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Isolation of Nitrate and Phosphate Removing Bacteria from Various Environmental Sites

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Abstract: Problem statement: Nitrate and phosphate are two major pollutants due to anthropogenic activity like excessive use of fertilizers in agriculture. Their contamination has emerged as a global problem and its potential threat is marked on the environmental sustainance as well as on the public health. **Approach:** The objective of the current study is to isolate efficient nitrate and phosphate removing microbes from various environmental sites that have been selected on the basis of the nature of polutants received by them and their water quality assessment. These well characterized isolates could in future be used for the remediation of waste water. 30 different sites were screened using culture based method. The nitrate and phosphate removing abilities of the microbes were checked in enriched medium (Himedia M439) after 16 h of incubation at 37°C. **Results:** 7 efficient isolates were obtained from rhizosphere of Water lily, Marine beaches, Paddy field and Raw sewage canal. The highest nitrate removal (88.3%) was shown by isolate (WBUNB009) from raw sewage canal and the highest phosphate removal (82.9%) was shown by isolate (WBUNB004) from rhizosphere of Water lily. Morphologically all the isolates were gram positive bacilli as reconfirmed by environmental scanning electron microscopy. Biochemically as well as physiologically they differ from each other. **Conclusion/Recommendation:** This study leads to the isolation of efficient nitrate and phosphate removers from environmental origin. The phosphate removing efficiency is much higher than the type strain under identical condition. These native microbes might be responsible for maintaining the phosphate and nitrate levels at the 30 sites investigated inspite of the received pollution load. These isolates could be the potential bioremedial agents for other sites with high nitrate and phosphate contamination level.

Key words: Nitrate, phosphate, agricultural runoff, bacteria, Eutrophication, bioremediation, efficient isolates, phosphate contamination, European Community (EC)

INTRODUCTION

Nitrate and Phosphate are recognized as the major nutrients which are required by living organisms for their physiological processes. They are most commonly added as fertilizer to enhance the quality of soil. However they have emerged as most abundant pollutants in the world due to their excess usage. The traditional agricultural practices like dry farming with marginal irrigation, flood plain farming and random application of fertilizers are considered as diffused sources of nitrate and phosphate in soil and aquifers. Besides this, the irregular rainfall during different seasons and the stream flow pattern causes seepage of these contaminants from soil to surface and ground water (Whitmore *et al*., 1992; Jorgensen, 1999; Giupponi *et al*., 1999; Agrawal, 1999; Krishnaswamy *et al*., 2009).

The cultivation patterns like terrace farming results in nitrate leaching into aquifers (Nakasone and Yamamoto, 2004; Kinoshita *et al*., 2003).

Increased levels of nitrate up to 400 ppm have been detected in groundwater (Filintas *et al*., 2008). Possible sources of nitrate pollution include manure, agricultural fertilizer, industrial effluent, domestic wastewater, septic systems, human waste lagoons, animal feedlots and native soil organic matter, as well as geologic

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sources (Jin *et al*., 2004) Other point sources of nitrate are municipal sewage canals, septic tanks, sewage dumping grounds (Wakida and Lerner, 2005; 2006). The mining tailings, industrial effluent from nuclear reactors, radioactive waste processing units mainly those dealing with compounds like plutonium or thorium nitrate (Singleton *et al*., 2005). Nitrate contamination is a global problem and stands as second most dangerous pollutant after the pesticides. High concentration of nitrate in drinking water is a threat especially to infants, causing methemoglobinemia, also called "blue baby syndrome. The carcinogenic effect of nitrate is also reported. Concentration higher than 10ppm in drinking water may also cause stomach cancer in infants (Jin *et al*., 2004). EPA has demarcated the maximum contaminant level to be 10 ppm for NO_3 - N and 45 ppm for NO_3 concentration. A similar guideline of 50 ppm as NO_3 has been set by the WHO and the European Community (EC). Several conventional technologies adopted for nitrate removal are ion exchange resins, electro dialysis, reverse osmosis and distillation which substantially increase the cost of operation. Therefore the cost-effective alternative lies in the biological denitrification process (Pinar *et al*., 1997; Eckford and Fedorak, 2002).

The addition of phosphorus as phosphate fertilizers in soil in excessive amount causes serious environmental problems in the form of eutrophication which uses up large amounts of oxygen. The main sources of phosphate in aquatic environment is through household sewage water containing detergents and cleaning preparations, agricultural effluents containing fertilizers as well as industrial effluent from fertilizer, detergent and soap industries (Pradyot, 1997). Phosphate is generally present as polyphosphate and orthophosphate. The concentration of phosphate in water bodies vary from 0.005-10 ppm depending on the source of phosphate near the water body. On one hand digestive problems occur from extremely high levels of phosphate, on the other, phosphate levels greater than 1.0 may interfere with coagulation in water treatment plants. The EPA has fixed standard phosphate levels as 0.015 ppm for water supply, 0.025 ppm for aquatic life, 0.05 ppm for lakes and 0.02 ppm for mountain lakes (Kotoski, 1997). Microbial strategies are currently being used for the removal of excess phosphate load in waste water since it is an attractive alternative to chemical processing (Krishnaswamy *et al*., 2009).

The objective of the present study is to isolate efficient nitrate and phosphate reducing microbes from different environmental water bodies. Further characterization of these isolates would lead to the development of an array of novel organisms which can reduce nitrate and phosphate load in water bodies of various environmental sites leading to bioremediation.

