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Development of microbial enrichments for simultaneous removal of sulfur and nitrogenous metabolites in saline water aquaculture

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Abstract

Aim: The aim of the study was to develop microbial enrichments from the nitrifying microbial consortia and the environment for simultaneous removal of ammonia, nitrate, and sulfide in aquaculture systems at varied salinities.

Methods and results: Sulfur and nitrogen metabolites are the major factors affecting the farmed aquatic animal species and deteriorate the receiving environments causing ecological damage. The present study reports the development of microbial enrichments from the nitrifying microbial consortia and the environment. The enrichments used thiosulfate or thiocyanate as an energy source and simultaneously removed sulfur, ammonia, and nitrite in spiked medium (125 mg/l ammonia; 145 mg/l nitrite). Further, the microbes in the enrichments could grow up to 30 g/l salinity. Metagenomic studies revealed limited microbial diversity suggesting the enrichment of highly specialized taxa, and co-occurrence network analysis showed the formation of three micro-niches with multiple interactions at different taxonomic levels.

Conclusions: The ability of the enrichments to grow in both organic and inorganic medium and simultaneous removal of sulfide, ammonia, and nitrite under varied salinities suggests their potential application in sulfur, nitrogen, and organic matter-rich aquaculture pond environments and other industrial effluents.

Significance and impact of study:

The enrichments could be used to degrade environmental pollutants and promote sustainable agriculture and aquaculture practices. Further, the ability of these enrichments to utilize thiosulfate and thiocyanate in addition to sulfide suggests their potential application in industrial wastewater treatment plant.

Keywords: bioremediation, aquaculture, SOB, desulfurization

Introduction

Aquaculture is the fastest-growing food production sector, with an average growth of more than 8% over decades (Handbook on Fisheries Statistics 2020). Nearly half of the fish produced is sourced from aquaculture; the international fish and fishery products market was worth USD 164 billion in 2018 (FAO 2020). Economic sustainability of the sector depends on farm intensification with the stocking of seeds in higher densities and the application of a higher quantity of feed and other growth promoters.

The expansion of aquaculture activities worldwide is limited by biotic and abiotic stress (Ciji and Akhtar 2021). Diseases are the complex outcome of the interaction of host, pathogen, and environment. In aquaculture, however, environmental stress plays a significant role in the health and production of farmed aquatic animals. In semi-intensive/intensive aquaculture operations, accumulated toxic metabolites in the pond bottom due to uneaten feed and excreta of the animals are considered the major factor responsible for environmental stress and increased susceptibility to opportunistic pathogens (Dauda et al. 2019). Further, zero water exchange during the culture period due to biosecurity concerns has led to the deposition of sludge in the pond bottom and the build-up of toxic metabolites in the culture environment (Lananan et al. 2014). Decomposing the accumulated organic waste (uneaten feed, faecal matter, algal debris, etc.) generates sulfide, which gets deposited in the pond sediments, affecting farmed aquatic animals, especially the Pacific whiteleg shrimp, *Penaeus vannamei*, which dwells in the benthic zone.

Under aerobic conditions, sulfur in the suspended pond water decomposes to sulfide and oxidizes to sulfate; under anaerobic conditions, this sulfate is metabolized to hydrogen sulfide (Avnimilech and Ritvo 2003, Antony and Philip 2006). Additionally, in environmental conditions with higher organic matter load and lower dissolved oxygen, sulfur-reducing bacteria decompose organic sulfur to produce H_2S. Brackishwater aquaculture is more prone to H_2S toxicity as seawater is rich in sulfate (Letelier-Gordo et al. 2020). Sulfur-oxidizing bacteria (SOB) in a dissimilatory sulfate reduction process produce H_2S by using sulfate as a terminal electron

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acceptor during the degradation of organic matter (Gerardi 2006). Pond environment parameters like pH, temperature, the concentration of sulfate, and bioavailability of organic matter determine the sulfide production rate (Muyzer and Stams 2008).

Formation of metal sulfides (iron sulfide or manganese sulfide, etc.) leads to blackening of water and pond bottoms, and dissipation of H_2S leads to rotten egg smell excreting stress on the farmed aquatic animal species and other living beings in the receiving natural water bodies. Further, direct discharge of shrimp pond water into the surroundings might lead to deterioration of receiving water bodies causing ecological damage. The reported toxic level of H_2S for fish was 0.002 mg/l and 0.0087 and 0.0185, respectively, for larval and juvenile stages of *P. vannamei* (Reynolds1976 , US Environment Protection Agency 2010). Toxicity due to sulfide depends on the concentration of H_2S, pH, species, and growth stage of shrimp. Shrimp exposed to H_2S suffer from hypoxia due to inhibition of cell respiration (Affonso et al. 2002). Chronic exposure can affect gluconeogenesis, proteins synthesis, and energy metabolism (Li et al. 2017), in addition to intestinal damage and altered gut microbial composition, while acute toxicity is reportedly disturbing the intestinal integrity and immunity (Duan et al. 2017) and microbiome diversity (Duan et al. 2019).

Further, constant exposure to H_2S at lower doses has reportedly suppressed the shrimp's immunity increasing its susceptibility to pathogens (Vismann 1996). The unionized H_2S, which readily crosses the cell membrane, increases with decreasing pH (Boyd 2017). Further, sulfide is known to inhibit nitrification, causing ammonium accumulation, leading to nitrogen toxicity in the *P. vannamei* shrimp ponds (Joye and Hollibaugh 1995).

Generally, the issue of black coloured and foul odours on pond bottoms is addressed by manual removal during the inter-cropping period, which is labor intensive. Alternatively, the application of probiotics was reportedly improving the water and sediment quality in aquatic environments (Antony and Philip 2006, Martinez Cruz et al. 2012, Boyd 2014). Studies have reported the role of microbial communities in maintaining environmental quality in natural and farming conditions (Boyd and Tucker 1998, Mischke 2003). Disruption of such microbial community structure in intensive/semi-intensive aquaculture operations has been suggested to be the buildup of toxic metabolites (Graslund et al. 2003, Schiller 2011, Arias-Moscoso et al. 2018). The biological removal of toxic sulfide from industrial and agricultural discharges with particular reference to the role of SOB has been extensively reviewed (Tang et al. 2009, Lohwacharin and Annachhatre 2010, Pokorna and Zabranska 2015, Lin et al. 2018). SOBs oxidize sulfide to sulfate and elemental sulfur (Li et al. 2017).

