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Characterization of exopolymeric substances from selected *Rhodopseudomonas palustris* strains and their ability to adsorb sodium ions

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ABSTRACT

Removal of Na^+ by binding with exopolymeric substances (EPS) from *Rhodopseudomonas palustris* TN114 and PP803 was investigated. The moderate negative correlation pairs (r_p) between remaining Alcian blue and amount of Na^+ adsorbed on EPS from strains TN114 and PP803 were −0.652 and −0.609. Both strains showed positive relationships between the amounts of EPS produced and bacterial growth. EPS from strain PP803 had a higher efficiency in removing Na^+ than the EPS from strain TN114 based on their EC_{50} values (1.79 and 1.49 mg/mL for TN114 and PP803, respectively). The principal component from EPS of strain PP803 which was responsible for salt removal was purified and it was identified as a polysaccharide (≈18 kDa) mainly composed of galacturonic acid. Overall results suggested that EPS is a key factor that our strains used to bind Na^+ allowing their survival in high NaCl concentrations.

1. Introduction

Approximately 20% of agricultural land is affected by salt, and accounts for more than 6% of the world's total land area (FAO, 2007). NaCl is the predominant chemical causing salinization (Munns & Tester, 2008). The tolerance of plants to salts depends mainly on the capability of roots to control the uptake of Na^+ and Cl^- and to limit the negative effects of Na^+ and Cl^- on the uptake of some essential elements, such as K^+ and NO_3^- (Bazihizina, Barrett-Lennard, & Colmer, 2012). Plant growth promoting rhizobacteria (PGPR) or bacteria (PGPB) have been widely used as inoculants for the enhancement of plant growth under various conditions including various conditions of stress (Yang, Kloepper, & Ryu, 2009). Recently, some of those bacteria have been extensively studied for their ability to facilitate the phytoremediation of soils contaminated with heavy metals (Ma, Prasad, Rajkumar, & Freitas, 2011). One of the most important characteristics of either PGPR or PGPB is their ability to secrete extracellular polymeric substances (EPS) that form biofilms or facilitate adhesion to the surfaces of plant roots.

EPS are a complex mixture of high molecular weight polymers ($M_W \geq 10{,}000$) secreted by microbes into the environment in response to some physiological stresses such as heavy metals and osmotic salts such as Na^+ (Qurashi & Sabri, 2011; Panwichian, Kantachote, Wittayaweerasak, & Mallavarapu, 2011; Upadhyay, Singh, & Singh, 2011). In addition to carbohydrates and proteins; some EPS, from various matrixes of activated sludge, contain lipids, nucleic acids, uronic acids and some inorganic components (Sheng, Yu, & Li, 2010). The ability of the microbes to bind cations is normally associated with the carboxyl, sulfhydryl, hydroxyl and phosphoryl groups of the EPS to bind ions (Watanabe, Kawahara, Sasaki, & Noparatnaraporn, 2003). Increasing the population of EPS-producing bacteria in root zones should mitigate salt stress for plants growing in saline environments. Amongst the bacteria that secrete EPS into the environment, purple nonsulfur bacteria (PNSB) have attracted considerable attention as effective biofertilizers

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because they can both fix N_2 and secrete plant promoting growth substances such as 5-aminolevulinic acid (ALA) and plant hormones (Lee, Koh, & Song, 2008; Kantha, Chaiyasut, Kantachote, Sukrong, & Muangprom, 2010; Nunkaew, Kantachote, Nitoda, Kanzaki, & Ritchie, 2014).

Our previous studies have demonstrated that ALA in the culture media of selected *Rhodopseudomonas palustris* strains have the ability to reduce salt stress in rice paddies (Kantha et al., 2010; Nunkaew et al., 2014). The ability of vascular plants, particularly cereals, to survive and flourish in saline environments under the influence of osmotic stress has received considerable attention. A role for bacteria that produce EPS that will bind Na^+ and protect plants from salt stress conditions together with improving the quality of biofertilizers has not yet been established. Hence, before field trials of whether our selected strains of *R. palustris* biofertilizer would improve the salinity tolerance of rice in saline paddy fields a better understanding of the likely mechanism would be desirable. The aims of the present study were to quantify binding of Na^+ with EPS and identify the components of EPS that facilitated Na^+ tolerance.

2. Materials and methods

2.1. PNSB strains used and culture preparation

In this study the *R. palustris* strains (TN114 and PP803) isolated from saline paddy fields in Phatthalung and Nakhon Si Thammarat provinces, Thailand, respectively were previously selected on the basis of their ability to produce ALA under microaerobic light conditions (Nunkaew et al., 2014). Paddy field soils have large amounts of acetate as a major available carbon source from decomposition of organic matter under anaerobic conditions (Nunkaew, Kantachote, Nitoda, & Kanzaki, 2012), and so a glutamate-acetate (GA) broth medium was used to grow the PNSB strains intended to be used as biofertilizers. One loopful of each pure culture from a stab culture was inoculated into a screw cap test tube (150 × 15 mm: 20 mL) containing 18 mL GA medium, leaving a small space on top of the medium to achieve microaerobic conditions. The culture was incubated with an incandescent tungsten light source (3500 lx) for 48 h and bacterial growth was monitored turbidometrically at 660 nm, with a spectrophotometer. For use as an inoculum the culture broth was adjusted to an optical density (OD_{660}) of 0.5 by diluting with GA broth.

2.2. Effect of NaCl on the amount and composition of EPS produced by PNSB and their ability to bind Na^+

The influence of NaCl on EPS production by the selected PNSB strains (TN114 and PP803) were investigated by inoculating a 10% (v/v) inoculum into the GA broth containing various concentrations of NaCl (0.00, 0.25, 0.50, 1.00, 2.00, 4.00, 6.00, 7.00, 8.00, 9.00, 10.00, 11.00 and 12.00%) for providing a final cell density of approximately 1.2×10^7 cells/mL and incubating under microaerobic light conditions as previously described for 48 h. The Alcian blue adsorption assay following the method of Vandevivere and Kirchman (1993) was used to detect the EPS. Briefly, 8 μL of 1% (w/v) Alcian blue 8GX (Sigma Chemical Co., St. Louis, MO) was added to 2 mL of culture supernatants collected by centrifugation from the PNSB culture broths with different NaCl concentrations. The mixture was shaken at 80 rpm for 15 min and finally was centrifuged at 2340 × *g* for 10 min to separate the sediment color that had been adsorbed by the EPS. The supernatant containing Alcian blue was measured by a spectrophotometer at a wavelength of $OD_{606\,nm}$ as the absorbance values decreased after EPS adsorbed the Alcian blue. By comparing with the absorbance of a control without inoculation (GA broth), the absorbance values show a negative correlation with the amount of EPS that was secreted by PNSB in different salt concentrations. An inductively coupled plasma-optical emission spectrometer (ICP-OES) was used to analyze the amount of Na^+ in the GA broth containing 0–12% NaCl both before and after 48 h of PNSB growth. The protocol used for the ICP-OES followed the instruction manual for the instrument. All results were calculated as disappearance of Na^+ or Na^+ adsorbed on EPS after PNSB growth as follows:

$$Na^+ \text{ adsorbed on EPS} = \frac{(\text{mg/L } Na^+)\text{before} - (\text{mg/L } Na^+)\text{after}}{(\text{mg/L } Na^+)\text{before}}$$

