Pakistan J. Zool., vol. 56(5), pp 2001-2008, 2024 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20221124101150

Efficient Sulphate Reduction by Cellulolytic and Sulphate-Reducing Bacterial Co-Culture Using Different Agro-Industrial Wastes as Growth Substrates

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Article Information
Received 24 November 2022
Revised 18 January 2023
Accepted 28 February 2023
Available online 29 April 2023 (early access)
Published 01 July 2024

Authors' Contribution
MM performed all experiments. QA co-supervised the work. AJ worked on collecting and arranging data. JH helped in statistical analysis and compilation of data. AH supervised the work and drafted manuscript.

Key words
Aquatic pollution, Economical bioremediation, Microbial consortium, Organic wastes, Sulphate-reducing bacteria

ABSTRACT

Aquatic resources are being devastating rapidly due to the continuous intrusion of untreated wastes into the environment due to rapid industrialization and causing severe problems to aquatic life. Different physicochemical methods have been used to reduce these pollutants but all have their own limitations including production of secondary pollutants. The current study was designed to show the effect of cellulolytic and sulphate-reducing bacterial species in the form of a co-culture to treat an in-vitro prepared sulphate-rich wastewater while employing various agro-industrial organic waste as economical growth substrates. A combination of sulphate-reducing and cellulolytic bacteria in a ratio of 1:1 (v/v) was proved to be efficient for the reduction of sulphate in controlled as well as in the experimental conditions. The implicated microbial co-culture reduced 96 and 93 % of the added sulphate ($5\ gL^{-1}$) while using rice straw and animal manure, respectively in a 60-day trial of anaerobic incubation. Mixture of industrial and agricultural waste reduced about 90 % of the total added sulphate. A trend of decrease in pH with time was observed in all the incubated cultures. Our findings will be helpful for devising sustainable waste management strategies.

INTRODUCTION

In addition to many positive aspects of modern industrialized life, its eco-destructive aspects cannot be ignored (Idris *et al*., 2007). There is a close relationship between health hazards and anthropogenic sources of generation of different metallic pollutants (Campbell, 2006; Scragg, 2006; Becker *et al*., 2010; Paithankar *et al*., 2021; Karunanidhi *et al*., 2022). Different industries including food processing, potato starch, edible oil production, pulp manufacturing, petroleum refineries, textile, tanneries and solid waste process plants are adding different metallic and non-metallic pollutants in the environment constantly. Among these pollutants, various compound forms of sulphates are considered as the major culprits of environmental pollution (Wang, 2002; Boshoff *et al*., 2004; Vaiopoulou *et al*., 2005; Huang *et al*., 2006). These forms of sulphates have been causing some severe health hazards including pulmonary oedema, renal failure, hepatotoxicity, loss of consciousness and nervous disorders (Christia-Lotter *et al*., 2006; Kucukatay *et al*., 2007; Mortazavi and Jafari-Javid, 2009).

A number of physicochemical methods to treat industrial wastewaters including but not limited to solvent removal, electro-dialysis, adsorption, reverse osmosis, electrochemical extraction and coagulation

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0030-9923/2024/0005-2001 $ 9.00/0

have been applied to reduce the level of metallic and non-metallic pollutants in the wastewaters (El-Samrani *et al*., 2008; Aguado *et al*., 2009; Rafique *et al*., 2022; Pereira *et al*., 2023). All these methods require energy and generate secondary pollutants. Thus, the application of physicochemical treatment methods for the remediation of wastes can be problematic (Rocha *et al*., 2009; Batool *et al*., 2019). In this connection, implication of metals-resistant sulphate-reducing bacteria (SRB) have been proved to be potential candidates of treating sulphates and metals ions concomitantly by utilizing sulphates as their terminal electron acceptors and converting metal ions into their respective sulphides (Gadd, 2000; Diels *et al*., 2002; Kaksonen *et al*., 2004; Van Roy *et al*., 2006; Hussain and Qazi, 2016a; Hussain *et al*., 2016; Muneeb *et al*., 2020).

