

ISOLATION AND CHARACTERIZATION OF PURPLE NONSULFUR BACTERIA (PNSB) FROM A RICE PADDY SOIL IN BULACAN, PHILIPPINES

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ABSTRACT

Purple nonsulfur bacteria (PNSB) are phenotypically diverse group of microorganisms and are known for their biological nitrogen fixation in flooded rice paddies. Our research study, then, aimed to isolate and characterize PNSB and determine their population count. Rice paddy soil samples were collected from San Jose del Monte, Bulacan, and were inoculated into completely filled culture vials pre-filled with different enrichment media, e.g. Larsen's medium, Van Niel's medium, and Acetate Yeast Extract (AYE) medium. Following incubation for 4 weeks under an incandescent bulb, only the AYE medium resulted to bright "red bloom" indicating growth of PNSB. Morphological and cultural characterization of the isolated PNSB showed pinpoint, red colonies and Gram negative, non-spore forming, thin, elongated (0.5 x 2.0 μm) rods. *In vivo* absorption spectrum using spectrophotometer showed the presence of bacteriochlorophyll a and carotenoids. The isolated PNSB utilized pyruvate, malate, glucose, lactate, citrate and soluble starch as its carbon sources, and ammonium sulfate, peptone and yeast extract as nitrogen sources. Preliminary identification identified the PNSB isolates as *Rhodopseudomonas* sp. MPN estimated low number of PNSB (2 cells per ml) in the collected rice paddy soil.

Keywords: purple nonsulfur bacteria, *Rhodopseudomonas* sp., enrichment culture, Most Probable Number (MPN), bacterial identification, rice paddy soil

INTRODUCTION

Chemical and organic fertilizers have become the main-stay of present-day agriculture in order to maintain optimal nutrients in soils. These practices have led to improved agricultural production. However, several

disadvantages associated with chemical fertilizers included its high-cost and low use-efficiency. These chemicals also supply only limited nutrients to plants. Excessive use of chemical fertilizers and/or organic fertilizers may even result to problems of soil, groundwater or atmospheric pollution and may promote the production of certain greenhouse gases, e.g. methane in agricultural soils, particularly observed in rice fields (Neue, 1993). The concern for environmental safety and quality necessitates a more judicious use of chemical or organic fertilizers and an investigation of alternative sources, especially “biological fertilizers” (Roger & Ladha, 1992; Peoples et al., 1995). Certain microorganisms, which can fix atmospheric nitrogen, were of great significance as potential “biofertilizers” (Rao et al., 1994).

The major free-living, nitrogen-fixing microbial systems include the photosynthetic bacteria that inhabit floodwater and surface soil. Photosynthetic bacteria like the purple nonsulfur bacteria (PNSB) were reported to occur in water columns of rice fields, in activated sludge systems, in wastewater environments, and in aquatic sediments (Watanabe, 1978; Hiraishi & Kitamura, 1984; Roper & Ladha, 1995; Oda et al., 2002; Okubo et al., 2005). Species of purple nonsulfur bacteria, e. g. *Rhodopseudomonas*, *Rhodospirillum* and *Rhodomicrobium*, were reported to be nitrogen-fixing microorganisms in flooded rice soils (Rao et al., 1998), though bacterial nitrogen fixation was probably greater in the anaerobic than in the aerobic layers of flooded soil (Wada et al., 1978). Their occurrence in paddy soil may perhaps contribute to rice productivity (Elbadry et al., 1999a). Elbadry & Elbanna (1999) reported that *Rhodobacter capsulatus* enhanced seedling growth, i.e. increasing shoot height of rice seedlings, regardless of rice variety. Inoculation of *Rhodopseudomonas palustris* increased the grain yield of rice (Harada et al., 2005) while *Rhodobacter sphaeroides* promoted root growth of *Brassica campestris* (Kensuke et al., 2004). The use of bio-fertilizers in rice production may prove to be more economical and environmentally friendly than the use of commercially available chemical fertilizers. Our research study then aimed to isolate through enrichment culture technique purple nonsulfur bacteria (PNSB) from rice paddy soil collected in San Jose del Monte, Bulacan, and characterize morphoculturally and physiologically the isolated bacteria. It also aimed to determine the number of PNSB present in the rice paddy soil by the Most Probable Number (MPN) method.

METHODOLOGY

Collection and physico-chemical analysis of rice paddy soil

Rice paddy soil was collected randomly within a rice field in San Jose del Monte, Bulacan. The pooled soil sample was immediately transported to the laboratory and the soil pH and nutrient content, e.g. NPK (nitrogen N, phosphorous P, and potassium K) were determined using the Department of Agriculture Soil Test Kit. The collected rice paddy soil was used for the enrichment culture.

