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The use of rice straw broth as an appropriate medium to isolate purple nonsulfur bacteria from paddy fields

Tomorn Nunkaew [1] · Duangporn Kantachote[1] ✉ · Teruhiko Nitoda[2] · Hiroshi Kanzaki[2]

1 Prince of Songkla University, Faculty of Science, Department of Microbiology, Hat-Yai, Thailand

2 Okayama University, The Graduate School of Natural Science and Technology, Okayama, Japan

✉ Corresponding author: duangporn.k@psu.ac.th
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Abstract The aims were to explore an appropriate isolating medium for obtaining purple nonsulfur bacteria (PNSB) for use as biofertilizers in saline paddy fields and to obtain pure cultures. We therefore chose a defined isolating medium containing 0.25% NaCl, (Glutamate-Acetate broth, GA) and a rice straw broth to compare them for numbers of PNSB obtained, time to obtain pure cultures, diversity and costs. A total of 30 water and 30 sediment samples were collected from saline paddy fields in southern Thailand and used to isolate PNSB in both the isolating media. Based on 60 samples and a period of 13 days incubation under anaerobic light conditions, a greater number of samples produced PNSB growth in GA broth after only day 3; however, after that the rice straw broth provided about a 2 fold increase in the number of samples that produced PNSB growth. Colonies isolated from GA broth required a significantly higher number of repeated streaking to obtain a pure culture (average 3.5) than those from rice straw broth (average 2.7) and the latter medium also produced significantly ($P < 0.05$) more isolates per sample. Sixty samples of water and sediment, from rice paddies with salinity (average, 3.43 ± 0.67 mS/cm) and slight acidity (average, pH 5.84 ± 0.42) provided 62 PNSB isolates by GA broth and 210 isolates by rice straw broth, and rice straw broth also produced a greater prevalence of PNSB. Estimates of the costs based on current prices of media, Gas Pak and electricity to obtain PNSB with the use of GA broth was roughly 6 times higher than for the rice straw broth.

Keywords: biofertilizer, isolation, paddy field, purple nonsulfur bacteria, rice straw broth.

INTRODUCTION

The purple nonsulfur bacteria (PNSB) are widely distributed in nature especially in most submerged conditions such as paddy fields, soils in riverbeds and also sewage disposal plants that are exposed to sunlight (Harada et al. 2003b; Takeno et al. 2005). It is well recognized that these bacteria have been isolated and selected for applications in the areas of environmental protection and agriculture because they are metabolically versatile organisms that are capable of photoautotroph/photoheterotroph growth under anaerobic light conditions and chemoorganotroph growth under aerobic dark conditions (Kim et al. 2004). In addition to using various organic compounds in wastewater treatment (Takeno et al. 2005) they can produce H_2 (Suwansaard et al. 2009), indole-3-acetic acid (IAA) and 5-aminolevulinic (ALA) (Koh and Song, 2007) and many can fix N_2 gas (Harada et al. 2005). Therefore, PNSB have been considered to be one of 'Natures Biofertilizers' and they also have the potential to reduce CH_4 emission by growing out competing methanogenic bacteria in the light (Harada et al. 2003a; Gamal-Eldin and Elbanna, 2011). Recently 'organic' agriculture is becoming popular in Thailand and thereby PNSB have already been sold as commercial biofertilizers for use in paddy fields and also for shrimp cultivation although they are very expensive as there is no competition.

Conventional methods for isolating PNSB have been developed using natural decomposition processes of organic matter such as hay or boiled eggs; or a defined medium containing a single

organic compound with a small amount of a mixture of organic growth factors; and source samples directly inoculated into or onto a solid defined medium and incubated under anaerobic light conditions. Such conditions provide for enrichment of PNSB as they can grow and out-compete other microbes (van Niel, 1971). Nowadays, the most common method used is liquid enrichment with anaerobic light conditions involving media such as G5 and GM (Glutamate-Malate medium) (Kantachote et al. 2005). However, this method has been most successful if source samples were taken from situations with a bloom of PNSB and in our experience it has proved to be very difficult to purify isolates when compared with other bacterial groups. The first problem has been partly solved by using a double strength medium *i.e*. GM to allow for a larger sample size (Kantachote et al. 2005); however, successful isolations are best achieved with samples from aqueous sources. Recently in our previous study we showed that we could readily isolate PNSB from a soil habitat like paddy fields using rice straw broth (Kantha et al. 2010) and therefore it would be worth investigating this medium as an alternative medium for isolating PNSB.

