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Nitrogen-Fixing Purple Nonsulfur Bacteria Originating from Acid Saline Soils of a Rice-Shrimp Farm

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Abstract. The study was conducted to (i) isolate, select, and identify strains of purple nonsulfur bacteria (PNSB), which can fix nitrogen (N), from soil and water in a rice-shrimp integrated system, (ii) to determine the capacity of the selected potent PNSB strains in producing plant growth promoting substances. The isolation resulted in 57 pure PNSB strains from 36 soil samples and 36 water samples of rice-shrimp paddy fields in Thanh Phu - Ben Tre. Among them, 49 strains survived under pH 5.0 conditions, 24 of which grew well under microaerobic light (ML) and aerobic dark (AD) conditions in a basic isolation medium (BIM) containing NaCl 5‰. Two strains (S01 and S06) with the greatest N fixation were identified by 16S rRNA techniques as *Rhodobacter sphaeroides*. Their N production was 16.9 mg L^{-1} under the ML condition and 32.1 mg L^{-1} under the AD condition. Moreover, two *R. sphaeroides* S01 and S06 strains performed P solubilization at 0.382-2.954 mg L^{-1} from Al-P, 3.81-4.28 mg L^{-1} from Fe-P, and 3.87-4.74 mg L^{-1} from Ca-P, and production of plant growth promoting substances, such as IAA (12.3-15.5 mg L^{-1}), EPS (1.09-1.58 mg L^{-1}), siderophores (10.7-53.6%) and ALA (1.68-2.82 mg L^{-1}) under both the incubating conditions.

Keywords: nitrogen-fixing bacteria, purple nonsulfur bacteria, *Rhodobacter sphaeroides,* rice-shrimp paddy fields

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1. Introduction

Since 2020, saline water has intruded further into farming lands and affected 10 out of 13 provinces in the Mekong Delta, Vietnam, which severely damages millions of hectares of farmlands and the livelihood of thousands of households [1]. Soils with an electrical conductivity (EC) from 250 to 750 µS cm^{-1} harms salt-susceptible crops, especially rice [2]. Therefore, in order to farm rice in those regions, farmers there converted from intensive rice farming to a rice-shrimp model to make use of rainwater for rice and to raise shrimp during the dry season [3]. This practice has had a negative impact on rice cultivation in coastal paddy fields [4]. Nevertheless, soil in

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Thanh Phu, Vietnam has been detected to be contaminated by acid sulfate matter with higher concentrations of H^+, Al^{3+}, $Fe^{2+,}$ and Mn^{2+}. Rice is vulnerable to salinity, so high Na^+ concentration in soil affects the processes of photosynthesis, respiration, and assimilation, resulting in withering, drying, and dying plants [5]. In some susceptible species, high salt concentration represses growth and development due to reductions in leaf area, photosynthesis, respiration rate, and protein synthesis, which altogether reduce productivity [6]. As a result, the yield and seed quality of rice grown on saline soils drop. This is caused mainly by the decline in filled seed ratio, available shoot, and number of seeds per panicle; and salinity also reduces the integrity rate of rice seeds, and enhances the chalky rice rate [7].

Nowadays, many approaches have been conducted to reduce damages caused by salinity on rice grain yield, such as the application of calcium compounds (gypsum) to alter Na^+ absorbed on the surface of soil colloids [8]. Farming approaches are modified, such as soil preparation, irrigation system, and biochar application [9] – [10], and application of salt-tolerant varieties [11]. Moreover, microbiological approaches are also promising, while purple nonsulfur bacteria (PNSB) are one of the most potent approaches, because PNSB can diversely habituate, such as photoautotroph, chemoautotroph, and chemoheterotroph [12] – [13]. Some species of PNSB belong to the genera *Rhodopseudomonas* spp. and *Rhodobacter* spp. which are able to fix N under both microaerobic and aerobic conditions [13]. PNSB is studied for being applied as a nutrient source for rice via N fixation in many types of soils [14] – [17]. Three catalytic isozymes facilitating the N fixation are molybdenum-iron, vanadium - iron and iron-iron nitrogenases. For instance, a PNSB strain, *Rhodopseudomonas palustris* TN10, possesses the three types of nitrogenase genes and performs the greatest N fixation [18]. However, these strains have not been evaluated for supplying N under saline conditions. In addition, PNSB also possesses other functions, such as P solubilization [16], and production of 5-aminolevulinic acid (ALA metabolite [17], indole-3-acetic acid (IAA), siderophores [18]-[20] and EPS [20]. Noticeably, PNSB produces more exopolymeric substances (EPS) under high saline conditions [16], [21]. Therefore, PNSB that can tolerate salinity, such as *Luteovulum sphaeroides* W01, W14, W22, and W32 can support rice to overcome saline stress [17], promote growth and increase rice grain yield [22]. Therefore, the aim of the current study was to isolate and selected potent strains of PNSB that can fix N in saline soils of the rice-shrimp integrated system. Some PNSB strains were expected to live, fix nitrogen, and produce plant growth-promoting substances under saline acidic microaerobic light and aerobic dark conditions.

2. Materials and Methods

2.1. Materials

Isolating media: The basic isolation medium (BIM) was used to isolate bacteria from soils, and in a liter of distilled water, it consisted of 1.0 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 2.0 g

NaCl, 5.0 g $NaHCO_3$, 1.5 g yeast extract, 1.5 g glycerol and 0.03 g L-cysteine. For solid culture, the medium was added with 15 g agar L^{-1}. A BIM with double concentrations (D-BIM) was used to isolate bacteria from saline water, with ranging of 0.65-8.89 mS cm^{-1}. Both BIM and D-BIM were adjusted to pH equal to 6.8.

Sample collection and processing: A total of 36 soil samples and 36 water samples were collected in rice-shrimp paddy fields in Thanh Phu, Ben Tre province which is a coastal area in the Mekong delta, Vietnam. The dry season with higher salinity, this resulted in culturing shrimp while wet season was used for rice growth due to fresh water from rain. Thus, samples were collected in wet season. Each soil and water sample of the same location were collected at 7 different sites, and combined into 1 sample. In particular, the soil sample was collected at 0-10 cm deep with a weight of 100 g $sample^{-1}$, and 100 mL of a water sample was collected from the bottom to 3 cm from the surface. Samples were stored cold until PNSB isolation, and soil samples were mixed in one to analyze soil chemical properties.