MATERIALS AND METHODS

Sampling: Water samples were collected from different environmental sites to screen for nitrate and phosphate removing organisms. The sites were selected on the basis of the pollutants received by them. Main focus was on sites, which according to pollutants received were expected to have high load of nitrate and phosphate but showed low concentrations of these. Presumably these are the sites which host the nitrate and phosphate removers. The list of sites has been given below Table 1.

Environmental parameters: Different physical, chemical and biological parameters were assessed for the water samples from each site to understand the different concentration of pollutants in them. The physical and chemical parameters were assessed as per the protocol laid down by Central Pollution Control board of India. The biological parameter of the sites were assessed by studying the normal population of the sites. 10 microbes were selected which are commonly present in waste water (viz *Escherichia coli*, *Enterobacter aerogenes*, *Shigella flexneri*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis*, *Salmonella sp.* and *Staphylococcus aureus*.) and the water from the sites were serially diluted and spread on media specific for the microbes. The different media used were HiChrome *E.coli* coliform Selective agar base (HiMedia M-1294 for *Escherichia coli*, *Enterobacter aerogenes*, *Shigella flexneri*, *Klebsiella pneumonia*); HiChrome UTI agar base (HiMedia M-1353 for *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis*); *Salmonella* differential agar base (HiMedia M-1078 for *Salmonella sp*) and HiChrome Aureus agar base (HiMedia M-1468 for *Staphylococcus epidermidis* and *Listeria monocytogenes*).

Cultivation medium and growth conditions: Since one of the main objectives of our study is to isolate nitrate reducing microbes therefore the screening for microbes were done in high nitrate containing medium. The water samples of all the above mentioned sampling sites were serially diluted and plated on media containing 2000ppm of nitrate followed by overnight incubation at 37°C to isolate microbes which can survive in high nitrate concentration.

Table 1: Water samples were collected from the following sites. The sampling sites were selected on the basis of the nature of the pollutatnts received by them. The co-ordinates of the sampling sites have been mentioned below

Types	Locations	CO-Ords	Source of waste
Marine coast	Udaypur Beach	21°36’31”N	Recreational activity, animal waste
	(West Bengal)	87°28’52”E	in low volume, natural waste.
	Digha Beach	21°36’59”N	Recreational activity, animal
	(West Bengal)	87°30’08”E	waste, natural waste.
	Mandarmani Beach	21°41’N	Recreational activity,
	(West Bengal)	87°33’E	animal waste, natural waste.
Artificial water	Haldiram fountain	22°37’40”N	Bathing, natural waste,
Bodies		88°26’01”E	washing activity.
	Baguiati fountain	22°36’36”N	Natural waste
		88°25’41”E	
River	Ganga (Naihati)	22°53’23”N	Industrial waste, idol immersion, domestic waste, recreational waste,
		88°24’40”E	oil spill from transportation,
			piggery waste, sewage waste from surrounding area.
	Ichamati (Taki)	22°36’01”N	Industrial waste, idol immersion, domestic waste,
		88°56’46”E	recreational waste, agricultural waste, erosion of banks
	Churni (Ranaghat)	23°14’35”N	Human bathing, domestic and sewage waste,
		88°36’23”E	transportation by boat, agricultural waste, industrial waste.
	Damodar (Bardhaman)	23°12’41”N	Bathing, recreational waste, domestic waste,
		87°50’48”E	agricultural waste, fish aquaculture, oil spillage from launch
	Mayurakshi (Tilpara)	23°57’04”N	Fishing, washing, small
		87°31’06”E	scale agricultural run off
Hotspring:	Agnikundu, Bakreswar	23°52’51”N	Rice and coins thrown by
		87°22’32”E	devotees, plastic packets,
	Dudhkundu, Bakreswar	23°52’51”N	Natural waste, rice and coins.
		87°22’34”E	Population of frogs and snails found
	Jibatsakundu, Bakreswar	23°52’52”N	Algal growth seen, natural waste,
		87°22’34”E	rice and coins by devotees.
	Swet ganga (Birbhum)	23°52’52”N	Washing of hands and feet,
		87°22’34”E	waste from rituals e.g., flowers, utensil washing.
	Taptapani (Orissa)	19°29’04”N	Human bathing, natural
		84°23’37”E	wastes, rice and coins
			thrown by devotees.
Paddy field	Berunanpukuria (Barasat)	22°44’34”N	Fertilizer waste,
		88°26’20”E	agricultural run off.
Rhizosphere	*Typha* sp	22°35’14”N	Dumping of wastes from the nearby region.
of Swampy	Aquatic grass	88°28’31”E	(beside the ONGC green building),
			animal grazing therefore
Plants	*Nymphaeae nouchali*		animal waste
	(violet flower, water lily)		
	Water Hyacinth	22°35’40”N	Bathing, dumping of waste
		88°23’51”E	from local shops and residence, washing of utensils,
			cleaning of buses and trucks,
Natural water	Under Ultadanga over	22°41’19”N	Domestic waste dumping from
Bodies	bridge (Kolkata) Kalyani	88°25’05”E	local residence, erosion of banks.
	Byepass		
	Expressway (Fordillapur)		
	Site reserved for Himangini	22°43’34”N	Aquaculture, dumping of waste from
		88°24’12”E	local residence, erosion of banks.
	Jute Retting Site	22°47’12”N	Used for jute retting,
	Thankurbari	88°30’12”E	dumping of waste from local shops and residence
	Kaikhali pond	22°38’03”N	Bathing, washing clothes,
		88°26’16”E	cleaning utensils, idol immersion.
	Rajarhat pond	22°35’52”N	Animal waste, Pisciculture,
		88°28’09”E	recreational activity, erosion of the banks.
	Captain Bheri	22°33’10”N	Sewage, domestic and garage waste
		88°24’43”E	passed through a crude filtration for removal of coarsensuspended
			solids. Primarily used for pisciculture. Bathing and cleaning
			also found, agricultural run-off from surrounding region.
	Private Bheri	22°33’19”N	
		88°24’41”E	
Raw sewage	Kestopur khal	22°35’50”N	Domestic and sewage waste,
Canal		88°25’37”E	waste from agricultural
			land, oil from garage
	Bagjola khal	22°37’16”N	Domestic and sewage waste,
		88°24’10”E	waste from agricultural land,
			oil from garage, effluent
			from rubber industry.
	Khal Next to	22°33’17”N	Sewage, domestic and garage waste.
	Captain Bheri	88°24’47”E	Dumping from local area.