Rice straw is agricultural waste produced in large amounts from rice cultivation and consists of different biopolymers such as cellulose (32-37%), hemicellulose (29-37%) and lignin (5-15%) (Conrad, 2007). The polysaccharides in the straw may serve as substrates for complex microbial communities. The microbial metabolic pathways for the anaerobic degradation of 'dead' organic matter is in principle well known and involves hydrolysis of organic polymers; fermentation of the resulting monomers to fatty acids, alcohols, CO_2 and H_2 (Conrad, 2007). Among the fatty acids, acetate is a major product from anaerobic digestion and this is an important carbon source for the growth of PNSB (Harada et al. 2005). Hence, GA medium (Glutamate-Acetate medium) was considered for use as a defined control medium in this study. According to the above information the purposes of this study were to investigate the possibility of producing protocols to allow rice farmers to isolate PNSB by themselves for use as alternative biofertilizers and to purify PNSB isolates for further studies by comparing the use of GA medium and a rice straw broth. Consequently, the criteria used for comparison were the ease with which PNSB could be obtained from paddy fields and purified, the varieties of isolated PNSB and the cost.

MATERIALS AND METHODS

Survey of salinity in paddy fields

With the aim of isolating PNSB for use as biofertilizers in saline paddy fields, the following areas were examined for their salinity and acidity. Paddy fields were located around Talay Noi and Songkhla Lake Basins, that cover the following districts; Pak Phanang, Chian Yai in Nakhon Sri Thammarat province; Khuan Khanun, Khao Chaison in Phatthalung province and Kuan Niang district in Songkhla province. During rice cultivation, sediment and water samples were collected from paddy fields. To obtain a representative sample of each paddy field, a total of 13 sediment subsamples each of about 100 g was collected from each paddy field at a depth of 5 cm from the top of the sediment along two diagonals and a half point from each bank. For water samples, 100 mL of water at about 10 cm from the surface water level was collected from the points used for collecting sediment samples. All samples of sediment and water from the 13 points in each field were mixed to obtain one sample each of sediment and water and kept in an ice box during transporting to our laboratory and promptly used to isolate PNSB followed by measuring the electrical conductivity (EC) and pH values determined by a conductivity-pH meter (Seven Multi, Mettler Toledo, USA). The water samples were directly measured while the sediment samples were mixed with 5 times the volume of distilled water to make a ratio of 1:5 prior to measuring the values.

Isolation of purple nonsulfur bacteria from paddy fields

Glutamate-Acetate (GA) medium as an isolation medium. Ten mL of each water sample was transferred into a screw cap test tube (150 x 15 mm: 20 mL) containing 10 mL of double strength GA medium (Kantachote et al. 2005) and its screw cap lid applied. As the tubes were now filled this also made for microaerobic conditions as there was no space left in the test tubes and they became anaerobic over time as the microbes used the dissolved oxygen. GA medium was modified from GM medium by replacing malate with acetate. 1000 mL therefore contained 3.8 g sodium L-glutamic acid, 5.4 g sodium acetate, 2.0 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.8 g $(NH_4)_2HPO_4$, 0.2 g

$MgSO_4.7H_2O$, 0.053 g $CaCl_2.2H_2O$, 0.001 g nicotinic acid, 0.001 g thiamine hydrochloride, 0.01 g biotin, 0.012 g $MnSO_4.5H_2O$, 0.025 g ferric citrate and 0.95 g $CoCl_2.6H_2O$, with deionized water added up to 1000 mL and adjustment of the pH made with 1 M HCl to 6.8 (Suwansaard et al. 2009). In this study 0.25% NaCl (w/v) was also added to the GA medium to provide EC values of approximately 4 mS/cm, which is commonly found in southern saline soil conditions (Onthong et al. 1999). All test tubes were incubated with light for 13 days using tungsten lamps that provided a light intensity of 3,500 ± 200 lux at room temperature. The colour of the GA medium changed to pink, red or brown. Culture broths were streaked onto GA agar plates to purify the PNSB. All plates were placed in an anaerobic jar with the light condition as previously described. Distinguishable colonies were isolated and each isolated colony was checked for its purity by a light microscope after Gram staining. Several repeated streaking were carried out to obtain a pure culture from most colonies. All pure cultures were kept by stab inoculation into GA agar at 4ºC until used. For each sediment sample one loopful of sediment was added into a screw cap test tube (150 x 15 mm: 20 mL) containing 10 mL GA broth with 0.25% NaCl and then covered with a 1 cm high sterile liquid paraffin layer to achieve anaerobic conditions. The same protocol as described above was used to incubate the sediment samples for obtaining anaerobic light conditions and purifying the PNSB.

Rice straw broth as an isolation medium. Rice straw broth was prepared as follows: a bulk (5 g) of chopped rice straw (roughly 1 cm pieces), 2.5 g paddy soil and 10 mL 0.25% (w/v) NaCl solution were placed in a screw cap test tube (150 x 15 mm: 20 mL) and then the medium was sterilized by autoclaving (121ºC, 15 min). A similar protocol as for the GA medium was used for the 10 mL of water sample or one loopful of sediment was transferred to a test tube broth; however, liquid paraffin was not added to the top of each test tube. The incubating condition with light and the purification step procedure used were the same as previously described for the GA broth.

