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ISOLATION, BIOCHEMICAL CHARACTERIZATION OF PURPLE NON SULFUR BACTERIA AND IN WASTE WATER TREATMENT.

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ABSTRACT

The purple non sulfur bacteria are one of the most diverse photosynthetic bacteria. They are adaptable phototropic organisms known to occur in aquatic sediments. This research study aimed to isolate, identify, and characterize Purple non sulfur bacteria. Morphological character of the isolated Purple non sulfur bacteria showed gram negative, oval and non motile. The isolated Purple non sulfur bacteria utilized starch, sugar, citrate, urease and nitrate. The isolated Purple non sulfur bacteria was used to treat waste water. The COD level was reduced from 2546.1 to 345.96 ppm and BOD level was reduced from 1275 ppm to 183 ppm and for 1 gm of sample over a period of twenty days. The pH also reduced from 8.7 to 7.1. The decrease in COD, BOD and pH was significant. The data clearly demonstrates that the bacterium is effective in transforming complex waste water into useful products.

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1. INTRODUCTION

Photo synthetic bacteria like the purple non sulfur bacteria to occur in water columns of rice fields, in activated sludge systems, in waste water environment and in aquatic sediments (Montano *et al* 2009). Low dissolved oxygen tension and the high availability of light and simple organic nutrients are important factors for promoting the proliferation of purple non sulfur bacteria in the environment (Okubo *et al* 2006). These bacteria are called non sulfur because it was originally thought that they were unable to use sulfides as an electron donor for the reduction of carbon dioxide to cell material. The PNSB are known to play an important role in the

circulation of carbon, nitrogen and sulfur (Kondo *et al* 2010). Some of the purple non sulfur bacteria eg Rhodospirillum, Rhodospirillum and Rhodospirillum are the nitrogen fixing microorganisms. Purple non sulfur bacteria contain valuable substances that have practical importance as feed protein (Paronyan *et al* 2009). These bacteria were used to supplement feed along with seaweed meal in some fish species which are used as a feed ingredient to reduce cost to increase the growth and survival of fish species (Azad and Xiang, 2012). The bacterium Rhodovulum, sulfidophilum when combined with commercial tilapia feed, improves the growth and survival of tilapia during grow out period

(Banerje et al 2000). Another study by (Shapa et al 2012) showed that the inclusion of purple non sulfur bacteria *Rhodovulum* species in formulated feed promoted the growth, feed conversion ratio and survival rate of Asian sea bass juveniles. Purple non sulfur bacteria were also used to improve water quality (Kim et al 2004). Remediation of waste water using microorganisms is being exploited worldwide due to several advantages (Singleton, 1994). Several of environmental factors affect the metabolism rate of biochemical activities, pH, and concentration of suspended solids, BOD, COD, dissolved oxygen. Number and species of microorganism present nutrient contaminations are of vital importance (Hamme et al 2003). Aerobic and anaerobic microorganisms are used for treating waste water. Anaerobic organism's especially oxygenic phototropic purple non sulfur bacteria after several advantages over an aerobic treatment for moderate to high strength wastes. These organisms have more advantages as they can grow at high BOD and need no dilution of waste water, require small treatment space, no sludge disposal problems, easy maintenance of treatment space, tolerance to cold and feasibility of nitrogen and grease removal and biomass rich in protein (60%) and vitamin B12 production are ideal organisms for waste water treatment. The aim of this research to examine their morphology, cultural characteristics as well as their biochemical characters. It was also aimed to assess the BOD, COD and pH in the waste water.

2. MATERIALS AND METHODS

Collection of sample

Sediment samples were collected from aquaculture pond of Puthuvypu, Cochin. It's a polyculture pond where Tilapia, mullets, milkfish, etroplus and shellfish like penacus indicus are cultured. The sample was collected aseptically in polythene bags and transported immediately to the bacteriology lab for isolation of photosynthetic bacteria.

