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Isolation and Characterization of Microorganisms from Edible Bivalves as Potential Agents for Bioremediation

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Abstract: *Bivalves are common marine species which are used as bio-indicators. These organisms are filter feeders and have a tendency to accumulate heavy metals and organic pollutants from sea water in their tissue. Their gills host microorganisms, which are also exposed to the heavy metals and other pollutants. The objective of the present study was to isolate and characterize these microorganisms and explore their potential for bioremediation of polluted waters. Eight different microbial cultures were isolated from edible clams (bivalve molluscs) collected from the coast of Mumbai and Alibaug. Biochemical and morphological tests were performed to characterize the isolated bacteria. All the eight cultures showed growth in 8% NaCl solution indicating that they are halotolerant. Gradient plate method was used to test resistance of the isolates to three dyes commonly used for painting of idols – Fluoorange, Fluo green and Fluo blue and Malachite green. The isolates were also tested for metal resistance against Zn, Cu, Co and Pb using agar cup method. It was observed that six isolates (AS1, AS3, MS3, MS4, MS9, MS11) were resistant to the three selected dyes while two isolates (AS2, MS1) were sensitive. AS1, AS3, MS9 and MS11 were found to be resistant to Malachite green up to 0.01 mg/ml. MS11, identified as Staphylococcus arlettae, showed the highest tolerance to metals – up to 80 mM of $ZnSO_4$, $CuSO_4$, $CoCl_2$ and $PbNO_3$. For the other isolates the resistance to metals was variable; MS4 was found to be most sensitive. The results suggest that the isolated bacteria, Staphylococcus arlettae, from bivalves has resistance to toxic chemicals and may find application in bioremediation processes.*

Keywords: Bivalves, Gradient plate method, Agar cup well method, *Staphylococcus arlettae*, Bioremediation.

Introduction

Water pollution is a major concern in today's life. To a large extent, uncontrolled and unregulated industrial activities along with cultural practices are the primary reason for deterioration of fresh water and sea water quality. Not only the aquatic life and environment are under a serious threat, but also these pollutants become a part of the food chain leading to serious health effects in the human population (Kaur, 2012; Sahasrabudhe and Pathade 2011).In various parts of India, idol immersion of deities is practiced as part of culture and tradition. Idols made of Plaster of Paris are painted with different coloured dyes and at the end of festivities, the idols in large numbers are immersed in nearby lakes, rivers and seas (Watkar and Barbate, 2014).Plaster of Paris is not biodegradable while coloured dyes used are industrial grade chemicals. Dyes with dark metallic colours such as green, orange, blue, gold and silver are preferred to make the idols look attractive and sparkling (Kaur, 2012). Paints which are used may also contain toxic and carcinogenic metals like Cu, Zn, Pb, Hg, As, Cr, Fe, Co and Cd (Kaur, 2012). After the immersion of the idols, these foreign chemicals become part of marine environment. Dyes reduce oxygen level and increase acidity level of water, causing death of marine life and disturbing the ecological balance. The effect of these chemicals has been previously looked

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by Reddy and Kumar (2001) and Dhote *et al*. (2001).They showed that non-biodegradable materials, synthetic paints and toxic chemicals used for colouring idols lead to increase in hardness of water and significantly alter the water quality, thus affecting the marine life.

Microorganisms can play an important role in degradation of toxic pollutants. Industrial dyes like Malachite green and Congo red are widely used in textile industry and agriculture, due to easy availability, cost effectiveness and greater efficiency (Cao, 2009). However, these dyes are a serious health hazard to aquatic flora and fauna (Hassanshahian and Mohamadian, 2011).Due to carcinogenic effect of Malachite green, developed countries like USA have banned its use. Previous studies have shown the ability of intestinal bacteria and *Staphylococcus aureus* to degrade Malachite green into colourless, less toxic compounds (Cao, 2009).