Statistical analysis

To compare the numbers of PNSB isolated from each medium, observation based on the number of samples that produced PNSB growth, how many streaking times were required for purification, and how many different colonies (number of isolates) were produced from each detected PNSB sample were used for statistical analysis. Moreover, a cost estimate for the use of each method was analyzed. Mean values and their standard deviations are presented. All the features were compared using the Wilcoxon signed ranks test which was performed by SPSS 11.5 (SPSS for windows, Version 11.5, USA). Differences with values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Salinity in paddy fields

Table 1 shows the levels of pH and EC in samples collected from paddy fields in the southern region of Thailand; Khuan Khanun, Pak Phanang and locations around Talay Noi. Values of the pH in the sediment samples were in a range of 4.81 ± 0.43 to 6.93 ± 0.36 whereas the water samples were between 4.97 ± 0.54 and 6.16 ± 0.58. The EC values were between 3.30 ± 0.31 and 3.80 ± 0.72 mS/cm in the sediment samples, and 3.09 ± 0.25 and 3.61 ± 0.72 mS/cm in the water samples. There were no significant differences in pH and EC values between samples of sediment and water from any one site. In contrast, among the studied areas the levels of pH in samples of water and sediment from Talay Noi were significantly ($P < 0.05$) lower than those in other areas but no significant difference was observed for the EC levels.

Isolation of PNSB from paddy fields

Duration of PNSB growth. Observations on the growth of PNSB in media supplemented with 0.25% NaCl (GA medium and rice straw broth) under anaerobic light conditions for 13 days, among 30 samples of water or sediment collected from rice fields in southern region of Thailand are presented in Figure 1 and Figure 2. Both water and sediment samples first showed detectable PNSB growth at day 3 of the incubation period in both isolating media and the number of samples producing PNSB growth in GA broth was significantly higher ($P < 0.05$) than that found in rice straw broth. However, after a

longer incubation time, starting from day 5, there was a marked increase the number of detected PNSB growth found in the rice straw medium while the number of detected PNSB that grew in GA medium increased slightly. After day 9 there was little further increase of the numbers of detected PNSB observed in either medium until the end of the incubation period at day 13. Over 13 days there was no significant difference for the numbers of detected PNSB growth found in each medium between the samples of water and sediment (Figure 3). However, different media produced a big difference in the numbers of samples (water and sediment) that showed PNSB growth. The percentage of samples producing PNSB growth in rice straw broth at the end of the incubation was an average of 73.33 ± 14.24 in water and 75.65 ± 14.12 in sediment samples. On the other hand in the GA broth an average percentage of producing PNSB growth was 40.25 ± 17.69 for water samples and 40.25 ± 23.53 for sediment samples (Figure 3).

Table 1. Values of pH and EC in samples collected from paddy fields in the southern región of Thailand.

Area (n)	Water		Sediment	
	pH	EC (mS/cm)	pH	EC (mS/cm)
Khuan Khanun n = 14	6.16 ± 0.58^{Aa}	3.13 ± 1.19^{Aa}	6.93 ± 0.36^{Aa}	3.66 ± 0.97^{Aa}
Pak Phanang n = 8	6.02 ± 0.21^{Aa}	3.09 ± 0.25^{Aa}	6.17 ± 0.52^{Aa}	3.30 ± 0.31^{Aa}
Talay Noi n = 8	4.97 ± 0.54^{Ab}	3.61 ± 0.72^{Aa}	4.81 ± 0.43^{Ab}	3.80 ± 0.72^{Aa}

Data represent a mean ± standard deviation. n = number of rice fields. The same upper-case letter in each row indicates no significant difference between samples of water and sediment, while the different lower-case letters in each column indicate significant differences among areas ($P < 0.05$).

Purification and number of PNSB isolates per sample. The PNSB that grew in the test tubes of both media was streaked onto GA agar containing 0.25% NaCl and incubated under anaerobic light conditions to select for single colonies and begin their purification. A total of 272 colonies from all 60 samples of water and sediment were picked for purification based on their different characteristics. In order to achieve a pure culture from each colony, the isolating medium had a significant effect ($P < 0.05$) on the numbers of streaking required. Colonies originally isolated from GA broth with 0.25% NaCl required an average number of times to streak for purification of 3.43 ± 1.04 and 3.46 ± 0.81 for the water and sediment samples, respectively (Figure 4). On the other hand, PNSB colonies that were isolated from rice straw broth required an average of 2.70 ± 0.46 and 2.73 ± 0.44, for the water and sediment samples, respectively. However, the sample sources of PNSB from either the water or sediment showed no differences in the numbers of streaking required to obtain pure cultures.

