

Purple Nonsulfur Bacteria (PNSB) Isolated from Aquatic Sediments and Rice Paddy in Iligan City, Philippines

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Abstract

The purple nonsulfur bacteria (PNSB) are one of the most diverse photosynthetic bacteria. They are adaptable phototrophic organisms known to occur in water columns of rice fields, wastewater environments, aquatic sediments and in activated sludge systems. This research study aimed to isolate, identify, and characterize PNSB from aquatic sediments and rice fields in Iligan City. Isolation of the source of organisms was obtained through a *Winogradsky's* column. After six weeks of incubation of the column, organisms were further incubated with the Acetate Yeast Extract Medium. Morphological characterization of the isolated PNSB showed Gram-negative, non-spore forming, thin, elongated rods. Cultural characterization showed orange colonies, an indication for the presence of carotenoids. The isolated PNSB utilized citrate, dextrose, and soluble starch as sole carbon sources. Initial identification of the isolated PNSB following evaluation with published articles and identification keys indicates that they belong to the genus *Rhodopseudomonas*.

Keywords: gram-negative, photosynthetic bacteria, phototrophic organism
Rhodopseudomonas, *Winogradsky's* Column

Introduction

Chemical fertilizers are commonly used in crop production nowadays to help increase yield. On the other hand, chemical fertilizers cause health risks to consumers as well as to the soil itself by altering the soil structure. Chemical fertilizers degrade the natural physical structure of the soil causing deficiency of oxygen in the plant root zones (Nagananda et al., 2010) that in a way may eventually fully obliterate soil fertility. Due to this soil degradation effect and high cost production experienced by farmers through using expensive chemical fertilizers it might be helpful to discover the use of biofertilizers as an alternative enhancer of soil fertility.

Biofertilizers are cost effective, ecofriendly and renewable source of plant nutrients to supplement chemical fertilizers in sustainable agricultural system. These are products containing living cells of different types of microorganisms which when applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promote growth by converting nutritionally important elements (nitrogen, phosphorus) from unavailable to available form through biological process such as nitrogen fixation (Mohammadi & Sohrabi, 2012). These microorganisms in biofertilizers accelerate and improve plant growth and protect plants from pests and diseases (El-Yazeid et al., 2007).

The major free-living, nitrogen-fixing microbial systems include the photosynthetic bacteria that inhabit floodwater and surface soil. These organisms absorb light through bacteriochlorophyll and carotenoid which are divided into two principal classes which include the green bacteria and the purple bacteria (Poretsky, 2003). They have a varied range of growth modes and are able to grow under photoheterotrophic, chemoheterotrophic and photoautotrophic conditions (Soto et al., 2010).

Photosynthetic bacteria like the PNSB were reported to occur in water columns of rice fields, in activated sludge systems, in wastewater environment, and in aquatic sediments (Montano et al., 2009). Low dissolved oxygen (DO) tension and the high availability of light and simple organic nutrients are important factors promoting the proliferation of purple nonsulfur bacteria in the environment (Okubo et al., 2006). These bacteria are called “nonsulfur” because it was

originally thought that they were unable to use sulfide as an electron donor for the reduction of CO₂ 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 PNSB, e.g. *Rhodopseudomonas*, *Rhodospirillum*, and *Rhodomicrobium* were reported nitrogen-fixing microorganisms in flooded rice soils. Several studies conducted have shown that the presence of PNSB in paddy soil may contribute to rice productivity. Harada et al. (2005) reported that inoculation of *Rhodopseudomonas palustris* increased the grain yield of rice while *Rhodobacter capsulatus* enhanced seedling growth, i.e. increasing shoot height of rice seedlings, regardless of rice variety (Elbadry & Elbanna, 1999). Purple nonsulfur bacteria were reported to 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 & Xiang, 2012). They are reported to contain valuable substances and has practical importance as feed protein. The bacterium *Rhodovulum sulfidophilum*, when combined with commercial tilapia feed, improves the growth and survival of tilapia during grow out period (Banerjee et al., 2000). Another study by (Shapawi et al., 2012), showed that the inclusion of purple nonsulfur bacteria *Rhodovulum sp.* in formulated feed promoted the growth, feed conversion ratio and survival rate of Asian sea bass juveniles. Purple nonsulfur bacteria were also reported to improve water quality (Kim et al., 2004).