Various researchers have identified different types of wastes used as source of carbon and energy for bacterial sulphate reduction and consequent precipitation of metals (Hussain *et al*., 2014a, b; Hussain and Qazi, 2016b; Hussain *et al*., 2019). As SRB have not the potential to utilize complex growth substrates that's why they can be employed in the form of co-culture along with those bacteria that have the potential to convert complex substrates into simpler substrates like cellulolytic bacteria (CB) to enhance the efficiency of such remedial processes. In this co-culture, CB will degrade the implicated waste and SRB will utilize the degraded waste as growth substrate and consequently reduce sulphates and metals from the wastewater to be treated. Therefore, the present study was designed to construct a cellulolytic and sulphate-reducing bacterial co-culture that will potentially utilize cellulosic wastes as growth substrates efficiently and reduce sulphate from artificially prepared sulphate-rich wastewater.

MATERIALS AND METHODS

Microorganisms and culture media

Two bacterial species namely, *Desulfovibrio desulfuricans*-HAQ3 (SRB) and *Bacillus cereus*-HA1 (CB) were employed as co-remedial agents for the co-culturing of cellulolytic and sulphate-reducing bacteria. Both of the two bacterial species were obtained from Microbial Culture Repository of Microbial Biotechnology Laboratory, Institute of Zoology, University of the Punjab, Lahore, Pakistan. The SRB culture was initially maintained in Postgate B medium (KH_2PO_4 0.5 gL^{-1}, NH_4Cl 1 gL^{-1}, $CaSO_4$ 1 gL^{-1}, $MgSO_4.7H_20$ 2 gL^{-1}, $FeSO_4.7H_2O$ 0.5 gL^{-1}, Sodium lactate 3.5 mL gL^{-1}, Yeast extract 1 gL^{-1}, Ascorbic acid 0.1 gL^{-1}, Thioglycolic acid 0.1 mL gL^{-1}, pH: 7.0 to 7.5), while the CB culture (KH_2PO_4 0.5 gL^{-1}, (NH_4Cl 2 gL^{-1}, $CaCl_2.6H_2O$ 0.06 gL^{-1}, Na_2SO_4 2, gL^{-1}, $FeSO_4.7H_2O$ 0.005 gL^{-1}, Organic waste* 2% gL^{-1}, Yeast extract 0.1 gL^{-1}, Sodium citrate 0.3 gL^{-1}, pH: 7.0 ± 0.5) was maintained in nutrient broth.

Storage of stock cultures

The stock culture of SRB was stored at room temperature in Postgate B medium and that of CB was preserved on nutrient agar slants. SRB growth was revived in Postgate B medium, while the growth of CB was revived in nutrient broth (CM0001B, Oxoid) for all further experiments.

Optimization of growth conditions of the bacterial co-culture

The culture conditions (temperature and pH) of the bacterial consortium were then optimized using a mixture of nutrient broth and Postgate B medium in equal proportions (v/v). A temperature of 30 °C and pH range of 7.0 and 7.5 was found optimum for the both bacterial cultures.

Co-culturing of cellulolytic and sulphate-reducing bacteria

In order to construct the co-culture, the sulphate-reducing and cellulolytic bacterial species were mixed in different proportions. The bacterial ratios in co-culturing such as inoculum size of SRB (%, v/v): Inoculum size of CB (%, v/v) = 1:1, 1:2, 2:1.

Table I. Collection and processing of organic wastes as economic growth substrates for biological sulphate reduction.