Bivalves are a class of marine and freshwater molluscs. They include clams, scallops, mussels and oysters. One of the subspecies, clams, is found in inter-tidal zone of seas and have long been part of diet of coastal population. Clams harbour microorganisms in their gills which are exposed to organic and metal pollutants. The aim of the present study is to isolate and characterize microorganisms present in gills of clams. Clam samples were collected from two different locations:1-Coast of Mumbai, a large metropolitan area with a history of large textile industry since 1865 (The Cotton Mills, 1997) and where idol immersion during Ganesh Chaturthi festival is practiced in large numbers (Bansal, 2010). 2-Alibaug which is a beach resort about 96 km from Mumbai with a small population and comparatively less pollution. A number of studies have been done on the effect of metals and other pollutants in coastal water, sediment and different tissues of bivalves (Sunita, 1987;Tendulkar, 1996); however, not much attention has been paid on the presence of microorganisms in gills of bivalves and their bioremediation potential. The present study focused on microorganisms isolated from gills of bivalves which were studied by Gram-nature, biochemical and enzyme tests to identify their characteristics. Further, the tolerance of the isolates to high salt concentration and to dyes and metals used for painting of idols was also looked at.

Materials and Methods

Sample Collection and Isolation of Bacteria

Bivalve samples were collected from the coast of Mumbai and Alibaug. Gills of bivalves were cut into pieces and added to sterile saline suspension under aseptic condition. The growth medium used for the isolation, activation and further studies of microorganisms was modified halophilic agar (Dundas, 1977; Gibbons, 1969). The medium consisted of casein acid hydrolysate (10 g/l), yeast extract (10 g/l), protease peptone (5 g/l), trisodiumcitrate (3 g/l), potassium chloride (2 g/l), magnesium sulphate (25 g/l), sodium chloride (80 g/l) and agar (20 g/l). Cultures were incubated at 37 ºC for 24 hours.

Characterization of Microbial Isolates

Microorganisms were characterized for their Gram-nature, motility and colony characteristics. The isolates were tested for their salt tolerance on halophilic agar medium supplemented with sodium chloride between 8–25%. Biochemical tests were conducted including glucose fermentation, acid production from glycerol, tryptophan utilization, Methyl Red and Voges-Proskauer (MR-VP) test, citrate utilization and reduction of nitrate to nitrite. Further, enzyme tests including catalase, starch hydrolysis, casein hydrolysis, urease and arginine decarboxylase were carried out.

Effect of Dyes on Strains

Resistance of the isolated cultures to dyes was qualitatively analysed using gradient plate method. Three different dyes commonly used for colouring Ganesh idols Fluo orange, Fluo green and Fluo blue were sourced from Matrix Speciality, Mumbai. The dye tests were run in parallel with Malachite green. Stock solution

of dyes were prepared at 1mg/ml and then serially diluted to 0.1, 0.01 and 0.001 mg/ml. Different growth media was prepared by mixing halophilic agar with 1 ml of the dye solution at different concentrations and plates were prepared with concentration gradient. Cultures were streaked from lower concentration side to higher concentration side and incubated at 37ºC; bacterial growth was studied after 24 hours. Continuous growth of the culture on the streaked area across the plate indicated resistance of the isolate to the dye incorporated in the halophilic agar. Cultures with growth only at lower concentration side of the plate were categorized as sensitive, while cultures without growth at both sides were categorized as highly sensitive.

Effect of Dyes on Marine Microorganisms

Sea water sample was collected from Mumbai coast and marine micro-organisms were tested against the three dyes–Fluo orange, Fluo green and Fluo blue along with Malachite green using spread plate method with halophilic agar media. A control plate without dye was also run in parallel. Total number of microorganisms was counted on each plate using colony count method in terms of Colony Forming Unit (CFU).

Tolerance of Strains to Heavy Metal Compounds

The isolated cultures from bivalves were tested for heavy metal tolerance using agar cup well method. Zinc sulphate ($ZnSO_4.7H_2O$), cobalt chloride ($CoCl_2.6H_2O$), copper sulphate ($CuSO_4.5H_2O$) and lead nitrate ($Pb(NO_3)_2$) solution were prepared at 10, 20, 40 and 80 mM; all salts were sourced from Loba Chemie, Mumbai. Plates were prepared by bulk seeding 1 ml of isolated culture in molten agar media, followed by adding 50 µl of metal salt solution of various concentrations in the agar cups. The plates were then placed in incubator at 37ºC and bacterial growth and Zone of Inhibition (ZOI) were studied after 24 hours. Cultures which showed growth without any inhibition were classified as resistant, cultures which showed zone of inhibition were classified as sensitive and cultures which showed no growth were classified as highly sensitive.