Isolation of microorganisms

Isolation and growth of microorganisms were done by bio - chemical characteristics and particular growth pattern. The bacterial growth usually recognized by the development of color on the selective media MSS broth (Rodina, 1972). During logarithmic phase of photosynthetic bacteria which was isolated following all the anaerobic techniques were used for different experimental procedures. Synthetic media was used for the isolation, purification and sub culture of photosynthetic bacteria. MSS agar was used to isolate individual colonies and adjusting the pH 7.2 by pour plate method.

Characterization and identification of isolates

The isolates were grown on selective media and were chosen for further characterization. Isolates were examined for colony and cell morphology. Colony morphology was described with special emphasis on pigmentation, colony elevation and opacity. These characteristics were described from cultures growth at optimum temperature and pH. In biochemical tests, catalase test, starch hydrolysis, gelatin liquefaction, sugar fermentation, indole test, citrate utilization, urease test, nitrate reduction test, dye reduction test were performed.

Gram staining

Gram staining is a differential staining technique employed for the studies of bacterial morphology.

Motility

Locomotion is an active process in which a cell or an organism moves from place to place. Brownian movement achieves motility. Motility enables the organism to respond to certain environment stimuli.

Biochemical test

The colonies were absorbed in MSS broth for growth and pigmentation

Catalase test

This test is used to differentiate those bacteria that produce an enzyme catalase from non catalase producing bacteria. Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is treated for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. It indicates the positive.

Starch hydrolysis

The starch medium was prepared and sterilized and poured into plates. The test culture was streaked on the medium and kept in the desiccated under anaerobic conditions. To make the test, place two or three iodine crystals in the petridish and slightly warm the plates. Iodine starts vaporizing and reacts with starch to produce the blue color.

Gelatin liquefaction

The gelatin medium was sterilized by tyndalization by keeping the medium in steaming stream for three consecutive days. Then the medium was inoculated heavily with the test organism by stab inoculation and incubated for 96 hours. The hydrolysis of gelatin was indicated by the liquefaction when the cultures kept in 40 c for 1 hour.

Sugar fermentation

It is tested for five sugars glucose, maltose, sucrose, lactose and mannitol. Acid and gas

production can be noted by the presence of gas in the Durham's tube and bromocresol purple is the indicator used which will give a yellow if the sugar has to be fermented.

Hugh and Leif sons test

This test is used to differentiate those organisms that oxidize carbohydrates (aerobic utilization), from those organisms anaerobic treatment of carbohydrates is carried over (anaerobic fermentation)

Indole test

Tryptophan is an essential amino acid that can undergo oxidation by the way of enzymatic activities of bacteria and converted into metabolic products is mediated by the enzyme tryptophanase. The presence of indole is detected by adding Kovacs reagent which produces cherry red color. The color is produced by the reagent which is composed of p- dimethyl aminobenzaldehyde yielding the cherry red color. Absence of red coloration demonstrates that the substrate tryptophan was not hydrolyzed and indicates an indole negative.

Citrate utilization

It is used to determine the ability of a bacterium to utilize citrate as source of carbon. Bacteria can break the conjugate base salt of citrate into organic acids and CO₂. The CO₂ can combine with sodium and water to form sodium carbonate. The presence of carbonate changes the Bromothymol blue indicator incorporated into the medium from green to blue. Citrate negative will show no growth and the medium will remain green.

Urease test

The urea or agar medium was prepared and sterilized. The sterile medium was poured into the tubes and slants were prepared using aseptic technique, the culture was inoculated on to the slant and adds pH indicator phenol red. The tubes were kept for incubation for 24 to 48 hours. During incubation, microorganisms possessing urease will produce ammonia that raises the pH of the medium. As the pH becomes higher the phenol red changes from a yellow color to a pink color.

