

Original Research Article

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Isolation and Screening of *Bacillus* Species from Sediments and Application in Bioremediation

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ABSTRACT

In the present study *Bacillus* species were isolated and screened for bioremediation applications. The quantifiable analysis showed *Bacillus* flora in septclean 154x10⁵ CFU/ml in nutrient agar and 243x10⁵ CFU/ml in nutrient agar containing glucose. In river sediment, the *Bacillus* flora in the nutrient agar was 59x10⁵ CFU/ml and 104x10⁵ CFU/ml in nutrient agar containing glucose while the marine sediment in nutrient agar 48x10⁵ CFU/ml and 69x10⁵ CFU/ml in nutrient agar containing glucose. The isolates were found to be gram positive rod shaped spore forming motile bacteria. The biochemical analysis was prepared to recognize the qualitative potential by the following conventional tests like starch hydrolysis, casein hydrolysis, gelatin liquefaction, cellulose test, urease test, carbohydrate fermentation, oxidation fermentation, Indole test, Methyl red, Voges Proskauer, Citrate utilization, Triple Sugar Iron, Nitrate reduction, Catalase and Oxidase test. *Bacillus* species were showed their richness in protease, amylase, gelatinase, catalase, cellulase and urease enzymes. Fermentation capability for different sugars like glucose, sucrose, lactose, maltose and mannitol was determined. The three species of *Bacillus* were predominant which include *Bacillus subtilis*, *Bacillus polymyxa* and *Bacillus megaterium* in septclean samples while in sediment sample *Bacillus subtilis* was found to be the common inhabitant. The *Bacillus* isolates from septclean flora and sediments showed good potential in reducing coliforms from polluted aquarium water, dye reduction and oil degradation. Efficiency for dye reduction and growth in oil was also high. The *Bacillus* flora proved an excellent agent for bioremediation in aquarium water tanks and their application can be extended further in treating waste water effluent from industries and to the polluted environment.

Keywords

Bioremediation,
Bacillus species,
Dye reduction,
Coliform
reduction,
Effluent treatment.

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Introduction

Bacillus is a genus of rod-shaped gram positive bacteria are usually either obligate or facultative aerobes, under stressful environmental conditions, they produce oval endospore that can stay dominant for

extended periods (Berkeley and Ali, 1994). *Bacillus* species produce numerous enzymes enabling them to degrade a variety of complex substrates and continuously changing environment (Beveridge and

Davies, 1983). Bioremediation of manmade contaminants in the subsurface depends upon type of the organisms, type of contaminants, geological and chemical condition at the contaminated area (You *et al.*, 2007). Microorganisms possess enzymes that allow them to use environmental contaminants as food (Sreenivasulu and Aparna, 2001). Biological cleaners are microbes which differ according to the type of microbial strains and enzyme produced by them (Srivastava *et al.*, 1980; Rahman *et al.*, 2006). The *Bacillus* species of cultural strains can be used for bioremediation of septic tank cleaning and water pollution reduction in aquaculture ponds (Debarati *et al.*, 2005). The aromatic degradation of 3-hydroxy benzoate is produced by using *Bacillus brevis*, *Bacillus sphaericus* and *Bacillus megaterium* (Ronald, 1975). Some bacteria can actively metabolize pollutants by consuming them as energy sources and others produce enzymes that break down toxins into less harmful substances (Lisa *et al.*, 2006; Mathias and Jan, 1992). *Bacillus subtilis* can selectively absorb gold from solutions containing copper, zinc, iron and gold (Yung-Guo *et al.*, 2006; Gee and Dudeney, 1988). Bioremediation of gasoline contaminated soil by *Micrococcus* species, *Bacillus* species and *Corynebacterium* species (Rahman Pattanathu *et al.*, 2002). Removal of dyes from the waste water has been reviewed with respect to biological decolorization as well as complete biodegradation of the dye molecules (Nuno *et al.*, 2012). Bioremediation of heavy metal in contaminated soil is occurred by *Pseudomonas*, *Sporophyticus*, *Bacillus* and *Phanerochate* (Congeevaram *et al.*, 2007). In the present study, *Bacillus* species were isolated from septic tank, river and marine sediments. They were comparatively studied and assessed qualitatively to know their biochemical potential. The septic tank flora was tested for their ability to reduce

coliforms in aquarium water. The isolates from the three samples were applied for dye reduction and utilization of hydrocarbon.