Though several studies have reported the bioremediation of nitrogenous metabolites in aquaculture, literature on the bioremediation of sulfur metabolites is limited. The purple and green photosynthetic sulfur bacteria present in the sediments are capable of utilizing H_2S under anaerobic conditions. Bacteria belonging to the *Chromatiaceae* and *Chlorobiaceae* can be mass-produced and applied for effective management of H_2S in aquaculture ponds (see Jasmin et al. 2020). Recently, biological removal of sulfur from soil and wastewater using heterotrophic, chemolithotrophic, chemoautotrophic, and mixotrophic SOB has reportedly been found to be efficient (see Sun et al. 2019). Similarly, biological oxidation using autotrophic denitrifying SOB has also been investigated (Ramadhani et al. 2017).

We have recently reported the development of nitrifying and denitrifying bacterial consortia (Baskaran et al. 2020) and evaluated the formulation's performance under commercial shrimp farming conditions to control the toxic nitrogenous metabolites (Patil et al. 2021b). Similarly, microbial enrichments containing SOBs could be explored to mitigate toxic sulfur metabolites in aquaculture operations. The metagenomic analysis of nitrifying consortia revealed the presence of several genera of bacteria with potential sulfur-oxidizing ability. Although several SOB groups' essential characterization, growth optimization, and utility in the biological removal of sulfur in polluted environmental waters are reported extensively, studies on sulfur-oxidizing enrichments, microbial profiling, and their ability to mitigate sulfur molecules to evaluate their potential application in shrimp farming operations have not been reported. The present study reports the development and microbial profile of six SOB enrichments from previously developed nitrifying consortia and brackish water environments using 16S rDNA sequencing analysis. Further, their sulfur oxidation efficiency was evaluated to develop a method of microbial bioremediation for the effective removal of toxic sulfur metabolites in anoxygenic conditions at the soil-water interface in shrimp culture ponds.

Materials and methods

Enrichment for SOB

Samples for SOB enrichment ($n = 6$) were selected from nitrification bacterial consortia (SOB1 to SOB4) developed previously (Dinesh Kumar 2013, Baskaran et al. 2020) and brackishwater environmental samples (SOB5 and SOB6) located in the East Coast region of India. Chemoautotrophic ammonia and nitrite-oxidizing bacteria consortia were developed for bioremediation of total ammonia nitrogen in the brackishwater shrimp farms using Koops medium and Watson and Waterbury medium, as described by Dinesh Kumar (2013). The SOB medium comprised $(NH_4)_2SO_4$ (0.04%), $MgSO_4$ (0.05%), $FeSO_4$ (0.001%), KH_2PO_4 (0.40%), $CaCl_2$ (0.025%), NaCl (1.00%), and yeast extract (0.05%) was sterilized by autoclaving at 121°C for 20 min, followed by adding filtered $Na_2S_2O_3$ (1.00%) (Starkey 1935). SOB medium was inoculated with 10% inoculum and maintained with vigorous aeration. For estimation of hydrogen sulfide, the SOB medium contained H_2S (100 mg/l) replacing sodium thiosulfate was used; the pH was adjusted to 7.5 by the addition of 0.1 M NaOH. Multiple samples from the six sources were enriched in the SOB medium, and cultures showing promising growth as indicated by observed turbidity and reduction of pH of the medium were harvested and processed further.

Growth and sulfide oxidation efficiency

SOB medium with and without 0.5% yeast extract as an organic carbon source was prepared in a serum bottle and inoculated with enrichments. The medium was inoculated with SOB enrichments and incubated at 30°C for 24 h under shaking at 100 rpm. The effect of the organic substance on the growth and oxidation efficiency was determined by measuring the sulfide concentrations and OD_{600}, respectively, at the end of the incubation period.

Oxidation of inorganic sulfur compounds

The sulfur oxidation ability of enrichments was tested using different sulfur compounds as the sole energy source. The sulfur compounds used in the study were thiosulfate (1%) or thiocyanate (1%) and the SOB enrichment medium containing H_2S. Medium was inoculated with each of the SOB enrichments and incubated at 30°C for 24 h with shaking at 100 rpm. The bacterial growth and sulfide oxidation efficiency were recorded by measuring the sulfide concentrations and OD_{600}, respectively, at the end of the incubation period.

Effect of ammonia and nitrate on growth and sulfide oxidation efficiency

The SOB medium was supplemented with Ammonia-N (125 mg/l) and nitrate (145 mg/l), inoculated with each of the SOB enrichments, and incubated at 30°C for 24 h with shaking at 100 rpm. The bacterial growth and sulfide oxidation efficiency were recorded by measuring the sulfide concentrations and OD_{600}, respectively, at the end of the incubation period.

Effect of salinity on sulfur oxidation efficiency

The SOB medium was prepared with different salinity (0, 5, 10, 20, and 30 g/l), inoculated with each of the SOB enrichments, and incubated at 30°C for 24 h with shaking at 100 rpm. The bacterial growth and sulfide oxidation efficiency were recorded by measuring the sulfide concentrations and OD_{600}, respectively, at the end of the incubation period.

Sulfide oxidase assay

The quantitative determination of the sulfide oxidase activity was determined by measuring the sulfate (SO_4^{2-}), a product of enzyme reaction (Hirano et al. 1996). The sulfate ion concentration as an indicator of sulfide oxidase activity was measured by recording the absorbance at 450 nm in the spectrophotometer (UV-1800, Shimadzu, Japan). The sulfate concentration in the sample was proportional to the turbidity formed, and one unit of sulfite oxidase activity was defined as an amount of the enzyme required to produce 1 µmol sulfate/h/ml (U/ml).

Analytical method

Sulfur oxidation efficiency of enrichments was estimated by methylene blue method, microbial growth by measuring OD at 600 (UV spectrophotometer, Shimadzu UV1800, Japan), while the concentrations of ammonia and nitrate were measured by Nessler's and Griess's methods, respectively (APHA 2012).