With the use of the 2 isolating media a total number of 272 PNSB were isolated from 60 samples from paddy fields (Table 2 and Figure 5). Based on the characterization of colonies such as differences in colour, 210 isolates were obtained from the rice straw broth with 0.25% NaCl with an average of 3.43 ± 2.25 and 3.56 ± 2.23 isolates per sample for water and sediment samples, respectively. Only 62 isolates were isolated from GA broth containing 0.25% NaCl with 0.96 ± 0.88 and 1.1 ± 0.88 isolates per sample of water and sediment, respectively. Again, the different sample sources, water and sediment, produced no significant differences in the numbers of isolates per sample.

The prevalence of isolated PNSB. The 272 isolates could be classified into 3 groups based on their colony colour and each group was calculated as a percentage on the basis of 30 samples (Table 2). The biggest group had red colour colonies with cells mostly rod shaped and they were in the range of 70.13 to 91.87% followed by brown colonies with mostly rod shaped cells (8.13-29.2%) and the smallest group was dark-brown colonies with spiral shaped cells (0-0.67%). In general the GA broth containing 0.25% NaCl produced a lower prevalence of PNSB isolates than those from the straw broth containing 0.25% NaCl and this was partly due to the dark-brown colonies with spiral shaped cells being obtained from the straw broth only. Based on Table 2, the prevalence of PNSB isolates was more influenced by the isolating medium than from the sample sources ($P < 0.05$).

Table 2. The percentage prevalence of PNSB isolates found in water and sediment samples collected from paddy fields using isolating medium (GA broth or rice straw broth) containing 0.25% NaCl.

Colony and cells characteristics	Water samples		Sediment samples	
	Rice straw (%)	GA (%)	Rice straw (%)	GA (%)
Red, mostly rod shaped cells	75.58 ± 4.98^{Ab} (78 isolates)	91.87 ± 4.45^{Aa} (27 isolates)	70.13 ± 4.02^{Bb} (77 isolates)	77.94 ± 4.65^{Ba} (30 isolates)
Brown, mostly rod shaped cells	24.09 ± 2.55^{Ba} (25 isolates)	8.13 ± 1.34^{Bb} (2 isolates)	29.2 ± 4.53^{Aa} (27 isolates)	22.06 ± 2.02^{Ab} (3 isolates)
Dark-brown, only spiral shaped cells[1]	0.33 ± 0.06 (1 isolate)	0	0.67 ± 0.12 (2 isolates)	0
Total	104	29	106	33

Data represent a mean ± standard deviation. N = 30.
[1]No statistical analysis as only 1 or 2 isolates compared with no isolates. The different upper-case letters indicate significant differences for sample sources while the different lower-case letters indicate significant differences for the isolating medium used ($P < 0.05$).

Cost comparisons for the isolation/purification of PNSB between GA and rice straw broths. Due to anaerobic light conditions being used to isolate PNSB the main costs were associated with the price of the media used, electricity and operation cost. However, the costs of the electricity (for each of 9 days based on results of Figure 1 and Figure 2) and also operation cost (man/hr) were virtually identical. The cost differences associated with the isolating medium used were based on the prices of chemicals used for GA broth (Sigma-Aldrich Co, St. Louis, USA) was 1.74 USD per 1 liter and 11.70 liters was used in this study of 60 samples. Therefore, the cost of GA broth used was 20.42 USD (Table 3). The rice straw price currently available in Thailand is 0.05 USD/kg this includes the price for chopping to make small pieces (Suramaythangkoor and Gheewala, 2010). As 3.90 kg rice straw was used, it cost 0.20 USD for rice straw broth in this work. According to the above information the cost to isolate PNSB in each sample by GA broth (0.39 USD) was significantly higher (7.8 times, $P < 0.05$) than that for rice straw broth (0.05 USD). In addition, as more PNSB isolates were observed from the rice straw broth the isolation cost of this medium per unit isolate was significantly cheaper ($P < 0.05$) than for the GA broth.

The cost incurred during the purification steps depended on GA agar, Gas Pak (to make anaerobic conditions), electricity and operation cost, hence more streaking repeats increased the costs for obtaining pure cultures. As the number of repeated streaking was less for the rice straw (Figure 4) this again was cheaper for the rice straw method (Table 3). Hence, the cost of purification for each pure culture (excluding operation cost) totally was an average of 0.16 USD for rice straw broth and 0.65 USD for GA broth. As there are 2 steps for obtaining pure cultures of PNSB, the total cost was the sum of the isolation and purification costs, and it was 0.18 and 1.03 USD for rice straw and GA broths, respectively. We can therefore conclude that rice straw broth was an appropriate isolating medium for obtaining pure PNSB cultures with a lower cost (roughly 6 times).

DISCUSSION

Results of the surveys indicated that most of the studied areas had pH values in the range of slightly acid to neutral with the exception of an area of Talay Noi that was acidic. However, EC values in all areas indicated only a slight saltiness (Dobermann and Fairhurst, 2000). Talay Noi had the lowest pH value but its EC value was the highest. It is likely that this is due to the fact that this area is a wetland and swamp forest that receives an effect from sea water by its connection to Songkhla Lake and the gulf of Thailand. Therefore, there is an accumulation of sodium and sulphate ions. Consequently, the reaction of sulphate and oxygen produces jarosite that is a major cause of acid sulphate soil (Osaki et al. 1998).