To determine the amount of EPS and also to analyze the composition of the EPS each PNSB strain was grown by adding 50 mL of inoculum into 450 mL GA broth in bottles with various concentrations of NaCl (0.00, 0.25, 0.50, 1.00 and 2.00%) to obtain a final cell density of roughly 1.2×10^7 cells/mL and incubating under microaerobic light conditions (3500 lx) at 30 °C for 48 h. The culture broths were centrifuged at 10,408 × *g* for 15 min and cell pellets were dried at 105 °C until a constant weight was obtained. The EPS in the culture supernatants was precipitated by adding two volumes cold ethanol and the solution was chilled at 4 °C overnight (Panwichian et al., 2011). The resulting EPS precipitate was recovered by centrifugation at 23,500 × *g* for 30 min. Each EPS precipitate was washed with ethanol–water mixtures by increasing concentrations of ethanol from 70 to 100%. To remove excess salts, the EPS was redissolved in distilled water and dialyzed using a molecular weight cut-off of 12,000 Da (Sigma-Aldrich, D0530-100FT) against distilled water for 2 days at room temperature. Excess water was removed under vacuum before lyophilization. The dry weight of the EPS was determined and total carbohydrate was measured by a phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956); total protein was determined by the Bicinchoninic assay (BCA) using BSA standards (Smith et al., 1985). The nucleic acid content was determined according to the method of Sheng, Yu, and Yue (2005) using a UV spectrophotometer (PowerWaveX, Biotek, US). The EPS Yield (*Yp*/*b*), i.e. the amount of EPS produced in mg/mg of bacterial cells, was calculated based on the weight of the dried EPS (*p*) and the dried cell weight (*b*).

To determine the half maximal effective concentration (EC_{50}), for the biosorption of Na^+ the following concentrations of EPS; 0.0, 0.75 1.5, 3.0, 6.0 mg/mL were added to a saline solution containing 20 mg/mL NaCl at a ratio of 1:1. This produced a final concentration of NaCl of 1% NaCl (10 mg/mL) and the EPS from 0 to 3.0 mg/mL. This NaCl concentration was selected because it was the concentration at which both PNSB strains produced the maximal amount of EPS. The maximum of EPS at 3.0 mg/mL was tested because amounts of EPS higher than this concentration cannot be completely dissolved in water. We did not use another organic solvent for increasing the solubility of EPS because PNSB will be applied on paddy fields with live rice plants already planted. Before adding EPS, the initial Na^+ concentration was measured using ICP-OES. The biosorption test condition was set at a pH of 7, 35 °C for 30 min (Panwichian et al., 2011). After biosorption, the EPS-Na^+ complex in each sample was separated by adding one volume of cold ethanol at 4 °C overnight and then centrifuged at 23,500 × *g* for 30 min and the Na^+ left in the supernatant was determined by the ICP-OES. The EC_{50} was used to evaluate the Na^+ biosorption ability of the EPS from each strain. The removal percentages of Na^+ at various concentrations of EPS were investigated and the amount of EPS at the half of the maximum removal percentage was the EC_{50} that was determined by Probit-analysis (MLS method) and this experiment was conducted in triplicate.

2.3. EPS characterization

To purify the EPS, each 250 mg of EPS was dissolved in 200 mL Tris–HCl buffer, pH 8 and applied to a column (1.6 × 10 cm) of DEAE-Sephacel (Amersham Pharmacia Biotech, Uppsala, Sweden), that was equilibrated with 20 mL of the same buffer and eluted sequentially with 0, 0.1, 0.2 and 0.3 M KCl dissolved in Tris–HCl buffer, pH 8 at a flow rate of 0.75 min/mL. A 100 mL or a five times volume of the column for each concentration of KCl from 0.1, 0.2, 0.3, and1.0 M was used to elute the column by step eluting. Each fraction (5 mL) from the column was collected for determination of the chemical components. Total sugar was analyzed by the phenol-sulfuric acid method (Dubois et al., 1956). The carbazole-sulfuric acid method using glucuronic acid as the standard solution (4–40 μg/mL) was used for analysis of the uronic acid content (Bitter & Muir, 1962) and total protein was determined by a spectrophotometric method using the absorbance at 280 nm (Jia, Yu, Lin, & Dai, 2007). The carbohydrate-containing fractions were combined, dialyzed and assayed for their Na^+ adsorption activity. The active fraction eluted with 0.3 M KCl was purified by repeated gel filtration on a Sephadex G-100 (Sigma chemical Co., St. Louis, MO, USA) column to remove protein and eluting with 0.3 M KCl, at a flow rate of 1 mL/min. This purified fraction was designated the name FP3.

To characterize a purified polysaccharide after lyophilization, 50 μg was used in this experiment and after sample hydrolysis, 5 μg myo-inositol was added to use as an internal standard. Each 10 μg of the following authentic monosaccharides; D-glucose, D-galacturonic acid and D-glucuronic acid were used to compare with the FP3 from EPS component because these monosaccharides are the main components of the EPS produced by the bacterial family Bradyrhizobiaceae that includes *Bradyrhizobium* sp. and *Rhodopseudomonas* sp. (Quelas et al., 2010; Sheng et al., 2010). The purified polysaccharide was hydrolyzed in 2.0 M trifluoroacetic acid for 6 h at 115 °C. Diethyldithioacetal-TMS derivatives were prepared by following the method of Pitthard and Finch (2001) and were then analyzed by GC–MS (Shimadzu GC–MS QP2010 Plus together with InertCap 1 MS column; 0.25 μm-thick, 30 m). A 50 μg TMS derivatives of sugar was dissolved in 100 μL of hexane and 2 μL of hexane solution was analyzed using the GC–MS. The injector was set to 300 °C and chromatographic conditions were run from 165 °C up to 231 °C at a 2 °C/min. Helium with purity 99.999% was used as a carrier gas at an inlet pressure of 107 kPa. The operating MS conditions were electron ionization, scan over the range *m/z* 35–650, and a source temperature of 200 °C.

The purified FP3 was also subjected to ^1H NMR analysis. ^1H NMR spectra were recorded on a Varian NMR System 600 at 600 MHz in D_2O at room temperature (22 °C). Acetone in D_2O (δ 2.22) was used as an external reference. In order to confirm the presence or absence of signal(s) under the HDO signal around δ 4.8, ^1H NMR measurement was performed at a series of temperature (23, 32, 42, and 52 °C) in D_2O using acetone (δ 2.22) as an internal reference. The molecular mass of the fraction portion 3 (FP3) obtained when eluting with 0.3 M KCl was determined by gel filtration with high performance liquid chromatography (HPLC) using an Agilent 1100 system equipped with a TSK-gel $GMPW_{XL}$ column (7.8 × 300 mm, Tosoh Corporation, Tokyo, Japan). HPLC was performed at room temperature, at a flow rate of 0.3 mL/min, with detection by refractive index. The column was saturated with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer (pH 7.2), and elution was carried out with the same buffer. The column was calibrated with a series of pullulans (maltotriose polymer) (molecular weight of 4.04×10^5, 1.12×10^5, 2.28×10^4 and 5.9×10^3), that were used as molecular mass markers.