Further selection was made on the basis of morphology. The cultures were re-streaked three or more times to obtain pure colonies. The isolates were also grown in Nitrate Broth (Himedia M439-500G) and maintained at 37°C in 150rpm shaking condition. The final selection of the pure isolates was on the basis of their nitrate removing efficiency from the liquid culture.

Morphological characterization: The initial morphology of the isolates were determined by using light microscope (1000X magnification on a Zeiss Axiostar Plus microscope) following simple staining using 5% Crystal Violet. The Gram nature of the isolate was determined by differential staining as per standard procedure.

Dimensions of the isolates were determined using Environmental scanning electron microscopy (FEI QUANTA 200 MARK 2 at 15 kV) as per the protocol.

Biochemical characterization: The ability of the isolates to produce enzymes like DNase, oxidase, lipase, catalase and amylase was determined. The tests for the first five enzymes were done according to the protocol of Nandy *et al*. (2007) the amylase test was done on 1% starch agar plate and incubated at 37°C for overnight followed by flooding of the plate with iodine solution. The substrate utilization profile of the isolates were checked as per manufacturer's protocol using substrate utilization kits (HiMedia KB009).

Antibiotic assay: The response of the isolates towards 18 different antibiotics (HiMedia) were checked according to the procedure reported by Nandy *et al*. (2007)

Nitrate removal: The isolates were inoculated (2% inoculum) in Nitrate broth and incubated for 16 h at 37°C in a shaking incubator 150 rpm. The cell free supernatant was taken for estimation of nitrate removal after harvesting the culture at 8609×g for 10min. 200 µL of Salicyalic acid (5% Salicylic acid in H_2SO_4) and 40 µL of cell free supernatant was added and vortexed. The tubes were incubated in dark for 10 min. The reaction was stopped by addition of 2 mL of 4N NaOH. Optical density of this solution was measured after 20 min at 420 nm. The O.D was then compared to the standard curve prepared with known concentrations of $NaNO_3$ (100-1000 ppm) to determine the concentration of Nitrate remaining in the medium (Cataldo *et al*., 1975).

Phosphate removal: Phosphate can be detected by spectrophotometric method by conversion of the phosphates to Molybdophosphoric acid Complex (MOP) by Ammonium molybdate, followed by reduction of the MOP Complex by Sn^{2+} of $SnCl_2$ to give a blue coloured complex. For our study, at first a standard curve of phosphate was prepared by using standard solutions of phosphate from 0.05-0.5 ppm. 10 mL of cell free supernatant of bacterial sample (2% inoculum grown in Nitrate broth for 16hrs at 37°C) was diluted in 60 mL of distilled water in a 250 mL conical flask. 2 mL of Ammonium molybdate reagent was added followed by 4 drops of Stannous chloride. The solution was shaken well and volume was made upto 100 mL. The blue colour which developed indicated presence of phosphate which could be measured spectrophotometrically at 660 nm. The unknown concentration of phosphate was determined by comparing with the standard curve (Krishnaswamy *et al*., 2009).

Statistical analysis: The objective of the study being isolation of efficient nitrate and phosphate removers, the ideal situation would be a single isolate performing both the functions. The relation between nitrate and phosphate removal was investigated by using the Correlation Co-efficient as a measure of the association between the two variables. The correlation co-efficient measures the strength of the linear relationship between the variables.

RESULTS

Environmental parameters: The physical parameters assessed were pH, Suspended solids, Turbidity level of the water body, Temperature, Odour and Colour (Table 2).

The Chemical parameters include Alkalinity, Hardness, Calcium, Magnesium, Chlorine, Flouride, Nitrate, Ammonia, Phosphate, Total Iron, Residual Chlorine, Biological Oxygen Demand (Table 3).

The biological parameters were assessed by the growth of microbes in four different kinds of medium HiChrome *E.coli* coliform Selective agar base (HiMedia M-1294 for *Escherichia coli*, *Enterobacter aerogenes*, *Shigella flexneri*, *Klebsiella pneumonia*); HiChrome UTI agar base (HiMedia M-1353 for *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis*); *Salmonella* differential agar base (HiMedia M-1078 for *Salmonella sp*); and HiChrome Aureus agar base (HiMedia M-1468 for *Staphylococcus epidermidis* and *Listeria monocytogenes*) (Table 4).