During the first 3 days of incubation a higher number of samples produced PNSB from GA broth but over the next 5 days this changed and more samples produced PNSB from the rice straw broth (Figure 1 and Figure 2). The GA medium is an enrichment medium for promoting PNSB growth under anaerobic light conditions because acetate is the main carbon source with glutamate and yeast extract acting as carbon and nitrogen sources including vitamins. In contrast, for rice straw broth the microbes present in the anaerobic sample took time to produce nutrients like acetate from the anaerobic.degradation of rice straw. The rice straw microbial degradation pathway of dead organic matter is in principle well known and involves hydrolysis of organic polymers; fermentation of monomers resulting in fatty acids, alcohols, CO_2, followed by acetogenesis to form acetate before being converted to CH_4 and CO_2 (Rui et al. 2009). Conrad (2007) reported that the acetate formed by the decomposition of rice straw increased rapidly from 5 to 10 days and the maximum acetate concentration was 23 mM. This corresponds to the results from this study in rice straw broth when PNSB grew well in this period (Figure 1 and Figure 2). Therefore, it took a longer time (2 days) than with GA broth for the PNSB to have sufficient nutrients for growth.

Table 3. Cost estimation to obtain PNSB based on isolation and purification steps.

Cost	Water samples		Sediment samples	
	Rice straw (USD)	GA (USD)	Rice straw (USD)	GA (USD)
Isolation				
Medium + Electricity	0.10 + 1.52	10.21 + 1.52	0.10 + 1.52	10.21 + 1.52
Cost/sample[1]	0.05 ± 0.01^{Ab}	0.39 ± 0.02^{Aa}	0.05 ± 0.01^{Ab}	0.39 ± 0.02^{Aa}
Cost/isolate[2]	0.02 ± 0.01^{Ab}	0.38 ± 0.02^{Aa}	0.02 ± 0.01^{Ab}	0.38 ± 0.02^{Aa}
Purification				
GA medium[3]	0.02 ± 0.001^{Ab}	0.05 ± 0.01^{Aa}	0.02 ± 0.001^{Ab}	0.04 ± 0.01^{Aa}
Gas-Pak[4]	0.08 ± 0.001^{Ab}	0.38 ± 0.04^{Aa}	0.08 ± 0.001^{Ab}	0.34 ± 0.04^{Aa}
Electricity[5]	0.05 ± 0.001^{Ab}	0.24 ± 0.04^{Aa}	0.05 ± 0.001^{Ab}	0.21 ± 0.02^{Aa}
Cost/isolate	0.16 ± 0.001^{Ab}	0.67 ± 0.01^{Aa}	0.16 ± 0.001^{Ab}	0.63 ± 0.01^{Aa}
Total cost/isolate	0.17 ± 0.001^{Ab}	1.05 ± 0.01^{Aa}	0.17 ± 0.001^{Ab}	1.01 ± 0.01^{Aa}

[1]T = 9 days, n = 30.
[2]Rice straw; n = 210, GA; n = 62; the cost of liquid paraffin for isolating PNSB from sediment samples using GA broth was excluded as it can be reused after being sterilized.
[3]Medium used depended on the number of streaking times.
[4]Anaerocult®, Merck KGaA, Germany; 3.25 USD/unit.
[5]Electricity for purification, 0.06 USD/power unit; T = 5 days/streaking timeThe different upper-case letters indicate significant differences for sample sources while the different lower-case letters indicate significant differences for the isolating medium used ($p \leq 0.05$).

One reason why PNSB in samples of sediment and water grew better than other microbes in rice straw broth under anaerobic light conditions could be that the presence of phenolic compound caused by rice straw degradation inhibited other microbes that included cyanobacteria and methanogens (Glissmann et al. 2005; Park et al. 2006). The allelochemicals released from rice straw consist of phenolic compounds, such as *p*-hydroxybenzoic, *p*-coumaric, ferulic, vanillic, salicylic, syringic and benzoic acid (Chung et al. 2001) and these compounds may be non toxic to PNSB (Gibson and Harwood, 2004). Thus, with the conditions of anaerobic light and fewer competitors PNSB became the dominant organism in rice straw broth in anaerobic conditions and in the presence of light. To explain why rice straw broth promoted PNSB growth roughly 2 times better than GA broth (Figure 3), it may be that in rice straw broth, available nutrients slowly increased and provided more suitable condition for PNSB growth whereas in the GA broth the higher amount of nutrients was most readily used immediately by other microbes in the samples. Hence, other anaerobic heterotrophs rapidly became the dominant organism in GA broth leaving less nutrient for the PNSB. This indicated that PNSB isolation using rice straw broth can be readily applied for both water and sediment samples.