Enrichment culture of the purple nonsulfur bacteria

From the pooled rice paddy soil, one gram was inoculated into culture vials pre-filled either with Van Niel's Medium (VNM: NH_4Cl , 1.0 g; MgCl_2 , 0.2 g; KH_2PO_4 , 1.0 g; NaHCO_3 , 5.0 g; $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, 1.0 g; 1 L distilled water), Larsen Medium (LM: NH_4Cl , 1.0 g; KH_2PO_4 , 1.0 g; CaCl_2 , 0.1g; MgCl_2 , 0.5g; NaHCO_3 , 2.0 g; $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, 1.0 g; NaCl , 1.0 g; $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$, 50 ppm; H_3BO_3 , 10 ppm; $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 10 ppm; $\text{Co}(\text{NO}_3)_2\cdot 6\text{H}_2\text{O}$, 5 ppm; $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 0.5 ppm; $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 0.5 ppm; 1L distilled water), and Acetate-Yeast Extract Medium (AYE: K_2HPO_4 , 1.0 g; MgSO_4 , 0.2 g; CaCl_2 , 0.02 g; $\text{Na}_2\text{S}_2\text{O}_3$, 0.10 g; Na-Acetate, 2.2 g; Yeast Extract, 4.0 g; 1 L distilled water). The inoculated culture vials were completely filled with their respective media and sealed with rubber stoppers. The cultures were then incubated under a 60 W incandescent bulb at room temperature. The vials placed at a distance of 20 cm from the light source. After 4 weeks of incubation, formation of red blooms was noted, indicating growth of PNSB.

Isolation and characterization of purple nonsulfur bacteria

Enrichment cultures exhibiting "bright red blooms" were streaked on petri dishes filled with AYE Agar and incubated for one week inside an anaerobic gas chamber (GasPak, BBL) under similar light conditions. Following incubation, isolated red colonies were purified by diluting a colony in a drop of sterile distilled water and subsequently streaking it on fresh AYE Agar. Inoculated culture plates were again incubated inside an anaerobic gas chamber under an incandescent light. Subsequent subcultures were done until pure cultures were obtained. Microscopic examination was also done to check for the purity of the isolated PNSB. Purified colonies were then maintained in culture vials filled with AYE medium.

To identify the isolated PNSB, morphological, cultural and physiological characterizations were done. For morphological characterization, pure cultures were initially stained using the Gram staining and Schaeffer-Fulton Endospore staining techniques. Cell shape and the presence of specialized cell structure, e.g. endospores, were then determined under a compound light microscope (OIO, 1000X). Cell sizes were also determined using an ocular and a stage micrometer under a microscope. Isolated colonies on AYE Agar were also observed for their cultural characteristics, e.g. colony margin, shape, color and elevation. The bacteriochlorophyll content of the whole-cell culture was also determined using a spectrophotometer (Jasco V530 UV/VIS Spectrophotometer, Japan) (Pfennig, 1969). The Jasco V530 UV/VIS spectrophotometer has a wavelength range of 190 nm to 1,100 nm provided by a deuterium lamp and a halogen lamp. Initially, cultures were agitated to suspend the cells and the Optical Density (OD) reading was then determined with wavelength set at 320 to 1,100 nm. Uninoculated culture media served as blank.

For physiological characterization, the isolated PNSB were grown on culture medium with different carbon and nitrogen sources. To determine their ability to utilize different carbon sources, AYE basal medium was prepared

with yeast extract as sole nitrogen source and the carbon source acetate replaced by citrate, glucose, lactate, malate, pyruvate or soluble starch. For their ability to utilize different nitrogen sources, AYE basal medium was also prepared with acetate as sole carbon source and the yeast extract replaced with either ammonium salt or peptone. All culture media were dispensed in culture vials to which 3.0 ml of the PNSB culture were added. Culture vials (in triplicates) were then filled to brim with excess culture media and incubated for 1 week at the light condition described above. Growth was measured by determining their OD values at 600 nm following incubation for 1 week.

Determination of PNSB population using the Most Probable Number (MPN) method

To determine the number of PNSB cells present in the rice paddy soil, ten gram soil samples were initially diluted in 100 ml sterile distilled water. Then, 1.0, 0.1, and 0.01 ml soil suspensions were each inoculated into 5 screw-capped tubes filled with 10 ml AYE medium. To the culture tubes, approximately 2 ml sterile paraffin wax was added on top of medium and allowed to harden to create an anaerobic condition. The cultures were then incubated at 20 cm distance from a 60 W incandescent bulb for 4 weeks. Following incubation, culture tubes with “red blooms” indicated PNSB growth. The number of positive culture tubes were noted and used to determine the PNSB population based on the MPN index.