Cultivation medium and growth conditions: The serial dilution of water samples from the above mentioned 30 sites on medium with 2000 ppm of nitrate gave 130 different colonies. Further selection was made on the basis of morphology since most of the nitrate reducers according to literature are bacilli therefore bacilli in long or short chain or isolated bacilli were selected.

Table 2: Water samples were collected from 28 different environmental sites which were then tested for different physical parameters like pH, suspended solids, turbidity level, Temperature, Odour and Colour

SITES	pH	Susp.solid (gm)	Turbidity level (cm)	Temp (°C)	Odour	Colour
Udaypur Beach (West Bengal)	6.30	0.008	Not applicable	15.0	Fishy	Normal
Digha Beach (West Bengal)	6.10	0.063	Not applicable	15.0	Normal	Normal
Mandarmani (West Bengal)	5.70	0.056	Not Applicable	15.0	Normal	Normal
Haldiram fountain	7.02	0.015	Not applicable	24.0	Fishy	Normal
Baguiati Fountain	7.25	0.030	Not applicable	24.0	Fishy	Normal
Ganga (Naihati)	7.42	0.010	5.1	21.0	Normal	Off white
Ichamati (Taki)	6.50	0.005	18.1	21.0	Unpleasant	dirty light brown
Churni (Ranaghat)	6.50	0.009	55.1	21.0	Normal	normal
Damodar (Bardhaman)	7.10	0.068	17.75	22.5	Normal	Normal
Mayurakshi (Tilpara)	7.50	0.013	Not applicable	20.0	Normal	Normal
Agnikundu, Bakreswar	8.84	0.002	100	72.0	Normal	Normal
Dudhkundu, Bakreswar	7.40	0.001	100	31.5	Fishy	Normal
Jibatsakundu, Bakreswar	7.20	0.003	100	22.0	Normal	Normal
Swet ganga (Birbhum)	7.90	0.017	28.3	34.0	Normal	Normal
Taptapani (Orissa)	7.30	0.016	100	40.0	Normal	Normal
West Bengal state university (Barasat)	6.40	0.018	Not applicable	22.0	Normal	Light brown
Rhizosphere of aquatic plants	7.10	0.003	Not applicable	22.0	Normal	Normal
Under Ultadanga over bridge	7.20	0.054	20.5	21.0	Fishy	Green
Byepass Kalyani Highway(Fordillapur)	7.30	0.014	18.1	22.0	Normal	Green
Site reserved for Himangini	7.60	0.028	49.5	22.0	Fishy	Very light green
Jute Retting Site Thankurbari	6.50	0.066	5.2	22.0	Unpleasant	Black
Kaikhali pond	7.80	0.006	49.0	23.0	Normal	Normal
Rajarhat pond	7.30	0.014	5.1	20.0	Normal	Green
Captain Bheri	7.20	0.028	28.1	21.0	Normal	Green
Private Bheri	7.30	0.036	20.5	24.0	Normal	Light green
Kestopur khal	6.50	0.011	22.0	21.5	Unpleasant	Black
Bagjola khal	6.80	0.043	18.1	21.0	Unpleasant	Grey
Khal Next to Captain Bheri	6.50	0.059	20.0	23.0	Unpleasant	Grey

Table 3: Water samples were collected from 28 different environmental sites which were then tested for different chemical parameters of the sampling sites like Alkalinity, Hardness, Calcium, Magnesium, Chlorine, Fluorine, Nitrate, Ammonia, phosphate, total Iron, Residual Chlorine and Biological Oxygen Demand in parts per million (ppm)

