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ANAEROBIC BIODEGRADATION OF SULPHATE EMPLOYING ANIMAL MANURE AS A COST EFFECTIVE GROWTH SUBSTRATE

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ABSTRACT

The toxicity of various compound forms of sulphates to animals is well known. Therefore, the sulphate rich industrial wastewaters must be treated before discharging them to the environment. In this context, the present study reports the implication and assessment of two different states of bovine and poultry manures as sources of carbon and energy in the treatment of artificially prepared sulphate rich wastewater while recruiting pure cultures of *Desulfovibrio desulfuricans*-HAQ3. About 73% sulphate reduction was achieved in a 60 days trial of anaerobic incubation when composted bovine manure was used as an electron donor. Sulphate reduction efficiency remained limited to 27%, 31% and 36% for fresh bovine manure, poultry manure and poultry litter as electron donors, respectively. The findings of this study will be very helpful in developing economical and environmental friendly bioremediation process(es) addressing the sulphate pollution.

Key words: Economical bioremediation, Electron donor, SRB, Bovine manure, Poultry manure, Poultry litter.

INTRODUCTION

A number of industries including edible oil production plants, tanneries, textile wastewaters, petroleum refineries, pulp manufacturing industries, potato starch factories, paper mills, food processing industries and solid waste processing plants have been introducing sulphates continuously into the environment (Boshoff *et al*., 2004; Vaiopoulou *et al*., 2005; Huang *et al*., 2006). Deleterious effects of compound forms of sulphates on human health are well known and include acute renal failure, coma, confusion, cough, dyspnea, hepatotoxicity, glutathione peroxidase (GPx), hippocampus superoxide dismutase (SOD), increase in catalase (CAT), late sequelae of interstitial fibrosis, loss of consciousness, metabolic acidosis, myocardial necrosis, prolonged apnea, pulmonary edema, seizures, shocks, severe intravascular hemolysis and severe neurological impairment (Duong *et al*., 2001; Mbaye *et al*., 2003; Christia-Lotter *et al*., 2006; Kucukatay *et al*., 2007; Mortazavi and Jafari-Javid, 2009). Therefore, treatment of sulphate rich industrial effluents is necessary before discharging them to the environment.

In the recent years, sulphate reducing bacteria (SRB) have been extensively employed in the treatment of acid mine drainage and other sulphate rich effluents (Steed *et al*., 2000; Lima *et al*., 2001; Burgess and Stuetz, 2002; Johnson and Hallberg, 2005; Neculita *et al*., 2007). As biological sulphate reduction is an energy requiring process, therefore, remediation of sulphate rich effluents using SRB demands an efficient energy rich reductant (Barnes, 1998). The ability of SRB to utilize only low molecular weight organic compounds is well known, whereas some have been documented capable of utilizing environmental contaminants such as constituents of petroleum hydrocarbons and other halogenated compounds as sources of carbon and energy (Fauque *et al*., 1991; Hao *et al*., 1996; Harms *et al*., 1999; Morasch *et al*., 2004). The most preferred carbon sources for culturing SRB at laboratory scale include ethanol and lactate but being too much expensive these can't be afforded for large scale operations (Barnes, 1998; El Bayoumy *et al*., 1999; Tsukamoto *et al*., 2004; Huisman *et al*., 2006). Several organic wastes have thus been identified as cheaper carbon sources for biological sulphate reduction which include sugarcane bagasse, leaf mulch, molasses, mushroom compost, sawdust, sewage sludge, vegetable compost, watermelon rind, whey, wood chips and many other similar agricultural wastes (Dvorak *et al*., 1992; Hammack *et al*., 1994; Christensen *et al*., 1996; Waybrant *et al*., 1998; Annachhatre and Suktrakoolvait, 2001; Costa and Duarte, 2005; Coetser *et al*., 2006; Hussain and Qazi, 2012).

Livestock and poultry wastes are substantial contributors of both point and non-point source pollutions and can affect wetland habitats and contaminate potable water sources. Application of organic pollutants for cultivation of SRB may lead to the development of inexpensive treatment of sulphate rich wastes with concomitant consumption / removal of these pollutants. The present study was thus intended to investigate electron donating potentials of composted as well as fresh bovine manure, poultry litter and poultry manure for biological sulphate reduction. The results represent an economical way for the concomitant treatment of sulphate and animal manure.