2.4. Data presentation and statistical analysis

All experiments in this study were carried out in triplicate. The mean values and standard deviations are presented. Statistical analysis was performed to investigate the EPS from the PNSB tested that affected the disappearance of Na^+ (mg/L). This was analyzed using linear correlation analysis. Pearson's product momentum correlation coefficient (r_p) was used for linear estimations of the strength and direction of the linear correlations between two parameters. The Pearson coefficient is always between −1 and +1, as $r_p = -1$ or +1 meaning a perfect correlation, 0 means the absence of a relationship (Jin, Wilen, & Lant, 2004). For Pearson coefficients (r_p) values <0.4 were taken to show a weak correlation, values 0.60–0.67 indicated a moderate correlation and values of 0.68–1.0 indicated a high correlation between the two parameters (Taylor, 1990). Correlations were considered statistically significant at the 95% confidence interval ($p < 0.05$). Statistical analysis, one way ANOVA was also used to analyze statistical differences at a *p*-value <0.05 and mean comparisons were performed by the Duncan's multiple range test.

3. Results

3.1. Effect of NaCl on the amount and composition of EPS produced by PNSB and their ability to bind Na^+

Based on the Alcian blue adsorption assay, the observed decrease in the amount of dye present in the centrifuged culture supernatants indicated that EPS was being secreted by the cells. Reduction of dye absorbance due to its binding to EPS was observed in culture supernatants containing various concentrations of NaCl with the biggest reduction occurring with 1% NaCl followed by 0.5, 0.25, 0.00, 2.00, 4.00 and 6.00% NaCl (data not shown). The results also showed that only surviving PNSB cells produced EPS as there was no decrease of dye absorbance when growth inhibitory NaCl concentrations of between 7% and 12% were tested. As GA broth itself containing Na^+ 1380 mg/L (467 mg from 3.8 g/L monosodium glutamate monohydrate and 913 mg from 5.4 g/L sodium acetate trihydrate), when the loss of Na^+ (adsorbed on EPS) from the GA broth was measured. The maximum loss was also observed in GA broth with 1% added NaCl (Na^+ 3932 mg/L), and thus total amount of Na^+ in the culture supernatant was 5312 mg/L. The amount of Na^+ adsorbed on EPS in GA broth with 1% added NaCl was 1964.16 mg/L by strain TN114 and 1996.15 mg/L by strain PP803, and the loss of Na^+ in solution was in the order of 1.0 > 0.5 > 0.25 > 0.0 > 2.0 > 4.0 > 6.0% with no loss when no cell growth occurred at NaCl concentrations from 7% to 12% added NaCl to GA broth (data not shown). There was a significant correlation between the remaining Alcian blue in supernatant (based on biosorption of Alcian blue by EPS) and the Na^+ adsorbed on EPS. As the Pearson correlation coefficient (r_p) was roughly −0.61 this indicates a moderate negative correlation between the two parameters (Fig. 1A and B). The negative correlation between the remaining Alcian blue in supernatant and the Na^+ adsorbed on EPS had $r_p = -0.652$ for TN114 and $r_p = -0.609$ for PP803 at $p < 0.05$.

Table 1 shows the amounts of EPS and biomass on the basis of dry weight including the EPS yield by the PNSB strains, TN114 and PP803 in GA broth with various concentrations of NaCl from 0 to 2%. The amount of added NaCl (%) into the GA broth significantly affected ($p < 0.05$) the amount of EPS produced by both PNSB strains in the order of 1 > 0 ~ 0.25 ~ 0.50 > 2.0. The maximum amount of EPS found at 1% NaCl was 1521.17 mg/L by strain TN114 and 1559.75 mg/L by strain PP803. However, no significant difference ($p < 0.05$) was found for the amount of NaCl at 0–1.00% on the growth of both PNSB strains but a significantly lower growth was

Table 1
Effect of NaCl concentrations in GA broth on biomass, amount of EPS and EPS yield of *R. palustris* TN114, PP803.

%NaCl (w/v)	Strain TN114			Strain PP803		
	EPS dry weight (mg/L)	Cell dry weight (mg/L)	Yield (*Yp*/*b*)	EPS dry weight (mg/L)	Cell dry weight (mg/L)	Yield (*Yp*/*b*)
0.00	1132.583 ± 6.257^{b}	1760.556 ± 80.005^{ab}	0.64 ± 0.02^{b}	1105.000 ± 9.165^{c}	1747.000 ± 58.796^{ab}	0.63 ± 0.00^{b}
0.25	1084.916 ± 10.830^{b}	1853.445 ± 20.870^{a}	0.58 ± 0.02^{b}	1200.000 ± 8.000^{b}	1873.445 ± 56.911^{a}	0.64 ± 0.02^{b}
0.50	1065.000 ± 16.703^{b}	1730.667 ± 55.548^{ab}	0.61 ± 0.01^{b}	1101.916 ± 11.501^{c}	1746.778 ± 66.541^{ab}	0.63 ± 0.01^{b}
1.00	1521.167 ± 15.237^{a}	1633.667 ± 51.189^{b}	0.93 ± 0.05^{a}	1559.750 ± 5.597^{a}	1710.000 ± 52.915^{b}	0.91 ± 0.03^{a}
2.00	348.334 ± 9.504^{c}	639.667 ± 50.003^{c}	0.54 ± 0.08^{b}	636.334 ± 5.500^{d}	743.333 ± 61.101^{c}	0.85 ± 0.05^{a}

Each value is the mean of three replicates and it is given with standard deviation. Different superscripts in the same column indicate significant differences ($p < 0.05$).

observed at 2.0% NaCl. Therefore, the maximum yield of both PNSB strains in GA broth was found at 1% NaCl with $Yp/b = 0.93$ for TN114 and $Yp/b = 0.91$ for PP803.

Fig. 2 presents the EPS content from strains TN114 and PP803 based on dry weight at varying concentrations of NaCl. The main component of the EPS produced by strains TN114 and PP803 in GA broth containing 0–2% NaCl was carbohydrate (>40% of the total EPS) followed by nucleic acid, proteins and unidentified compounds. There was no significant difference in the EPS content at the different NaCl concentrations from strain TN114. In contrast, there was a remarkable increase in the carbohydrates content of EPS from strain PP803 at 2.0% NaCl as this had significantly reduced amounts of nucleic acid and protein.