Results and Discussion

Characterization of Isolates

Eight different microbial cultures (five from Mumbai and three from Alibaug) were identified from bivalves samples based on colony shape, size and morphology. The isolates from Mumbai samples were named as MS1, MS3, MS4, MS9, MS11 and isolates from Alibaug as AS1, AS2 and AS3. The isolates from Mumbai exhibited variable colony characteristics, Gram-nature and morphology. MS4 and MS11 were found to be Gram-positive and cocci-shaped, MS9 was Gram-positive and rod-shaped and MS1 and MS3 were Gram-negative and rod-shaped. All the isolates from Alibaug were Gram-negative and rod-shaped. Except MS4, MS9 and MS11, all isolates were found to be motile. All the eight cultures showed growth at 8% NaCl concentration but no growth was observed at 15% and 25% NaCl concentration. Hence, the cultures are halo tolerant but not extremophiles. The results of biochemical and enzyme tests for the eight isolates are presented in Table 1.

Effect of Dyes on Isolates

Qualitative results of the dye tests are summarized in Table 2. Photographs of culture after dye tests are shown in Figure 1, 2, 3 and 4 for Fluo orange (0.1 mg/ml), Fluo green (0.1 mg/ml), Fluo blue (0.1 mg/ml) and Malachite green (0.01 mg/ml) respectively. Isolated cultures AS1, AS3, MS3, MS4, MS9 and MS11 were found to be resistant to Fluo orange, Fluo green and Fluo blue at 0.1 mg/ml, while AS2 and MS1 were found to be highly sensitive at 0.01 mg/ml. AS1, AS3, MS9 and MS11 were also found to be resistant to Malachite green at 0.01 mg/ml but were sensitive at 0.1 mg/ml. AS2 and MS1 were found to highly sensitive even at 0.001 mg/ml to Malachite green.

Table 1 Biochemical, enzymatic and other characteristics of the isolated strains.

Biochemical Tests	AS1	AS2	AS3	MS1	MS3	MS4	MS9	MS11
Morphology	rod	rod	rod	rod	rod	cocci	rod	cocci
Gram staining[1]	–ve	–ve	–ve	–ve	–ve	+ve	+ve	+ve
Motility[2]	+	+	+	+	+	–	–	–
Acid production from glucose	+	–	+	–	+	–	–	–
Acid from glycerol	+	–	+	–	+	–	–	–
Indole production from tryptophan	–	–	+	–	+	–	–	–
Methyl Red (MR)	–	+	+	–	+	–	–	–
Voges-Proskauer (VP)	–	–	–	–	–	+	–	–
Citrate	–	–	–	–	–	–	+	+
Reduction of nitrate to nitrite	+	+	+	–	–	–	+	+
Catalase	+	+	+	+	+	+	+	+
Starch hydrolysis	–	+	+	+	+	–	–	–
Casein hydrolysis	–	+	+	+	+	–	–	+
Urease	–	–	–	+	–	+	–	–
Arginine Decarboxylase	–	–	–	–	–	–	–	–

[1] "–ve": Gram-negative, "+ve": Gram-positive

[2] "–": no growth, "+": growth

Table 2 Sensitivity of the isolates to test dyes using gradient plate method.

