	3.3	Zagury et al. 2006
8	Chitin	2.85	6.42	Rbinson-Lora and Brennan 2010
9	Urea	46.62	0.428	Neculita and Zagury 2008
10	NH_4Cl	26.17	-	Kuo and Shu 2004
11	$(NH_4)_2HPO_4$	10.6	-	

Table 1 *Nitrogen content and C/N ratio of several substrates used for the treatment of AMD*

of nutrient specifically in the form of nitrogen source required for the growth of SRB.

- Protein rich fraction extracted from marine waste may serve as suitable nitrogen source for SRB.
- The liquid extract promoted better growth and sulfate reduction efficiency to SRB than the Postgate B medium and other growth substrates containing nitrogen.
- The extract can be used as suitable and available source of nitrogen for SRB. The costly nitrogen source used in commercial growth medium can be substituted with this liquid extract.

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