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Isolation and characterisation of sulphur oxidising bacterial isolates from two different pacific white shrimp, *Penaeus vannamei* culture systems

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Abstract
Sulphur reducing bacteria (SRB) and sulphur-oxidizing bacteria (SOB) are predominately involved in reduction and oxidation of sulphates and hydrogen sulphide, respectively, in pond and assist in maintaining healthy pond environment for shrimp culture. Hence, the present study aims to find out SOB two different *Penaeus vannamei* culture systems. Total 43 isolates from earthen ponds and 38 from plastic-lined ponds were obtained using thiosulphate agar and Starkey medium. The isolates were tested for their efficacy to reduce the pH of the growth media from 8.0 to ≤5.0 within 15 days of incubation. Data revealed the sulphate production was in the range of 2.5 to 195.36 mg/100 ml. The bacterial isolates from earthen pond showed significantly higher ($P < 0.05$) sulphate ion production compared to bacteria isolated from the plastic-lined pond. Among all these isolated bacteria, fifteen isolates possess autotrophic metabolism. SOBs were able to tolerate salt up to 13%. Morphological and biochemical characterization of isolated SOB revealed that as *Bacillus* species and *Micrococcus* sp. were present in the plastic-lined pond isolates whereas, *Bacillus* sp., *Geobacillus* sp., *Amphibacillus* sp., *Anoxibacillus* sp. and *Alkalibacillus* sp. were present in the earthen ponds. Thus, the earthen pond has more diverse SOB than in the plastic-lined pond.

Keywords: Sulphur-oxidizing bacteria, shrimp culture, biochemical test, *Bacillus*, *Penaeus vannamei*

Introduction
Aquaculture ponds receive large amounts of phosphorus, nitrogen and organic matter from the uneaten feed, animal excreta and dead plankton. Build-up of organic matter in high concentrations in the pond bottom results in the formation of anaerobic conditions in the subsurface layer and soil-water interface (Boyd, 1995) [9]. The microbial processes convert the toxic nitrogenous, sulphur and other compounds to non-toxic/favourable compounds, thereby improving water quality to a large extent (Abraham *et al*., 2004) [2]. Microbes play a pivotal role in nutrient recycling in the pond ecosystem through the process biodegradation and convert the organic molecules into inorganic molecules. During this biodegradation process, microbes produced hydrogen sulphide, which is toxic to shrimp (Rao *et al*., 2000; Antony and Philip, 2006; Hsu and Chen, 2007) [35, 4, 22].

Moreover, it also inhibits the nitrification, which leads to accumulation of ammonia and thus increases ammonium toxicity in aquaculture ponds (Avnimelech and Ritvo, 2003) [6]. The shrimps avoid pond bottom regions with the sulphide containing sediment, and feed acceptances were considerably low in such deposits (Gopakumar & Kutiyama, 1996) [19]. Heterotrophic bacteria oxidize organic matter, and the autotrophic nitrifying and sulphur bacteria oxidize complex compounds like ammonia, nitrite or sulphide (Moriarty, 1997) [31]. Sulphur reducing bacteria (SRB) and sulphur-oxidizing bacteria (SOB) are predominately involved in the reduction and oxidation of sulphates and hydrogen sulphide, respectively at the pond bottom (Syed *et al*., 2006) [47]. They assist in maintaining a healthy pond environment in commercial shrimp culture ponds (Abraham *et al*., 2015; Fernandes *et al*., 2010; Patil *et al*., 2012) [17, 1, 33].

Several authors have reported the abundance and diversity of SOBs from marine (Kambam and Sridar, 2012) [23], brackish water (Saravanakumar *et al*., 2012) [36], mangrove (Behera *et al*., 2014) [8] and aquaculture pond (Watanabe *et al*., 2003; Zhou *et al*., 2007; Krishnani *et al*., 2010; Mukkata *et al*., 2016) [51, 55, 29, 32]. Apart from sulphur bacteria, most of the heterotrophic bacteria belonging to the genera *Pseudomonas* (Schook and Berk, 1979) [37], *Xanthobacter* and *Escherichia* (Das *et al*., 1996) [15] and even fungi (Grayston *et al*., 1986; Xu *et al*., 2018) [20, 54] were reportedly involved in sulphur oxidation. However, the diversity of SOB in *P. vannamei* shrimp culture systems is not reported yet. Hence, present work deals with isolation and identification of sulphur oxidizing bacteria from earthen and plastic-lined shrimp culture ponds of Gujarat, India.