MATERIALS AND METHODS

Isolation of pure cultures of SRB from sewage water: Wastewater samples were collected in sterile, screw capped, air-tightened containers from the bed of a sewage channel, carrying domestic as well as industrial effluents from the city Lahore and were transported to Microbial Biotechnology Lab, University of the Punjab, Lahore for further processing. The collected samples were enriched anaerobically in Postgate B medium (Postgate, 1984). All enrichments were made after Hussain and Qazi (2012) by seeding 2 ml sample in sterilized serum bottles of 20 ml capacity. The bottles were filled up to the brim with fresh Postgate B medium and then sealed with rubber stoppers and aluminium crimps not allowing any air to be trapped in. The bottles were incubated at 30°C till blackening of the medium. SRB growth was confirmed by the formation of black precipitates in addition to production of rotten egg smell of H_2S, which was checked by withdrawing and smelling gas using a sterilized syringe. These enrichments were used to isolate pure cultures of SRB following the method of Postgate (1984). Following the procedure, eight different SRB species were isolated. Of these eight species, the one which showed best growth and highest sulphate reduction using animal manure as a carbon source was selected for the present study.

Molecular characterization of the bacterial isolate

a. Extraction of DNA: Total genomic DNA was extracted from the freshly grown culture of the selected bacterial isolate in Postgate B medium. About 100 µl of two days incubated culture was used to sediment the cells in an eppendorf tube. The pellet was then suspended in 20 µl of lysis solution which was composed of NaOH (0.05M) mixed in 25% sodium dodecyl sulfate (SDS). The cell suspension was then heated (95ºC) for 15 min and cooled rapidly. Then sterile Milli-Q water was added to this mixture to make the volume 200 µl and centrifuged for 5 min. The lysate was saved in fresh eppendorf tubes which were subsequently stored at -20°C till further use.

b. PCR amplification: PCR was performed in 50 µl total reaction volume containing 5 µl DNA extract, 5 µl each of 25 mM $MgCl_2$, 1 mM dNTPs, 5 pmol forward and reverse primers (universal primers), 2 U/ml DNA *Taq* polymerase and 1X *Taq* buffer. The remaining volume of this mixture was adjusted with DNA free water. The PCR cycle with denaturation for 3 min at 94°C following 35 cycles of denaturation for 30 sec at 95°C, annealing step of 2 min at 60°C and 1 min extension at 72°C with a final extension step of 30 min at 72°C was run in a thermal cycler (Hamburg 22331, Germany). The PCR product was separated by electrophoresis on 1% agarose gel stained with ethedium bromide in TAE buffer. Amplified band of 1.5 kb was visualized under UV (Gel Doc, Bio-Rad Laboratories, USA) and excised for purification using Gene Purification Kit (Fermentas).

c. Sequencing of 16S rRNA gene: The amplicon was then got sequenced using Big Dye Terminator v3.1 cycle sequencing ready reactions (Macrogen, Korea) at the DNA Sequencing Facility, Korea. 16S rRNA gene sequence was assembled with phrap (version 0.990319). Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). 16S rDNA sequence determined in this study was submitted to GenBank (a worldwide database of nucleotide sequences) for obtaining an accession number.

Batch experiments: Batch experiments were performed in triplicate in serum bottles of 120 ml capacity. The growth medium used was the modified Postgate B medium containing sulphate (2.0 g/l) and an organic waste (2%) as a carbon source instead of sodium lactate. Oven dried (60°C), meshed and sieved fine powder of a given organic waste was used to replace sodium lactate. Experiments without any carbon source served as control. The inoculum size used was 5% (v/v) harbouring around 1.7×10^6 CFU/ml. pH of the medium was adjusted at 7.0 for each experiment. Diffusion of oxygen in inoculated media was prevented by adding a layer of autoclaved liquid paraffin. The inoculated bottles were sealed with fine rubber stoppers and aluminium crimps and incubated at 30°C for 60 days.

Analytical procedures: Periodically after every 10 days, 5 ml samples were withdrawn with the help of a syringe for measuring pH, sulphate and colony forming units (CFU/ml). pH was measured with the help of a digital pH meter (InoLab pH7110) while sulphate was estimated following the method as described by Cha *et al.* (1999). The bacterial CFU were estimated by cultivating 0.2 µl of a culture in Postgate E molten medium (Postgate, 1984). The embedded black colonies were then counted after 5 days incubation at 30°C.