DNA extraction, PCR amplification, and high-throughput sequencing

DNA was extracted from SOB enrichments by phenol: chloroform method (Sambrook et al. 1989), and DNA concentration and purity were determined using Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, MA, USA). High-quality DNA was subjected to PCR amplification and next-generation sequencing (Macrogen, South Korea), targeting the 16S rRNA V3–V4 hyper-variable region using primers F: CCTACGGGNGGCWGCAG and R: GACTACHVGGGTATCTAATCC to profile bacterial communities. The amplicon libraries were prepared as per the metagenomic sequencing library preparation protocol. The amplicons were sequenced using the Illumina MiSeq platform.

Bioinformatics and statistical analysis

The raw sequences were processed using the MOTHUR pipeline (v. 1.42) (Schloss et al. 2009) to filter reads, create contigs, and reduce noise as per the standard MiSeq procedure. Sequences were aligned, clustered, and identified taxonomically with the SILVA database (http://arb-silva.de) release 138. Sequences with a 97% identity threshold were classified into operational taxonomic units at genetic distances of 0.03. For visualization, the gene abundance data output was subjected to detailed statistical and meta-analysis using an online tool, MicrobiomeAnalyst (Dhariwal et al. 2017). Heatmaps were generated with the Euclidean distance matrix and Ward clustering. Venn diagram was prepared using a web-based tool, interactiVenn (Heberle et al. 2015). Co-occurrence network analysis was performed to understand the relationship with different genera, which was analyzed using Spearman rank correlation based on the relative abundance of genera from the MOTHUR output. Only those interactions that had a P-value $< .05$ were considered. All correlation relationships which passed the significance level were admitted into the network and visualized using Cytoscape software (Version 3.8.3),and the MCODE plugin for Cytoscape was applied to detect network clusters in the data (modules). The topological parameters were calculated as the most important statistical descriptors of the network using the Cytohubba plugin.

Results

Effect of organic and inorganic media on growth and sulfide oxidation efficiency

All the SOB enrichments showed microbial growth and oxidized the sulfide in organic and inorganic media (Table 1, Fig. 1). However, the growth, as observed through the increase in OD600 and the proportionate increase in the sulfide removal efficiency was significantly higher in medium with organic supplementation. The observed higher growth might be due to the energy required for the bacterial synthesis from organic sources being considered one-eighth of the inorganic source. The specific activity of sulfide oxidase for each of the enrichments was proportional to the growth of the bacterial enrichments. Among the six enrichments, higher growth was observed in enrichments SOB2 and SOB5 in both media. Further incorporation of yeast increased their efficiency to remove sulfur from 20.30% to 57.35% and from 20.18% to 54.18% (Fig. 1).

Similarly, a general improvement in the enzyme-specific activity was observed following the incorporation of organic substances in the medium. Results suggest that SOB2 and SOB5 could grow both in organic and inorganic environments and efficiently remediate sulfur metabolites in aquaculture pond bottoms.

Effect of different sulfur sources on growth and sulfide oxidation efficiency

Enrichments SOB1, SOB2, and SOB5 showed significantly higher growth and proportionally higher H_2S removal efficiency than other enrichments (Fig. 2). The observed higher growth and the efficiency in thiocyanate medium suggest their

Table 1. Sulfide oxidase activity of SOB enrichments.

Strain No.	Inorganic media			Organic media		
	Sulfide oxidase activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Sulfide oxidase activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
SOB1	4.09	4.21	**0.97**	5.59	4.11	**1.36**
SOB2	4.72	3.67	**1.29**	5.96	3.88	**1.54**
SOB3	2.27	3.81	**0.60**	5.22	4.60	**1.14**
SOB4	3.96	4.25	**0.93**	5.55	4.12	**1.35**
SOB5	3.86	4.19	**0.92**	5.18	4.60	**1.13**
SOB6	5.69	4.21	**1.35**	5.89	4.35	**1.35**

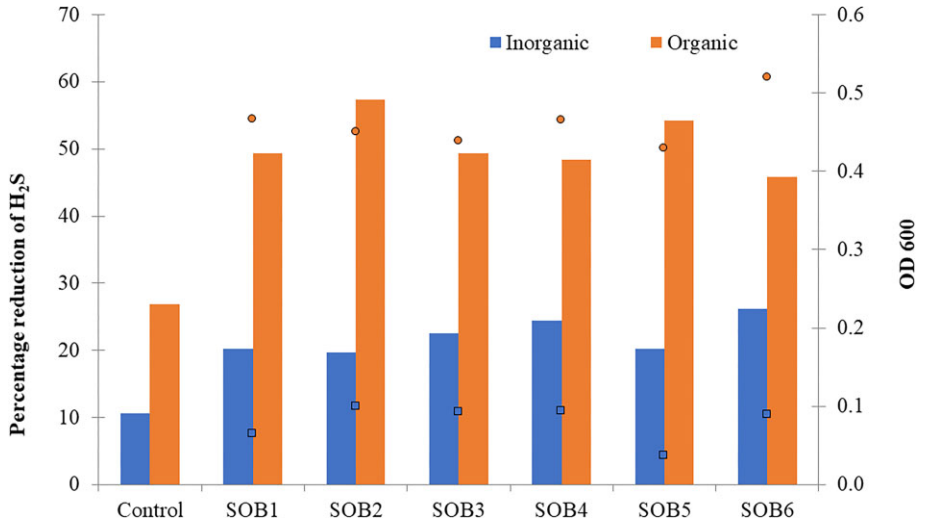

Figure 1. Growth and efficacy of SOB enrichments in organic and inorganic media.