The ability of the EPS from PNSB strains TN114 and PP803 to remove Na^+ at 1% NaCl (w/v) by varying the concentrations of EPS from 0 to 3.00 mg/mL is shown in Table 2. Increasing amounts of both EPS from strains TN114 and PP803 significantly increased Na^+ removal and thus a concentration of 3 mg/mL EPS produced the best removal of Na^+ (72.38 ± 1.33% and 77.00 ± 0.37%, respectively). The EPS secreted by strain PP803 was more efficient in removing Na^+ from the solution than the EPS from strain TN114 ($p < 0.05$). This was also supported by the EC_{50} values for both EPS as EC_{50} of EPS from strain PP803 1.49 ± 0.01 mg/mL and from strain TN114 was 1.79 ± 0.02 mg/mL (Table 2). Hence, the results confirmed that the Na^+ removal by the EPS from strain PP803 was significantly higher than that from strain TN114. This was the main reason for selecting the EPS from the strain PP803 (EPS-PP803) for purification and for investigating its major components.

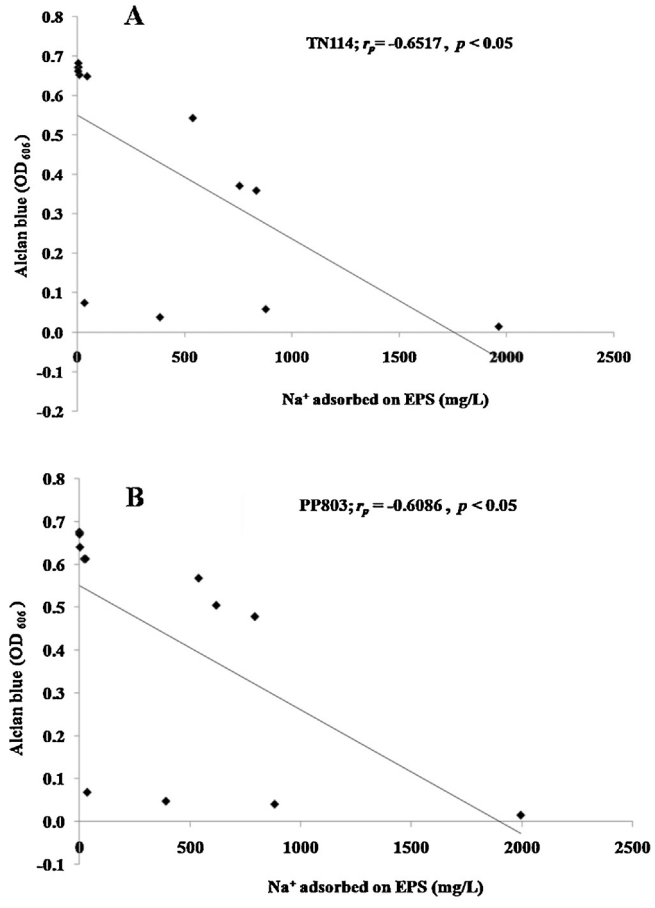

Fig. 1. Correlations between remaining alcain blue ($OD_{606\,nm}$) in the culture supernatant and Na^+ adsorbed by the EPS (mg/L) produced by *R. palustris* strains (A) TN114 and (B) PP803.

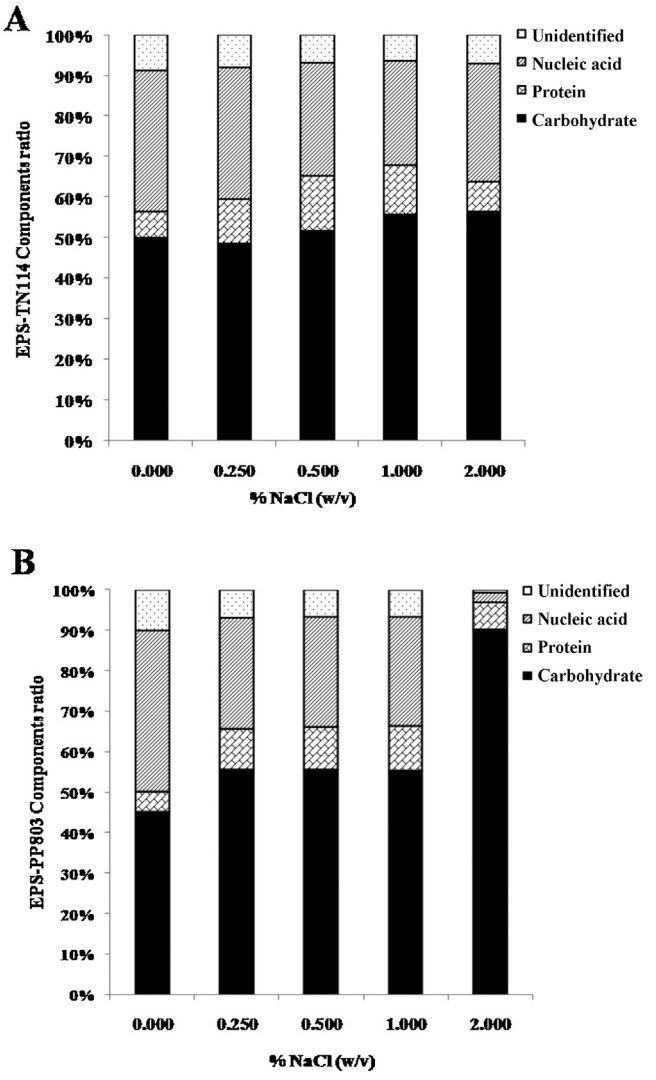

Fig. 2. Effect of NaCl concentrations in GA broth on the components of EPS produced by *R. palustris* strains, (A) TN114 and (B) PP803.

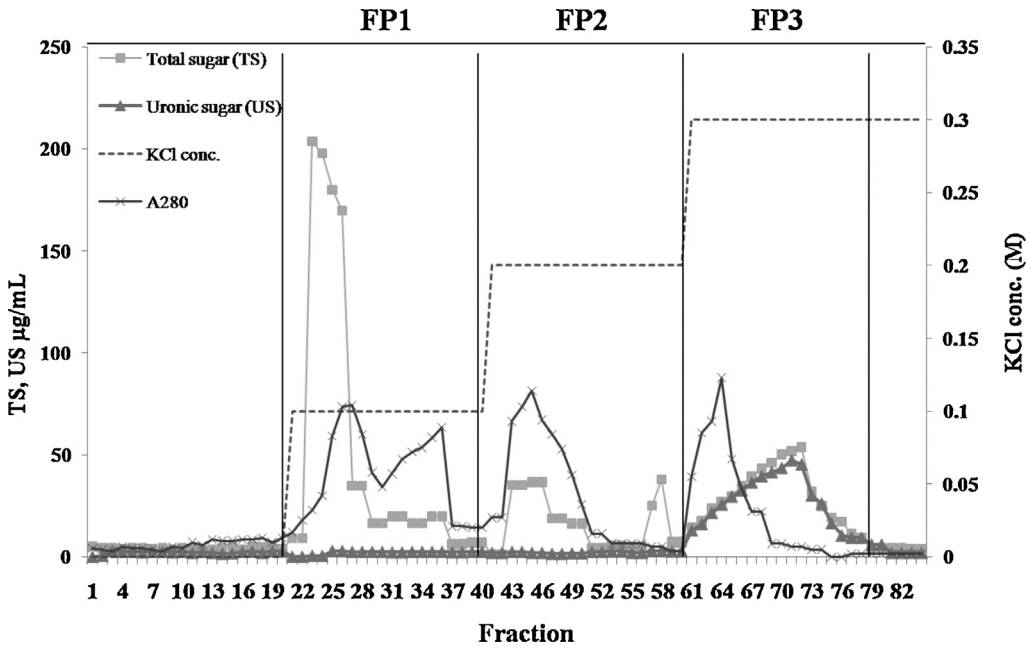

Fig. 3. Separation the FP3 from the EPS of *R. palustris* PP803 by DEAE-sephacel anion exchange chromatography.