Sr. No.	Major group of organic waste	Sub-type	Collection and processing of waste for experimental trial
1.	Industrial wastes	Sugarcane bagasse Molasses Animal manure	All these wastes were collected separately in clean and dry containers, oven-dried completely (60 °C), ground well and sieved to get fine powder and stored at room temperature (± 25 °C) in air-tightened glass containers.
2.	Agricultural wastes	Cotton sticks Maize sticks Rice straw Wheat straw Potato peels	
3.	Mixture	Sugarcane bagasse Molasses Animal manure Cotton sticks Maize sticks Rice straw Wheat straw Potato peels	

Effectiveness of agricultural wastes as growth substrates for the bacterial co-cultures

The capability and efficacy of various local agro-industrial organic wastes to support the biological sulphate reduction was estimated. For this purpose, agro-industrial wastes were collected and processed as shown in Table I. The experiments for this purpose were performed under batch cultivation conditions.

Batch experiments

Sterile serum vials (120 mL) were used to perform the experiment in triplicate. Different agro-industrial wastes were used independently as growth substrates instead of sodium lactate in Postgate B medium. Initially, the concentration of SO_4^{2-} was adjusted to 5 gL^{-1} in all the vials using sodium sulphate. The wastes (2 %) were added to the medium independently except the control group. Different proportions (v/v) of inocula of CB and SRB were made for the experiments. To make the conditions an-aerobic, a layer of autoclaved and cooled liquid paraffin (about 3−5 mm thick) was put over the vials. These vials were kept at 30 °C in an incubator (Memmert, IN55plus, Germany) for 60 days after inoculation and sealing of vials with butyl rubber stoppers and aluminium crimp seals.

Analysis of various parameters

To analyse SO_4^{2-} and pH, after every ten days, a sample of 5 mL was taken out with the help of sterilized syringes and centrifuged for 10 min at 4000 rpm. Percentage of SO_4^{2-} was estimated after Cha *et al*. (1999), while pH was measured using InoLab 7110 basic pH/mV/T bench top meter (Germany).

Statistical analysis

The data were analysed according to Completely Randomized Design (CRD, α = 0.05) under factorial arrangement using General Linear Model (GLM) procedures. Means were separated out using Duncan's Multiple Range (DMR) test with the help of SAS 9.1 for windows.

RESULTS

Growth conditions of the bacterial co-culture

Bacterial growth was optimized on the basis of C.F.U. counts and quantity of sulphate reduced. Bacterial strain showed a direct relationship between sulphate reduction and C.F.U. counts. The bacteria showed maximum growth and sulphate reduction at 30 °C with pH 7.0−7.5 using an inoculum of 1:1 (SRB: CB). That's why all further experiments were performed using this proportion of the bacterial species.

Biological sulphate reduction

There was 100 % reduction of SO_4^{2-} under control conditions using sodium lactate as a source of carbon within ten days of the incubation (Tables II and III).

In the vials where agricultural wastes were used carbon sources, during first 10−30 days of incubation, the sulphate was reduced up to 70% but in the later stagesof incubation (40−60 days), sulphate reduction became slower. Among different employed agricultural wastes, rice straw appeared as the most suitable carbon source for biological reduction of sulphate (Tables II and III). A trend of decrease in pH was observed in all the cultures (Table IV).

Table II. Pattern of biological sulphate reduction following periodic incubation of 10 days using agro-industrial wastes as growth substrates.