Materials and Methods

Collection of Samples

Probiotic culture concentrate of Septclean sample and sediment sample from Kauvery River as well as water samples from Sea were collected in sterile polythene bag and transported immediately to the laboratory in ice baskets for bacteriological investigations.

Enumeration of *Bacillus* Colonies from Septclean and Sediments

The total *Bacillus* flora was enumerated by serial dilution technique, enumerated and expressed in number of CFU per milligram of the samples using nutrient agar and nutrient agar with 0.5% glucose.

Serial Dilution Technique

99 ml of distilled water was taken in three conical flasks, each were plugged with cotton and sterilized. About 1 gram each of the two sediment samples and 1ml of the septic tank sample were added to the three conical flasks respectively and shaken well by placing in a mechanical shaker for five minutes. The conical flasks with sediment samples were boiled in water bath at 80°C for 10 minutes, after that 1 ml each of the three samples was transferred to the test tubes containing 9ml of sterile distilled water by using sterile pipette and followed by serial dilutions up to 10⁻⁷. From each 1 ml of the required dilutions were transferred into petriplates and 20 ml of nutrient agar medium was poured into them aseptically. To another set of petriplates with the same dilutions as above, nutrient agar medium

with 0.5% glucose added. The petriplates were rotated clockwise and anticlockwise direction for uniform mixing and after solidification plates were incubated in an inverted position at 37°C for 24 hours. The number of bacterial colonies was calculated using the formula:

$$\text{Number of the flora per gram of the sample} = \frac{\text{Total bacterial count}}{\text{Volume of the sample}} \times \text{dilution factor}$$

Isolation and Cultivation Technique

The colonies randomly selected from the plates were isolated and sub-cultured both in nutrient agar slants and nutrient broth. Further purification was done by repeated sub-culturing on nutrient agar slants and nutrient broth. The parameters were observed by morphological and Biochemical tests.

Morphological Characterization

Microscopic examination such as gram's staining, spore staining, motility of bacteria by hanging drop method were done to identify the isolated organism.

Biochemical Characterization

The biochemical tests was used to determine the ability of the organism to produce Indole, Methyl Red test, Voges Proskauer test, Citrate Utilization test, Triple Sugar Iron, Amino acid Utilization test for identification of isolates.

Carbohydrate Fermentation (Acid and Gas Production)

Carbohydrate fermentation tests were performed to detect the ability of the isolates to ferment sugars like glucose, sucrose, lactose, maltose and mannitol with the

production of acid and gas. A colour change from pink to yellow indicates that sugar has been fermented and gas production was indicated by the presence of air bubble in the Durham's tube.

Enzyme Hydrolysis Test

Enzyme hydrolysis tests were performed to check the ability of the organisms to utilize substrate by producing respective enzymes. The enzyme hydrolysis tests such as starch hydrolysis, casein hydrolysis, cellulose hydrolysis, gelatin liquefaction test, urea test, catalase test, oxidase test and nitrate reduction test were carried out for the identification of the isolates.

Bioremediation Applications

Most Probable Number (MPN) Method

The Most Probable Number (MPN) method is a statistical multistep assay for estimating the concentration of viable organisms (usually bacteria) in a suspension. The test includes presumptive, confirmed and completed phases. The MPN calculations are commonly used to enumerate particularly physiological types of the microorganisms such as lactose fermenters in coliform counts. Presumptive test is used to determine the concentration of total coliforms in the water sample. Confirmed test determines the concentration of fecal coliforms in the sample using the Brilliant Green Lactose Bile broth media and followed by completed test. A loopful of culture from all positive tubes of the confirmed test were inoculated into EC broth and incubated at 44°C for 48 hours.