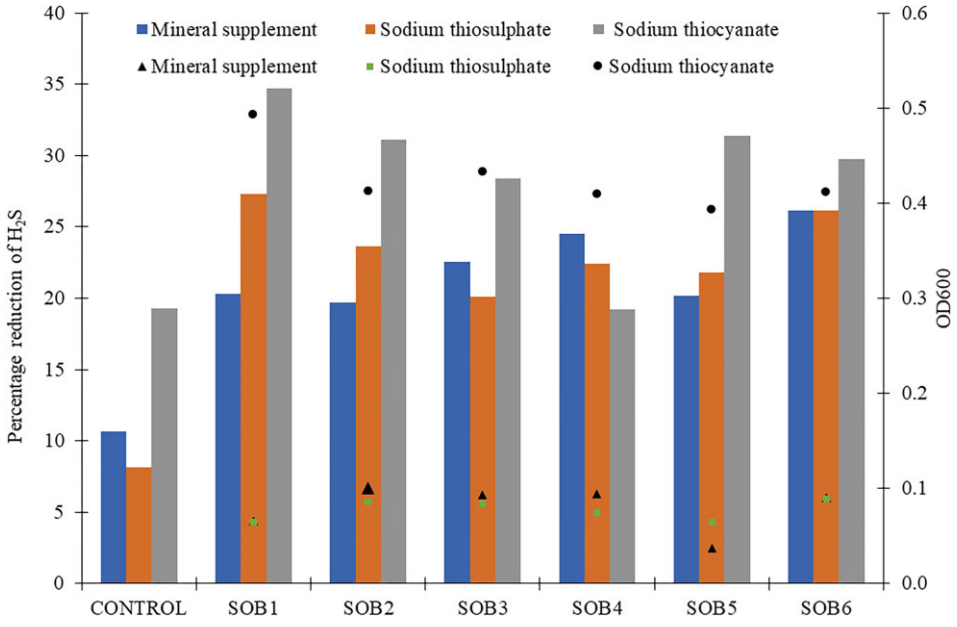

Figure 2. Growth and efficacy of SOB enrichments in different sources of sulfur.

ability to use thiocyanate as an energy source compared to thiosulphate.

Simultaneous removal of sulfide, nitrate, and ammonia

There was a general increase in the efficacy of SOB enrichments in the presence of nitrate. Three of the enrichments, SOB1, SOB2, and SOB4, showed more than 50% efficiency in reducing the levels of H_2S. There was a general improvement in the nitrate removal efficiency at lower levels (up to 2-fold) (Fig. 3). At the concentration of 125 mg/l tested in the present study, a 3–4-fold increase in the sulfide removal ability in three enrichments (SOB1, SOB2, and SOB5) was observed. Interestingly, the isolates were also efficient in reducing ammonia concentration between 86% and 63% (Fig. 3).

Effect of salinity on growth and sulfide oxidation efficiency

Growth and sulfur oxidation efficiency were evaluated up to 30 ppt to evaluate the salinity tolerance of enrichments. Higher growth was observed at 10 ‰ and efficiency of sulfur oxidation between 5 and 10 ‰ among all the enrichments (Fig. 4).

Community composition and diversity profile

The chimaeras and undesirable sequences were removed to obtain 1466 392 high-quality reads from 18 samples with an average of 81 466 reads. The raw sequences obtained in this study are available at NCBI BioProject ID PRJNA751554. The sequences with 97% similarity were taxonomically classified into 131 features at 20% prevalence (≥2). The taxonomic analysis revealed the presence of 6–11 phyla and 57–112 genera among the SOB enrichments. The richness indices in Chao and ACE ranged between 42.1 ± 5.2 to 97.9 ± 5.6 and 44.3 ± 3.2 to 97.3 ± 4.7, respectively. The diversity indices in Shannon and Simpson ranged between 0.3 ± 0.01 to 2.26 ± 0.07 and 0.10 ± 0 to 0.85 ± 0.02, respectively (Table 2). Unique and shared SOB genera among the six enrichments are depicted in Venn diagram (Fig. 5). *Proteobacteria* and *Firmicutes* were the dominant phyla in all the SOB enrichments (Fig. 6). *Bacillus, Brucella, Pseudomonas*, *Acidihalobacter*, *Pseudoxanthomonas*, *Alcaligenaceae*_unclassified at the genus level *Bacillaceae*_unclassified were dominating in addition to *Brevibacillus, Paenibacillus*, and *Nitratireductor* (Fig. 7).

Network analysis

The network was used to visualize the correlation of dominant genera based on Spearman rank correlation ($-1 < \rho < -0.5$) and ($0.5 < \rho < 1$) and was significant (P-value < .05), which gives both symbiotic and commensal relationships of microbes in SOB enrichments. Network analysis revealed complex relationships among the different genera; the blue and red lines indicate positive and negative correlations. The network grouped 72% of the microbes into three specific "microniches"; module 1 (37%), module 2 (17%), and module 3 (14%), while 28% of the microbes were unclustered. The seed genus in each cluster is depicted in a rhomboid shape. The co-occurrence patterns revealed the complex grouping and association between the microbial taxa in the SOB enrichments (Fig. 8).

Discussion

In aquaculture practice, the organic matter builds up in the pond bottom as the culture progress due to the accumulation of uneaten feed, animal faeces, and dead plankton leading to increased microbial activity (Boyd 2017) and H_2S formation. This study reports the development of microbial enrichments to mitigate sulfur toxicity in the aquatic systems. The ability to grow both in organic and inorganic media suggests the versatile nature of the enrichments and their bioremediation potential in aquaculture environments.

SOBs are chemolithoautotrophic bacteria that obtain energy from the oxidation of inorganic sulfur compounds. These substrates act as electron donors, and nitrate, nitrite, and nitrous oxide function as terminal electron acceptors in autotrophic denitrification (Kelly and Wood 2000). The observed higher growth and the efficiency in thiocyanate medium suggest their ability to use thiocyanate as an energy source compared to thiosulphate. Chemical or microbial oxidation of sulfides in the sulfur cycle involves intermediate sulfur species like elemental sulfur, polysulfides, thiosulfate, and sulfite. At the same time, industrial discharges and abiotic decomposition of organic matter are the primary sources of thiocyanate to water bodies (see Kurashova et al. 2018, Jørgensen et al. 2019). SOBs utilize all types of reduced sulfur compounds as energy sources and mostly oxidize them to sulfate. Further, several species of bacteria have been shown to degrade thiocyanate using different biochemical pathways and obtain energy or a source of sulfur or nitrogen for their growth (see Gould et al. 2012). The higher growth and H_2S removal efficacy among the enrichments following the supplementation of thiocyanate and thiosulphate suggest the possible utility of these enrichments in the mitigation of different sulfur compounds.