2.2. Methods

Sample analysis: All parameters were analyzed according to the methods gathered by Spark *et al.* [23]. Soil samples were extracted with KCl 1.0 M (to measure pH_{KCl}) or with water (to measure pH_{H2O}) at a 1-to-5 ratio. The extracted solution was measured by a pH and an electrical conductivity (EC) meter. Total nitrogen (N_{tot}) was analyzed according to the Kjeldahl distilling method after organic N was converted to inorganic N. Analysis of NH_4^+-N in soil was conducted by extracting soil with KCl 2.0 M, and measured by the blue phenol method at 650 nm wavelength. Total carbon (C_{tot}) was determined by the ash content determination method. Soils were extracted with $MgSO_4$ 0.005 M and titrated with EDTA 0.01 M to determine the cation exchange capacity (CEC). The Al^{3+} concentrations among soil samples were extracted with KCl 1.0 M, analyzed with 8-hydroxyquinoline and butyl acetate, and measured at the 395 nm wavelength. The ferrozine method was used to determine Fe^{2+} concentrations with the 1, 10-phenanthroline indicator, and measured at the 562 nm wavelength. Mn^{2+} concentrations were determined by a colorimeter at the 545 nm wavelength.

Isolating PNSB: The PNSB isolation was conducted according to the method of Brown [24], a popular protocol to isolate PNSB, and proceeded as follows: 1.0 g of soils or 9.0 mL of water sample was added to 23mL-tubes with 18.0 or 9.0 mL of BIM or DBIM, respectively. Then, 1.5 mL of sterilized liquid paraffin was used to cover the surface of the medium to ensure a completely anaerobic condition. All tubes were continuously incubated under an illuminated condition; whose light intensity was roughly 3,000 lux for 7 days. Tubes with red, purple, brownish yellow, or brown colors, were all used to purify bacteria on the solid BIM. All inoculated dishes were incubated in jars, which were made anaerobic by using gas-pak. The purity of single colonies was determined by observing the morphology, shape and size of colonies and

bacteria with a microscope after Gram staining Gram. All colonies were stored at 4 ºC, or kept in glycerol 20% solution at -80ºC.

Selecting PNSB isolates that can live under microaerobic light and aerobic dark conditions: All bacterial isolates were incubated under microaerobic light condition (MLC) and aerobic dark condition (ADC) (pH 7.0). Under the MLC, the bacteria were raised in capped tubes, and illuminated with light at 3,000 lux. Under the ADC, the bacteria were tubes covered with aluminium foil, and shaken at 150 rpm at 30°C. Bacterial suspensions were cultured for 48 h in BIM, and adjusted to the OD_{660} = 0.5. 10% of the OD_{660} = 0.5 bacterial suspension was transferred into a tube containing 90% of BIM (pH 7.0). After 72 h of culture under 3,000 lux illuminated condition, or under 150 rpm shaking and ADC, the growth of bacteria was measured at the 660 nm wavelength, and bacterial isolates with $OD_{660} \geq 1.0$ were selected.

Selecting PNSB isolates that can tolerate pH 5.0 and NaCl 5‰ conditions: All PNSB isolates that grew well under the MLC and ADC were used to examine their tolerance under acidic conditions. 10% of 48-hour-cultured bacterial suspension under BIM of each isolate (adjusted to OD_{660} = 0.5) was transferred into a tube of 90% BIM (pH 5.0). After 72 h of culture, the growth of bacteria under both the incubating conditions were measured at the 660 nm wavelength, and PNSB isolates that had $OD_{660} \geq 1.0$ were selected for the next experiment. PNSB isolates that could grow well under the pH 5.0 condition were used to test for viability under Na^+ condition (at 5 g L^{-1} due to normal concentration in paddy field of rice shrimp system) which was prepared from the NaCl compound. 10% of the bacterial culture after 48 h was adjusted to OD_{660} = 0.5 and added to tubes containing 90% of BIM pH 5.0. After 72 h, the growth of bacteria was measured under both MLC and ADC at the 660 nm wavelength. Bacteria isolates that had $OD_{660} > 1.0$ were chosen for the further experiment.

Selecting PNSB isolates that can fix N: The salt-tolerant bacterial isolates were tested for N fixing capacity. After 48 h of culture in BIM, 10% of bacterial suspension was adjusted to OD_{660} = 0.5, and transferred to tubes containing 90% BIM without N (pH 5.0, NaCl 5‰). Then, the bacteria were cultured under MLC with 3,000 lux light intensity, and under ADC with 150 rpm shaking at 30 ˚C. After 72 h of culture, 1.0 mL of centrifuged bacterial suspension at 3,000 rpm for 15 min had its N content fixed by the blue phenol colorimetry, and measured by a spectrophotometer at the 650 nm wavelength [25]. In particular, 10 mL of sample was centrifuged at 3,500 rpm for 5 min. Subsequently, 2.0 mL of sample was added to a tube which was then added with 0.5 mL of the reagent A, and 0.5 mL of the B reagent, capped and well shaken, and measured at the 650 nm wavelength. The A reagent contained four chemicals (0.05 g sodium nitroprusside, 13 g sodium salicylate, 10 g sodium citrate, and 10 g sodium tartrate), dissolved and adjusted to 100 mL volume by distilled water, while the B reagent (6 g sodium hydroxide in 100 mL of distilled water and added with 2 mL of sodium hypochlorite).

Determining capacity of the selected isolates in providing soluble P: The procedure was conducted according to the above incubations. However, in BIM, the P source as KH_2PO_4 was replaced by insoluble P compounds, $AlPO_4•2H_2O$, $FePO_4•2H_2O$ and $Ca_3(PO_4)_2$ at 0.3, 1.0 and 5.0 g L^{-1}, respectively. After 120 h of culture, 1.0 mL of bacterial culture was centrifuged at 3,000 rpm in 15 min. The centrifuged solution was used to determine the P content solubilized by bacteria by reacting with the ascorbic acid to form a color complex, and measured by a spectrophotometer at the 880 nm wavelength [26].