SITES	Alk (ppm)	Hard (ppm)	Ca (ppm)	Mg (ppm)	Cl (ppm)	F (ppm)	NO3 (ppm)	NH3 (Ppm)	PO4 (Ppm)	Total Iron (ppm)	Res Cl (ppm)	BOD (ppm)
Udaypur Beach (West Bengal)	88	7400	4070.4	809.090	15095.320	1.5	17.73	0.5	0.15	0.00	0.00	2.4
Digha Beach (West Bengal)	96	7216	4032.0	773.710	15295.250	1.0	19.02	0.5	0.16	0.00	0.00	2.0
Mandarmani (West Bengal)	88	1248	793.0	110.565	8678.000	1.5	19.30	0.5	0.04	0.00	0.00	2.8
Haldiram fountain	340	360	112.0	60.260	207.080	1.0	0.00	0.0	0.20	0.00	0.10	2.0
Baguiati Fountain	180	240	92.8	35.760	262.410	1.5	8.28	0.0	0.00	0.00	0.10	6.6
Naihati (Ganga)	164	184	54.4	31.490	33.980	3.0	20.89	0.0	1.57	0.20	0.00	0.0
Taki (Ichamati)	180	600	115.2	117.800	1099.659	1.5	22.73	0.0	0.00	0.00	0.00	4.4
Ranaghat(Churni)	348	360	112.0	60.260	19.990	2.0	16.71	0.0	0.00	0.00	0.00	1.2
Bakreswar (Damodar)	108	120	48.0	17.490	45.980	1.5	29.70	0.0	0.609	0.00	0.00	1.2
Tilpara(Mayurakshi)	72	200	44.8	37.710	13.990	1.5	25.60	0.0	0.00	0.00	0.00	2.4
Agnikundu,	124	8	0.0	1.940	52.868	1.0	32.90	0.0	0.00	0.00	0.00	0.8
Dudhkundu,	140	80	16.0	15.550	129.950	1.0	29.76	0.0	1.54	0.20	0.00	1.6
Jibatsakundu,	180	104	48.0	13.60	129.950	0.0	33.93	0.0	0.00	0.20	0.00	2.0
Swet ganga (Birbhum)	140	40	9.6	7.38	121.960	1.0	17.73	0.0	0.14	0.20	0.00	2.8
Taptapani (Orissa)	140	480	41.6	106.53	23.990	0.5	16.15	0.0	0.02	0.00	0.00	1.6
West Bengal state university (Barasat)	228	176	64.0	27.21	154.950	0.5	19.95	0.5	0.00	0.00	0.00	1.2
Rhizosphere of aquatic plants	172	200	51.2	36.15	119.960	1.5	20.23	0.5	0.45	0.20	0.00	2.8
Ultadanga over bridge	404	560	176.0	93.31	624.800	2.0	19.40	0.5	0.00	0.20	0.00	0.4
Byepass Kalyani Highway (Fordillapur)	172	216	76.8	33.82	74.940	1.5	10.78	0.0	0.00	0.00	0.00	2.8
Site reserved for Himangini	340	360	112.0	60.26	187.440	1.5	18.00	0.5	0.00	0.00	0.00	4.4
Jute Retting Site Thankurbari	320	240	96.0	34.99	49.980	2.0	22.00	0.5	2.22	0.20	0.10	2.8
Kaikhali pond	280	224	51.2	41.99	54.980	1.5	18.00	0	0.00	0.20	0.00	7.2
Rajarhat pond	356	360	89.6	65.70	149.950	2.0	27.45	0.5	1.04	0.40	0.10	2.4
Captain Bheri	80	120	96.0	5.83	249.920	1.5	6.80	0.5	0.20	0.40	0.10	2.8
Private Bheri	85	600	96.0	122.47	189.940	0.5	3.10	1.0	0.00	0.40	0.15	3.2
Kestopur khal	168	192	64.0	31.10	87.470	1.5	15.00	0.0	0.33	0.20	0.00	0.4
Bagjola khal	280	280	89.6	46.26	149.950	2.0	26.25	0.0	0.62	0.60	0.00	2.4
Khal Next to Captain Bheri	200	384	131.2	61.43	252.40	1.0	10.00	0.5	1.50	0.80	0.00	2.0

Alk-Alkalinity; Hard-Hardness; Ca-Calcium; Mg-Magnesium; NO_3- Nitrate; PO_4- Phosphate; Res Cl-Residual Chlorine; BOD- Biological Oxygen demand