Autotrophic denitrifying and some mixotrophic SOBs generally use nitrate as electron acceptors (Yu et al. 2014). A recent study reporting the concentration-dependent increase in the efficiency of SOBs following the incorporation of nitrate suggested the ability of SOBs to drive energy by autotrophic denitrification (Juang et al. 2015, Sun et al. 2019). The addition of nitrate into the aquaculture ponds decreased the built-up H_2S in the system (Torun et al. 2020). Studies have also reported the utility of sulfur-based autotrophic and mixotrophic denitrifiers to remove nitrate from the drinking water (Sahinkaya et al. 2011, 2012); sulfur and nitrate act in the process as electron donors and acceptors, respectively. However, the present study did not observe the inhibitory effect of nitrate on a few of the SOB isolates (Sun et al. 2019).

The inhibitory effect of ammonia at higher concentrations (120 mg/l) on denitrifying sulfide removal in biofilters was reported earlier (Chung et al. 2001, Tsang et al. 2015) however, at concentrations between 50–110 mg/l, a new pathway was proposed suggesting the use of ammonia instead of nitrate or nitrite as nitrogen (Juang et al. 2015). The presence of sulfide improved the ammonia removal efficiency of the enrichments. In a similar earlier study, improvement in the ammonia removal efficiency in the presence of sulfide was suggested to be due to a chemical reaction with SO_4, and no interference of H_2S on the enzymatic activity of nitrifying bacteria was reported (Tsang et al. 2015).

Generally, the salinity in brackishwater aquaculture varies from 5 to 30 ‰. Bioremediation of sulfur metabolites in aquaculture farming systems requires salinity-specific microbial

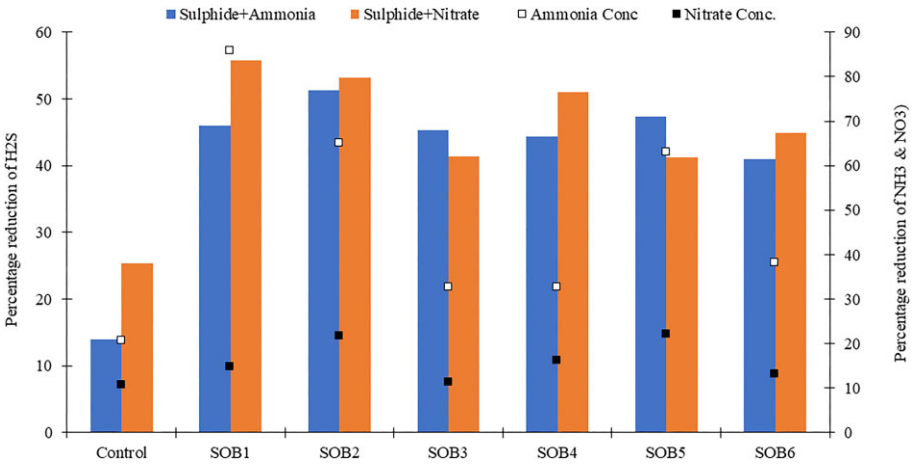

Figure 3. Simultaneous removal of sulfide, ammonia, and nitrate by SOB enrichments.

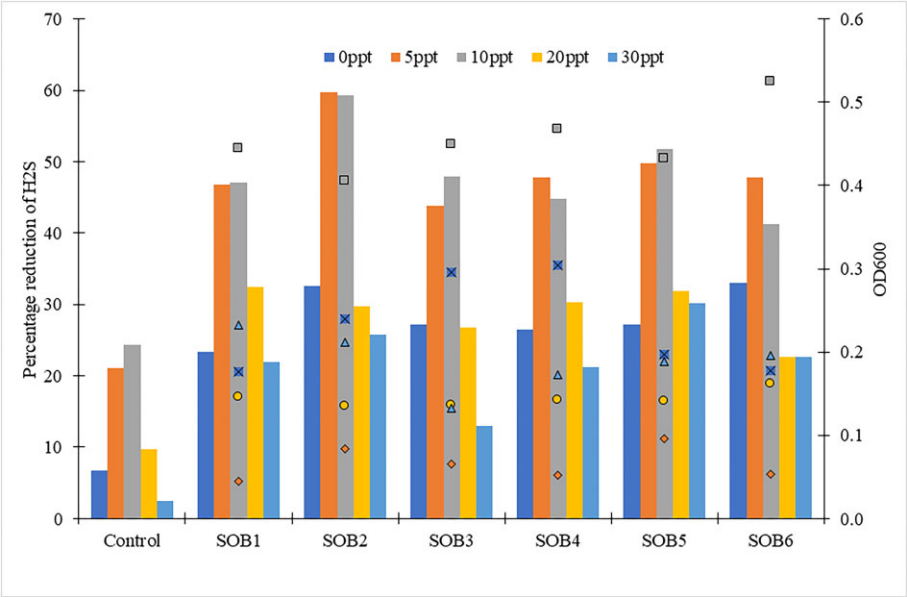

Figure 4. Growth and efficacy of SOB enrichments at different salinities.

Table 2. Summary of sequence reads, alpha diversity, and taxonomy.

Sequence_ID	Av reads ($n = 3$)	Alpha diversity				Taxonomy				
		Chao1	ACE	Shannon	Simpson	Phylum	Class	Order	Family	Genus
SOB1	77 616	74.1 ± 7.8	71.2 ± 4.0	1.96 ± 0.01	0.82 ± 0.0	9	15	35	52	69
SOB2	84 478	64.9 ± 4.4	65.8 ± 3.9	2.13 ± 0.15	0.83 ± 0.03	10	15	42	61	84
SOB3	91 666	42.1 ± 5.2	44.3 ± 3.2	0.30 ± 0.01	0.10 ± 0.0	8	12	34	50	66
SOB4	72 151	97.9 ± 5.6	97.3 ± 4.7	2.26 ± 0.07	0.85 ± 0.02	6	9	23	39	57
SOB5	84 947	52.4 ± 8.2	52.7 ± 3.6	1.3 ± 0.12	0.65 ± 0.04	9	11	28	46	64
SOB6	77 941	92.8 ± 4.2	95.1 ± 4.8	1.57 ± 0.13	0.65 ± 0.06	11	16	42	67	112

consortia. Salinity is known to influence SOBs' diversity and community structure (Yang et al. 2016). Earlier studies have reported the SOBs from coastal aquacultures (Krishnani et al. 2010), hypersaline and soda lakes (Tourova et al. 2013), coastal (Lenk et al. 2012), and salt marsh sediments (Thomas et al. 2014). We have identified a diverse group of SOBs surviving in aquaculture environments with varying salinity (unpublished data) Ability of the enrichments developed in the study to grow and oxidize the H_2S under varying salinities efficiently suggests their suitability for application in brackishwater systems.