Determining capacity of the selected isolates in plant growth promoting substances: The IAA content produced by bacteria was measured in BIM supplied with 100 mg L^{-1} tryptophan. After 48 h of culture, 1 mL of the bacterial solution was centrifuged at 3,000 rpm for 15 min. The clear solution was used to determine IAA content by the Salkowski's colorimetry at the 535 nm wavelength [27]. The ALA quantification was determined similarly, the BIM contained the glycine precursor (0.563 g L^{-1}) and sodium acetate (5.44 g L^{-1}). After 48 h of culture, the transparent extract was used to determine ALA content by a spectrophotometer at the 553 nm wavelength [28]. The siderophores production was determined according to the method of Schwyn and Neilands [29]. It was via an orange clear zone surrounding the bacteria colony after 4 days of culture under MLC and ADC in BIM supplied with 1 g L^{-1} of succinate and 0.5 µM of $FeCl_3•6H_2O$ (30 ˚C, 150 rpm). The bacterial solution was centrifuged at 3,000 rpm, for 15 min. 1.0 mL of this solution was mixed with 1.0 mL of CAS (Chrome azurol S), and measured at the 630 nm wavelength. Determination of EPS released by PNSB was conducted as follows: 50 mL of bacterial solution cultured for 48 h in BIM and adjusted to OD_{660} = 0.5 was transferred into 450 mL of BIM with NaCl 5‰, and was incubated under MLC (3,500 lux) at 30 ˚C for 48 h. Subsequently, the incubated solution was centrifuged at 3,000 rpm for 15 min. The centrifuge solution was precipitated by adding cold (4 ˚C) with a ratio of 1: 2.2 for 24 h to precipitate EPS [30].

Identifying N-fixing PNSB: The selected isolates were cultured in solid BIM for 48 h. Two mL of colonies were centrifuged at 6,000 rpm for 15 min to collect cells to extract DNA by the Genomic DNA Prep Kit (BioFACT™) based on the manufacturer's instruction. Concentration and purity of the extracted DNA was checked by electrophoresis on 1.0% w/v agarose gel. DNA was amplified at the 16S rRNA coding sequence by PCR technique. The forward primer 8 F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and the reverse primer 1492 R (5′-GGT TAC CTT GTT ACG ACT T-3′) [31] were used as described in the iProof™ High-Fidelity PCR Kit - Bio-Rad (BioRad, Hercules, CA) by T100™ thermo cycler (BioRad). The PCR products were purified by the TIANquick Midi Purification Kit (Tiangen Biotech Ltd., Beijing, China) according to the manufacturer's instructions. The purification of the amplicon was checked by the electrophoresis on 1.0% w/v agarose gel. The PCR products were then sequenced by the automatic sequencing machine in Macrogen DNA Sequencing Service (Macrogen, Seoul, Korea). The sequences were

analyzed by the BioEdit software, version 7.0.5.3 [32] and the ChromasPro software, version 1.7 (http://technelysium.com.au/wp/chromaspro). To determine similarity, sequences of the selected isolates were compared to available sequences in GenBank by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

Statistical analysis: All means were applied with variance analysis (ANOVA), and compared by the Ducan's test at $P < 0.05$ in the SPSS software, version 13.0.

3. Results and Discussion

3.1. Determining Soil Characteristics of Rice-Shrimp System in Thanh Phu-Ben Tre for Isolating PNSB

Based on the result of soil analysis of sample for isolation, the median of pH_{H2O} (6.05) and pH_{KCl} (5.28), the soil was classified to be slightly or moderately acidic. Average EC was 1.99 mS cm^{-1}, but the highest value was 8.89 mS cm^{-1}, which a few crops can tolerate. Median values of Al^{3+}, Fe^{2+} and Mn^{2+} toxins were determined as 5.15 meq Al^{3+} 100 g^{-1}, 27.6 mg Fe^{2+} kg^{-1} and 587.9 mg Mn^{2+} kg^{-1}, respectively (Table 1). Therefore, the soil characteristics of sampling sites were acidic and had high EC (Table 1), which damaged crop yield. In acid sulfate soil, concentrations of Al^{3+}, Fe^{2+} and Mn^{2+} are high, which causes damage to crop roots, growth and yield [17], [33], [34]. In addition, the N content was 0.21%, and was considered to be moderate (Table 1). Therefore, rice farming on rice-shrimp soil with acidity, and saline properties requires suitable approaches to improve N nutrient to increase rice grain yield. Approaches using microorganisms which can grow well under adverse conditions to provide nutrients for crops are prioritized for development of sustainable agriculture [35].

Tabel 1. Characteristics of Soils Used for Isolating Purple Nonsulfur Bacteria in Thanh Phu - Ben Tre

Parameter	pH_{H2O}	pH_{KCl}	EC	N total	NH_4^+	Organic matter	Al^{3+}	Fe^{2+}	Mn^{2+}	CEC
	-	-	mS cm^{-1}	%	mg/kg	%	meq/ 100g	mg/kg	mg/kg	meq/ 100g
Maximum	6.74	6.22	8.89	0.33	36.6	5.13	19.6	98.7	1456.3	13.5
Mean	5.75±0.75	5.39±0.41	2.91±2.26	0.21±0.05	23.1±6.47	3.38± 1.02	6.37±3.95	35.5±19.4	691.2±324.3	10.3±1.76
Median	6.05	5.28	1.99	0.21	22.4	3.31	5.15	27.6	587.9	10.0
Minimum	4.12	4.75	0.65	0.11	11.2	1.27	1.21	14.3	245.9	6.98

Note: Sample size n=36. EC: Electrical Conductivity; CEC: Cation Exchange Capacity

Isolation and purification resulted in 57 PNSB isolates from the salt-contaminated rice-shrimp system. Therein, there were 46 isolates originating from water samples, and 11 isolates isolated from soil samples, accounting for 80.7% and 19.3%, respectively. Additionally, morphological observation revealed that PNSB isolates from water samples were brown or brownish yellow, while isolates from soils were red or brownish yellow. All 57 PNSB isolates survived in BIM (pH=7.0) with $OD_{660} > 1.0$ under both MLC and ADC for 72 h. Therefore, isolates were chosen for evaluation in the following experiments. PNSB can accumulate energy from light under

anaerobic conditions as well as from chemicals under aerobic conditions because under both conditions, there are C sources from CO_2 or organic matter [36]. Moreover, PNSB exist in different phototrophic environments such as photoautotroph, photoheterotroph, and chemotroph [37]-[40], so they are well adapted to harsh environments, such as acidic or saline soils. Thus, these bacteria should be further studied under saline conditions.