Metals	Conc. (mg/ml)	Cultures[1]							
		AS1	AS2	AS3	MS1	MS3	MS4	MS9	MS11
Fluo orange	0.001	R	HS	R	S	R	R	R	R
	0.01	R	HS	R	HS	R	R	R	R
	0.1	R	HS	R	HS	R	R	R	R
Fluo green	0.001	R	S	R	S	R	R	R	R
	0.01	R	HS	R	HS	R	R	R	R
	0.1	R	HS	R	HS	R	R	R	R
Fluo blue	0.001	R	HS	R	S	R	R	R	R
	0.01	R	HS	R	HS	R	R	R	R
	0.1	R	HS	R	HS	R	R	R	R
Malachite green	0.001	R	HS	R	HS	R	R	R	R
	0.01	R	HS	R	HS	HS	HS	R	R
	0.1	S	HS	S	HS	HS	HS	S	S

[1]R: Resistant, S: Sensitive, HS: Highly Sensitive

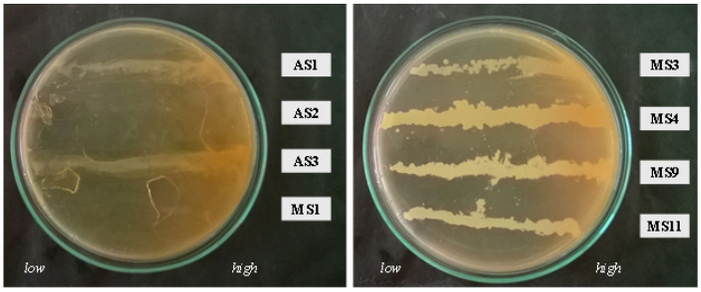

Fig. 1 Resistance of microbial isolates to Fluo orange at 0.1 mg/ml. Growth was observed at both low and high concentration side for all isolates except AS2 and MS1.

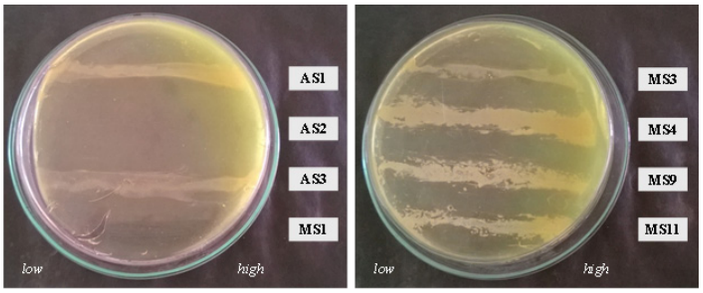

Fig. 2. Resistance of microbial isolates to Fluo green at 0.1 mg/ml. Growth was observed at both low and high concentration side for all isolates except AS2 and MS1.

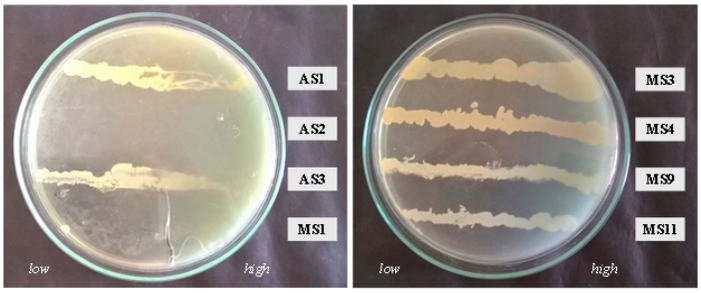

Fig. 3 Resistance of microbial isolates to Fluo blue at 0.1 mg/ml. Growth was observed at both low and high concentration side for all isolates except AS2 and MS1.

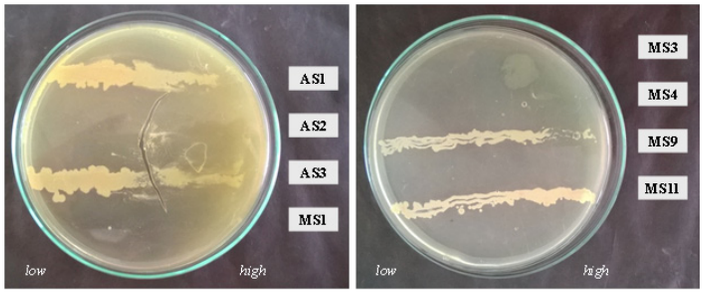

Fig. 4 Resistance of microbial isolates to Malachite green at 0.01 mg/ml. Growth was observed at both low and high concentration side for AS1, AS3, MS9 and MS11.