The study observed the limited microbial diversity in all the enrichments. The selective pressure of thiosulfate medium on the enrichments might be the observed limited diversity. The metagenomic studies on the effect of enrichments on change in the microbial diversity for SOBs are limited. However, PCR-

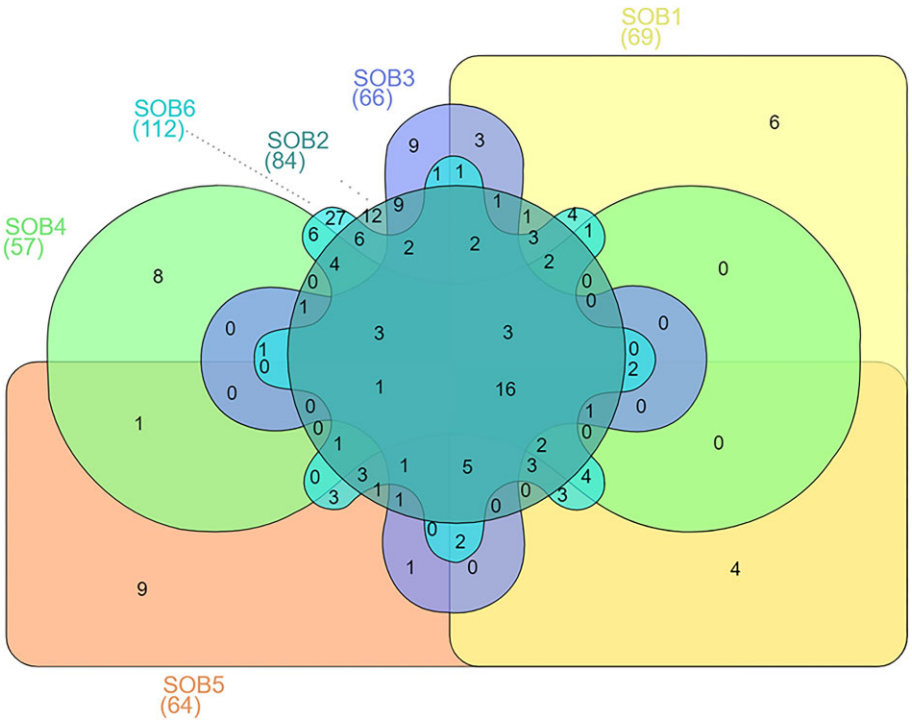

Figure 5. Venn diagram depicting the shared and unique genera among various SOB enrichments.

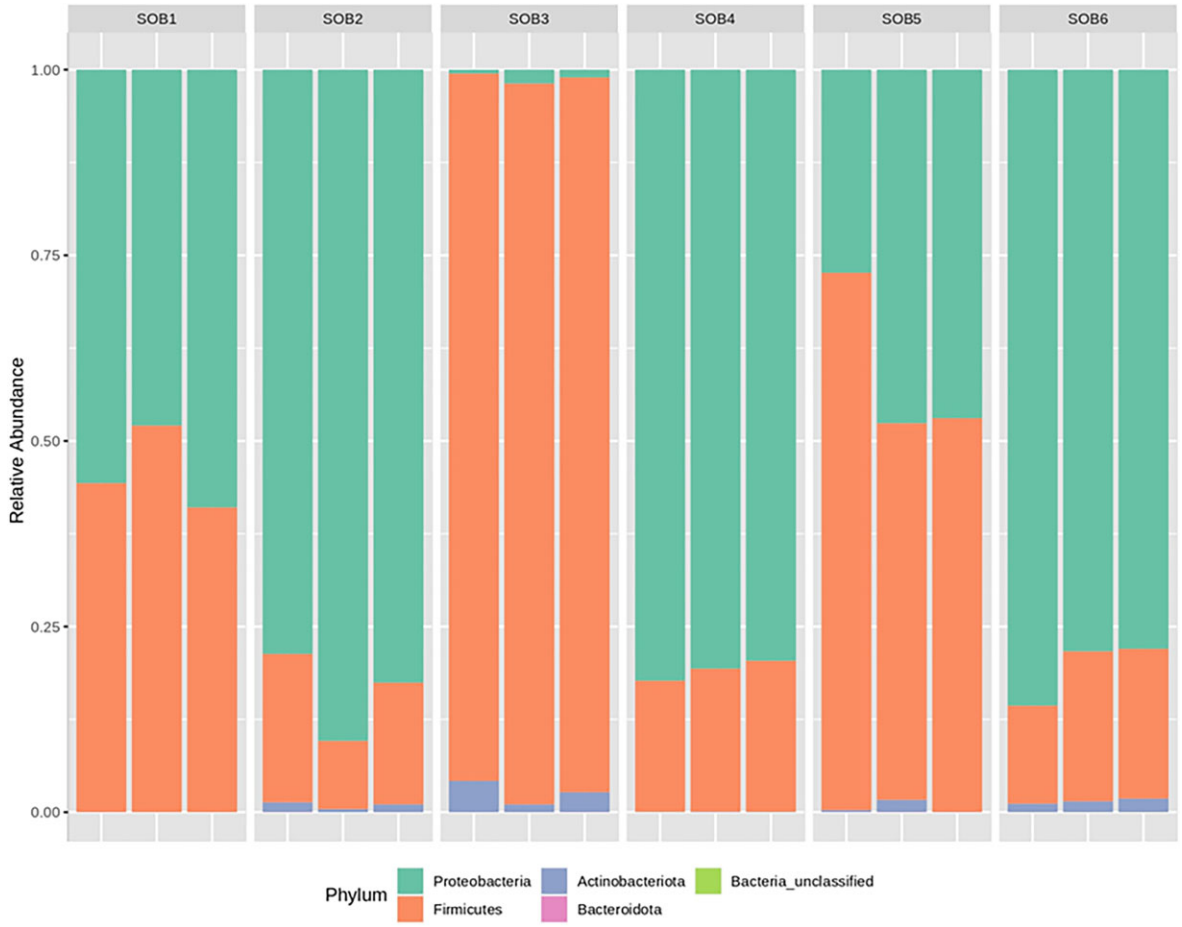

Figure 6. Relative abundance of major phylum in various SOB enrichments.