Materials and Methods

Sample collection

A total of six different ponds were selected, out of six three were plastic-lined, and three were earthen ponds. Three earthen ponds were from Onjal village (Lat. 20°49’23.01’’ N, Long. 72° 50’53.19’’ E), Navsari district, Gujarat and was 2.5 km from Gulf of Cambay. While, three plastic-lined ponds (300 microns; GSE Lining Technology Co. Ltd., Thailand) were selected from Chijgam village (LAT 20° 49’58.26’’ N, LONG 72° 52’21.90’’ E), Navsari district, Gujarat and was 2 km from Gulf of Cambay.

Fortnightly water and sediment samples were collected in sterile plastic bottles and sterile plastic bags, respectively, from both the types of culture systems. Samples were transported in a laboratory in an icebox and processed within 4 hrs of sampling.

Isolation of SOB

For isolation of sulphur oxidizing bacteria, the method described by Vidyalakshmi and Sridar (2007) [50] was used. Briefly, 1 g or 1 ml of sample was inoculated in 50 ml in Starkey (1935) [45] broth (3.0 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$ and 0.2 g $CaCl_2{\cdot}2H_2O$, in 1000 ml distilled water with pH 8.0) supplemented with 10 g of elemental sulphur per litre for three days. Subsequently, the sample was taken from Starkey broth and streaked on thiosulphate agar medium (5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$ and 0.1 g NH_4Cl with final pH 8.0). Morphologically different colonies were selected, purified and preserved on thiosulphate slants. Bacteria isolated from earthen pond culture systems were labelled as “S” series and plastic-lined pond systems as "L" series.

The pH reduction test

The isolates were inoculated in the Starkey medium (0.2 g $CaCl_2{\cdot}2H_2O$ and $FeSO_4$ (in traces) in 1000 ml distilled water with pH adjusted to 8.0) as well as in the thiosulphate broth media (initial pH adjusted to 8.0) and incubated at 32° C for 15 days.

The isolates were screened for their ability to reduce the pH of the growth media from 8.0 to 5.0 or less than 5.0. Isolates which were able to lower the pH were selected for further studied for their morphological and biochemical characterization.

Estimation of sulphur oxidizing efficiency

The amount of sulphate ion (SO_4^{2-}) produced during the growth of sulphur-oxidizing bacteria on thiosulphate media was determined using the UV spectrophotometer as per the procedure described by Kolmert *et al*. 2000 [28].

Morphological characterization

For morphological characterization, isolates were grown on Thiosulphate agar medium. Colony characteristics like shape, size, arrangement, elevation, margin, consistency, surface and opacity were studied. Cell shape and Gram's reaction was examined using Gram's staining.

Biochemical Characterization

The biochemical characterization of the isolated bacteria was performed according to Bergey’s Manual of Determinative Bacteriology (Brenner *et al.*, 2005) [10]. All the isolated bacteria were tested for MR, VP, citrate utilization, urease production, oxidase, catalase, sugar fermentation and amino acid decarboxylase tests.

Salt tolerance

Effect of different salt concentrations (0, 2, 3, 5, 8, 10, 11and 13%) on the isolated bacteria was studied. Trypton broth with varying salt levels was used against the growth of the bacterial culture. With the help of a sterilized loop, the bacterial cultures were inoculated into this broth containing varying levels of sodium chloride and incubated at 37ºC for 18-24 hours. The salt tolerance was tested by observing the growth of the isolates in different salt concentrations.

Statistical Analysis

Statistical analysis was performed by SPSS version 2.0 for windows.

Results and Discussion

Isolation of bacteria

Morphologically diverse colonies were observed from both earthen and lined pond samples. A total of 43 different bacteria were isolated from the earthen pond and were labelled with S series. Form line pond samples, a total of 38 different bacteria were isolated and marked with L series.