Effect of Dyes on Marine Microorganisms

Figure 5 presents the number of microorganisms grown on marine sample plates with and without dye. Highest number of colonies (296 CFU/ml) was seen in sample without any dye. The plates which had the test dyes incorporated showed significantly less number of colonies as compared to the control. 39 microbial colonies were observed on the plate with Fluo orange and 108 colonies on the plate with Fluo green. No growth was observed in plates containing Fluo blue and Malachite green. These results suggest that the dyes have a strong toxic effect on microorganisms found in sea water.

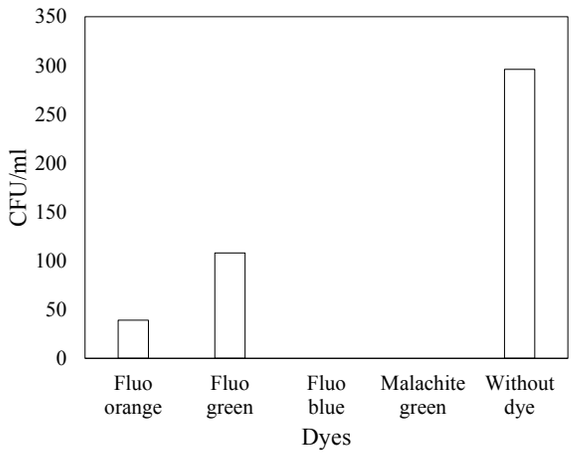

Fig. 5 Number of Colony Forming Units (CFU) of marine microorganisms with and without test-dye.

Effect of Heavy Metals on Isolates

Resistance of isolates from bivalves to heavy metal compounds is presented in Table 3. Among the eight isolates, MS11 showed the highest tolerance – up to 80 mM of $ZnSO_4$, $CuSO_4$, $CoCl_2$ and $PbNO_3$. For the other isolates the resistance was variable. MS4 was

Table 3 Sensitivity of the isolates to heavy metal compounds using agar cup method.

Metals	Conc. (mM)	Cultures[1]							
		AS1	AS2	AS3	MS1	MS3	MS4	MS9	MS11
$ZnSO_4$	10	HS	HS	R	R	R	HS	HS	R
	20	HS	HS	R	R	R	HS	HS	R
	40	HS	HS	S (22 mm)[2]	S (18 mm)	S (19 mm)	HS	HS	R
	80	HS	HS	HS	S (21 mm)	S (20 mm)	HS	HS	R
$CuSO_4$	10	HS	HS	R	R	HS	HS	HS	R
	20	HS	HS	S (19 mm)	S (19 mm)	HS	HS	HS	R
	40	HS	HS	HS	HS	HS	HS	HS	S (15 mm)
	80	HS	HS	HS	HS	HS	HS	HS	S (18 mm)
$CoCl_2$	10	HS	HS	R	R	HS	HS	HS	R
	20	HS	HS	R	S (19 mm)	HS	HS	HS	R
	40	HS	HS	S (20 mm)	HS	HS	HS	HS	S (20 mm)
	80	HS	HS	HS	HS	HS	HS	HS	S (25 mm)
$PbNO_3$	10	R	R	R	HS	R	HS	R	R
	20	R	R	R	HS	R	HS	R	R
	40	S (17 mm)	R	R	HS	HS	HS	R	R
	80	S (19 mm)	S (20 mm)	R	HS	HS	HS	R	R

[1] R: Resistant, S: Sensitive, HS: Highly Sensitive

[2]Size of Zone of Inhibition

found to be the most sensitive – no growth was observed with $ZnSO_4$, $CuSO_4$, $CoCl_2$ and $PbNO_3$ even at 10 mM concentration.