Table 2
Percent of Na^+ removal with various EPS concentration and EC_{50} value of EPS produced by *R. palustris* TN114, PP803.

EPS (mg/mL)	% Na^+ removal	
	TN114	PP803
0.000	0.000 ± 0.000^{Ea}	0.000 ± 0.000^{Ea}
0.375	12.004 ± 0.611^{Db}	16.384 ± 1.021^{Da}
0.750	23.125 ± 0.305^{Cb}	32.944 ± 0.675^{Ca}
1.500	46.073 ± 0.917^{Bb}	54.363 ± 0.696^{Ba}
3.000	72.376 ± 1.332^{Ab}	76.995 ± 0.372^{Aa}
EC_{50} (mg/mL)	1.791 ± 0.093^{b}	1.493 ± 0.095^{a}

Each value is the mean of three replicates and it is given with standard deviation. Different capital letters of superscripts indicate significant differences in the same column ($p < 0.05$). Different lowercase of superscripts indicate significant differences in the same row ($p < 0.05$).

3.2. EPS characterization

EPS-PP803 was initially purified by DEAE-Sephacel anion exchange chromatography and eluted with 0.1–0.3 M KCl. Results showed that total sugar was separated into 4 peaks and 1 peak in the 0.3 M KCl fraction (FP3) was an uronic sugar (Fig. 3). Only the FP3 had ability to remove Na^+ (21.02%) as no Na^+ removal was measureable by the other fractions and the FP3 was roughly 14% of the total EPS by weight. Therefore, after lyophilization, FP3 was tested for Na^+ removal again and this fraction removed Na^+ with an EC_{50} 0.023 mg/mL (Table 3). Protein was also detected in the partially purified FP3 from the DEAE-anion exchange chromatograph. It was thus possible that the protein had a role in binding the Na^+. However, the efficiency of Na^+ binding only decreased slightly (EC_{50} 0.022 mg/mL) with consequent repeated gel filtration when the protein content decreased (Table 3). These results indicated that the protein component had little effect on the removal of Na^+, and FP3 was thus used for identification of the polysaccharide.

Table 3
Concentration of total sugar and EC_{50} in EPS produced by *R. palustris* PP803 during process of purification.

EPS purified step	Total sugar (mg/mL)	EC_{50} (mg/mL)
Crude extract	0.867 ± 0.002^{a}	1.493 ± 0.095^{a}
DEAE-anion exchange	0.554 ± 0.012^{b}	0.023 ± 0.001^{b}
Gel filtration	0.244 ± 0.009^{c}	0.022 ± 0.001^{b}

Each value is the mean of three replicates and it is given with standard deviation. Different superscripts in the same column indicate significant differences ($p < 0.05$).

GC–MS was used to examine the monosaccharide composition of FP3 from EPS-PP803. Retention times of the following authentic monosaccharides; D-galacturonic acid (GalA), D-glucuronic acid and D-glucose were 24.3, 25.6 and 27.5 min, respectively (data not shown). The retention time for myo-inositol used as the internal standard was 19.9 min (Fig. 4A). GC–MS analysis of the trimethylsilylated diethyldithioacetal (TMS-diethyldithioacetal) derivatives of FP3 showed 1 major peak with a retention time at 24.3 min (Fig. 4A), which was identical to that of the TMS-diethyldithioacetal derivatives of the authentic D-galacturonic acid, but not D-glucose and D-glucuronic acid (Fig. 4A). In addition, the mass spectrum of the peak at 24.3 min of the TMS-diethyldithioacetal derivative of FP3 was also identical to that of the peak at 24.3 min of the TMS-diethyldithioacetal derivative of the authentic D-galacturonic acid. These results indicated that FP3 was composed mainly of galacturonic acid. The ^{1}H-NMR spectrum of FP3 showed broad signals between δ 3 and δ 5.5 (Fig. 4B). The signals at δ 5.07, 4.42, 4.00 were detected clearly with similar integration values. The signal at δ 3.75 was partially covered with the sharp intense signal at δ 3.73. In order to confirm the presence or absence of signal(s) under the HDO signal around δ 4.8, the ^{1}H-NMR spectra of FP3 were obtained at a series of temperatures. As shown in Fig. 5, one additional broad signal was found at δ 4.77 with an integration value similar to those of the signals at δ 5.07, 4.42 and 4.00. Finally, these major broad signals at δ 5.07, 4.77 4.42, 4.00, 3.75 were found to be, respectively assigned to the H-1, H-5, H-4, H-3, H-2 of de-esterified pectin namely, polygalacturonic acid composed of (1 → 4) linked α-galacturonic acid residues (Tamaki, Konishi, Fukuta, & Tako, 2008). In addition, the molecular weight of FP3 was estimated to be ≈18 kDa by gel filtration HPLC. Hence, the FP3 was concluded to be a polysaccharide mainly composed of galacturonic acid.