Nitrate reduction test

The ability to reduce nitrate was tested in an ordinary nitrate medium with peptone broth containing 0.3% potassium nitrate. The prepared broth was inoculated with the organism and sealed with a layer of paraffin oil. Turbidity was checked after the inoculation period and the nitrate reduction was done with the help of the nitrate reagent. Alphanaphthylamine and sulphanilic acid were mixed in equal proportions at the time of making the test. After mixing the red color obtained was recorded indicated the reduction of nitrate to nitrite

Dye Reduction Test

Dye reduction test was performed with given bacterial culture. A known concentration of dye solution was prepared and bacterial culture was inoculated. Then kept for incubation. The absorbance values for the dye solution were observed on the spectrophotometer. A graph was plotted against the absorbance value and concentration of the dye.

Collection of sample

The waste water was collected from the HLL Prawn hatchery. It was treated by purple non sulfur bacteria (PNSB). Treatment of the sample using purple non sulfur bacteria. The growth medium contained 20 ml of minimal salt medium and 80 ml of filtered sterilized sample. To maintain the anaerobic conditions, layering with paraffin oil was carried out. Glucose served as the carbon source and ammonium sulphate as the nitrogen source. After incubation with no shaking, the samples were analyzed every 5, 10, 15 and 20 days.

Determination of COD (Chemical Oxygen Demand)

Take two 250 ml conical flask and one was blank and other for the test. In a blank conical flask was taken and to that 10 ml distilled water and 10 ml of 0.025N potassium dichromate was added. In test conical flask were taken and add 10 ml sample and 10 ml of 0.025 N potassium dichromate. Then add 30 ml of acid reagent in both the flask. Refluxed the reaction mixture for 3 hours in Soxhlet apparatus and cooled to reach the room temperature. Then add 2 drops of ferroin indicator to the 2 conical flasks. Titrated against ferrous ammonium sulphate to get red color as the end point.

$$\text{COD} = \frac{(A-B) \times M \times 8000}{\text{ml of sample}}$$

(A = ml FAS used for blank, B= ml FAS used for sample, M= Molarity of FAS)

Determination of BOD (Biological Oxygen Demand)

Two flasks were taken; labeled one as initial and the other as final. About 15 ml was taken and then aerated sample was added to overflowing. To the initial alone, added 2 ml each of sodium azide and manganese sulphide. Then kept for five day incubation. After the incubation period the sample is tested for BOD. Add the above two reagents to the other flask. Also add 2 ml of acid reagent to all the flasks. Then titrated with thio reagent using starch as indicator.

$$\text{BOD} = \frac{\text{Initial value} - \text{Final value}}{\text{Total volume}} \times 300$$

3. RESULTS AND DISCUSSION

Purple non sulfur bacteria was isolated from aquaculture pond. Purple non sulfur bacteria require an external electron donor for growth and biomass. Since they have the capacity to utilize a wide range of organic and inorganic compounds as electron donors, the use of waste water containing unused organic and inorganic compounds might provide cheap raw materials for biomass. The waste water generally contains high concentration of nutrients which support the growth of bacteria. In the present study the biochemical identification and degrading potential of PNSB was determined.

Cell Morphology

The isolate was found to be gram negative, oval shaped organism. On addition of Lugols iodine pink color stain was observed. This showed that isolates was gram negative. Motility of the isolates was checked by hanging drop method. It was found to be non motile. Results obtained are shown in Table: 1

Biochemical tests

Isolated purple non sulfur bacteria was tested for biochemical characteristics.

Catalase test

Catalase test was performed for PNSB was found to negative. Catalase an extracellular enzyme secreted by several microorganisms helps in degradation of hydrogen peroxide to produce molecular oxygen, generates vigorously while producing effervescence absence of effervescence is taken as indicative negative for catalase production. Kavitha *et al* 2016 reported that lactobacilli produce the negative result for catalase production.

Starch hydrolysis

Purple non sulfur bacteria showed the positive result for the starch hydrolysis.

R.Sphaeroides had recorded utilization of methyl cellulose and starch in complete anaerobic condition (Wilson *et al* 2008).

Gelatin liquefaction

Purple non sulfur bacteria showed the gelatinase test positive.