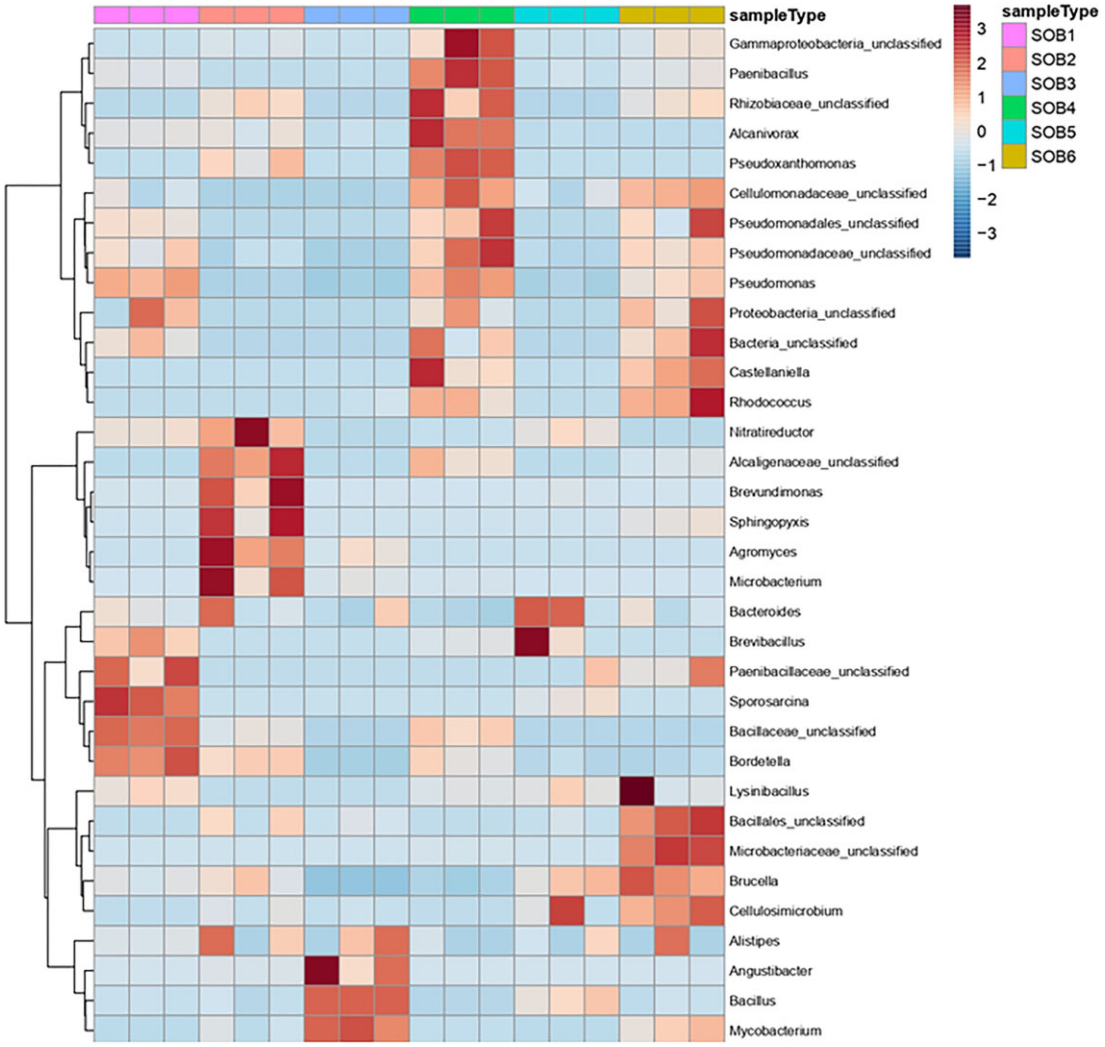

Figure 7. Heatmap showing the relative abundance of genera in each enrichment.

DGGE analysis reported a reduction in diversity following the enrichment of bioreactor sludge using SOB-specific medium (Luo et al. 2013). Similar dominance of *Firmicutes*, *Proteobacteria,* and *Actinobacteria* was reported from the SOBs of the Northern Indian Ocean (Menezes et al. 2020) and thermal springs (Jaffer et al. 2019) using 16 rRNA phylogeny. Similar studies in saline water RAS systems revealed the presence of gamma *Proteobacteria, Bacteroidia, Firmicutes,* and *Bacilli* in fish wastes (Letelier-Gordo et al. 2020) and biofilters (Rojas-Tirado et al. 2021). Role of *Acidihalobacter* spp. (Khaleque et al. 2017a,b), Bacillus spp. (Behera et al. 2014), *Pseudomonas* sp. (Behera et al. 2014, Liu et al. 2017), *Pseudoxanthomonas* spp. (Singh et al. 2012), and *Alcaligenaceae* spp. (Ghosh et al. 2011) sulfur metabolism, hydrogen sulfide biosynthesis, oxidation/reduction, transportation, alkali soil reclamation, and desulfurizing lignite coal have been reported. Some of these genera were reportedly played a major role in the simultaneous removal of nitrite and sulfur through Anammox and autotrophic desulfurization-denitrification process (Xia et al. 2019).

Functionally closely related microbial communities often show similar variation in abundance and may be divided into different co-abundance groups. Consequently, potential bacterial network interactions occur. The co-occurrence network tools are being applied to investigate the specie/genus level interactions such as syntropy, competitive interactions, and symbiotic relationships in the microbial communities (Röttjers and Faust 2018, Yuan et al. 2021). Revealing interactions between taxa is helpful to ascertain the function of microorganisms and evaluate the influence of interference on the bacterial community composition (Barberán et al. 2012, de Vries et al. 2018). During the sulfur cycle in aquatic systems, the connections among phyla reportedly change among *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* (Zhou et al. 2020). Since the laboratory tests revealed the capability of enrichments to remove sulfur simultaneously and nitrogenous metabolites, the interaction between denitrifies and sulfur oxidizer communities is worth investigating.