3.2. Selecting N-Fixing PNSB that Can Live in Saline Soil

Selecting PNSB that can tolerate acidic saline conditions: The PNSB isolates continued to be cultured in BIM (pH 5.0) for 72 h. For $OD_{660} > 1.0$, there were 49 isolates. In particular, there were 3 isolates with $OD_{660} > 2.0$ (W12, W31, and S69) under ADC. On the other hand, there were 46 isolates with $OD_{660} > 1.0$ under MLC (Table 2). PNSB isolates that grew well in BIM (pH 5.0) continued to be cultured in BIM added with NaCl 5‰, and there were 24 isolates that could live under this condition. OD_{660} values were from 0.344 to 3.140 under MLC, while they were from 0.772 to 1.740 under ADC. However, the abilities of the PNSB isolates to tolerate salinity were equivalent among isolates (Table 2).

Table 2. Viability of Purple Nonsulfur Bacteria Under Acidic and Saline Conditions

No.	Isolate	pH = 5.0		pH = 5.0 and NaCl 5‰	
		MLC	ADC	MLC	ADC
1	W01	1.338^{e-i}±0.043	1.247^{d-k} ±0.123	0.895±0.121	0.990±0.146
2	W04	1.505^{c-h}±0.051	1.294^{c-k} ±0.204	0.870±0.112	0.980±0.100
3	W05	1.315^{e-i}±0.025	1.069^{g-l} ±0.073	-	-
4	W07	1.596^{c-g}±0.036	1.708^{ab} ±0.049	0.888±0.485	0.930±0.244
5	W08	1.759^{b-e}±0.217	1.341^{b-k} ±0.039	-	-
6	W09	1.335^{e-i}±0.023	1.143^{e-l} ±0.508	-	-
7	W10	1.402^{d-i}±0.039	1.472^{b-g} ±0.035	0.988±0.102	1.110±0.243
8	W11	1.082^{h-k}±0.047	0.993^{h-l} ±0.068	0.991±0.058	1.187±0.187
9	W12	2.150^{ab}±0.145	1.843^{a} ±0.383	0.913±0.090	1.195±0.126
10	W14	1.305^{e-i}±0.290	1.424^{b-h} ±0.083	0.716±0.483	0.892±0.442
11	W16	1.272^{f-i}±0.073	1.137^{e-l} ±0.111	-	-
12	W17	1.339^{e-i}±0.081	1.449^{b-h} ±0.025	-	-
13	W19	1.603^{c-g}±0.223	1.471^{b-g} ±0.052	1.706±0.813	1.220±0.151
14	W20	1.161^{g-k}±0.017	1.433^{b-h} ±0.085	0.997±0.794	1.252±0.274
15	W21	1.360^{e-i}±0.055	1.454^{b-h} ±0.026	0.714±0.647	1.060±0.127
16	W22	0.820^{k}±0.062	1.129^{e-l} ±0.080	1.450±1.937	1.145±0.210
17	W23	1.567^{c-g}±0.284	1.444^{b-h} ±0.025	1.253±1.955	1.734±0.341
18	W24	1.008^{ik}±0.207	1.334^{b-k} ±0.210	3.140±3.357	1.326 ±0.095
19	W25	1.388^{d-i}±0.096	1.294^{c-k} ±0.088	2.918±2.129	0.988±0.702
20	W26	1.192^{f-k}±0.139	0.959^{kl} ±0.088	-	-
21	W27	1.491_{c-h}±0.124	1.340^{b-k} ±0.071	-	-
22	W28	1.328^{e-i}±0.226	1.538^{a-e} ±0.097	-	-

Table 2. Continued

No.	Isolate	pH = 5.0		pH = 5.0 and NaCl 5‰	
		MLC	ADC	MLC	ADC
23	W29	1.404 $^{d-i}$±0.203	1.503^{a-f} ±0.007	-	-
24	W30	1.598^{c-g}±0.085	1.336^{b-k} ±0.022	-	-
25	W31	2.134^{ab}±0.077	1.632^{a-d} ±0.280	1.024±0.328	1.236 ±0.198
26	W32	1.588^{c-g}±0.300	1.328^{b-k} ±0.038	-	-
27	W34	1.323 $^{e-i}$±0.277	1.320^{c-k} ±0.027	0.344±0.176	0.881±0.554
28	W36	1.019^{ik}±0.310	1.396^{b-h} ±0.060	-	-
29	W37	1.007^{ik}±0.496	1.564^{a-e} ±0.321	-	-
30	W40	1.160^{g-k}±0.136	1.257^{d-k} ±0.083	0.770±0.443	0.814±0.363
31	W42	1.552^{c-g}±0.393	1.452^{b-h} ±0.096	-	-
32	W43	0.983^{ik}±0.066	1.182^{e-k} ±0.133	-	-
33	W44	1.190^{f-k}±0.032	1.416^{b-h} ±0.018	-	-
34	W45	1.261^{f-i}±0.070	1.252^{d-k} ±0.207	1.716±1.059	1.158 ±0.088
35	W46	0.978^{ik}±0.109	1.245^{d-k} ±0.153	-	-
36	W47	1.357 $^{e-i}$±0.083	1.116^{e-l} ±0.017	1.436±1.357	0.772±0.466
37	W49	1.346 $^{e-i}$±0.271	0.816^{m} ±0.483	-	-
38	W51	1.348 $^{e-i}$±0.233	1.358^{b-i} ±0.515	-	-
39	W58	1.834^{a-d}±0.062	1.229^{e-k} ±0.078	-	-
40	W60	1.314 $^{e-i}$±0.550	1.226^{e-g} ±0.054	-	-
41	W62	1.532 $^{c-h}$±0.092	1.548^{a-e} ±0.108	-	-
42	W75	1.253^{f-k}±0.335	1.083^{f-l} ±0.123	-	-
43	S01	1.075^{h-k}±0.135	1.633^{a-d} ±0.282	0.997±0.794	1.252 ±0.274
44	S06	1.501^{c-h}±0.445	1.498^{a-f} ±0.102	1.750±1.669	1.740±0.341
45	S28	1.522 $^{c-h}$±0.067	1.670^{a-c} ±0.182	0.714±0.647	1.060±0.127
46	S29	1.887^{a-c}±0.510	1.289^{c-k} ±0.133	1.450±1.937	1.145±0.210
47	S69	2.190^{a}±0.114	1.452^{b-h} ±0.163	0.940±1.477	0.999±0.600
48	S70	1.649^{c-f}±0.123	1.313^{c-k} ±0.079	-	-
49	S71	1.426^{d-i}±0.444	1.458^{b-h} ±0.438	-	-
Level of significance		**	**	ns	ns