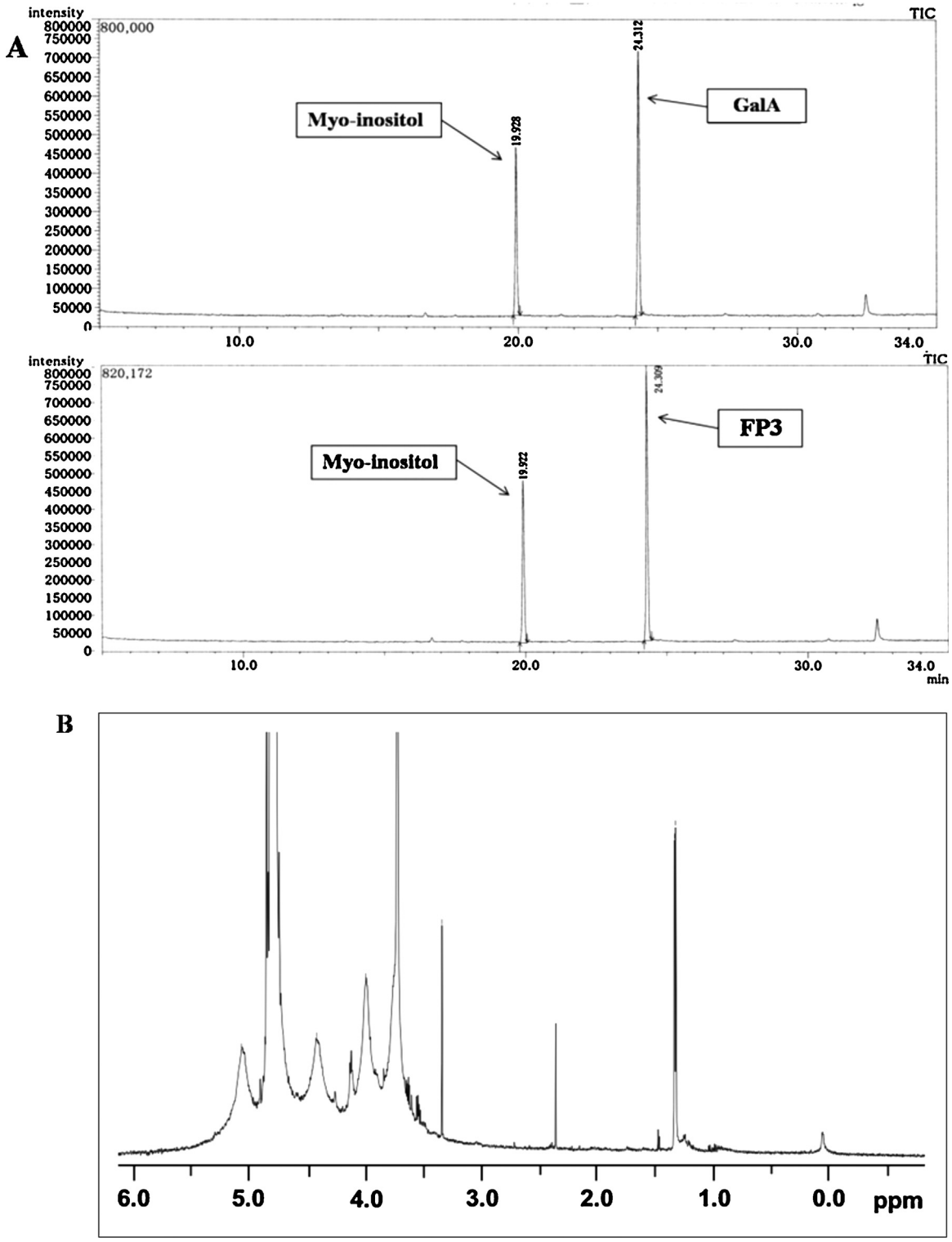

Fig. 4. (A) Retention times from GC–MS for the trimethylsilylated diethyldithioacetal galacturonic acid (GalA) and the FP3 samples and (B) ^{1}H NMR spectrum of the purified FP3 (in D_2O at 22 °C). Acetone in D_2O (δ 2.22) was used as an external reference.

4. Discussion

Although the role of EPS to flocculate or bind with metal ions has been reported by several research groups (Watanabe et al., 2003; Panwichian et al., 2011), the effect of NaCl on EPS production in PNSB has been less studied. Hence, in this study we examined the role of EPS on the NaCl tolerance of PNSB. We have shown that the EPS secreted by the PNSB strains reduced the levels of free Na^+ in the aqueous solution (Fig. 1 and Table 2). The presence of 1% NaCl in the growth medium had little effect on bacterial growth but it significantly increased the amount of EPS excreted ($p < 0.05$) (Table 1). However, 2% NaCl significantly inhibited the growth of the PNSB strains tested and significantly reduced the amount of secreted EPS (Table 1). The basic growth medium contained both 5.4 g/L sodium

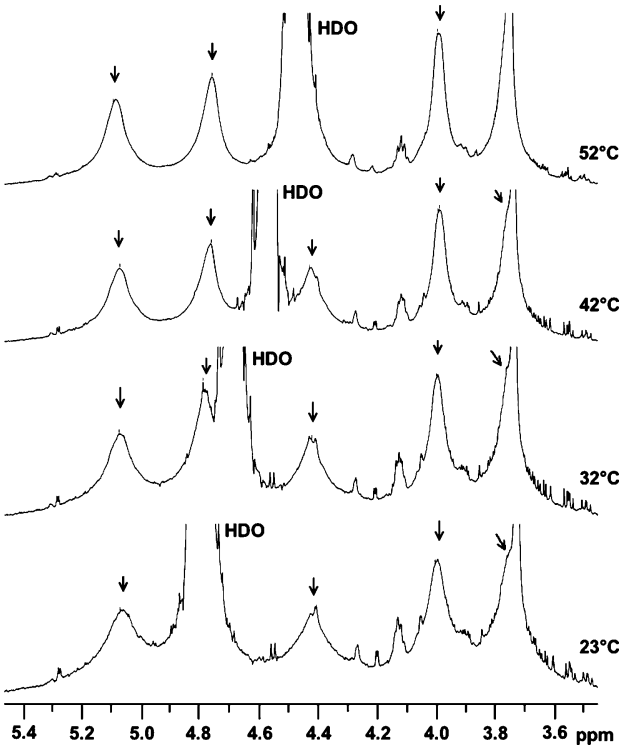

Fig. 5. ^{1}H NMR spectra of the purified FP3 (in D_2O) at a series of temperatures (23, 32, 42, and 52 °C). Acetone (δ 2.22) was used as an internal reference. The signals indicated with arrows can be assigned to the protons of polygalacturonic acid.

acetate trihydrate and 3.8 g/L monosodium glutamate monohydrate (Fig. 1 and Table 1) and so there was some baseline production of EPS; it is a constitutive capacity of the PNSB to produce EPS, and this is supported by the results of no addition of NaCl and adding NaCl until 0.50% no significant differences for amount of EPS produced by both strains. However, increasing concentrations of NaCl until met the salt stress significantly increased EPS production but only up to a point. EPS production increased as the salinity increased to 1% NaCl but higher concentrations inhibited growth and even more on the EPS yield (Table 1).

Previous studies have reported a limited adaptation of phototrophic bacteria such as *Rhodovulum* sp. and *Rhodopseudomonas acidophila* to high salinity (6.0% and 12.6% w/v, respectively) that resulted in the loss of ability to produce EPS, and reduced bacterial growth (Watanabe et al., 2003; Sheng, Yu, & Yue, 2006). Similar results were found for both our PNSB isolates in the present study (Fig. 1) as the correlation between Na^+ adsorbed on the EPS and the remaining Alcian blue in the culture supernatants was at a moderate level, while non-saline tolerant PNSB isolates were completely inhibited by 1% (w/v) NaCl (Hougardy, Tindall, & Klemme, 2000; Ramana et al., 2012). Toxic ions or osmotic stress conditions arising from elevated salt levels decrease bacterial metabolism and lead to inhibition of growth (Sheng et al., 2005; Upadhyay et al., 2011; Zhang, Chen, Wang, & Luo, 2011). Under high salt conditions bacteria consume significantly more energy to respond to the high osmotic environment and, hence might reduce the production of EPS (Vyride & Stuckey, 2009). Several research groups have reported that EPS not only protected bacterial cells but also helped them to reduce the toxicity of sodium in the soil to plants leading to increase the plant growth under saline conditions (Qurashi & Sabri, 2011).