Sugar fermentation:

PNSB showed the fermentation of sugars with production of acid and gas. Montano *et al* 2009 attested that growth was best with the carbon sources such as pyruvate, malate, acetate, glucose, soluble starch and citrate

Hugh and Leifsons test:

This test was found to be positive.

Indole test

Red ring was produced. It showed the positive result.

Citrate utilization

Purple non sulfur bacteria was found to be citrate utilization test positive as the color of media turned blue. Citrate is the only carbon source available to the bacteria if it can metabolize citrate, then the bacteria will grow and the media will turn into blue as a result increase in the ph of the media.

Urease test

Purple non sulfur bacteria found to be urease positive as the media color changes from yellow to pink.

Nitrate reduction test

Red color was formed it indicate the reduction of nitrate to nitrite. Chitin degradation was found to be positive which indicated the active chitinolytic activity of the consortium of photosynthetic bacteria. Biochemical characteristics results are given in table 2.

Table 1: Antimicrobial activity of *Mango ginger* rhizome extract

S. No	Characteristics	Purple non sulfur bacteria
1	Color	Brownish red pink
2	Cell shape	Oval
3	Gram staining	Negative
4	Motility	Non Motile

Table: 2 Biochemical characteristics of isolated purple non sulfur bacteria

S. No	Biochemical Test	PNSB
1	Catalase	-ve
2	Starch hydrolysis	+ve
3	Gelatin liquefaction	+ve
4	Sugar fermentation	+ve
5	Hugh and Leifsons test	+ve
6	Indole test	+ve
7	Citrate utilization	+ve
8	Urease test	+ve
9	Nitrate reduction test	+ve

+ve = positive, -ve = Negative.

Table: 3 Waste water analysis

S. No	Parameters	Values given by the sample
1	Coli forms	1100+MPN/100 ml
2	BOD	1275 PPM
3	COD	2546 PPM
4	PH	8.7

Sample analysis

The waste water was analyzed to determine its biochemical parameters immediately after the sample collection. The result was tabulated in 2.

The pH of the sample was measured immediately after its collection using pH meter. pH of the water is known to influence the availability of micronutrients as well as trace metals (Krishanan 2006). It is well known that the pH is an important parameter in evaluating the acid base balance of water. (Apha1995). Initial coli forms were estimated by MPN method the count of 1100++.

Waste water is any water that has been adversely affected in quality by anthropogenic influences. It comprises liquid waste discharged by domestic residences, commercial properties, industries and agriculture and can encompass a wide range of potential contaminants and concentrations. (Suliman *et al* 2010)

From the data it was clear that the sample was successfully treated by the PNSB species. The isolate was able to decrease the organic content to about 70% i.e., the BOD level was reduced from 1275 ppm to 183 ppm for 1 gm of sample over a

period of twenty days. COD level was reduced from 2546.1 to 345.96 ppm for 1 gm of sample over a period of twenty days. The pH of sample was also reduced from the alkaline range (8.7) to a slightly acidic range (7.1). All the data (COD, BOD and pH) was given in table 4, 5 and 6.

Akil (2003) reported the transformation of toxic compounds by bacteria into less toxic compounds. Vasavi *et al* (2008) and Ramachander *et al* (2007) used photosynthetic bacteria for remediation of waste water. Removal of heavy metals and sodium from contaminants of shrimp ponds Saijai *et al* (2010). Micro aerobic biodegradation of high organic load of waste water have also been reported Madukasi and Zhang (2010). Remediation of sewage waste water by the phototropic bacterial consortium isolated from sewage water was reported by Ramachander *et al* (2015)

Odour of the sample was only slightly reduced while after complete treatment a completely clear solution was obtained. Choorit *et al.* (2002) used that isolated, identified and cultivated photosynthetic bacteria in waste water from a

Table: 4 COD values obtained before and after treatment

S.NO	Contents	Sample volume	Volume of standard	Blank Volume	Volume of FAS for raw water	Normality of FAS	COD Value
1	Before treatment	4 ml	27.1	26.9	13.1	0.09225	2546.1 ppm
2	After treatment(1 gm)	7ml	25.6	25.2	22.1	0.09765	345.96ppm