Agro-industrial waste	Incubation period (days)					
	10	20	30	40	50	60
Sugarcane bagasse	2.84 ± 0.11[bcd]	0.59 ± 0.21[bc]	0.74 ± 0.06[b]	0.77 ± 0.14[ab]	0.54 ± 0.11[b]	0.43 ± 0.02[ab]
Animal manure	0.71 ± 0.16[e]	0.40 ± 0.10[bc]	0.69 ± 0.09[b]	0.52 ± 0.18[b]	0.59 ± 0.28[b]	0.34 ± 0.05[b]
Rice straw	2.89 ± 0.22[bc]	0.29 ± 0.04[bc]	0.48 ± 0.38[b]	0.20 ± 0.03[c]	0.70 ± 0.13[ab]	0.34 ± 0.03[b]
Wheat straw	2.85 ± 0.17[bcd]	0.83 ± 0.08[bc]	0.56 ± 0.13[b]	0.64 ± 0.04[ab]	0.51 ± 0.22[ab]	0.39 ± 0.13[ab]
Maize straw	3.35 ± 0.64[b]	1.10 ± 0.34[b]	0.75 ± 0.15[b]	0.71 ± 0.02[ab]	0.96 ± 0.12[b]	0.40 ± 0.02[ab]
Cotton sticks	2.22 ± 0.28[d]	0.47 ± 0.12[bc]	0.79 ± 0.02[b]	0.64 ± 0.20[ab]	0.59 ± 0.11[b]	0.52 ± 0.16[ab]
Potato peels	2.44 ± 0.31[cd]	0.98 ± 0.77[b]	0.50 ± 0.11[b]	0.52 ± 0.16[b]	0.65 ± 0.20[ab]	0.65 ± 0.20[ab]
Mixture	2.91 ± 0.24[bc]	1.12 ± 0.50[b]	0.76 ± 0.16[b]	0.86 ± 0.23[a]	0.67 ± 0.17[ab]	0.51 ± 0.06[ab]
Molasses	4.09 ± 0.63[a]	3.74 ± 1.15[a]	1.33 ± 0.11[a]	0.81 ± 0.26[ab]	0.75 ± 0.28[ab]	0.64 ± 0.32[a]
Sodium lactate	0.00 ± 0.00[f]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[c]

Values represent sulphate concentration (gL^{-1}) and are means ± S.E. of three replicates. Those not sharing a common alphabet within a respective column are significantly different from each other. Single factor analysis of variance at $P<0.05$

Table III. Percentage of biological sulphate reduction following periodic incubation of 10 days using agro-industrial wastes as growth substrate.

Agro-industrial waste	Incubation period (days)						
	0	10	20	30	40	50	60
Sugarcane bagasse	0	43.00	84.00	85.14	88.04	89.00	91.00
Animal manure	0	85.76	86.14	88.00	89.40	92.00	93.00
Rice straw	0	42.10	86.00	90.22	93.00	94.00	96.00
Wheat straw	0	44.00	83.40	87.00	89.00	90.00	92.00
Maize sticks	0	33.00	78.00	81.00	85.00	86.00	92.00
Cotton sticks	0	55.42	84.00	87.00	88.00	90.00	92.00
Potato peels	0	52.00	80.24	87.00	89.00	90.00	92.00
Mixture	0	42.00	78.00	83.00	85.00	87.00	90.00
Molasses	0	18.04	25.02	73.38	81.42	83.00	90.00
Sodium lactate	0	100.0	100.0	100.0	100.0	100.0	100.0

Table IV. Pattern of pH following periodic incubation of 10 days using agro-industrial wastes as growth substrates.