Reported studies on increased rice production through the presence of purple nonsulfur bacteria show the use of biofertilizers as an alternative form of soil fertility enhancement and may demonstrate to be more economical and ecologically friendly than the use of commercially available chemical fertilizers. The utilization of PNSB as supplement to commercial tilapia feed proves its valuable use to reduce cost and increase growth and survival of fish species. This research study aimed to isolate, identify, and characterize PNSB from aquatic sediments and rice fields in Iligan City. This study could serve substantial baseline data as to the status of the rice paddy soil and beach sediments in the area. Through the information provided by this study, further research on improving optimum growth of rice or rice

productivity itself in the area using purple nonsulfur bacteria as biofertilizers can be conducted.

Materials and Methods

Source of Organisms

The organism was isolated from the prepared *Winogradsky's* column sampled from beach sediments in Reg's beach resort and rice paddy soil from Steeltown, Iligan City. The *Winogradsky's* column was prepared with the use of a two-liter translucent plastic container filled with mud/moist soil, water from the source of the mud/moist soil, shredded newspaper, powdered chalk and egg yolk, sealed, and incubated approximately 4-6 weeks in an area that received bright, indirect light at room temperature 28 - 30°C.

Media

The mineral base used was the Acetate – Yeast Extract (AYE) medium which contained (per liter distilled water): K₂HPO₄, 1.0 g; MgSO₄, 0.2 g; CaCl₂, 0.02 g; Na₂S₂O₃, 0.10 g; Na-Acetate, 2.2 g; Yeast Extract, 4.0 g, and agar, 15.0 g. The AYE medium is formulated for the growth of PNSB (Montano et al., 2009).

Isolation and Characterization

From the pooled samples in incubated *Winogradsky's* column, one milliliter was aseptically transferred to sterile tubes. *Winogradsky* column is an efficient device for culturing microorganisms for it provides numerous nutrients from which a variety of microbial organisms can grow. Approximately, 20 µL was pour-plated into AYE agar and incubated for one week inside an improvised anaerobic glass chamber. Following incubation, isolated colonies were purified by subsequently streaking it on fresh AYE agar. Inoculated culture plates were further incubated for two weeks; the white colonies turned into orange colonies. Subsequent subcultures were done until pure cultures were obtained. Microscopic examination was performed to check the purity of the isolated PNSB. Purified colonies were maintained in AYE agar culture plates. To identify the isolated PNSB, morphological, cultural, and physiological characterizations were conducted. For morphological characterization, pure cultures were

initially stained using the Gram staining technique. Cell shape and the presence of specialized structure were then determined using LEICA light microscope (OIO, 1000X). The isolated colonies on AYE agar were also observed for their cultural characteristics such as the colony color and shape. The motility was observed using the Motility Test Semisolid Medium (peptone, 10.0 g; NaCl, 5.0 g; agar, 4.0 g; beef extract, 3.0 g, 1L distilled water) (MacFaddin, 2000). Prepared Motility Test Semisolid Medium was dispensed into sterile tubes subsequently inoculated with isolated PNSB and then incubated for 24 hours in an anaerobic chamber.

For the physiological characterization, the isolated PNSB were grown on culture medium with different carbon and nitrogen sources. To determine their ability to utilize citrate/acetate, Acetate Differential Agar (Simmon's Citrate Agar) was prepared. For their ability to utilize glucose and soluble starch, Phenol Red Dextrose Agar and Starch Agar were used respectively. All culture media were pour-plated into separate sterile petri plates subsequently inoculated with isolated purple nonsulfur bacteria and incubated for 24 hours with exception to Phenol Red Dextrose Agar which took 24-48 hours.