The pH reduction test

All the isolates were able to reduced pH as indicated by a colour change from purple (pH 8.0) to reddish-purple or yellow colour. Six of the earthen pond isolates (S8, S11, S21, S28, S35 and S41) and fourteen of the lined pond isolates (L5, L8, L9, L11, L13, L15, L20, L23, L24, L36, L37, L40, L43 and L46) reduced the pH to below 6.5 (control 8.0) of the three growth media (Starkey, NCL and Thiosulphate) within 15 days of incubation. Thirteen of the earthen pond isolates (S3, S4, S10, S16, S22, S27, S31, S34, S36, S37, S42, S43 and S46) and eight of the lined pond isolates (L2, L3, L7, L14, L21, L27, L30 and L35) did not show a remarkable reduction in pH of either Starkey or NCL broth media. All other isolates showed a decrease in pH of thiosulphate agar growth medium.

Estimation of sulphur oxidizing efficiency

Isolates from both the culture systems, i.e., earthen and line pond were able to oxidize sulphur. The bacterial isolates from earthen pond showed significantly higher ($P<0.05$) sulphate ion production compared to bacteria isolated from the plastic-lined pond. Among the earthen pond isolates, S11 (199.80 mg/100 ml) produced highest, followed by S28 (198.88 mg/100 ml) and S8 (195.36 mg/100ml) (Fig 1a). Least was reported in S30 (5.72 mg/100 ml). In the case of the isolates from lined ponds, highest sulphur was observed in L11 (98.48), followed by L13 (97.16 mg/100ml) and L40 (93.20 mg/100 ml) (Figure1b). Least was observed in L42 (2.76 mg/100ml). Among both the samples, bacteria isolated from the earthen pond showed significantly higher sulphate ion production compared to bacteria isolated from the lined pond.