Agro-industrial waste	Incubation period (days)					
	10	20	30	40	50	60
Sugarcane bagasse	7.10 ± 0.00^{a}	6.20 ± 0.20^{ef}	5.76 ± 0.55^{d}	5.40 ± 0.10^{d}	5.43 ± 0.15^{d}	5.43 ± 0.15^{e}
Animal manure	7.13 ± 0.05^{a}	7.13 ± 0.06^{ab}	6.93 ± 0.05^{ab}	7.06 ± 0.05^{a}	7.10 ± 0.00^{a}	7.23 ± 0.05^{a}
Rice straw	7.03 ± 0.32^{a}	6.90 ± 0.10^{bc}	6.26 ± 0.11^{c}	6.13 ± 0.05^{c}	6.16 ± 0.12^{c}	6.30 ± 0.10^{c}
Wheat straw	6.56 ± 0.32^{b}	6.43 ± 0.30^{de}	6.43 ± 0.28^{c}	6.30 ± 0.26^{bc}	6.30 ± 0.17^{c}	6.40 ± 0.26^{bc}
Maize straw	6.33 ± 0.15^{b}	6.70 ± 0.10^{cd}	6.20 ± 0.20^{c}	6.26 ± 0.05^{c}	$6.40 \pm 0.00b^{c}$	6.66 ± 0.20^{b}
Cotton sticks	7.03 ± 0.05^{a}	6.93 ± 0.06^{bc}	6.56 ± 0.05^{bc}	6.60 ± 0.00^{b}	6.66 ± 0.06^{b}	6.60 ± 0.00^{bc}
Potato peels	5.43 ± 0.30^{d}	5.30 ± 0.30^{g}	4.60 ± 0.17^{f}	4.50 ± 0.17^{f}	4.70 ± 0.10^{e}	4.66 ± 0.11^{f}
Mixture	5.90 ± 0.10^{c}	5.93 ± 0.25^{f}	5.53 ± 0.32^{de}	5.70 ± 0.40^{d}	5.66 ± 0.35^{d}	5.80 ± 0.36^{d}
Molasses	5.23 ± 0.15^{d}	5.46 ± 0.28^{g}	5.13 ± 0.25^{e}	4.83 ± 0.20^{e}	5.40 ± 0.26^{d}	5.36 ± 0.305^{e}
Sodium lactate	7.30 ± 0.00^{a}	7.30 ± 0.00^{a}	7.30 ± 0.00^{a}	7.30 ± 0.00^{a}	7.30 ± 0.00^{a}	7.30 ± 0.00^{a}

Values represent pH and are means ± S.E. of three replicates. Those not sharing a common alphabet within a respective column are significantly different from each other. Single factor analysis of variance at $P<0.05$

Among different industrial wastes, the most efficient carbon source was animal manure (93 %) for the reduction of sulphate followed by sugarcane bagasse (91 %) and molasses (90 %) (Tables II and III).

Almost same trend of sulphate reduction was observed for the mixture of agro-industrial waste as seen for the individual wastes. Up to 85 % sulphate was reduced at the end of 60 days, however, there was more than 70 % sulphate reduction in the first 30 days of incubation (Tables II and III).

DISCUSSION

In the controlled experiments, there was 100 % reduction of sulphate within first 10 days of incubation as there were optimum conditions for SRB growth having sodium lactate as a source of carbon and slight basic pH of the medium. Several researchers have concluded parallel observations for SRB growth using simpler growth substances and basic pH (Zagury *et al*., 2006; Martins *et al*., 2009). Agro-industrial organic wastes proved to be good sources of carbon for the growth of SRB, though sulphate reduction appeared slow when compared to cultures amended with sodium lactate. During the first half of incubation (up to 30 days), the rate of sulphate reduction was higher compared to the latter half (30−60 days) possibly due to low sulphate contents and accumulation of metabolic wastes of the bacteria in the latter half (Hussain and Qazi, 2016b; Hussain *et al*., 2019; Zhang *et al*., 2019).