NaCl concentrations in the culture medium may have an effect on the composition of EPS produced by both PNSB strains; particularly at higher concentrations. In 2% NaCl strain PP803 produced EPS with a 90% carbohydrate content (Fig. 2). The second major component of the EPS was nucleic acids and this is in agreement with several research workers that some EPS normally contains nucleic acids (Dignac et al., 1998; D'Abzac et al., 2010) and other unidentified compounds that might be lipids. Some inorganic components have also been found in EPS from bacteria for example, insoluble metal oxides (Sheng et al., 2010). Protein levels in the EPS produced by the two PNSB tested in the present study were very low. In contrast, other PNSB isolates from wastewater such as *R. acidophila* and *Rhodovulum* sp. produced EPS which did contain protein as a major component (Watanabe et al., 1998; Sheng et al., 2006). Higgins and Novak (1997) found that the protein content in sludge EPS increased at higher Ca^{2+} or Mg^{2+} concentrations, while higher Na^+ concentrations led to a lower protein content. However, the EPS produced by bacteria that were isolated from the rhizosphere of maize species contained carbohydrates as their main component and a low protein content (Qurashi & Sabri, 2011). Whilst, our results showed that the EPS from the PNSB tested was similar to the EPS from PGPR of maize species as it had high amount in carbohydrates but a lower amount of protein. Hence, the variations in the compositions of the secreted EPS from PNSB or other bacteria may depend on the species of bacterium and environmental factors such as the types of salts present.

The presence of many functional groups in EPS such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups, can bind various cations with varying affinity for different cations and have different exchange capacities at different pH (Ritchie & Larkum, 1982; Liu & Fang, 2002; Joshi & Juwarkar, 2009). The carboxylic and phosphoric groups seem to play a major role in the formation of complexes with metal ions at approximately pH 7 (Guibaud, Tixier, Bouju, & Baudu, 2003). This was in agreement with the FP3 (EPS from strain PP803 that was eluted in the 0.3 KCl fraction during DEAE-anion exchange purification); this fraction contained both uronic acid and protein as its EC_{50} was lower than that of the EC_{50} crude extract (Table 3). However, there was no significant difference in the EC_{50} values after removal of protein by gel filtration hence the uronic acid groups were the main component binding Na^+. Our results are in agreement with Liu and Fang (2002) that the binding of various cations to EPS occurs through electrostatic interaction with negatively charged carbohydrates such as uronic acids including polygalacturonic acid as a majority component.

The results indicated that the FP3 was a polysaccharide as the sample was purified by gel filtration chromatography so low molecular weight compounds were removed (<12 kDa). Determination of the monosaccharide composition of the polysaccharide of the EPS isolated from *R. palustris* PP803 is the first step toward understanding its role in NaCl tolerance. The main monosaccharide was identified as galacturonic acid which has a great potential for binding Na^+ because its COOH groups are fully ionized at pH values found in most soils. Standard methods for characterizing the chemical composition of the polysaccharide including GC–MS and ^{1}H-NMR spectra (Fig. 4) indicated that the polysaccharide of the FP3 was mainly composed of uronic acid. Our result is in agreement with Fazio, Uhlinger, Parker, and White (1982) who reported that extracellular polymer or EPS from two marine pseudomonads, *Pseudomonas marina*, was rich in galacturonic acid, while EPS produced by a pseudomonas isolated from the sediment consisted of large amount of galacturonic acid with a trace of mannuronic acid and a lesser amount of glucuronic acid. As our results do not exclude the possibility of a uronic acid other than D-galacturonic acid as the major component sugar of the polysaccharide of FP3. Therefore, further analyses of other uronic acid sugars such as mannuronic acid and L-glucuronic acid are required. The carboxyl group was identified as the important functional group in the uronic acid (Bitter & Muir, 1962) and this led to a significant binding of cations due

to the presence of hydrophilic carboxyl groups near the microbial surface.

The results in this present study have indicated that the EPS secreted by both PNSB strains would support these cells as biofertilizers to become dominant in a root zone in saline soil because of an ability of the EPS to bind to Na^+, and thus could increase their activity. This hypothesis is currently being tested in green house experiments with a future expectation for their use in saline paddy fields in Thailand that generally have an average NaCl content of 0.25% (Nunkaew et al., 2012, 2014).

5. Conclusions

Overall the results confirmed that EPS is a key factor that PNSB use to adsorb Na^+ to improve their survival in high NaCl concentrations. Galacturonic acid was identified as the most important part in EPS of *R. palustris* PP803 for binding Na^+ in aqueous solution.

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References

Bazihizina, N., Barrett-Lennard, E. G., & Colmer, T. D. (2012). Plant growth and physiology under heterogeneous salinity. *Plant and Soil*, *354*(1–2), 1–19.

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.

D'Abzac, P., Bordas, F., Joussein, E., van Hullebusch, E., Lens, P. N. L., & Guibaud, G. (2010). Characterization of the mineral fraction associated to extracellular polymeric substances (EPS) in anaerobic granular sludge. *Environmental Science and Technology*, *44*, 412–418.

Dignac, M. F., Urbain, V., Rybacki, D., Bruchet, A., Snidaro, D., & Scribe, P. (1998). Chemical description of extracellular polymeric substances: Implication on activated sludge floc structure. *Water Science and Technology*, *38*(8), 45–53.

Dubois, M., Gilles, K. A., Rebers, J. K., Hamilton, A., & Smith, F. (1956). Colorimetric method for determination of sugar and related substances. *Analytical Biochemistry*, *28*, 350–356.

FAO, Fisheries and Aquaculture Department. (2007). *The state of world fisheries and aquaculture*. Rome: Electronic Publishing Policy and Support Branch.

Fazio, S. A., Uhlinger, D. J., Parker, J. H., & White, D. C. (1982). Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. *Applied and Environmental Microbiology*, *43*(5), 1151–1159.

Guibaud, G., Tixier, N., Bouju, A., & Baudu, M. (2003). Relation between extracellular polymers composition and its ability to complex Cd, Cu and Pb. *Chemosphere*, *52*, 1701–1710.

Higgins, M. J., & Novak, J. T. (1997). Characterization of exocellular protein and its role in bioflocculation. *Journal of Environmental Engineering*, *123*(5), 479–485.

Hougardy, A., Tindall, B. J., & Klemme, J. H. (2000). *Rhodopseudomonas rhenobacensis* sp. nov., a new nitrate-reducing purple non-sulfur bacterium. *International Journal of Systematic and Evolutionary Microbiology*, *50*(3), 985–992.