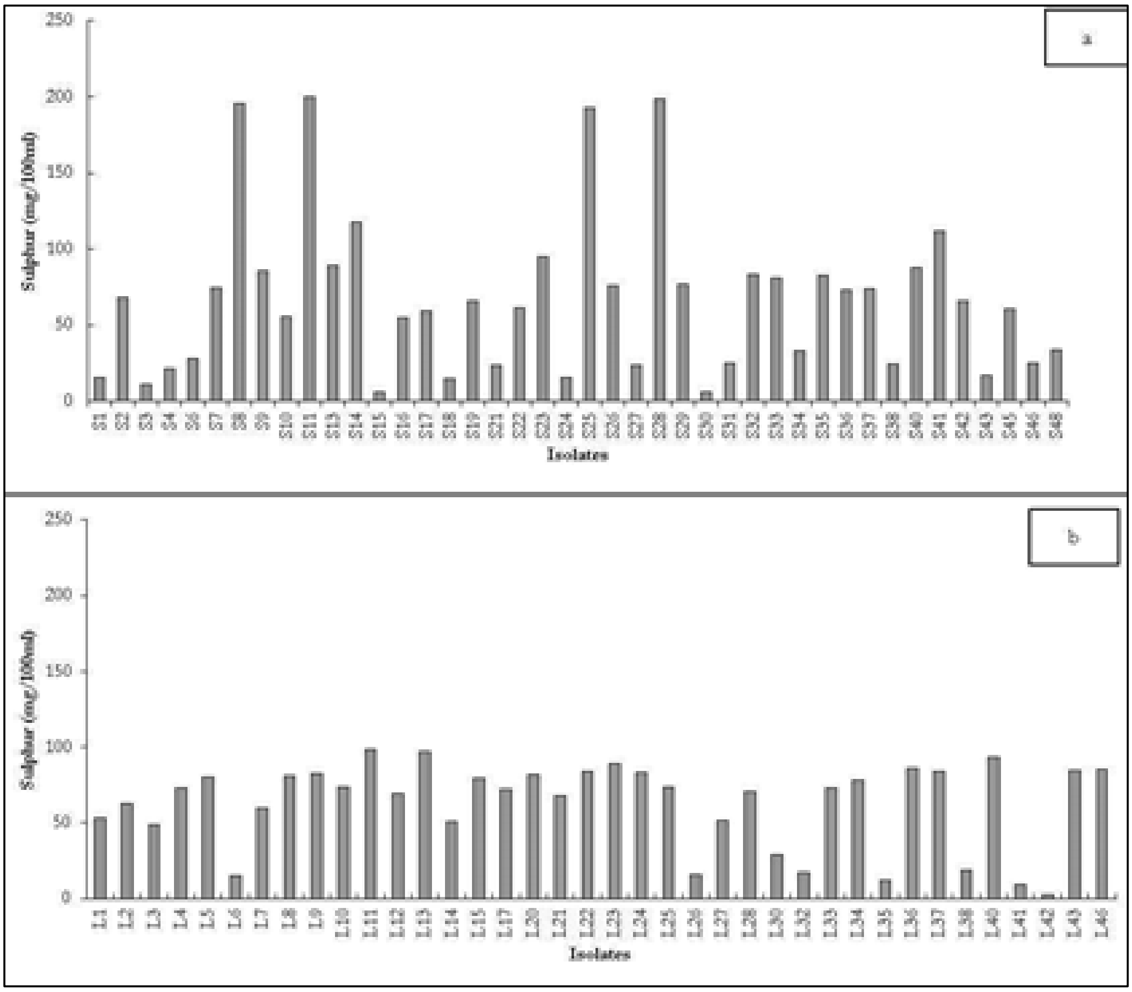
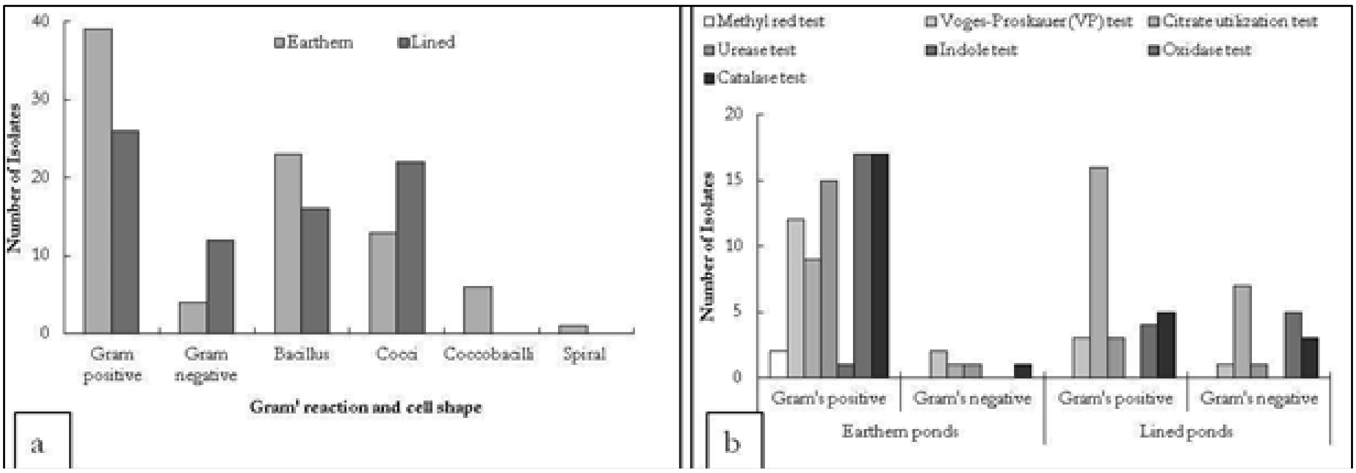


Fig 1: Sulphate ion production by earthen pond isolates (a) and lined pond isolates (b)

Morphological characteristics

All the isolates were observed for their colony characteristics and morphological characteristics. The colonies of the isolates were different in their shape, cell, elevation, margin, consistency, surface and optical characteristics. Most of the colonies were convex, smooth, opaque, sebaceous and moist. Morphological characterization was done using Gram's staining. Data revealed that out of 43 isolates from the earthen pond, 39 were Gram-positive, whereas four isolates were Gram-negative (Figure 2a). Whereas in the plastic-lined pond, 26 isolates were Gram-positive, and 12 were Gram-negative (Figure 2a). Plastic lined pond isolates contained more of gram-negative and cocci shaped bacterial isolates compared to earthen ponds. Also, the earthen pond isolates had more diverse shapes in terms of shapes and cell arrangements than the counterpart arrangement.