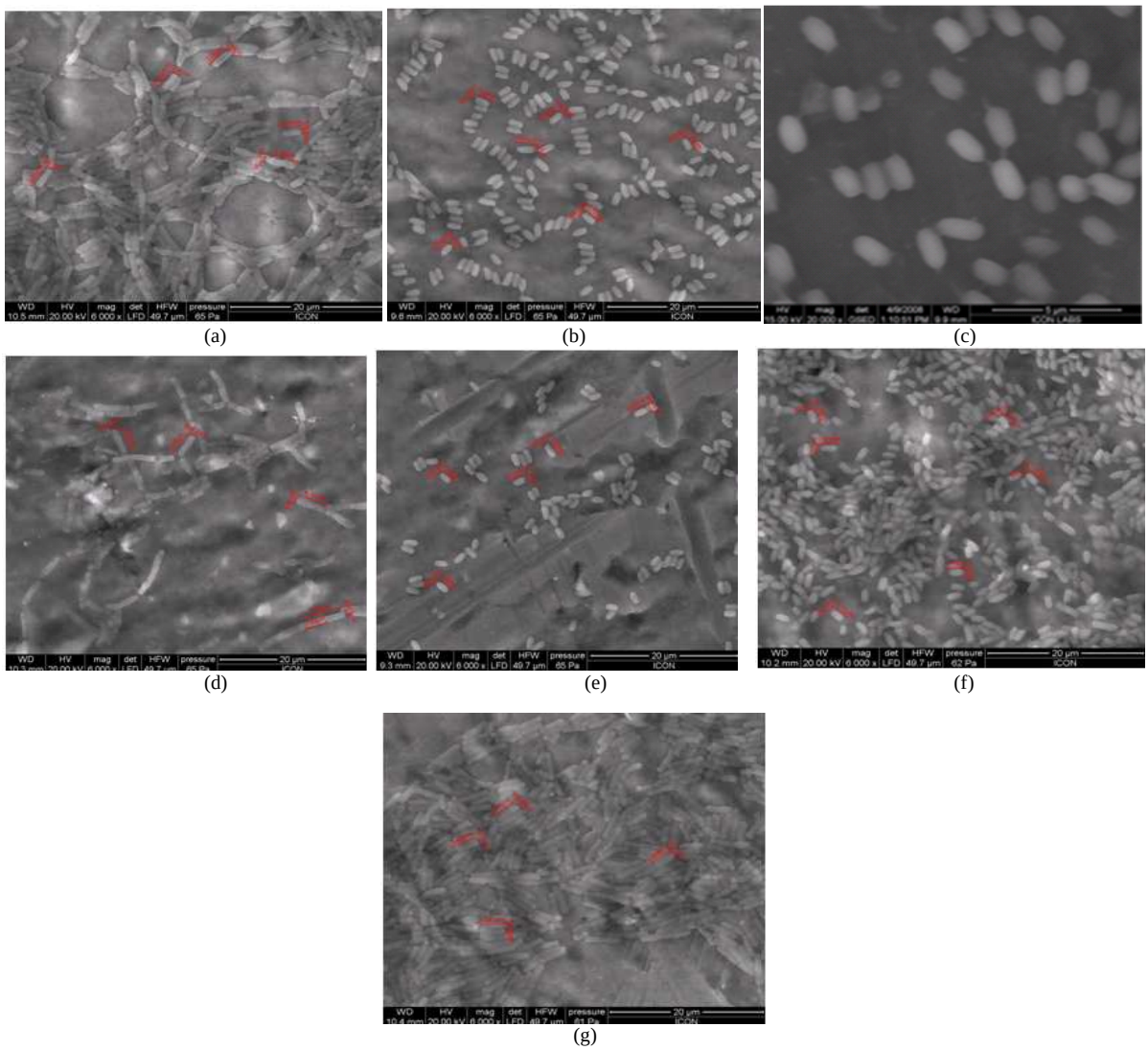

Fig. 1: (a) (WBUNB005) (b) WBUNB008 (c) SM2 (d) WBUNB006 (e) WBUNB009 (f) WBUNB004 (g) WBUNB007 The figures represent the Scanning electron micrographs of the isolates at different magnifications. WBUNB005 (6000X, 2.54µmX966.5nm), WBUNB008 (6000X, 2.26µmX996.5nm), SM2 (20000X), WBUNB006 (3000X, 3.44µmX936.1nm), WBUNB009 (6000X, 1.97µmX922.6nm), WBUNB004 (6000X, 1.95µmX923.3nm), and WBUNB007 (6000X, 3.18µmX791.6nm) These were visualized by Scanning electron microscope (FEI QUANTA 200 MARK 2)

19 such strains were then checked for their efficiency of nitrate removal from liquid culture. 7 bacilli were selected on the basis of nitrate removing efficiency for further characterization.

Morphological characterization: Simple staining of the microbes showed different morphology of microbes. The Environmental Scanning Electron Micrographs of the isolates (Fig. 1) show that WBUNB004, WBUNB005 and WBUNB006 (from rhizosphere of water lily) are bacilli in chain whereas WBUNB008 (from paddy field), SM2 (from marine beach) and WBUNB009 (from raw sewage canal) are short isolated bacilli and WBUNB007 (from marine beach) is long bacilli.Gram staining showed that all the isolates were gram positive in nature.

Biochemical characterization: The biochemical characterization of the strains is represented in Table 5. All isolates except WBUNB007 produced oxidase and protease.

Table 4: Water samples were collected from 28 different environmental sites which were then tested for various microbial populations by incubating diluted water samples on medium specific for the growth of Escherichia coli, Enterobacter aerogenes, Shigella flexneri, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus faecalis, *Salmonella* sp. and Staphylococcus aureus

SITES	*E.coli It*	*E. Aerogenes*	*S. Flexneri*	*K. Pneumonia*	*P.aer uginosa*	*P.mir abilis*	*E.fae calis*	*Salmonella sp*	*S. epide*	*L.monocy togenes*
Udaypur Beach (West Bengal)	0	20	0	0	0	0	0	0	0	0
Digha Beach (West Bengal)	80	160	740	60	320	0	100	20	20	40
Mandarmani (West Bengal)	0	0	13	40	27	0	40	0	0	13
Haldiram fountain	4000	2480	3120	6000	114000	0	0	0	40	160
Baguiati fountain	2000	200	3600	100	160	0	160	200	0	0
Naihati (Ganga)	300	420	2400	220	960	0	720	80	400	0
Taki (Ichamati)	120	280	800	320	2400	0	160	1040	2480	0
Ranaghat(Churni)	0	0	0	160	60	0	20	80	0	0
Bakreswar (Damodar)	1020	220	0	480	0	0	280	320	1000	0
Tilpara(Mayurakshi)	160	940	120	200	420	0	720	40	0	0
Agnikundu, Bakreswar	0	0	0	0	0	0	0	0	20	0
Dudhkundu, Bakreswar	1200	3680	240	80	800	0	2480	3300	0	0
Jibatsakundu, Bakreswar	2400	4800	800	800	160	400	3280	160	280	140
Swet ganga (Birbhum)	240	0	40	80	420	0	260	60	0	40
Taptapani (Orissa)	0	0	17760	0	20560	0	0	0	0	0
West Bengal state university (Barasat)	0	200	1300	140	900	0	240	2740	0	60
Rhizosphere of aquatic plants	1600	0	0	540	1040	0	160	800	0	20
Under Ultadanga over bridge	150000	1600	0	10000	66000	0	42000	50000	0	1920
Byepass Kalyani Highway (Fordillapur)	440	300	0	140	0	0	0	0	160	0
Site reserved for Himangini	40000	1120	0	800	320	0	0	0	0	1560
Jute Retting Site Thankurbari	312000	40000	112000	48000	56000	0	24000	40000	100	220
Kaikhali pond	80	120	220	100	0	0	380	20	0	120
Rajarhat pond	4000	1740	0	8000	0	0	0	4000	0	0
Captain Bheri	1800	520	8800	160	300	0	2120	0	0	0
Private Bheri	800	1400	500	1200	1200	0	1120	0	0	1440
Kestopur khal	72000	32000	64000	72000	90000	0	192000	48000	140	420
Bagjola khal	240000	464000	88000	16000	208000	0	192000	0	2600	1340
Khal Next to Captain Bheri	328000	0	56000	64000	272000	0	320000	0	2560	5760