Note: In the same column, numbers with identical letters are insignificantly according to Duncan's test. *different level of significance of 5%. MLC: Microaerobic light condition; ADC: Aerobic dark condition

Evaluating the capacity of PNSB in fixing N: There were 24 PNSB isolates selected for growing well under pH = 5.0 and NaCl 5‰. These isolates produced N content from 0.45 to 32.1 mg L^{-1} under MLC, and from 1.61 to 16.9 mg L^{-1} under ADC. The S06 isolate fixed the highest amount of N under MLC (32.1 mg L^{-1}), while the S01 isolate did best under ADC (16.9 mg L^{-1}) after 72 h of culture (Figure 1). In other words, the two isolates S01 and S06 were selected for fixing the greatest N content under MLC and ADC from 57 isolates derived from soil and water of saline paddy fields. PNSB is used as a nutrient-providing source for rice via N fixation processes [14], [15].

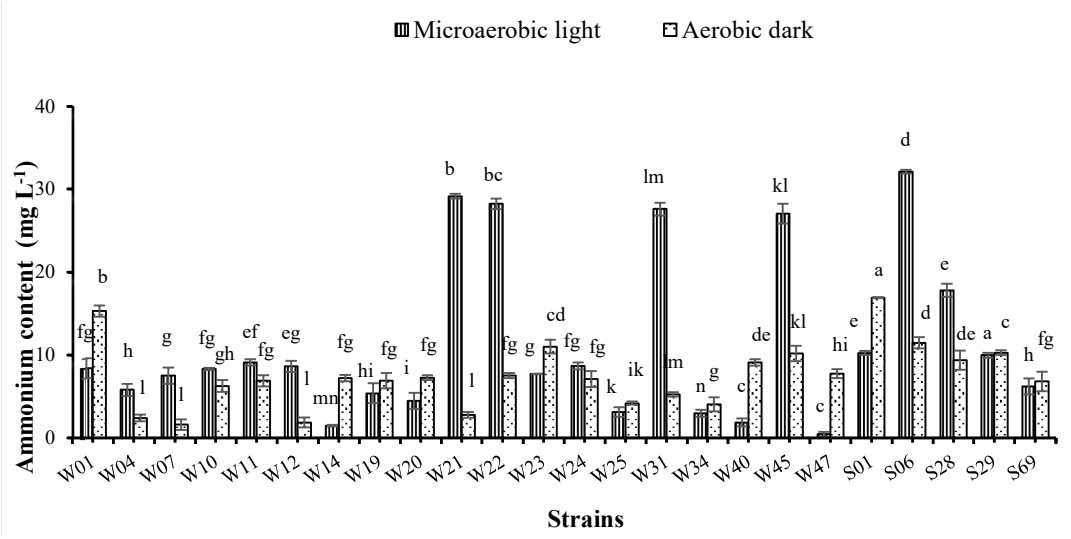

Figure 1. Production of NH_4^+ Based on N_2 Fixation by Selected Purple Nonsulfur Bacteria under Saline-Acidic Stress in Microaerobic Light and Aerobic Dark Conditions

Genera, *Rhodopseudomonas,* and *Rhodobacter*, belong to N-fixing PNSB under microaerobic or aerobic conditions [13], [41]. In particular, *R. palustris* TLS12, VNS19, VNS32, VNS62, and VNW95 strains have increased content of available NH_4^+ in acid sulfate soil [21]. Subsequently, the two N-fixing selected isolates, S01 and S06, were identified by the 16S rDNA technique, with 100% similarity, as *Rhodobacter sphaeroides* based on GenBank database. Their accession numbers were OR055984 and OR055985, respectively (Figure 2).

Figure 2. Neighbor-Joining Phylogenetic Trees Based on 16S rDNA Sequences of Two Selected PNSB Strains Compared to the Closely Related Strains in the GenBank Database. The Percentage Levels of Bootstrap Analysis of 1000 Replicated are Indicated at Each Node. Bar, 0.1 Substitutions per Nucleotide Position. Access Number of GenBank Sequences is Implied in Brackets.

3.3. Evaluating the Capacity of N-Fixing PNSB in Providing P, and Plant Growth Promoting Substances

In addition, PNSB can be considered as a type of plant growth promoting substances-providing PNSB [15], [21], [42]. Previous studies have stated that PNSB is present in a wide range of conditions, even in saline ones [16], [43]. Particularly, the four *Luteovulum sphaeroides* W01, W14, W22 and W47 isolates provide plant growth promoting substances, and originated from rice-shrimp paddy fields, this can be referred that on salt-contaminated soil, PNSB are used as biofertilizer to reduce Na^+, and to promote growth and yield of rice [17]. In addition, *L. sphaeroides* W22 and W47 provide δ-aminolevulinic acid, which contributes to increasing rice grain yield by 27.2%, and decreasing 31.3% proline content compared with the control, when rice is being irrigated four times at 5‰ concentration on salt-contaminated soil [44]. In the current study, the two N-fixing *L. sphaeroides* S01 and S06 isolates were able to provide IAA, ALA, EPS and siderophores (Table 3).