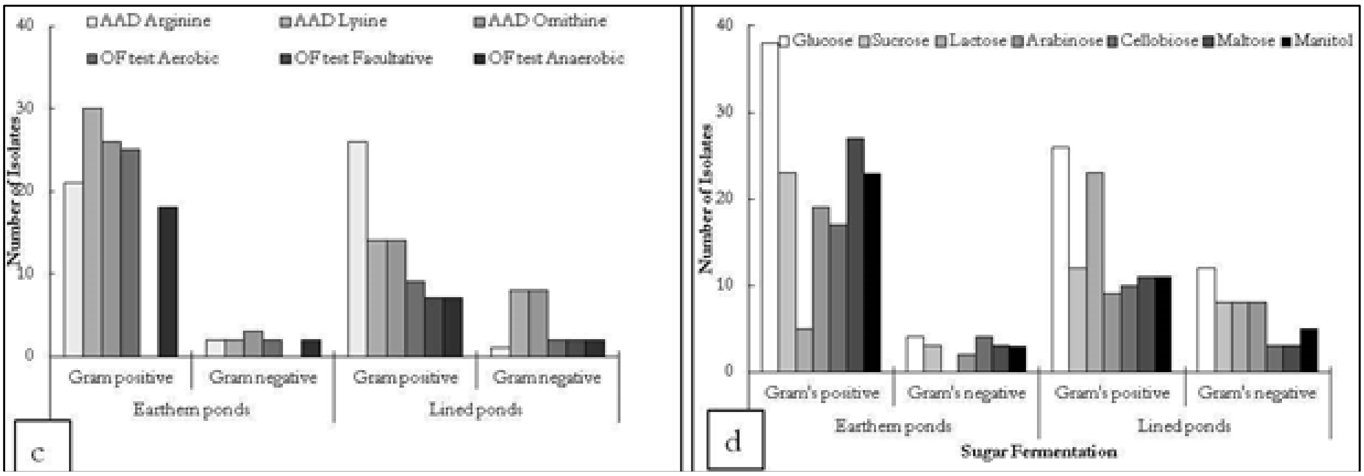


Fig 2: Biochemical and morphological characteristics. Gram's reaction and morphology of the isolated bacteria (a); Biochemical test (b); Oxidation Fermentation (OF) and Amino Acid Decarboxylation (AAD) (c); Sugar fermentation (d)

Biochemical characteristics

Various biochemical tests were performed to identify the isolated bacteria. All the isolates were able to ferment glucose (Fig 2d). Among them, most earthen pond isolates were maltose fermentative (62.79%) while the plastic-lined pond isolates were more of lactose fermentative (81.58%) type. Plastic lined ponds were able to utilize citrate (60.53%) more efficiently than the earthen pond isolates (23.26%) whereas; the latter responded positively for catalase (41.86%) and Urease tests (37.21%) than the former (21.05% and 10.53% respectively) (Fig 2b). Among the total 81 isolates, only two isolates from earthen ponds showed positive results for Methyl red and Indole tests. Among all the isolates, the isolate S36 was found positive for indole production. Among the isolates, earthen pond isolates (39.53%) and plastic pond isolates (23.68%) showed positive reactions for oxidase tests indicating the presence of cytochrome oxidase enzymes in the isolates.

In case of oxidation fermentation tests (Fig 2c), most of the earthen pond isolates were aerobic (62.79%) while few isolates showed anaerobic (46.51%) reactions but none showed facultative results. Plastic lined ponds isolates were aerobic (28.95%), facultative (23.68%) and anaerobic (23.68%) in nature. Among the isolates, a higher percentage of earthen pond isolates showed decarboxylation of the amino acid lysine (74.42%). In comparison, 71.05% of the plastic-lined ponds isolates could decarboxylate the amino acid arginine (Fig 2c).