Jia, S., Yu, H., Lin, Y., & Dai, Y. (2007). Characterization of extracellular polysaccharides from *Nostoc flagelliforme* cells in liquid suspension culture. *Biotechnology and Bioprocess Engineering*, *12*(3), 271–275.

Jin, B., Wilen, B. M., & Lant, P. (2004). Impacts of morphological physical and chemical properties of activated flocs on dewaterbility of activated sludge. *Chemical Engineering Journal*, *98*, 115–126.

Joshi, R. M., & Juwarkar, A. A. (2009). In *vivo* studies to elucidate the role of extracellular polymeric substances from *Azotobacter* in immobilization of heavy metals. *Environmental Science and Technology*, *43*, 5884–5889.

Kantha, T., Chaiyasut, C., Kantachote, D., Sukrong, S., & Muangprom, A. (2010). Selection of photosynthetic bacteria producing 5-aminolevulinic acid from soil of organic saline paddy fields from the Northeast region of Thailand. *African Journal of Microbiology Research*, *4*(17), 1848–1855.

Lee, K. H., Koh, R. H., & Song, H. G. (2008). Enhancement of growth and yield of tomato by *Rhodopseudomonas* sp. under greenhouse conditions. *Journal of Microbiology*, *46*(6), 641–646.

Liu, H., & Fang, H. H. P. (2002). Characterization of electrostatic binging sites of extracellular polymers by linear programming analysis of titration data. *Biotechnology and Bioengineering*, *80*, 806–811.

Ma, Y., Prasad, M. N. V., Rajkumar, M., & Freitas, H. (2011). Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnology Advances*, *29*(2), 248–258.

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, *59*, 651–681.

Nunkaew, T., Kantachote, D., Nitoda, T., & Kanzaki, H. (2012). The use of rice straw broth as an appropriate medium to isolate purple nonsulfur bacteria from paddy fields. *Electronic Journal of Biotechnology*, *15*(6) http://dx.doi.org/10.2225/vol15-issue6-fulltext-8

Nunkaew, T., Kantachote, D., Nitoda, T., Kanzaki, H., & Ritchie, R. (2014). Effects of 5-aminolevulinic acid (ALA)-containing supernatants from selected *Rhodopseudomonas palustris* strains on rice growth under NaCl stress, with mediating effects on chlorophyll, photosynthetic electron transport and antioxidative enzymes. *Electronic Journal of Biotechnology*, *17*(1), 19–26.

Panwichian, S., Kantachote, D., Wittayaweerasak, B., & Mallavarapu, M. (2011). Removal of heavy metals by exopolymeric substances produced by resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds. *Electronic Journal of Biotechnology*, *14*(4) http://dx.doi.org/10.2225/vol14-issue4-fulltext-2

Pitthard, V., & Finch, P. (2001). GC–MS analysis of monosaccharide mixture as their diethyldithioacetal derivative: Application to plant guns used in art works. *Chromatographia*, *53*, 317–321.

Quelas, J. I., Mongiardini, E. J., Casabuono, A., López-García, S. L., Althabegoiti, M. J., Covelli, J. M., et al. (2010). Lack of galactose or galacturonic acid in *Bradyrhizobium japonicum* USDA 110 exopolysaccharide leads to different symbiotic responses in soybean. *Molecular Plant-Microbe Interactions*, *23*(12), 1592–1604.

Qurashi, A. W., & Sabri, A. N. (2011). Osmoadaptation and plant growth promotion by salt tolerant bacteria under salt stress. *African Journal of Microbiology Research*, *5*(21), 3546–3554.

Ramana, V. V., Chakravarthy, S. K., Raj, P. S., Kumar, B. V., Shobha, E., Ramaprasad, E. V. V., et al. (2012). Descriptions of *Rhodopseudomonas parapalustris* sp. nov., *Rhodopseudomonas harwoodiae* sp. nov. and *Rhodopseudomonas pseudopalustris* sp. nov., and emended description of *Rhodopseudomonas palustris*. *International Journal of Systematic and Evolutionary Microbiology*, *62*, 1790–1798.

Ritchie, R. J., & Larkum, A. W. D. (1982). Cation exchange properties of the cell walls of *Enteromorpha intestinalis* (L.) Link (Ulvales Chlorophyta). *Journal of Experimental Botany*, *33*, 125–139.

Sheng, G. P., Yu, H. Q., & Yue, Z. B. (2005). Production of extracellular polymeric substances from *Rhodopseudomonas acidophila* in the presence of toxic substances. *Applied Microbiology and Biotechnology*, *69*(2), 216–222.

Sheng, G. P., Yu, H. Q., & Yue, Z. B. (2006). Factors influencing the production of extracellular polymeric substances by *Rhodopseudomonas acidophila*. *International Biodeterioration and Biodegradation*, *58*, 89–93.

Sheng, G. P., Yu, H. Q., & Li, X. Y. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. *Biotechnology Advances*, *28*(6), 882–894.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*(1), 76–85.

Tamaki, Y., Konishi, T., Fukuta, M., & Tako, M. (2008). Isolation and structural characterisation of pectin from endocarp of *Citrus depressa*. *Food Chemistry*, *107*, 352–361.

Taylor, R. (1990). Interpretation of the correlation coefficient: A basic review. *Journal of Diagnostic Medical Sonography*, *6*(1), 35–39.

Upadhyay, S. K., Singh, J. S., & Singh, D. P. (2011). Exopolysaccharide-producing plant growth-promoting rhizobacteria under salinity condition. *Pedosphere*, *21*(2), 214–222.

Vandevivere, P., & Kirchman, D. L. (1993). Attachment stimulates exopolysaccharide synthesis by a bacterium. *Applied Environmental Microbiology*, *59*(10), 3280–3286.

Vyrides, I., & Stuckey, D. C. (2009). Adaptation of anaerobic biomass to saline conditions: Role of compatible solutes and extracellular polysaccharides. *Enzyme and Microbial Technology*, *44*(1), 46–51.

Watanabe, M., Sasaki, K., Nakashimada, Y., Kakizono, T., Noparatnaraporn, N., & Nishio, N. (1998). Growth and flocculation of a marine photosynthetic bacterium *Rhodovulum* sp. *Applied Microbiology and Biotechnology*, *50*, 682–691.

Watanabe, M., Kawahara, K., Sasaki, K., & Noparatnaraporn, N. (2003). Biosorption of cadmium ions using a photosynthetic bacterium, *Rhodobacter sphaeroides* and a marine photosynthetic bacterium, *Rhodovulum* sp. and their biosorption kinetics. *Journal of Bioscience and Bioengineering*, *95*(4), 374–378.

Yang, J., Kloepper, J. W., & Ryu, C. M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science*, *14*(1), 1–4.

Zhang, Z. J., Chen, S. H., Wang, S. M., & Luo, H. Y. (2011). Characterization of extracellular polymeric substances from biofilm in the process of starting-up a partial nitrification process under salt stress. *Applied Microbiology and Biotechnology*, *89*(5), 1563–1571.