Tabel 3. Capacity of PNSB in Solubilizing P and Providing Plant Growth Promoting Substances under Microaerobic Light and Aerobic Dark Conditions

Parameter (mg L^{-1})	Condition	Isolate		Level of significance
		S01	S06	
Soluble P from Al-P	MLC	0.382±1.36	0.870±1.44	ns
	ADC	2.954±0.49	2.756±1.04	ns
Soluble P from Fe-P	MLC	4.279±0.38	3.811±0.97	ns
	ADC	3.953±0.06	4.004±0.24	ns
Soluble P from Ca-P	MLC	3.954±0.67	3.867±0.69	ns
	ADC	4.735±0.23	4.303±0.40	ns
IAA	MLC	15.5±1.33	14.7±1.22	ns
	ADC	12.3±0.29	14.1±0.95	ns
EPS	MLC	1.09^{b}±0.06	1.44^{a}±0.03	**
	ADC	1.31^{b}±0.07	1.58^{a}±0.05	**
Siderophores	MLC	10.7^{b}±1.11	18.0^{a}±2.16	**
	ADC	53.6^{a}±3.26	48.3^{b}±4.27	**
ALA	MLC	1.68^{b}±0.05	2.82^{a}±0.11	**
	ADC	2.42^{b}±0.11	2.81^{a}±0.07	**

Note: In the same column, numbers with identical letter are different insignificantly according to Duncan's test. **: different at level of significance of 1%. MLC: Microaerobic light condition; ADC: Aerobic dark condition

The two PNSB isolates were able to produce plant growth promoting substances, i.e., IAA, EPS, siderophores and ALA under the two conditions. IAA production of the *R. sphaeroides* S01 and S06 was statistically identical, and fluctuated from 14.7 mg L^{-1} to 15.5 mg L^{-1} under MLC, and from 12.3 mg L^{-1} to 14.1 mg L^{-1} under ADC. However, the S06 isolate performed a greater EPS production (1.44 mg L^{-1}; 1.58 mg L^{-1}) and ALA production (2.82 mg L^{-1}; 2.81 mg L^{-1}) than the other isolate (1.09 mg L^{-1}; 1.31 mg L^{-1}) and (1.68 mg L^{-1}; 2.42 mg L^{-1}) under the two conditions. For siderophores, the S06 isolate (18.0%) had a greater value than the S01 isolate (10.7%) under

MLC, but under ADC, the S01 isolate produced a greater siderophores content (53.6%) than the other one (48.3%) under ADC (Table 3). Therefore, the two *L. sphaeroides* S01 and S06 isolates are potent in providing N as well as some plant growth promoting substances under saline soil condition. Previous studies have proved that PNSB release ALA, EPS, IAA and siderophores to regulate growth under stress conditions, such as saline, acidic, and heavy metals-contaminated soils [15], [17], [21], [22], [44]. Bacteria can live in salt-contaminated acid sulfate soil because of their ability to produce EPS possessing functional groups, -OH and -COOH, binding with Na^+ in saline soils [16]. EPS can contain galacturonic acid (1.09-1.58 mg L^{-1}) which is highly potent in binding with Na^+ [45]. Moreover, the greater the salinity is (up to 6%), the more EPS PNSB produce [46]. Consequently, EPS producing PNSB reduce salinity of the environment. Furthermore, PNSB are also capable of solubilizing insoluble P compounds, Al-P, Fe-P and Ca-P [14]. In the current study, the P solubilizing capacity of the two PNSB isolates, S01 and S06, was identical (Table 3). Acid sulfate and saline tolerant PNSB strains can promote growth and yield of rice [16], [17]. Last but not least, the two PNSB isolates can also solubilize insoluble P compounds, such as Al-P, Fe-P and Ca-P. After 120 h of culture in BIM containing insoluble P compounds under the two incubating conditions, two PNSB isolates, *R. sphaeroides* S01 and S06, were able to solubilize P under MLC and ADC. Under MLC, solubilized P content from Al-P ranged from 0.382 to 0.870 mg L^{-1}, that from Fe-P was from 3.811 to 4.279 mg L^{-1}, and from 3.867 to 3.954 mg L^{-1} from Ca-P. Under ADC, the solubilized P content was 2.756-2.954 mg L^{-1}, 3.953-4.004 mg L^{-1}, 4.303-4.735 mg L^{-1}, in the same order (Table 3). This is in accordance with the study by Khuong *et al.* [16]. Ultimately, the two *L. sphaeroides* S01 and S06 isolates are promising in use as a biofertilizer.

4. Conclusion and Recommendation

Fifty-seven PNSB isolates were found in rice-shrimp systems in Thanh Phu, Ben Tre, and lived well under MLC and ADC. Among them, 49 isolates can survive under pH 5.0 condition, and 24 isolates grew well under 5‰ saline condition. Two isolates, S01 and S06, were selected for fixing N best under MLC (32.1 mg L^{-1}) and ADC (16.9 mg L^{-1}) at pH 5.0 and salinity 5‰. The two selected isolates were also able to provide P at 0.382-2.954 mg L^{-1} from Al-P, at 3.811-4.279 mg L^{-1} from Fe-P, and at 3.867-4.735 mg L^{-1} from Ca-P, and to produce IAA (12.3-15.5 mg L^{-1}), EPS (1.09-1.58 mg L^{-1}), siderophores (10.7-53.6%) and ALA (1.68-2.82 mg L^{-1}). The S01 and S06 isolates were identified according to 16S rRNA as *Rhodobacter sphaeroides*, with 100% similarity. The newly isolated PNSB isolates should be further tested under greenhouse and field conditions for their plant growth promoting and soil remediating capacities.

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REFERENCES

[1] N. Van Tho, "Salinity intrusion in the Vietnamese Mekong delta, a threat: possible causes, effects on people's life and production, and temporary solutions and adaptable strategies," in *Sustain. Dev. Water Environ. Proc. ICSDWE2022*. Cham, Springer International Publishing, 2022, pp. 1-10, doi: 10.1007/978-3-031-07500-1_1.

[2] M. El-Sayed and W. M. Salem, "Hydrochemical assessments of surface Nile water and ground water in an industry area–South West Cairo," *Egypt. J. Pet.*, vol. 24, no. 3, pp. 277-288, 2015, doi: 10.1016/j.ejpe.2015.07.014.

[3] J. Kruse, M. Koch, C. M. Khoi, G. Braun, Z. Sebesvari, and W. Amelung, "Land use change from permanent rice to alternating rice-shrimp or permanent shrimp in the coastal Mekong Delta, Vietnam: Changes in the nutrient status and binding forms," *Sci. Total Environ.,* vol. 703, pp. 134758, 2020, doi: 10.1016/j.scitotenv.2019.134758.

[4] L. T. H. Phan and A. Kamoshita, "Salinity intrusion reduces grain yield in coastal paddy fields: case study in two estuaries in the Red River Delta, Vietnam," *Paddy Water Environ.,* vol. 18, pp. 399-416, 2020, doi: 10.1007/s10333-020-00790-y.

[5] Otlewska *et al.*, "When salt meddles between plant, soil, and microorganisms," *Front. Plant Sci.,* vol. 11, pp. 1429, 2020, doi: 10.3389/fpls.2020.553087.