RESULTS AND DISCUSSION

Purple nonsulfur bacteria (PNSB) are Gram negative, motile or non-motile, non-spore forming, rod-shaped bacteria, generally with cell sizes varying from 0.5 - 1.5 μm wide and 2 - 10 μm long. Representative species have either bacteriochlorophyll a or bacteriochlorophyll b, with the red pigmentation in cultures attributed to the presence of carotenoids. PNSB grow chemotrophically under microaerophilic or aerobic conditions, although most species display photoheterotrophic growth under anaerobic conditions. They grow as photoautotrophs in the presence of light, as chemoheterotrophs in the presence of organic compounds, and as photoheterotrophs in the presence of both light and organic substrates. Hydrogen, thiosulfate, or sulfide, though only in low concentration, generally serves as the electron donor under these instances. Growth of PNSB is optimal at 25 - 35°C and at pH 6.5 - 7.0. 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).

PNSB were isolated from rice paddy soil collected in San Jose del Monte, Bulacan. The soil pH was determined to be slightly acidic (pH 5.8). The soil had low phosphorous level and was determined to have medium-content nitrogen and sufficient-content potassium based on the DA Soil Test Kit. Inoculation of different enrichment media with soil samples yielded growth (“red bloom”) only on the Acetate-Yeast Extract Medium after 2

weeks of incubation (Figure 1.0). Culture vials with Larsen and Van Niel's media did not manifest any PNSB growth. Since both media contained mainly inorganic salts, the isolated PNSB perhaps favored organic substrates for their growth.

Subsequent subculture of the AYE cultures exhibiting "red blooms" yielded one type of colonies. Pure cultures were then characterized morphoculturally. Results showed the isolated colonies to be deep red, pinpoint and convex with entire margins. Cells were Gram negative, thin, elongated or rod-shaped, and did not show any spore formation (Fig. 1). The cell size was determined to be 0.5 μm in width and 2.0 μm in length.

Determination of the major photosynthetic pigments by spectrophotometric analysis showed the presence of bacteriochlorophyll a in the whole-cell culture of PNSB and with peaks at 590, 805 and 870 nm (Fig. 2). Peaks at 475 and 525 nm showed the presence of carotenoids. Assay for the carbon and nitrogen sources utilization showed growth in all organic and inorganic chemicals tested (Fig. 3). Growth, however, was best with pyruvate as the main carbon source, followed by malate, acetate, glucose, soluble starch and citrate. Dönmez et al. (1999) showed also utilization of pyruvate and acetate by *Rhodopseudomonas palustris* isolated from an alkaline lake. But, this organism did not utilize glucose, citrate and malate in contrast to our isolate. Acetate metabolism is known among purple nonsulfur bacteria (Blasco et al., 1989). Other substrates utilized by PNSB included sucrose, trehalose, taurine, aromatic compounds and even alcohols (Fujii et al., 1983; Shoreit & Shabeb, 1994; Welsh et al., 1998; Novak et al., 2004). Differences were also noted on growth at different nitrogen sources, e.g. yeast extract, ammonium sulfate and peptone. Our PNSB isolate grew better with ammonium sulfate, followed by peptone and then, by yeast extract. Preliminary identification of the isolated PNSB following comparison with published literatures and identification keys indicate that it belongs to the genus *Rhodopseudomonas*.

The population count of PNSB in the rice paddy soil was estimated using the Most Probable Number (MPN) method (Table 1.0). Following incubation, all culture tubes exhibited turbidity indicating bacterial growth. The total bacterial count was determined to be $\geq 1,600$ cells per ml of soil suspension. However, only one tube gave positive result for PNSB, i.e. the presence of "red bloom". Thus, only low number of PNSB (2 cells per ml) was detected from the collected rice paddy soil in Bulacan. This contrasted with the PNSB count determined by Elbadry et al. (1999b). They reported the highest number of PNSB in the rhizosphere ($10^3 - 10^6$ cells / gram dry weight of soil, gdw), followed by soil ($10^3 - 10^5$ cells / gdw), and floodwater ($10 - 10^2$ cells per ml). Archana et al. (2004) also found $10^5 - 10^8$ CFU / g dry soil PNSB with their paraffin wax-overlay pour plate method. But, PNSB number in rice fields varied also in relation to field conditions, habitat and growth rice stage (Elbadry et al., 1999b). Furthermore, PNSB populations were relatively low in number at the transplanting stage, but their number increased gradually and reached a slightly higher level at the maturity stage of the rice plants

(Elbadry et al., 1999b). The soil sample used in the study was collected at the pre-planting stage.