Statistical analysis: Statistically the whole data regarding sulphate reduction were analysed using GLM procedures and means were compared using Duncan's Multiple Range test with the help of SAS 9.1 (Cary, 2002). Differences between means were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The present study reports the isolation and implication of pure culture of an indigenous SRB for economical decontamination of sulfate rich effluents while employing animal manure as a carbon source. Homology searches of 16S rDNA sequence revealed that the isolate belonged to genus *Desulfovibrio*. The

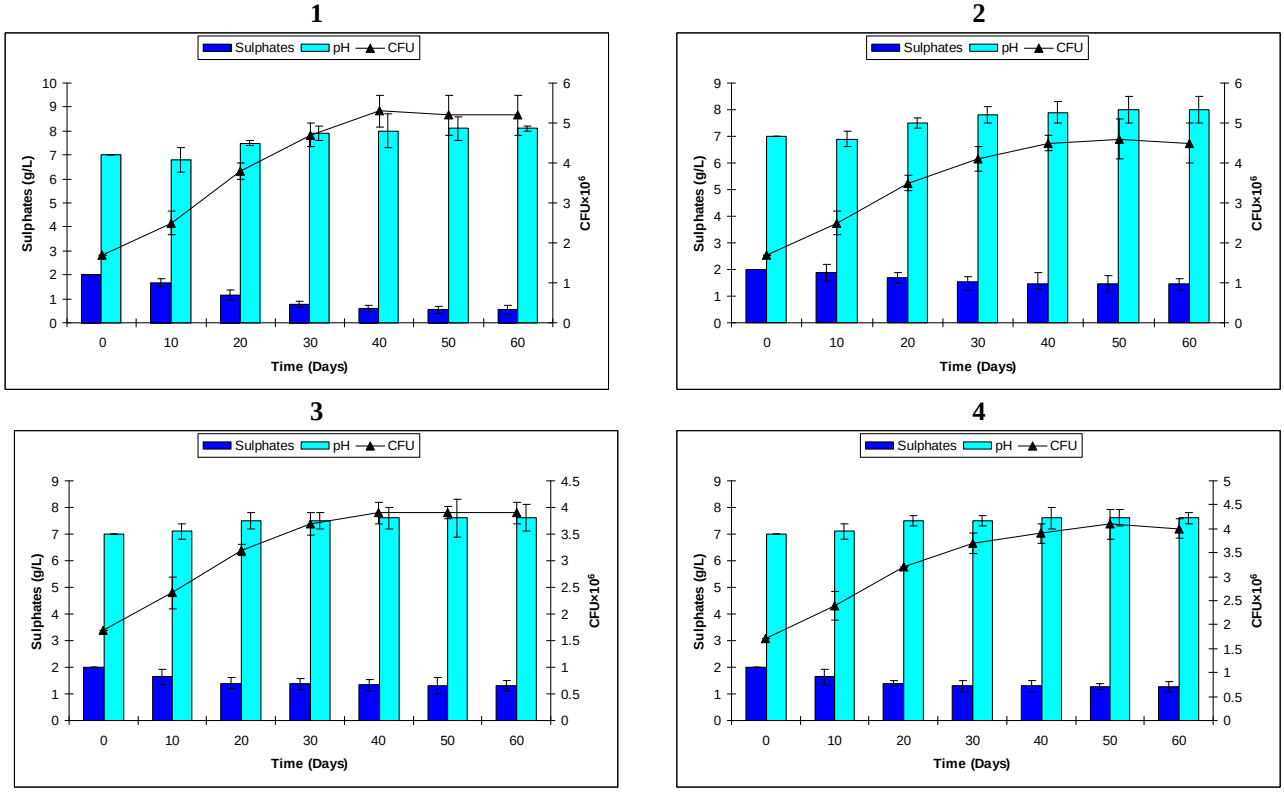
dominance of the *Desulfovibrio* genus in wastewaters has been reported by various researchers (Santegoeds *et al*., 1998; Baena *et al*., 1998; Dar *et al*., 2005; Martins *et al*., 2009). The sequence was allotted an accession number; KF536742 by the GenBank. Growth potential and consequent sulphate reduction rate of the bacterium varied considerably for each type of animal manure.