[6] W. Taratima, T. Chomarsa, and P. Maneerattanarungroj, "Salinity stress response of rice (*Oryza sativa* L. cv. Luem Pua) calli and seedlings," *Scientifica*, vol. 2022, Art. no. 5616683, 2022, doi: 10.1155/2022/5616683.

[7] Zheng *et al.*, "Effect of salinity stress on rice yield and grain quality: A meta-analysis," *Eur. J. Agron.*, vol. 144, Art. no. 126765, 2023, doi: 10.1016/j.eja.2023.126765.

[8] F. Gonçalo Filho *et al.*, "Reclaiming tropical saline-sodic soils with gypsum and cow manure," *Water*, vol. 12, no. 1, pp. 57, 2019, doi: 10.3390/w12010057.

[9] P. Gangwar, R. Singh, M. Trivedi, and R. K. Tiwari, "Sodic soil: Management and reclamation strategies." in *Environmental Concerns and Sustainable Development: Volume 2: Biodiversity, Soil and Waste Management*, V. Shukla, N. Kumar, Ed., Singapore, Springer, 2020, pp. 175-190, doi: 10.1007/978-981-13-6358-0_8.

[10] Kumar, S. Singh, A. Mukherjee, R. P. Rastogi, and J. P. Verma, "Salt-tolerant plant growth-promoting *Bacillus pumilus* strain JPVS11 to enhance plant growth attributes of rice and improve soil health under salinity stress," *Microbiol. Res.,* vol. 242, Art. no. 126616, 2021, doi: 10.1016/j.micres.2020.126616.

[11] P. Sheoran *et al.*, "Ameliorants and salt tolerant varieties improve rice-wheat production in soils undergoing sodification with alkali water irrigation in Indo–Gangetic Plains of India," *Agric. Water Manag.*, vol. 243, Art. no. 106492, 2021, doi: 10.1016/j.agwat.2020.106492.

[12] M. Cerruti, "Harnessing the metabolic versatility of purple non-sulfur bacteria," PhD dissertation, Delft Univ. Technol., The Netherlands, 2022, doi: 10.4233/uuid:c98876c1-aa83-4c8d-9816-191daf5cc423.

[13] E. Touloupakis, A. Chatziathanasiou, D. F. Ghanotakis, P. Carlozzi, and I. Pecorini, "Poly (3-hydroxybutyrate) production by *Rhodopseudomonas* sp. S16-VOGS3 cells grown in

digested sludge," *Environ. Technol. Innov.*, vol. 30, Art. no. 103058, 2023. doi: 10.1016/j.eti.2023.103058.

[14] N.Q. Khuong, D. Kantachote, J. Onthong, L. N. T. Xuan, and A. Sukhoom, "Enhancement of rice growth and yield in actual acid sulfate soils by potent acid-resistant *Rhodopseudomonas palustris* strains for producing safe rice," *Plant Soil,* vol. 429, pp. 403-501, 2018, doi: 10.1007/s11104-018-3705-7.

[15] N. Q. Khuong, D. Kantachote, P. Nookongbut, J. Onthong, L. N. T. Xuan, and A. Sukhoom, "Mechanisms of acid-resistant *Rhodopseudomonas palustris* strains to ameliorate acidic stress and promote plant growth," *Biocatal. Agric. Biotechnol.*, vol. 24, Art. no. 101520, 2020, doi: 10.1016/j.bcab.2020.101520.

[16] N.Q. Khuong *et al.*, "Two strains of *Luteovulum sphaeroides* (purple nonsulfur bacteria) promote rice cultivation in saline soils by increasing available phosphorus," *Rhizosphere*, vol. 20, Art. no. 100456, 2021, doi: 10.1016/j.rhisph.2021.100456.

[17] N. Q. Khuong *et al.*, "Potential of potent purple nonsulfur bacteria isolated from rice-shrimp systems to ameliorate rice (*Oryza sativa* L.) growth and yield in saline acid sulfate soil," *J. Plant Nutr.*, vol. 46, no. 3, pp. 473-494, 2023, doi: 10.1080/01904167.2022.2087089.

[18] J. Sakpirom, D. Kantachote, T. Nunkaew, and E. Khan, "Characterizations of purple non-sulfur bacteria isolated from paddy fields, and identification of strains with potential for plant growth-promotion, greenhouse gas mitigation and heavy metal bioremediation," *Res. Microbiol.*, vol. 168, no. 3, pp. 266-275, 2017, doi: 10.1016/j.resmic.2016.12.001.

[19] P. Nookongbut, D. Kantachote, M. Megharaj, and R. Naidu, "Reduction in arsenic toxicity and uptake in rice (*Oryza sativa* L.) by As-resistant purple nonsulfur bacteria," *Environ. Sci. Pollut. Res.*, vol. 25, pp. 36530-36544, 2018, doi: 10.1007/s11356-018-3568-8.

[20] N. Q. Khuong *et al.*, "Potential of Mn^{2+}-resistant purple nonsulfur bacteria isolated from acid sulfate soils to act as bioremediators and plant growth promoters via mechanisms of resistance," *J. Soil Sci. Plant Nutr.*, vol. 20, pp. 2364-2378, 2020, doi: 10.1007/s42729-020-00303-0.

[21] J. Zeng *et al.*, "Composition and aggregation of extracellular polymeric substances (EPS) in hyperhaline and municipal wastewater treatment plants," *Sci. Reports*, vol. 6, no. 1, Art. ID. 26721, 2016, doi: 10.1038/srep26721.

[22] N. Q. Khuong *et al.*, "Improvement of nutrient uptake, yield of black sesame (*Sesamum indicum* L.), and alluvial soil fertility in dyke by spent rice straw from mushroom cultivation as biofertilizer containing potent strains of *Rhodopseudomonas palustris*," *Sci. World J.,* vol. 2023, Art. no. 1954632, 2023, doi: 10.1155/2023/1954632

[23] L. Sparks, A. L. Page, P. A. Helmke, and R. H. Loeppert, Ed., *Methods of soil analysis, part 3: Chemical methods*, vol. 14, John Wiley & Sons, 2020.

[24] J. W. Brown, *Enrichment and isolation of purple non-sulfur bacteria*, Raleigh: Department of Biological Sciences, College of Sciences, North Carolina State University, NC, USA, 2017.