In the present paper, we studied Gram-nature and phenotypic character of bacteria isolated from gills of clams. The current results are comparable with the published work (Schweinemanns and Felbeck, 1985; Baldi *et al*., 2013; Espinosa *et al*., 2013). Bivalves carry large number of Gram-negative and Gram-positive bacteria within their gills, siphons and hepatopancreas (Baldi *et al*., 2013). Dando *et al*. (1985) showed that *Myrteaspinifera*clam collected from Ypsesund, Norway carrylarge number of Gram-negative, sulphuroxidizing bacteria. Other strains of clam like *Lucinid* collected from inshore lagoon in Bermuda contain chemoautotrophic bacteria in their gills (Schweinemanns and Felbeck, 1985).

Eight cultures were isolated in the current study – five from coast of Mumbai and three from coast of Alibaug. It is interesting to note that not only the cultures found in the two samples (Mumbai, Alibaug) were different but also their resistance to dyes and heavy metals were different. It indicates that the local marine environment is affecting the microflora. Compared to Alibaug, the marine environment in Mumbai is more polluted. We have obtained more resistant strains of microorganisms from Mumbai samples indicating survival and growth of microorganisms which have adapted to the local environment. Among the eight isolates, MS11 was found to be most resistant to the dyes (Fluo orange, Fluo green, Fluo blue, Malachite green) and metals (Zn, Cu, Co, Pb) used in this study. MS11 isolate was found in Mumbai sample and is Gram-positive and cocci-shaped. Detailed biochemical analysis (Table 4) identified MS11 as *Staphylococcus arlettae*. Schleifer *et al*. (1984) isolated *Staphylococcus arlettae* from skin of mammals and bird and found it to be Gram-positive, non-motile and cocci-shaped bacteria, which matches with the current results.

Microbial biodegradation is a natural and environment friendly process, compared to chemical processes, to convert toxic compounds into non-toxic end products (Saratale *et al*., 2011). Microorganisms use carbon and nitrogen as source of energy and lead to biodegradation of synthetic dyes. *Klebsiella* and *Bacillus* have been found to use glucose, starch, sucrose and yeast extract as carbon source and peptone as nitrogen source resulting in degradation of Turquoise blue and Malachite green dyes (Joshi *et al*., 2013; Ramezani *et al*., 2013). Cultures isolated in the current work use yeast extract and peptone from halophilic agar as nitrogen source and casein and beef extract as carbon source.

Table 4 Detailed biochemical analysis for identification of MS11.

Test	**AMY**	**PIPLC**	**dXYL**	**ADH1**	**BGAL**	**AGLU**	**APPA**	**CDEX**	**AspA**
Result	–	–	+	(+)	+	+	–	–	–
Test	BGAR	AMAN	PHOS	LeuA	ProA	BGURr	AGAL	PyrA	BGUR
Result	–	–	–	–	–	(–)	–	–	+
Test	AlaA	TyrA	dSOR	URE	POLYB	dGAL	dRIB	ILATk	LAC
Result	–	–	–	–	–	+	+	–	+
Test	NAG	dMAL	BACI	NOVO	NC6.5	dMAN	dMNE	MBdG	PUL
Result	–	+	–	+	+	+	–	+	–
Test	dRAF	O129R	SAL	SAC	dTRE	ADH2s	OPTO		
Result	–	+	+	+	+	–	+		

Elisangela *et al*. (2009) isolated *Staphylococcus arlettae* from activated sludge produced by textile industry in Brazil and showed that the isolate has the ability to decolourize and degrade azo dyes using a sequential microaerophilic/aerobic process. *Staphylococcus aureus*has been demonstrated to degrade Malachite green rapidly without the detection of leucomalachite green (Cao, 2009). With respect to heavy metals, Kumar *et* al. (2013) studied the resistance of *Staphylococcus sp*. and showed their potential to remove chromium and lead from solid waste. *Staphylococcus arlettae* isolated from arsenic contaminated site of West Bengal, India has the ability to remove arsenic from liquid media and to promote plant growth (Srivastava *et al.*, 2013). In the present study, *Staphylococcus arlettae* was isolated from gills of bivalves collected from coast of Mumbai. Halotolerance and resistance to toxic chemicals suggest that the isolated bacteria *Staphylococcus arlettae* could be useful in bioremediation processes in marine environments.

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