Dye Reduction Test

Dye reduction test was performed with the given bacterial culture (Tariq *et al.* 2015). A

known concentration of dye (Red, Violet, Yellow, Orange and Green) solutions was prepared and bacterial culture was inoculated followed by incubation. The absorbance value for the dye solutions were observed after each interval in spectrophotometer by following dye reduction assay,

Note where D = decolourization, A_{ini} = Initial absorbance, A_{fin} = Final absorbance

Test for the Capability of *Bacillus* to Grow in Oil Spills $D = \frac{A_{ini} - A_{fin} \times 100}{A_{ini}}$

The ability of *Bacillus* to grow in oil contaminated area was checked using Bushnell Hass medium. The media was prepared and poured into test tubes followed by sterilization. Diesel oil was sterilized separately and 0.5ml was added to the tubes containing Bushnell-Hass medium. The tubes were then inoculated with test organism and maintaining one as control. All the tubes were incubated for 7 days at 37°C and the OD value was taken by using spectrophotometer, each day by taking about 1 ml from the test tubes using sterile pipette and 3 drops of TTC were added to observe the colour change.

Results and Discussion

Total Plate Count

The total plate count was retrieved using nutrient agar and also with nutrient agar containing 0.5% of glucose following standard procedure. The *Bacillus* count for Septclean in nutrient agar was 154×10^5 CFU/ml, for River sediment was 104×10^5 CFU/ml and Marine sediment 48×10^5 CFU/ml.

Morphological Characterization

The cultural morphological characters of isolates showed slightly raised, flat, regular,

irregular, white, and cream coloured colonies. In microscopic examination by gram staining the isolates were found to be gram positive rod shaped bacteria while in motility test isolates were showed motility and in spore staining the isolated bacteria possessed terminal and subterminal spores.

Biochemical Characterization

The biochemical characterizations of isolates for Indole, Methyl Red, Voges Proskauer, Citrate Utilization, Triple Sugar Iron, Nitrate reduction and Amino acid Utilization tests which confirmed isolates were *Bacillus subtilis*, *Bacillus polymyxa* and *Bacillus megaterium* (Table 1).

Carbohydrate Fermentations

The isolates showed colour change from pink to yellow indicates that sugars like glucose, sucrose, lactose, maltose and mannitol has been fermented by production gas bubble in the Durham's tube (Table 2).

Enzyme Hydrolysis

The isolates found to be capable of hydrolyzing the substrates of starch, casein, gelatin, cellulose by producing zone of hydrolysis around the colonies and positive reactions for the urease; catalase while oxidase test showed both positive and negative reactions thus made it further confirmed that the isolates were belongs as *Bacillus subtilis*, *Bacillus polymyxa* and *Bacillus megaterium* (Table 3).

Analysis of Bio-Remediation Parameters

Most Probable Number (MPN)

The aquarium water was subjected to most probable number test to estimate the number of coliforms present in the sample. The results indicated that the highest number of

coliforms associated with sample before treatment were found 1400+ cells per 100 ml and no cell was found after treatment with septclean flora. These values indicated that the most probable number of polluted water decreased after the treatment with *Bacillus* species.

Dye Reduction Assay

Dye reduction test for red, yellow, violet, green and orange colour dyes was performed with the bacterial isolates of *Bacillus subtilis*, *Bacillus polymyxa* and *Bacillus megaterium*. After incubation, the

absorbance values for the dye solution for each interval were observed in the spectrophotometer (Table 4).

Growth Efficiency of *Bacillus* in Oil

The isolates from the septclean and the two sediment samples were checked for a period of 7 days for their ability to grow and utilize oil. An increase in the intensity of red colour was observed in these days when TTC was added. The rate of growth indicated that *Bacillus* species used oil as substrate for multiplication (Table 5).