All agricultural organic wastes appeared to be a good source of carbon for the activity of SRB, however, rice straw appeared as the most efficient organic waste in this regard which caused reduction of sulphate up to 96 % in the anaerobic trial of 60 days. It indicates that cellulose fibbers of rice straw are an easy target for the cellulolytic bacteria as compared to wheat straw, maize wastes, potato peels etc. However, while studying the efficiency of different agro-industrial wastes as cost-effective carbon sources for biological sulphate reduction, a reverse relation between waste complexity and sulphate reduction has been reported by a number of researchers (Martins *et al*., 2009; Hussain and Qazi, 2012). Among industrial organic wastes, CB can efficiently decompose comparatively simpler/semi-digested molecules found in animal manure (Nagpal *et al*., 2000; Gibert *et al*., 2004; Tsukamoto *et al*., 2004; Zagury *et al*., 2006; Hussain and Qazi, 2012; Dhakal *et al*., 2020). Moreover, these simple organic molecules also create low redox potential which in turn is responsible for creating an appropriate environment for the growth of SRB (Cohen, 2006). Semi-digested organic molecules in animal manure also provides the reasonable ratio between carbon and nitrogen which should be between 44−120 (Gibert *et al*., 2004) as less carbon to nitrogen ratio in organic waste resists to provide carbon for the SRB and consequently causes less efficiency of these bacteria (Munwar and Riwandi, 2010). Moreover, higher the lignin contents in the organic waste lower will be the activity of the SRB as lignin is comparatively harder for biodegradation and providing carbon (Gibert *et al*., 2004; Zhang and Wang, 2014; Chai *et al*., 2023). Most likely, all these were the reasons that in the presence of animal manure SRB and CB consortium showed maximum reduction (93 %) of sulphate as compared to molasses and sugarcane bagasse.

There was 91 % reduction of sulphate by SRB after the completion of incubation period (60 days). Sugarcane bagasse reduced 91 % after 60 days of incubation. There is complex as well as simple sugars in sugarcane bagasse and during the lag phase of incubation, SRB might have taken time in acclimation. In a complex nutrient medium containing different complex soluble carbon source, diauxic growth pattern of microbes is a common phenomenon (Crueger and Crueger, 2005; Hussain and Qazi, 2016b; Laraib *et al*., 2021a, b). Among industrial organic wastes, molasses showed lesser sulphate reduction (73 %) at the end of incubation period. A number of researchers concluded that there are several non-biodegradable substances along with high amount of volatile unsaturated fatty acids which might be unfavourable for the reduction of sulphate by SRB (Maree *et al*., 1991). Several researchers believe in that a mixture of SRB cultures can use molasses as an acceptable source of carbon for sulphate reduction (Maree *et al*., 1986; Maree and Hill, 1987; Annachhatre and Suktrakoolvait, 2001; Hussain *et al*., 2014a).

A mixture of all the organic wastes, i.e., molasses, animal manure, cotton sticks, wheat straw, potato peel, sugarcane bagasse, rice straw and maize sticks caused sulphate reduction at the end of 60th day of anaerobic incubation period up to 90 %. Here sulphate reduction was same as it was for the individual organic waste, indicating that there was no antagonistic nor supporting phenomenon was involved in the mixture.

In the start of the experiment, pH of all the media was neutral, i.e., 7.0 ± 0.5, however, as far as there was sulphate reduction and release of H_2S gas during the first half of the incubation period, a decreasing trend (slightly acidic) of pH was observed. It was again close to neutral or slight basic in the 2nd half of the incubation period (40 to 60 days) due to a decrease in rate of the sulphate reduction reaction. Martins *et al*. (2009) also reported the relationship between neutral to slight basic pH of the medium to the sulphate reduction ability of SRB.

For agricultural countries like Pakistan there is huge production of agri-wastes per day that is manic to the environment. Our study will provide a baseline data for the bioremediation of industrial sulphates along with the consumption of agro-industrial organic wastes which are themselves sources of point and non-point source pollution. For speedy bioremediation process, it is suggested that there should be natural substrates composed of simple molecules with minimum lignin contents for the effective growth of SRB. On the other hand, the existing substrate should be modified with appropriate additions for the optimum carbon to nitrogen ratio and de-lignified in addition to source of simple molecules for speedy growth of SRB for the effectively sulphate elimination strategies.

ACKNOWLEDGEMENT

The support of Dr. Shahid Ali, Associate Professor, F.G. College, Gujranwala Cantt., is highly acknowledged for carefully editing the manuscript.

Funding

No funding was received for this study.

IRB approval

The research work was approved by Departmental Board of Studies of Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore, Paki-stan.

Ethical approval

Not applicable.

Availability of data and materials

These will be provided by the corresponding author on reasonable request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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