Table: 5 BOD values obtained before and after treatment

S.NO	Contents	Sample volume	Initial Volume	Final Volume	BOD Values
1	Before treatment	1 ml	7.8	3.55	1275ppm
2	After treatment(1 gm)	5ml	7.6	4.55	183 ppm

Table:6 pH range of the sample

Quantity of sample	Incubation period	pH
	Initial level before treatment	8.7
	0-5 Days	8.4
250 ml	5-10 Days	7.6
	10-15 Days	7.3
	15-20 Days	7.1

concentrated latex processing factory. Izu *et al* (2001) found that aeration condition affecting the growth of PNSB in an organic waste water treatment process. Kim *et al* (2004) used PNSB like *Rhodospseudomonas palustris* isolated from eutrophicated ponds to reduce the odor from swine waste water treatment pond. Kobayashi *et al* (1996) studied the utilization of photosynthetic bacteria in the bioremediation of soil manure.

Paultry slatter house waste water was found to be very rich in *Rhodocyclus gelatinosis* was studied by Ponsano *et al* (2002). *Rhodospirillum* as well as other purple non sulfur bacteria can be found in natural settings such as pond water, mud or sewage samples (Brock, 1992).

Phototropic purple non sulfur bacteria can be used in sewage treatment processes for biomass production as a source of animal food or agriculture fertilizers and for the production of hydrogen gas by evolution from nitrogenase enzyme. They may also used as a source of cell system performing

photosynthesis and ATP formation and for the production of vitamins and other organic molecules. The hydrogen ion concentration is an important quality parameter of waste water. Table 4 shows that the pH of the influent was measured to be 8.7 compared to 7.1 with treated waste water. Low value of pH is due to the metabolism of bacteria and also metabolic production of acids by indigenous micro flora. (Khan *et al* 2011)

The dye decolourisation was done by adding 25 ml of the inoculums broth of PNSB to 225 ml of the dye sample. Orange color dye obtained from the commercial market was used in the present study and the values were observed spectrophotometrically. Decolourisation was observed within one week. The results was present in table 7.

Table: 7 Dye decolourization assay

Sample inoculated	Incubation Period	Spec value at 620 nm
PNSB	Initial	2.526
	1 day	2.403
	2 days	1.265
	3 days	1.137
	4 days	0.725
	5 days	0.510

Dye decolourization assay by photosynthetic bacteria has also been done and the flora was found to be very efficient in decolourization of orange dye. Results of the spectrophotometric analysis were even comparable with the percentage of dye decolourization exhibited by the white rot fungus *Trametes versicolor* (Yange *et al* 2009).

Multiple Tube Method (MPN)/Most Probable Number Method:

Initial monitoring study showed MPN as 1100+. After the PNSB treatment the coli forms was reduced, so the PNSB was found to be efficient to degrade the waste with 50% efficiency showing its antagonistic potential. The data was given in table 8.

Table: 8 Most Probable Number

Parameter	MPN values MPN /100 ml before treatment	MPN values MPN /100 ml after treatment
Coli forms	1100++	450+

Bioremediation procedure was adopted in the organic effluent sample using photosynthetic bacteria. Initial coli forms were estimated by MPN method after subjecting them with PNSB for 24 to 48hours. The initial count of 1100++ was reduced to 500+ after 48 hours of incubation which showed the bioremediation potential of photosynthetic bacteria.

4. CONCLUSION

Purple non sulfur bacteria was isolated characterized and used to treat waste water in this study. The results indicated that Purple non sulfur bacteria were efficient in treating waste water. The COD, BOD and pH values were reduced from pre treatment analysis. Purple non sulfur bacteria are excellent microbial source rich in proteins, variety of minerals and coenzymes. Apart from waste water recycling, Purple non sulfur bacteria are used in the following field like Animal husbandry and pet fish breeding. They may be used as a source of cell free systems performing photosynthesis and ATP formation and for the production of vitamins and other organic molecules.

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