There was no significant difference found for the average number of repeated streaking required to obtain pure cultures from samples of water compared to that from the sediment samples using either

the GA broth or rice straw broth (Figure 4). However, there was a significant difference when comparing the results of isolates from the GA broth and rice straw broth with the latter being better. This was caused by the different nutrient composition. Most single colonies on the GA agar showed either a red, pink or brown colour surrounded by a white colour, and they required repeated streaking to remove the white coloured culture. Presumably the GA broth allowed the growth of other heterotrophs although the anaerobic light conditions encourage the growth of PNSB. In contrast, the PNSB from rice straw broth were more easily purified because nutrients for other heterotrophs was less available immediately and required microbes able to degrade the complex substrates to substrates like acetate suitable for the growth of PNSB. Individual bacterial groups such as *Clostridium* spp. can hydrolyze polymers and convert the monomers to fatty acids including acetate under anaerobic conditions (Conrad, 2007) which then promotes the growth of PNSB to become the dominant population. Another key factor is the light condition, that promotes PNSB growth to be dominant during a period of incubation because its competitors such as methanogens and sulphate reducing bacteria would be out-competed by PNSB in the light conditions (Harada et al. 2003a; Tada et al. 2005). Because of this, PNSB became the dominant organism in rice straw broth and this allowed for easier purification when compared to the GA broth.

The finding that roughly 3.5 times more PNSB were isolated from the water and sediment samples from rice straw broth than from GA broth (Figure 5) indicated that rice straw broth medium might contain more different nutrients than those present in the defined GA broth medium. This was also indicated by the finding that the prevalence of PNSB isolates from the rice straw broth was much greater than that from the GA broth (Table 2), so the increased variety of nutrients increased the prevalence of PNSB isolates. GA broth has acetate and glutamate as the main carbon substrates, thus these substrates may limit the prevalence of PNSB from paddy samples (Table 2). The diversity of PNSB in paddy soil has been studied and most of them are *Rhodopseudomonas* spp., *Rhodoplanes* spp. with red and pink colonies (Feng et al. 2011) and *Rhodobacter* spp. with brown colonies (Nakayama et al. 2006). These genera are typically photoheterotrophic PNSB found in paddy soil (Harada et al. 2003b; Lakshmi et al. 2009; Gamal-Eldin and Elbanna 2011) and they can use various fatty acids (Kim et al. 2004). However, in this work we have not yet identified the isolated PNSB except for their different coloured colonies; red colonies like with rod shapped cells 75%, brown colonies with rod shapped cells 24%, and dark-brown colonies with spiral shape cells 1%.

It is well recognized that PNSB are regarded as aquatic organisms (Imhoff and Trüper, 2005); however, in this study it was shown that the sediment samples from paddy fields were also a good source of PNSB when compared with the water samples (Figure 3 and Figure 5). To may be that the shallow paddy fields allows light to penetrate to the sediment and perhaps provides higher nutrients through the degradation of organic matter provided by the rice. In addition, the oxidation-reduction potential (ORP) in the sediment of paddy fields is in the range of -200 to -250 mV that is a suitable condition for promoting PNSB growth (Overmann and Garcia-Pichel, 2006). These factors can encourage the growth of PNSB as they are photoorganotroph/photoautotroph; thereby rice sediments are suitable to sustain life of PNSB.

Based on cost estimations for obtaining PNSB including the purification of the cultures, rice straw broth as an isolating medium was much cheaper than GA broth (Table 3). In addition, this isolating medium also produced a higher prevalence of PNSB. It can be concluded that the rice straw broth was an effective isolating medium for PNSB and this might be applied to various sample sources. Considering rice straw broth as an appropriate medium for obtaining PNSB to use in paddy fields by farmers it is possible that they can do it by themselves. According to results in this study we propose that farmers can scale up the isolating rice straw broth into a shallow pond or a large container and the light source can be the sun in the daytime and tungsten lamps provided in night time (data not shown). It is well recognized that PNSB could be dominant organisms under anaerobic/microaerobic light conditions; therefore, to achieve the best conditions for promoting PNSB a small amount of fermented plant broth (1-2%) should be added to rapidly reduce the ORP value (Kantachote et al. 2010; preliminary work). The use of rice straw broth by farmers could produce blooms of PNSB for use as biofertilizers in their paddy fields and this method would be an easy way to use indigenous PNSB (autochthonous bioaugmentation). This technology will be available to farmers in the near future. In parallel to obtain promising PNSB strains, our pure cultures in this study have been currently selected based on their high salt tolerance and also ability to produce ALA and reduce CH_4 emission for possible use in saline paddy fields to enhance rice yield and reduce global warming.

CONCLUDING REMARKS

This study proved that rice straw broth was an appropriate isolating medium to obtain PNSB with a cheap price and high efficiency. In addition, this isolating medium allows farmers to produce biofertilizers by themselves and thus this method will encourage farmers to continue organic agriculture that is environmentally friendly and at a cheaper cost.