Table 5: Morphological and biochemical characterization of 7 isolates. The morphological characterization includes the Gram nature whereas the Biochemical Characterization includes the capability of the isolates to produce enzymes such as Catalase, Oxidase, Protease, Amylase, lipase and DNase

Strain	Gram nature	Catalase	Oxidase	Protease	Amylase	Lipase	Dnase
WBUNB005	Gram positive bacilli	+	+	+	+	-	-
WBUNB008	Gram positive bacilli	-	+	+	+	-	+
SM2	Gram positive bacilli	+	+	+	+	-	+
WBUNB006	Gram positive bacilli	+	+	+	+	+	-
WBUNB009	Gram positive bacilli	+	+	+	+	+	+
WBUNB004	Gram positive bacilli	+	+	+	+	+	+
WBUNB007	Gram positive bacilli	-	-	-	+	-	+

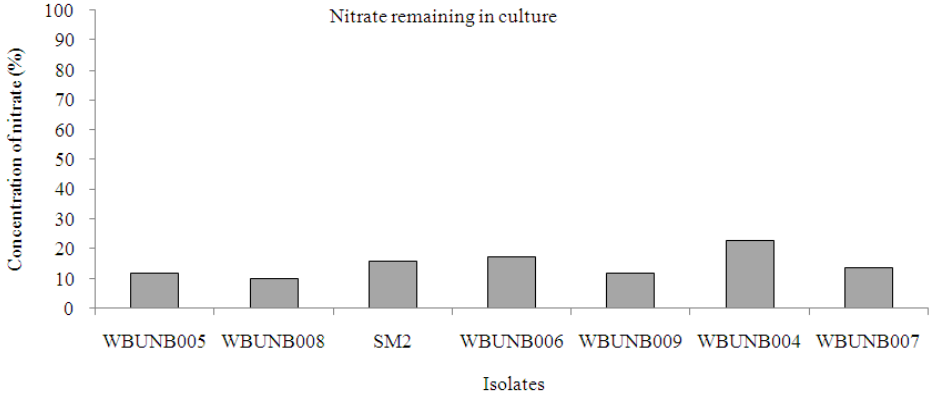

Fig. 2: Graph representing the percentage of nitrate remaining in the medium after incubation with the isolate for 16 h

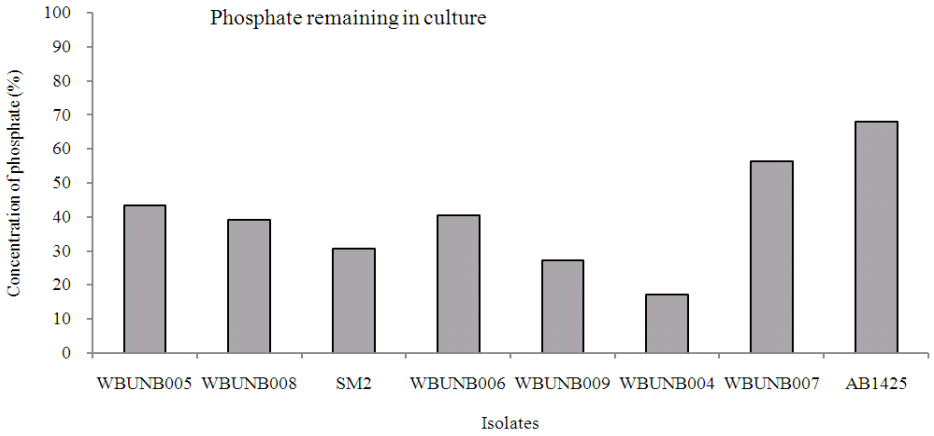

Fig. 3: Graph representing the percentage of phosphate remaining in the medium after incubation with the isolate for 16 h

Table 6: 7 isolates were tested for the preference of the substrate they required for growth by using Hi-Media Carbohydrate kit (KB 009). The substrate preference of the isolates are given below

STRAIN	SUBSTRATE UTILISED
WBUNB005	Maltose, Fructose, Dextrose, Trehalose, Sucrose, LArabinose, Mannose, Glycerol, Rhamnose, Cellobiose, Melezitose, α Methylmannoside, Xylitol, Esculin, D-Arabinose, Citrate, Malonate, Sorbose
WBUNB008	Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, Mannose, L-arabinose, Glycerol, Ribose Rhamnose, Cellobiose, Melezitose, α Methyl mannoside, Xylitol, Esculin, D-Arabinose, Citrate, Malonate, Sorbose
SM2	Maltose, Fructose, Dextrose, Trehalose, α Methyl-D-glucoside, Esculin
WBUNB006	Maltose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, LArabinose, Mannose, Glycerol, Ribose Rhamnose, Cellobiose, Melezitose, α Methyl mannoside, Xylitol, Esculin, D-Arabinose, Citrate, Malonate, Sorbose
WBUNB009	Maltose, Fructose, Dextrose, galactose, Raffinose, trehalose, Melibiose, Sucrose, Glycerol, Ribose Rhamnose, Cellobiose, Melezitose, α Methyl mannoside, Xylitol, Esculin, D-Arabinose, Citrate, Malonate, Sorbose
WBUNB004	Maltose, Raffinose, Melibiose, L-Arabinose, Mannose, Ribose Rhamnose, Cellobiose, Melezitose, α Methyl mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malonate, Sorbose
WBUNB007	Dextrose, Trehalose, Glycerol, Ribose Rhamnose, Cellobiose, Melezitose, α Methyl mannoside, Esculin, D Arabinose, Citrate, Malonate, Sorbose