Salt tolerance

The results showed that the organisms had a wide range of tolerance for NaCl concentration. In earthen ponds, isolates S27 and S45 showed salt tolerance up to 10% salt concentration whereas, isolates S10 and S48 could grow only at 2% salt concentration. The isolate S11 could tolerate salt concentrations up to 13% but could not tolerate lower salt concentrations. Similarly, in plastic-lined ponds, ten isolates (L1, L4, L5, L6, L21, L30, L32, L33, L37 and L42) could tolerate from 0 to 5% salt concentration whereas, 16 isolates could tolerate up to 3% salt concentration (Fig 3). The six isolates (L6, L7, L10, L11, L13 and L14) could tolerate salt concentrations up to 10% whereas; L14 and L11 isolates tolerated salt concentrations up to 11%.

The present study revealed the occurrence of diverse SOB groups in *Penaeus vannamei* culture ponds which have shown the potential to reduce the pH of growth medium from 8.0 to 5.5 and below. The six earthen pond isolates and fourteen lined pond isolates reduced the pH to below 6.5 (control 8.0) of all the three growth media indicating the ability of the strains of utilizing thiosulphate. The pH reduction of the medium was due to the production of sulphuric acid (Donati *et al*., 1996; Ashraf *et al*., 2018) [16, 5]. Isolates were able to lower the pH from 8 to 5 (Kambam *et al*., 2015) [24]. Some of the SOBs are even able to reduce pH up to 4 - 3.5 (Vidhyasri and Sridar, 2011; Priyanka *et al*. 2014) [39, 34]. Even SOB isolated from sewage sludge factory was able to reduce pH up to 3.7 (Ashraf *et al*. 2018) [5].

Similar diverse SOBs were isolated from various marine ecosystem supports the present findings (Anandham *et al*., 2005; Veerender and Sridar, 2012; Behera *et al*., 2014; Kambam *et al*., 2015) [3, 8, 24]. However, in one study finding reported 14 isolates with the short rod (Vidyalakshmi and Sridar, 2007) [50] while in other reported nine isolates with rod shape morphology (Vidhyasri and Sridar, 2011) [49]. Few of the earthen pond isolates were characterized as *Bacillus* sp. (S6, S22, S35, S46 and S48), *Geobacillus* sp.(S33), *Amphibacillus* sp. (S1, and S40), *Anoxibacillus* sp.(S28) and *Alkalibacillus* sp. (S11) whereas; the plastic-lined pond isolates were identified as *Bacillus* sp. (L1, L7, L11, L13, L23, L34 and L46) and *Micrococcus* sp.(L14 and L32). Behera *et al*. (2014) [8] identified *Micrococcus* spp., *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas* sp. and *Klebsiella* spp. having sulphur oxidizing efficiency. Heterotrophic bacteria *Escherichia coli*, *Xanthobacter* spp, *Micrococcus* spp., *Bacillus* spp. and *Pseudomonas* spp. were also able to oxidize sulphur (Starkey, 1935; Cho *et al*., 1992; Chattopadhyaya *et al*., 1993; Sorokin *et al*., 1999) [45, 13, 12, 39]

Generally, sulphur oxidation is attributed to few members of the genus *Thiobacillus*, *Beggiatoa*, *Chlorobium*, *Thiothrix*, *Acidothiobacillus*, *Starkeya*, *Thiocapsa*, *Rhodobacter* etc. (Wakesman, 1922; Strohl and Larkin., 1978; Lee and Kim, 1998; Williams and Unz, 1985; Kelly and Wood, 2000; Kelly *et al*., 2000; Caumette *et al*., 2004; Sievert *et al*., 2007) [46, 52, 30, 25, 26, 11, 38]. Thus present findings suggest some unique genera to the sulphur oxidation.

Anandham *et al*. (2005) [3] isolated nine chemolithoautotrophic SOBs and twelve chemolitho-heterotrophic SOBs from different ecological niches using modified Starkey's medium while, Vidyalakshmi and Sridar (2007) [50] isolated twenty-eight isolates from pulse rhizosphere, paddy rhizosphere, biogas slurry, sewage, tannery effluent and mine soil. Behera *et al*. (2014) [8] isolated

30 isolates while, Sridar *et al.* (2015) [41] separated 24 isolates from different ecosystems *viz*., marine, tanneries, sewage, rhizosphere soil, paper effluent, beverage and industrial wastes.