The Acetate Differential Agar (Simmon's Citrate Agar) contained $(\text{NH}_4)_2\text{H}_2\text{PO}_4$, 1.0 g; Na-acetate, 2.0 g; NaCl, 5.0 g, K_2HPO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; Bromothymol blue, 0.08 g; Agar, 20.0 g, 1L distilled water (Atlas, 2006). The Phenol Red Dextrose Broth is composed of casein peptone, 10.0 g; NaCl, 5.0 g; dextrose, 5.0 g; phenol red, 0.018 g; agar, 1.0 g; and 1L distilled water (Baron et al., 1994). The Starch Agar is composed of beef extract, 3.0 g; soluble starch, 10.0 g; and bacteriological agar, 12.0 g (Murray et. al., 1999).

Results and Discussion

Purple nonsulfur bacteria were isolated from Winogradsky's columns sampled from beach sediments in Reg's Beach Resort and rice paddy soil from Steeltown, Iligan City. Inoculation of the soil samples on the AYE agar yielded optimal growth of orange pigmented bloom of bacteria after two weeks of incubation in anaerobic chamber (Figures 1 and 2). Consequent subculture of the AYE cultures exhibiting "orange blooms" yielded the same colonies (Figure 3).



Figure 1. Winogradsky's Column. *Left*, sampled from Rice Paddy in Steeltown. *Right*, sampled from Reg's Beach Resort.



Figure 2. Improvised anaerobic chamber for the incubation of the inoculated plates.



Figure 3. Inoculated PNSB culture.

Purified cultures were characterized based on the morphological, cultural, and physiological structures of the bacteria. Results obtained are shown in Table 1.

Table 1. Tests to determine observed bacterial growth do belong to the genus *Rhodopseudomonas*

Characteristics	Observations	Test/Method
Rod-shaped, Gram-negative Technique	+	Gram-staining
Growth in anaerobic condition chamber	+	Grown in anaerobic glass
Produce accessory pigment from brownish yellow to deep red, when kept in light	+	Culture Growth Examination
Motile or Non-motile	+	Motility Test Medium, Semisolid
Ability to utilize carbon/acetate from different carbon sources	+	Test for Acetate Metabolism Using Simmon's Citrate Agar (Acetate Differential Agar)
	+	Test for utilization of Sugar as the sole carbon source using the Phenol Red Dextrose Broth
	+	Test for utilization of starch using the Starch Agar

Results showed isolated white colonies after one week of incubation and turned orange after further incubation. The isolated colonies appeared to be Gram-negative, thin, elongated, rod-shaped as viewed at 1000X OIO magnification under the LEICA light microscope (Figure 4). Cultural characteristics of isolated bacteria exhibited photosynthetic pigment in orange color which indicated the presence of carotenoid substances. Growth of PNSB is optimal at 25 - 35°C. Although, chemotrophy may be less favorable for bacterial growth; facultative chemotrophic growth is an adaptation that enables the PNSB to survive in the absence of light (Butow & Bergstein-Ben Dan, 1991). Montano et al. (2009) attested that growth was best with the carbon sources such as the pyruvate, malate, acetate, glucose, soluble starch, and citrate.

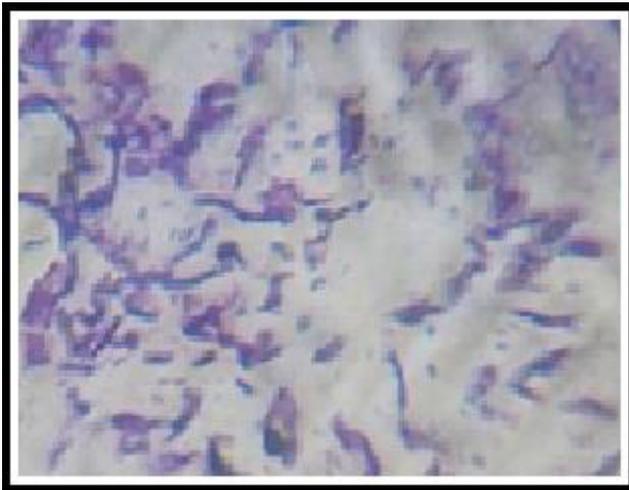


Figure 4. Gram stained cells, 1000x Leica Light Microscope.