Table.1 Biochemical Characterization of *Bacillus* Isolates from Septclean and Sediments of Kauvery

Biochemical activity	Septclean Isolate	Sediment River Isolate	Sediment Sea Isolate
Indole test	Negative	Negative	Negative
Methyl red test	Positive	Positive	Positive
Voges Proskauer test	Positive	Positive	Positive
Citrate test	Positive	Positive	Positive
Triple sugar iron agar test	K/A	K/A	K/A
Identified as	<i>Bacillus subtilis</i>	<i>Bacillus polymyxa</i>	<i>Bacillus megaterium</i>

River and sea

Table.2 Carbohydrate Fermentation of *Bacillus* Isolates from Septclean and Sediments of Kauvery

Carbohydrate fermentation	Septclean Isolate	Sediment River Isolate	Sediment Sea Isolate
Glucose	Positive	Positive	Positive
Lactose	Positive	Positive	Positive
Maltose	Positive	Positive	Positive
Mannitol	Positive	Positive	Positive
Sucrose	Positive	Positive	Positive
Oxidation fermentation test	Positive	Positive	Positive
Identified as	<i>Bacillus subtilis</i>	<i>Bacillus polymyxa</i>	<i>Bacillus megaterium</i>

River and sea

Table.3 Enzyme Characterization of *Bacillus* Isolates from Septclean and Sediments of Kauvery

Biochemical Tests	Septclean Isolate	Sediment River Isolate	Sediment Sea Isolate
Enzyme characterization	Positive	Positive	Positive
Casein hydrolysis	Positive	Positive	Positive
Gelatin hydrolysis	Positive	Positive	Positive
Cellulose test	Positive	Positive	Positive
Urease test	Positive	Positive	Positive
Catalase test	Positive	Positive	Positive
Oxidase test	Positive	Negative	Negative
Nitrate reduction test	Positive	Positive	Positive
Identified as	<i>Bacillus subtilis</i>	<i>Bacillus polymyxa</i>	<i>Bacillus megaterium</i>

River and Sea

Table.4 Rate of Dye Reduction by Various Isolates

Sample	Organism	Red	Violet	Yello	Orange	Green
Septclean	<i>Bacillus subtilis</i>	37%	73%	78%	52%	67%
River Sediment	<i>Bacillus polymyxa</i>	22%	60%	86%	42%	52%
Marine Sediment	<i>Bacillus megaterium</i>	27%	65%	84%	40%	68%

Table.5 O.D Values Showing Growth Rate of *Bacillus* Species in Oil

Sample	Organism	Initial value (Before treatment)	Final value (After treatment)
Septclean	<i>Bacillus subtilis</i>	0.224	0.425
River sediment	<i>Bacillus polymyxa</i>	0.121	0.317
Marine sediment	<i>Bacillus megaterium</i>	0.048	0.341

The present investigation on applications of *Bacillus* species for bioremediation has been done on laboratory based experiments employing *Bacillus* from sediments and a bio-based product, containing *Bacillus subtilis*, *Bacillus megaterium* and *Bacillus polymyxa*. All three floras are mixed in the bioproduct so that they can work effectively in septic tank where bioremediation is required. BTS septic tank cleaner (septclean) is a liquid form of millions of bacteria (*Bacillus subtilis*, *Bacillus polymyxa*, *Bacillus*

megaterium) which are highly active (Masahiro *et al.*, 2005). Their treatment in a septic tank twice a year is essential to clean the septic tank system which will ensure the sanitation by killing the pathogenic flora in human faecal matter like worms, bacteria, viruses and fungi (Tariq *et al.*, 2013). The morphological characters of *Bacillus* species were noted as: *Bacillus subtilis* of 5mm size, slightly raised, regular and cream coloured colonies; *Bacillus polymyxa* of 3mm size, flat, irregular and white coloured colonies;