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Figures

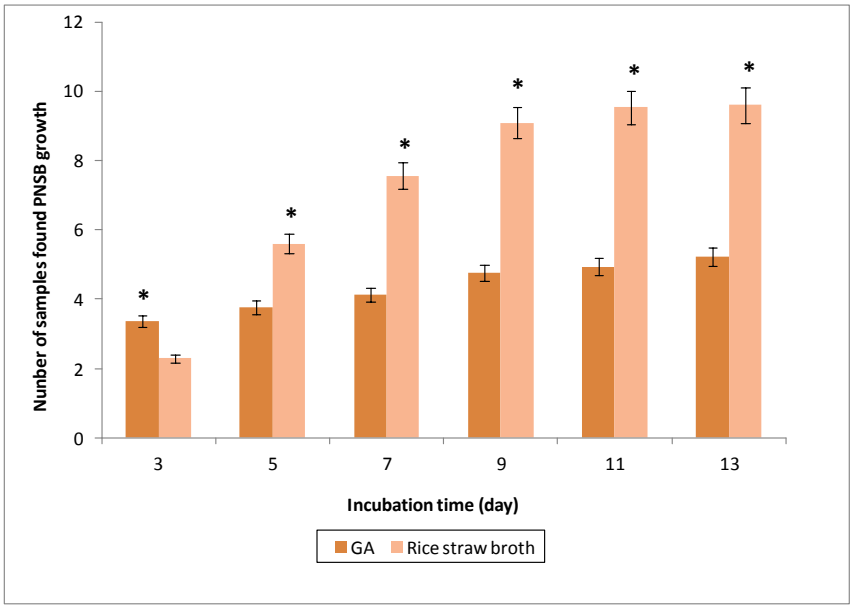

Fig. 1 The average number of water samples showing growth of PNSB on different media. Data are given as a mean ± standard deviation (n = 30). *significant differences between media (P < 0.05).

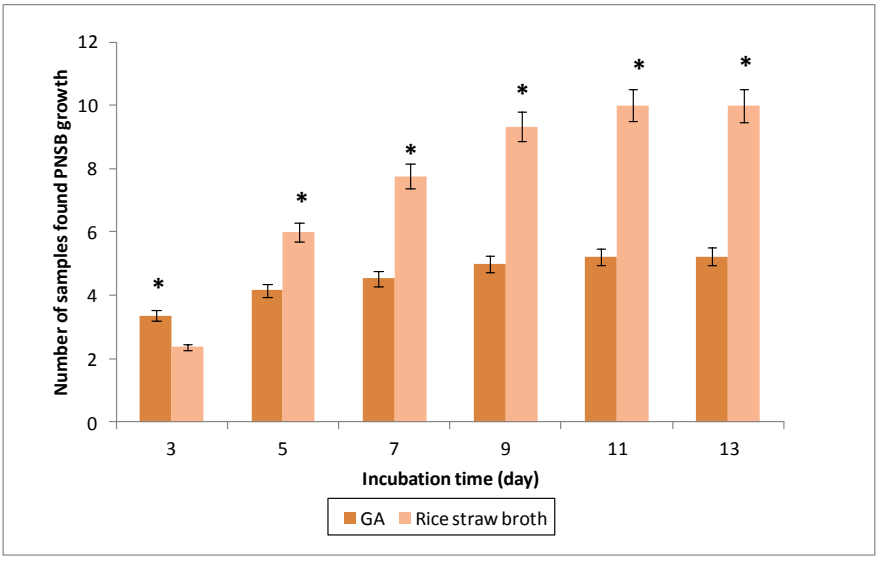

Fig. 2 The average number of sediment samples showing growth of PNSB on different media. Data are given as a mean ± standard deviation (n = 30). *significant differences between means (P < 0.05).