Organic wastes like feed, fertilizer, metabolic waste, and decaying matter in aquaculture sludge are the sources of sulfur, nitrogen, and phosphorous compounds in the aquaculture environments (Jasmin et al. 2020). Microbial biodegradation of ammonia and sulfide in industrial wastewater and aquacul-

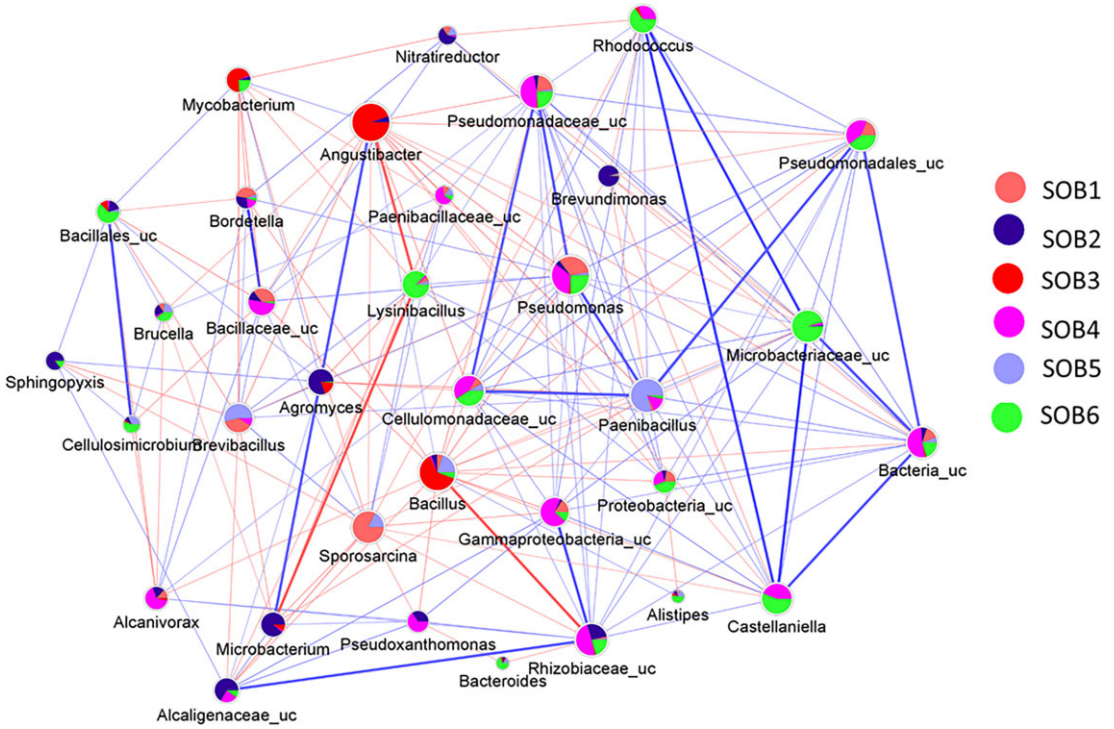

Figure 8. Network analysis based on Spearman rank correlation ($-1 < \rho < -0.5$) and ($0.5 < \rho < 1$) and is significant (*P*-value < .05) showing symbiotic and commensal relationships of microbes.

ture systems has been extensively reviewed (Divya et al. 2015, Bharagava and Saxena 2020, Jasmin et al. 2020, Ojha et al. 2021). Simultaneous conversion of organic matter to carbon dioxide, nitrate to nitrogen gas, and sulfide to elemental sulfur was proposed (Show et al. 2013). Previously, we have reported the isolation, characterization, and successful mitigation of nitrogenous metabolites in saline water aquaculture systems (Baskaran et al. 2020, Patil et al. 2021a). The *Halothiobacillus, Thiobacillus, Sulfuricurvum, Sulfurimonas, Limnohabitans, Hydrogenophaga*, and *Hyphomicrobium* species present in the nitrifying enrichments were known to be sulfur oxidizers. Additionally, heterotrophic communities like *Bacillus, Pseudomonas, Paracoccus,* and *Rhodococcus* were also present in the enrichments, and were reportedly mixotrophic sulfur oxidizers. The simultaneous removal of sulfur, ammonia, and nitrite by the bacterial consortia confirms the enrichment of microbes with multiple bioremediation activities.

Synergistic activity between these groups and marked enhancement of gene expression involved in sulfur metabolism were revealed in the recent report on their co-occurrence in nitrate-rich environments. Similar simultaneous removal of H_2S and NH_3 by microbes in the biofilters has also been reported (Chung et al. 2000, 2005, Hernandez et al. 2013). However, the development of microbial enrichments for simultaneous removal of sulfide, ammonia, and nitrate from natural water bodies and aquaculture operations is not available. We report the development of enrichments for simultaneously removing H_2S, nitrate, and ammonia. The ability of enrichments to simultaneously oxidize both nitrogenous and sulfur metabolites suggests the potential for developing microbial formulations for bioremediation of wastewater originating from aquaculture and industrial activities

The study reports the development and characterization of microbial enrichments for simultaneous removal of ammonia, nitrate, and sulfide in aquaculture systems at varied salinities under laboratory conditions. The enrichments could be used to degrade environmental pollutants and promote sustainable agriculture and aquaculture practices. Further, the ability of these enrichments to utilize thiosulfate and thiocyanate in addition to sulfide suggests their potential application in industrial wastewater treatment plant.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

Prasanna Kumar Patil (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing– review & editing), Vinay Tharabenahalli Nagaraju (Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft), Viswanathan Baskaran (Formal analysis, Investigation, Methodology, Writing – original draft), Satheesha Avunje

(Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft), Rajesh R (Formal analysis, Investigation, Methodology), Sudeep D. Ghate (Formal analysis, Methodology, Software, Visualization), and H. G. Solanki (Conceptualization, Resources, Writing – review & editing)

Data availability

The raw sequences obtained in this study are available at NCBI BioProject ID PRJNA751554.

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