CONCLUSION

Purple nonsulfur bacteria were isolated from rice paddy soil sample using the AYE enrichment medium. The isolated PNSB exhibited deep red, pinpoint, convex colonies. The cells were Gram negative, non-spore forming, thin, elongated rods occurring singly. Bacteriochlorophyll a and carotenoids were detected as the major photosynthetic pigments in whole-cell culture. The isolated PNSB can utilize pyruvate, malate, acetate, glucose, soluble starch and citrate as carbon sources, and yeast extract, ammonium sulfate and peptone as nitrogen sources. Morphological, cultural, and physiological characterization revealed the isolate as belonging to the genus *Rhodospseudomonas*. The PNSB count in the rice paddy soil was estimated to be low.

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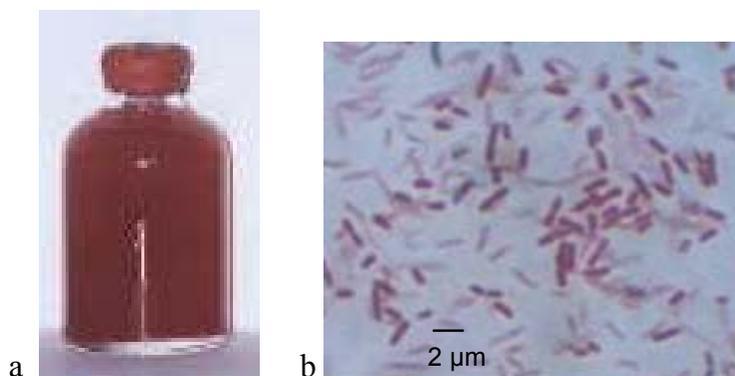


Figure 1. Growth in Acetate-Yeast Extract (AYE) Medium (a) and Gram-stained cells, 1000X (b) of *Rhodospseudomonas* sp. isolated from rice paddy soil.