For all the carbon sources employed, sulphate reduction rates were higher during first half of the observational period (between 10 to 30 days) and then became gradually low at the terminal stages (between 40 to 60 days) of anaerobic incubation. No growth was observed in control experiments and thus sulphate was not reduced. More than 50% of the total sulphate reduction occurred between 10 to 30 days of incubation. Generally, sulphate reduction rates decreased gradually with an increase in duration of incubation. This type of inverse relation has already been reported by many researchers while studying the effectiveness of different agro-industrial wastes as economical carbon sources for biological sulphate reduction (Martins *et al*., 2009; Hussain and Qazi, 2012).

Sulphate was reduced efficiently when composted bovine manure was used as a carbon source. About 73% of the total added sulphate was reduced using this substrate following an incubation period of 60 days (Fig. 1). Sulphate reduction potential of the bacterium dropped around 3 folds in the presence of fresh bovine manure (Fig. 2). Ability of the SRB to utilize only simple organic molecules is well known (Dvorak *et al*., 1992; Nagpal *et al*., 2000; Gibert *et al*., 2004; Tsukamoto *et al*., 2004; Zagury *et al*., 2006; Hussain and Qazi, 2012). Composted manure contains simpler forms of organic molecules that could be easily utilized by SRB and may provide conditions for generation of low redox potentials and thus create an ideal environment for SRB growth (Cohen, 2006). Higher growth of the SRB as well as maximum sulphate reduction in the presence of composted bovine manure is attributed to semi-digested nature of complex organic molecules of this cellulosic waste and vice versa.

Increase in pH was observed in all the cultures at the completion of the incubation period. Maximum sulphate reduction was also observed at this study point. Similar findings regarding efficient sulphate reduction in media exhibiting neutral to basic range have been reported by various researchers (Martins *et al*., 2009; Singh *et al*., 2011).

A rapid decline in sulphate reduction was observed when poultry manure and poultry litter were used as electron donors. Only 31% and 36% sulphate was reduced with the usage of poultry manure and poultry litter, respectively (Figs. 3, 4). Existence of low C/N ratios (20-24) and high lignin contents in these two



Figs. 1-4. Assessment of sulfidogenic bacterial (*D. desulfuricans*-HAQ3) growth and sulphate reduction with accompanying pH changes at different incubations using composted bovine manure, fresh bovine manure, poultry manure and poultry litter respectively, as carbon sources.

wastes have been reported by various researchers (Bakayoko *et al*., 2009; Munawar and Riwandi, 2010). According to Gibert *et al*. (2004) carbon to nitrogen ratio must be in the range of 45-120 for efficient sulphate reduction. An organic substrate with a lower C/N ratio can't provide sufficient carbon to SRB for reducing sulphate continuously (Munawar and Riwandi, 2010). Complete cessation of sulphate reduction after an incubation period of 30 days is attributable to low C/N ratios and / or higher lignin contents of these two organic wastes. Lower the lignin contents in an organic substrate higher will be its biodegradability and easily biodegradable substrates remove sulphate more effectively (Gibert *et al*., 2004). Poultry litter is amended with some additional carbon containing components. These include bedding material (rice or wheat straw / paper waste), feathers and spilt feed (Kelley *et al*., 1996; Tasistro *et al*., 2004). A slight difference in sulphate reduction efficiencies between these two wastes might be attributed to the differences of incorporated additional carbon containing stuffs.

A direct relationship between colony forming units (CFU) and sulphate reduction was observed during the initial stages of incubation. In the later stages of incubation, SRB growth became stationary. This might had happened due to insufficient availability of simple organic molecules in the final stages of incubation. These results are consistent with those of Hussain and Qazi (2012) who reported similar trends while assessing bacterial sulphate reduction using watermelon rind as a carbon source.

In this study, maximum 73% sulphate reduction occurred when composted bovine manure was used as a carbon source. It is important to note that implication of mixed cultures is advantageous over the use of pure cultures as bacterial consortia that facilitate the development of reducing conditions and these are also more easily available (Gibert *et al*., 2002). Similarly, multiple organic wastes perform better than a single waste (Waybrant *et al*., 1998 and 2002; Zagury *et al*., 2006). Further work is required to delineate sulphate reduction potential of mixed SRB cultures employing multiple organic wastes.

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