[25] R. S. Nelson, S. A. Ryan, and J. E. Harper, "Soybean mutants lacking constitutive nitrate reductase activity: I. Selection and initial plant characterization," *Plant Physiol.,* vol. 72, no. 2, pp. 503-509, 1983, doi: 10.1104/pp.72.2.503.

[26] J. A. M. E. S. Murphy and J. P. Riley, "A modified single solution method for the determination of phosphate in natural waters," *Anal. Chim. Acta*, vol. 27, pp. 31-36, 1962, doi: 10.1016/S0003-2670(00)88444-5.

[27] Glickmann and Y. Dessaux, "A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria," *Appl. Environ. Microbiol.*, vol. 61, no. 2, pp. 793-796, 1995, doi: 10.1128/aem.61.2.793-796.1995.

[28] B. F. Burnham, "[14] δ-Aminolevulinic acid synthase (*Rhodopseudomonas spheroides*)," in *Methods in enzymology*, H. Tabor and C. W. Tabor, Ed., The US, Academic Press, 1970, vol. 17, pp. 195-200, doi: 10.1016/0076-6879(71)17179-0.

[29] B. Schwyn and J. B. Neilands, "Universal chemical assay for the detection and determination of siderophores," *Anal. Biochem.*, vol. 160, no. 1, pp. 47-56, pp. 1987, doi: 10.1016/0003-2697(87)90612-9.

[30] K. E. Eboigbodin and C. A. Biggs, "Characterization of the extracellular polymeric substances produced by *Escherichia coli* using infrared spectroscopic, proteomic, and aggregation studies," *Biomacromolecules*, vol. 9, no. 2, pp. 686-695, 2008, doi: 10.1021/bm701043c.

[31] Y. Suzuki, S. D. Kelly, K. M. Kemner, and J. F. Banfield, "Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment," *Appl. Environ. Microbiol.*, vol. 69, no. 3, pp. 1337-1346, 2003, doi: 10.1128/AEM.69.3.1337-1346.2003.

[32] T. A. Hall, "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT," *Nucleic Acids Symp. Ser.*, vol 41, no. 41, pp. 95-98, 1999.

[33] J. K. Guo *et al.*, "Effects of EDTA and plant growth-promoting rhizobacteria on plant growth and heavy metal uptake of hyperaccumulator *Sedum alfredii* Hance," *J. Environ. Sci.*, vol. 88, pp. 361-369, 2020, doi: 10.1016/j.jes.2019.10.001.

[34] X. Cui *et al.*, "A review on the thermal treatment of heavy metal hyperaccumulator: Fates of heavy metals and generation of products," *J. Hazard. Mater.*, vol. 405, Art. no. 123832, 2021, doi: 10.1016/j.jhazmat.2020.123832.

[35] S. Elnahal *et al.*, "The use of microbial inoculants for biological control, plant growth promotion, and sustainable agriculture: A review," *Eur. J. Plant Pathol*, vol. 162, no. 4, pp. 759-792, 2022, doi: 10.1007/s10658-021-02393-7.

[36] J. F. Imhoff JF, "The phototrophic alpha-proteobacteria," in *The Prokaryotes*, M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt, Ed., New York, NY, Springer, 2006, pp. 41-64, doi: 10.1007/0-387-30745-1_2.

[37] T. Nunkaew, D. Kantachote, T. Nitoda, and H. Kanzaki, "The use of rice straw broth as an appropriate medium to isolate purple nonsulfur bacteria from paddy fields," *Electron. J. Biotechnol.*, vol. 15, no. 6, pp. 7-7, 2012, doi: 10.2225/vol15-issue6-fulltext-8.

[38] J. F. Imhoff JF and H. G. Truper, *Bergey's manual of determinative bacteriology*, Michigan, Michigan State University, 2005.

[39] M. T. Madigan, "Microbiology of nitrogen fixation by anoxygenic photosynthetic bacteria," in *Anoxygenic photosynthetic bacteria*, R. E. Blankenship, M. T. Madigan, C. E. Bauer, Ed., Dordrecht, Springer, 1995, pp. 915-928, doi: 10.1007/0-306-47954-0_42.

[40] M. George, A. S. Vincent, and H. R. Mackey, "An overview of anoxygenic phototrophic bacteria and their applications in environmental biotechnology for sustainable resource recovery," *Biotechnol. Rep.*, vol. 28, Art. no. e00563, 2020, doi: 10.1016/j.btre.2020.e00563.

[41] W. Larimer *et al.*, "Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*," *Nat. Biotechnol.*, vol. 22, no. 1, pp. 55-61, 2004, doi: 10.1038/nbt923.

[42] K. E. Luxem, A. M. L. Kraepiel, L. Zhang, J. R. Waldbauer, and X. Zhang, "Carbon substrate re-orders relative growth of a bacterium using Mo-, V-, or Fe-nitrogenase for nitrogen fixation," *Environ. Microbiol.*, vol. 22, no. 4, pp. 1397-1408, 2020, doi: 10.1111/1462-2920.14955.

[43] Hiraishi, "Distribution of phototrophic purple nonsulfur bacteria in massive blooms in coastal and wastewater ditch environments," *Microorganisms*, vol. 8, no. 2, pp. 150, 2020, doi: 10.3390/microorganisms8020150.

[44] N. Q. Khuong, D. P. T. Minh, L. T. M. Thu, and L. V. Thuc, "The potential of bacterial strains of *Luteovulum sphaeroides* W22 and W47 for producing δ-aminolevulinic acid to

improve soil quality, growth and yield of saline-irrigated rice cultivated in salt-contaminated soil," *Agronomy*, vol. 13, no. 5, pp. 1409, 2023, doi: 10.3390/agronomy13051409.

[45] T. Nunkaew, D. Kantachote, T. Nitoda, H. Kanzaki, and R. J. Ritchie, "Characterization of exopolymeric substances from selected *Rhodopseudomonas palustris* strains and their ability to adsorb sodium ions," *Carbohydr. Polym.*, vol. 115, pp. 334-341, 2015, doi: 10.1016/j.carbpol.2014.08.099.

[46] Z. Wang *et al.*, "Effect of salinity on extracellular polymeric substances of activated sludge from an anoxic–aerobic sequencing batch reactor," *Chemosphere*, vol. 93, no. 11, pp.2789-2795, 2013, doi: 10.1016/j.chemosphere.2013.09.038.