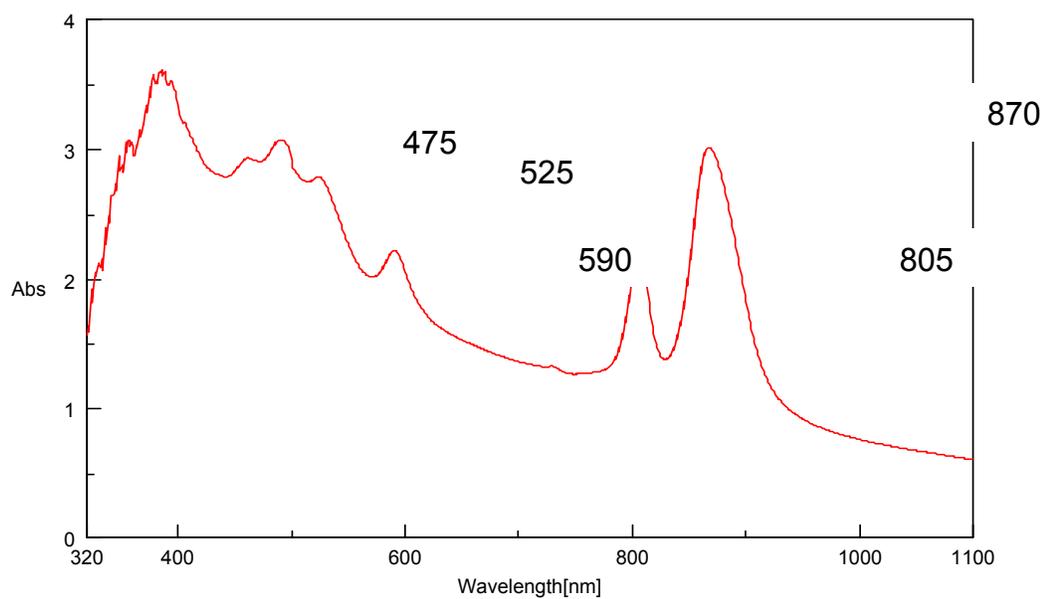
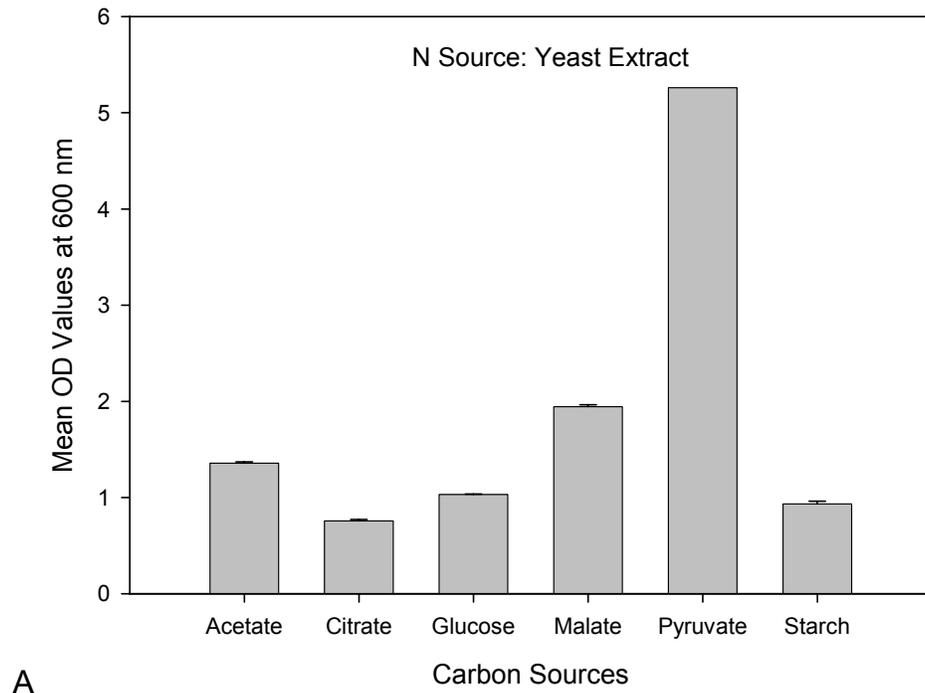
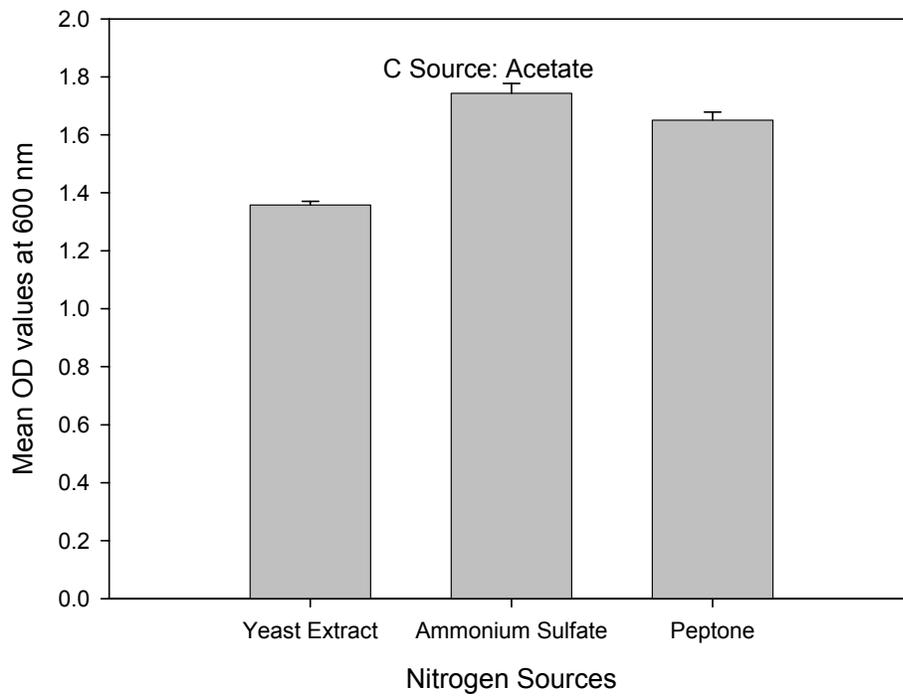


Figure 2. *In vivo* absorption profile of the whole-cell culture of *Rhodospseudomonas* sp..



A



B

Figure 3. Growth of *Rhodopseudomonas* sp. in various carbon and nitrogen sources.

Table 1. PNSB population in rice paddy soil collected in San Jose del Monte, Bulacan.

	Number of Positive Tubes			MPN/ml
	1.0 ml	0.1 ml	0.01 ml	
total bacterial count	5	5	5	≥ 1600
total PNSB count	1	0	0	2