The amount of sulphate ion production by the isolates in growth media is a measure of the sulphur oxidizing efficiency (Hirano *et al*., 1996) [21]. All the strains had sulphate ion production ability by utilizing various sources of sulphur and from Na_2SO_3 supplied in the medium. Some of the earthen ponds isolates had significantly higher ($P<0.01$) sulphate ion production capacity compared to the rest of the strains. Some isolates have the ability for the highest sulphate production of 200 – 460 mg/100ml (Teske *et al*. 2000) [48], while some can produce 43.21 mg/100 ml (Anandham *et al*. 2005) [3]. A similar observation was also reported where 52 mg/100ml sulphate production was reported (Priyanka *et al*. 2014) [34]. In marine environments, the highest sulphate production reported was 245 mg/ml (Behera *et al*. 2014) [8]. In the present study, the earthen pond might have supported the growth and nourishment of beneficial probably, which was lacking in lined ponds. Moreover, selective pressure at the pond bottom must have favoured the isolates for their higher sulphate production traits.

Isolated SOBs were able to tolerate high salt concentration. Halophilic SOB isolated from oil field brine were able to tolerate up to 40.91% salt concentration (Gevertz *et al*. (2000) [18]. Some SOB isolated from hypersaline lakes were able to tolerate 23.37% salt concentration (Wood *et al*., 2005) [53]. The occurrence of an SOB from saline and the hypersaline environment was well reported (Watanabe *et al*., 2003; Zhou *et al*., 2007; Srinivas *et al*., 2007a, 2007b; Krishnani *et al*., 2010; Kambam and Sridar, 2012; Saravanakumar *et al*., 2012; Behera *et al*., 2014; Mukkata *et al*., 2016; Srinivas *et al*., 2016; Knittel *et al*., 2005; Sorokin *et al*., 2006) [51, 55, 32, 43, 44, 23, 36, 8, 27, 39, 29, 18, 40]. In the present study, isolates were able to tolerate up to 13% salt concentration which was lower than other reported research. Despite the isolate habitat where salinity was ranged from 1.4 – 4%, their ability to higher salt tolerance may be attributed to the brackish water environment. This unique feature of sulphur oxidation and salt tolerance create a favourable environment for *P. vannamei* by reducing sulphur toxicity.

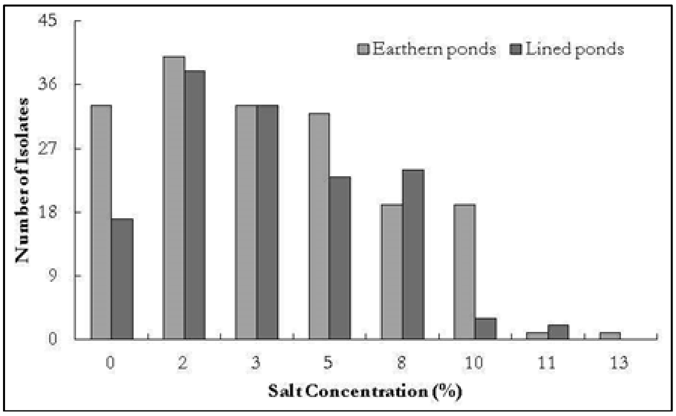


Fig 3: Salt tolerance of the bacteria isolated from earthen and lined pond.

Conclusion

The present study shows the occurrence of diverse sulphur oxidizing bacterial groups involved in the oxidation of sulphide in *P. vannamei* culture systems. The growth of these bacterial species on Starkey and thiosulphate agar medium with the ability to reduce the pH of growth medium and *in vitro* sulphate ion production clearly shows the sulphur oxidizing ability of these species. The isolates obtained from these environments could tolerate the salinity levels from 0 to 13%. Bacteria were able to oxidize sulphur and thus prevent its toxicity to *P. vannamei.* Therefore, they maintain sulphur in aquaculture pond for the healthy growth of *P. vannamei.*

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