Table 7: The 7 isolates were tested for their resistance and susceptibility to a common range of antibiotics. The antibiotic susceptibility profile of the isolates is given below

Antibiotics	Symbol	Wbunb 005	Wbunb 008	SM 2	Wbunb 006	Wbunb 009	Wbunb 004	Wbunb 007
Chloramphenicol (30 mcg)	C 30	S	S	I	S	S	S	S
Ceftazidime (30 mcg)	CA 30	R	R	R	R	R	R	R
Ampicillin (10 mcg)	A 10	R	R	R	R	R	R	R
Methicillin (4mcg)	MT 4	R	R	R	R	R	R	R
Rifampicin (15 mcg)	R 15	R	R	R	R	I	R	R
Norfloxacin (10 mcg)	N 10	S	S	S	S	S	S	S
Roxithromycin	Ro	S	S	I	S	S	S	S
Trimethoprim (5 mcg)	Tr	R	R	R	R	R	R	R
Doxycycline Hydrochloride (30mcg)	DO 30	S	S	S	S	S	S	S
Vancomycin (30 mcg)	VA 30	I	I	I	I	I	I	R
Cloxacillin (10 mcg)	CX 10	R	R	R	R	R	R	R
Polymyxin-B (300 units)	PB 300	R	R	R	R	R	R	R
Gentamycin (10 mcg)	G 10	S	S	S	S	S	S	S
Ciprofloxacin (5 mcg)	CF 5	S	S	S	S	S	S	S
Cefotaxime (30 mcg)	CTX 30	R	I	I	R	R	R	R
Cephadroxil (30 mcg)	CQ 30	S	S	I	S	S	S	S
Teicoplanin (30 mcg)	TE 30	S	S	S	S	S	S	S
Neomycin (30 mcg)	N 30	S	S	I	I	I	R	R

All except WBUNB007 and WBUNB008 produced catalase. Amylase is produced by all the isolates, whereas lipase is produced by WBUNB006, WBUNB009 and WBUNB004 and DNase by WBUNB008, SM2, WBUNB009 and WBUNB004. The substrate utilization profile of the isolates was performed by using different carbohydrate sources (Himedia KB009). This study gives us an array of substrates used by an isolate. Most of them could grow in presence of Maltose, Dextrose, Trehalose, Xylitol, Cellobiose, α-Methyl Mannoside, Melezitose, D-Arabinose, Citrate, Malonate and Sorbose (Table 6).

The antibiotic susceptibility profile of the isolates were performed by using different antibiotic discs (Himedia). This study shows that the strains were resistant to antibiotics like Ceftazidime, Ampicillin, Methicillin, Rifampicin, Trimethoprim, Polymixin-B and Cefotaxime and sensitive to antibiotics like Chloramphenicol, Norfloxacin, Roxithromycin, Doxycycline hydrochloride, Gentamycin, Ciprofloxacin, Cefadroxil and Teicoplanin (Table 7).

Nitrate removal: The nitrate removal from the medium is the primary step for the reduction of nitrate though after removal the bacteria may use the nitrate by assimilatory or dissimilatory pathway. The result is represented in the form of percentage of nitrate remaining in the medium after incubation with the isolate for 16 h at 37°C (Fig. 2).

Phosphate removal: The phosphate removal capacities of the isolates were checked in enriched medium in comparison with a type strain, *Acinetobacter baumanii* (MTCC 1425) known for phosphate removal obtained from MTCC. The result is represented in the form of percentage of phosphate remaining in the medium after incubation with the isolate for 16hrs in 37°C. The result indicates that all the isolates show better phosphate removal than *Acinetobacter baumanii* under the given set of conditions (Fig. 3).

Statistical analysis: The nitrate removal by the isolates were found to be within 77-88%, the average removal being 85.3%. The phosphate removal by the isolates were found to be within 43.8-82.9%, the average being 63.71% while that of the type strain under similar conditions showed 31.9% removal. The correlation study of nitrate and phosphate showed a negative moderate correlation of (-) 0.5584 which implies that an efficient nitrate remover is not necessarily an efficient phosphate remover.

DISCUSSION

In this study we report the isolation of 7 strains with potential for nitrate removal. They could be used for bioremediation of nitrate contaminated sites leading to environmental protection. Phosphate removers isolated during the study were found to be more efficient than the type strain (*Acinetobacter baumanii*) under identical conditions. Here we report 7 gram positive bacterial isolates which are highly efficient in phosphate removal. Since the mechanism of phosphate removal in bacteria leads to the intracellular accumulation of polyphosphate granules, these could be used as potential candidates for sequestration of phosphate from environmental sites.

CONCLUSION

The study is a successful attempt to isolate efficient nitrate and phosphate removing bacteria from various environmental sites for remediation of waste water by reducing the nitrate and phosphate load. In addition optimization of the waste water treatment parameters by these isolates in future could not only lead to environmental protection but also sequestration of essential plant growth nutrients from the waste which in turn could be re used.

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