and *Bacillus megaterium* of 6mm size, flat, irregular and cream coloured colonies. The range of *Bacillus* flora was more in septclean than in sediments. While comparing the *Bacillus* flora among the two sediments, the count was more in river sediment than in marine sediment. The organisms were motile, Gram positive long rods and produced oval endospores at subterminal and terminal positions (Yumoto *et al.*, 2005). The biochemical characters studied showed the richness in the extracellular enzymes and the flora can be used for the bioremediation of groundwater and waste treatment ponds apart from the septic tank cleaning (Pandey and Jain, 2002). They can also be used as pond probiotics in the aquaculture ponds as they possess stable alkaline protease, amylase, lipase, cellulose with the potential features they can grow in fresh, brackish and marine environment as bioremediators but the growth will be slower at salinity over 32-40 ppt (Breure and Andel 1984). The isolates fermented sugars like glucose, lactose, maltose, mannitol and sucrose which were fermented with the production of acid (Chang *et al.*, 2004). Triple sugar iron agar test with the isolates showed their ability to utilize glucose efficiently without the production of H₂S (Clements *et al.*, 2002). The bacterial culture from depths of freshwater sediments, capable of reducing nitrate to nitrite, or of complete denitrification. Indole test showed the absence of tryptophan enzyme to break down tryptophan and produce indole. The acidic product was formed in methyl red test and which turned to alkaline as end product by the formation of acetoin in Voges Proskauer's test (Sterling and Patrick 1965). The citrate of as a sole source of carbon for growth which confirmed by citrate utilization test (Manuel and Harold 1963). The isolates from septclean and river sediment were revealed to be *Bacillus subtilis*, *Bacillus polymyxa* and *Bacillus*

megaterium while that from marine sediment were *Bacillus subtilis* and *Bacillus megaterium*. The effectiveness of septclean flora in cleaning purpose, initial coliform count was found to be 1400+ cells per 100 ml. After treatment with *Bacillus* to 48 hours, the count was effectively reduced to zero which showed 100% reduction (Sekaran *et al.*, 2007). It showed that the bacterial count (MPN) was reduced by 99.9997%. The potential of *Bacillus* flora isolated from sediments and septclean for dye reduction was assessed and a final reduction in the colour of the dyes was obtained (Amit *et al.*, 2008). *Bacillus subtilis* from septclean was found to be more efficient in reducing red, violet and orange dyes while *Bacillus polymyxa* from river sediment reduced yellow dye efficiently. *Bacillus megaterium* from marine sediment was effective in reducing green dye. The azo dyes and redox dyes can be reduced using enzymes from *Bacillus subtilis* and *Bacillus cereus* (Mojca *et al.*, 2007). *Bacillus subtilis* from septclean was more efficient in utilizing oil followed by *Bacillus megaterium* from marine sediment and then by *Bacillus polymyxa* from river sediment. This shows that the *Bacillus* species would be effective in bioremediation in the areas of oil-spills. In previous literature *Bacillus* species DS6-86 was found to degrade 59% of the oil at 1% concentration and the mixed bacterial consortium degraded a maximum of 78% of BH crude oil. The sediment flora used in the present study was found to be equally active when compared to septclean flora, according to their biochemical characters studied which indicates the possibility of producing Efficiency Microbes (EM) preparation with the indigenous flora of *Bacillus* species which are abundantly available in sediments. Thus in the future, there will be no need to import the preparation if these microbial floras can be formulated into useful preparations. The study can be extended for

further bioremediation applications for proper maintenance of the ecosystem.

In conclusion, Bioremediation using *Bacillus* species was selected for their ability to produce more carbon dioxide than biomass from organic carbon pollutants. Indigenous septic tank or pond bacteria often generate an excess of biomass usually, mucous or slimy, which increases pond pollution. In aquatic environment, the bioremediation can reduce their severity of pollution during the period of low light. By reducing the pond pollution, aquatic diseases associated with polluted water can be reduced and the mortality can be prevented in the farmed species of fish and shrimps. Oil spills, especially in marine environment, can be effectively uptaken by *Bacillus* by producing a biosurfactant through a process of emulsification. Biodegradation of azo dyes is another main feature of *Bacillus* species.

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