Physiological characteristics of isolated PNSB based on its motility and carbon sources utilization were assayed through different several biochemical tests. The motility test (Figure 5) showed positive and negative, which follows the motile or non-motile characteristics of PNSB (Montano, et. al., 2009). If the bacteria have migrated away from the original line of inoculation, the test organism is motile (positive test); while the lack of migration away from the line of inoculation indicates a lack of motility (negative test result)

(Norman, n.d.). The isolated colonies were grown on Simmon's Citrate Agar (Acetate Differential Agar) as shown in Figure 6. Acetate metabolism is among purple nonsulfur bacteria (Blasco et al., 1989). In this media, 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 of increase in the pH of the media (Norman, n.d.).



Figure 5. Test for motility using the Motility Test Medium, Semisolid. Inset from the left showing a non-motile tube. Inset from the right showing a motile tube.



Figure 6. Test for Acetate Metabolism. Simmon's Citrate Agar (Acetate Differential Agar) turned blue when inoculated with PNSB.

In determining the ability of the organism to metabolize sugar and starch, the isolated colonies were grown on Phenol Red Dextrose Broth and Starch Agar, respectively. Positive utilization of sugar by the microorganism was observed, indicated by a change in color of the Phenol Red Dextrose Broth from red to yellow as shown in Figure 7. The isolated colonies grown in Starch Agar showed a positive result, which indicates a clearing surrounding to the inoculum (Figure 8). Initial identification of the isolated purple nonsulfur bacteria following evaluation with published articles and identification keys indicates that they belong to the genus *Rhodopseudomonas*.

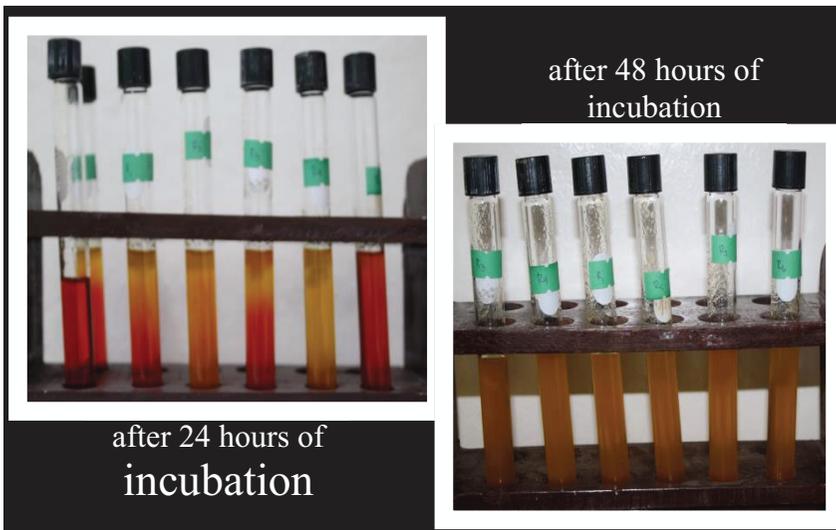


Figure 7. Test for utilization of sugar as the sole carbon source using the Phenol Red Dextrose Broth. A positive reaction is indicated by the appearance of a yellow color.



Figure 8. Test for utilization of starch using the Starch Agar. Positive result is indicated by a clearing surrounding the inoculum.

Conclusion and Recommendation

Purple nonsulfur bacteria were isolated from prepared *Winogradsky* column sampled from beach sediments in Reg's Beach Resort and rice paddy in Steeltown, Iligan City using the AYE medium (Acetate Yeast Extract) enrichment and selective medium for *Rhodopseudomonas*. Cells were Gram-negative, thin, elongated rods and non-spore forming as viewed under the microscope. Carotenoid was detected as the major photosynthetic pigment in whole-cell culture. Isolated PNSB can utilize citrate, dextrose and starch as sole carbon sources. Physiological, cultural and morphological characterization revealed the bacterial isolate as belonging to the genus *Rhodopseudomonas*. Further biochemical tests on thiosulfate, propionate, mannitol and sorbitol utilization are recommended to validate the observed characteristics of the isolated purple nonsulfur bacteria from aquatic sediments and rice paddy in Iligan City.

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