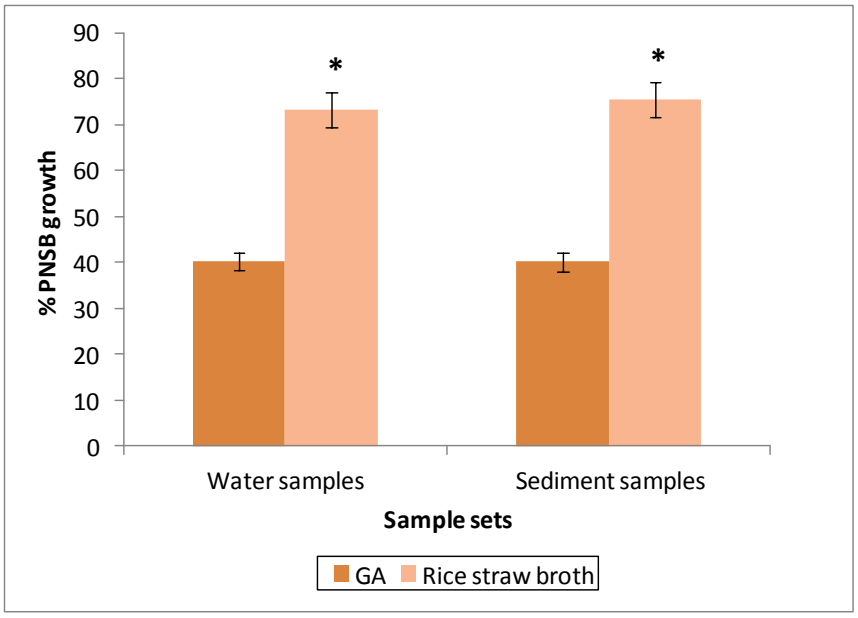

Fig. 3 Percentage of samples showing growth of PNSB after 13 days in GA or in rice straw broth containing 0.25% NaCl (n = 30). *significant differences between means ($P < 0.05$).

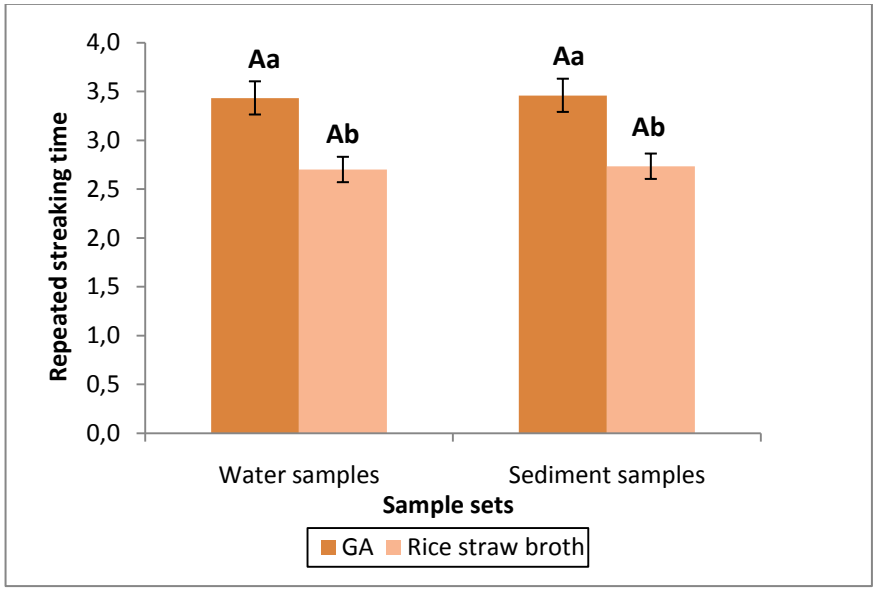

Fig. 4 The number of repeated streaking times, given as a mean ± standard deviation, required to obtain pure cultures from each colony isolated from GA broth (n = 62) or rice straw broth (n = 210) medium containing 0.25% NaCl. The same upper-case letter on the bars indicates no significant difference for sample sources while the different lower-case letters indicates a significant difference for isolating in the medium used ($P < 0.05$).

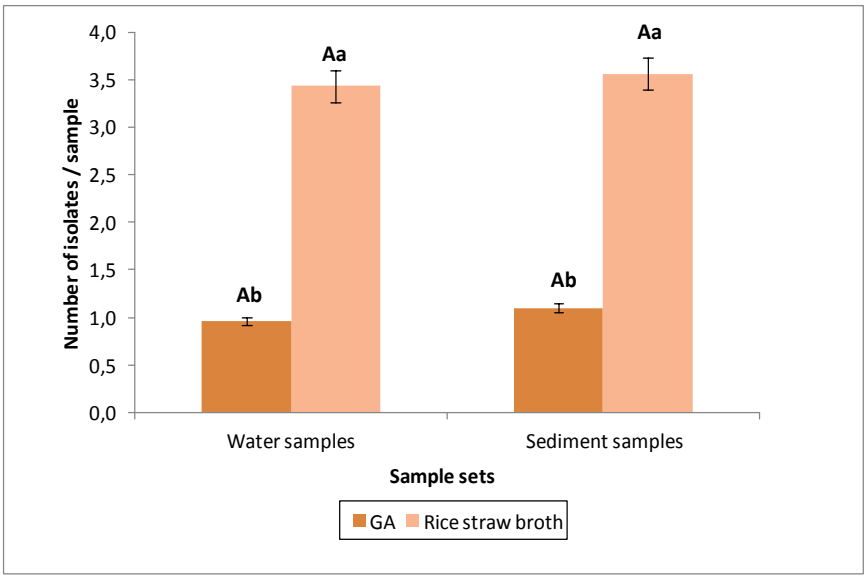

Fig. 5 The average number of PNSB isolates per sample found in GA broth or rice straw broth (n = 30) medium containing 0.25% NaCl. The same upper-case letter on bars indicates no significant difference for sample sources while the different lower-case letters indicate significant differences for the isolating medium used ($P < 0.05$).