Saudi Journal of Biological Sciences (2018) **25**, 642–650

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Use of wood vinegar to enhance 5-aminolevulinic acid production by selected *Rhodopseudomonas palustris* in rubber sheet wastewater for agricultural use

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Received 25 July 2015; revised 28 December 2015; accepted 15 January 2016
Available online 21 January 2016

KEYWORDS

5-Aminolevulinic acid;
Levulinic acid;
Response surface methodology;
Rhodopseudomonas spp.;
Rubber wastewater;
Wood vinegar

Abstract This study aimed to produce inexpensive 5-aminolevulinic acid (ALA) in a non-sterile latex rubber sheet wastewater (RSW) by *Rhodopseudomonas palustris* TN114 and PP803 for the possibility to use in agricultural purposes by investigating the optimum conditions, and applying of wood vinegar (WV) as an economical source of levulinic acid to enhance ALA content. The Box–Behnken Design experiment was conducted under microaerobic-light conditions for 96 h with TN114, PP803 and their mixed culture (1:1) by varying initial pH, inoculum size (% v/v) and initial chemical oxygen demand (COD, mg/L). Results showed that the optimal condition (pH, % inoculum size, COD) of each set to produce extracellular ALA was found at 7.50, 6.00, 2000 for TN114; 7.50, 7.00, 3000 for PP803; and 7.50, 6.00, 4000 for a mixed culture; and each set achieved COD reduction as high as 63%, 71% and 75%, respectively. Addition of the optimal concentration of WV at mid log phase at 0.63% for TN114, and 1.25% for PP803 and the mixed culture significantly increased the ALA content by 3.7–4.2 times (128, 90 and 131 μM, respectively) compared to their controls. ALA production cost could be reduced approximately 31 times with WV on the basis

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2016.01.028

of the amount of levulinic acid used. Effluent containing ALA for using in agriculture could be achieved by treating the RSW with the selected ALA producer *R. palustris* strains under the optimized condition with a little WV additive.

1. Introduction

In agriculture, 5-aminolevulinic acid (ALA) can be an effective and non-toxic biodegradable herbicide and insecticide (Sasaki et al., 2002) including an ability to regulate several key physiological processes associated with plant growth under saline conditions through improvements to the antioxidative defense systems and photosynthetic electron transport under stress conditions (Nunkaew et al., 2014). ALA is known as an essential biosynthetic precursor for all heterocyclic tetrapyrroles such as chlorophyll, vitamin B_{12}, and other specialized compounds in higher plants, algae, and bacteria (Akram and Ashraf, 2013). In phototrophic bacteria, it is synthesized via the Shemin pathway with two precursors, succinate and glycine, brought together by the enzyme aminolevulinic synthase (ALAS) (Liu et al., 2005). ALA itself is used for the synthesis of porphobilinogen (PBG) via the action of aminolevulinic dehydratase (ALAD) (Heinemann et al., 2008); thereby levulinic acid as an inhibitor of ALAD has been used to increase ALA production.

It is also well recognized that ALA at low concentrations can promote the growth of many plants including rice (Hotta et al., 1997; Nunkaew et al., 2014). Unfortunately, commercial ALA is too expensive for use in agricultural applications. Hence, the production of ALA by microorganisms has been suggested as an attractive alternative source because microbes can use a variety of raw materials to produce ALA with a high productivity but a low pollution (Kamiyama et al., 2000; Kang et al., 2012). Among the microbes that produce ALA, purple nonsulfur bacteria (PNSB), are good candidates for ALA production as they are versatile organisms that grow phototrophically and/or heterotrophically in various wastewaters under anaerobic–microaerobic/light conditions and aerobic dark conditions, respectively (Kantachote et al., 2005; Kars and Ceylan, 2013). They are often found in the wastewater that is being treated in open air lagoons (Kornochalert et al., 2014a,b). Selected PNSB strains have been extensively used to study ALA production by optimizing the amounts of succinate, glycine and volatile fatty acids (Saikeur et al., 2009; Sattayasamitsathit and Prasertsan, 2014). Nevertheless, those compounds are expensive to use; and thus perhaps we should try to use a much cheaper alternative substrate such as wastewater to reduce the costs of ALA production and to facilitate its use in agriculture.

Thailand has become one of the world's major producers of natural rubber and rubber plantations now exist in most parts of the country; consequently there has been a huge increase in the numbers of cooperative smoked rubber sheet factories (CSRFs) throughout Thailand. Wastewater from CSRFs is normally treated by anaerobic lagoons as they are of low cost and easy to operate (Chaiprapat and Sdoodee, 2007; Kornochalert et al., 2014a,b). However, this can produce serious problems of rotten egg odors (H_2S) thus more appropriate technologies have been developed to use PNSB such as *Rhodopseudomonas blastica* DK 6 and *Rhodopseudomonas palustris* P1 to remove 90% and 98% COD in non sterile latex rubber sheet wastewater (RSW), respectively without the production of H_2S (Kantachote et al., 2005; Kornochalert et al., 2014b). At the same time, the shortage of water for agricultural use is due to the increasing urbanization and industrialization; therefore, the concept to reuse wastewater after treatment could lead to solve this problem. Fortunately, our previous work with the use of 3% *R. palustris* P1 could treat RSW to produce effluent that met Thai standard guidelines, and the effluent could directly be used as irrigation water (Kantachote et al., 2010).

Hence, it should be possible to produce inexpensive ALA in RSW by concomitant with treating RSW. However, to increase the amount of ALA production by PNSB, levulinic acid has been used as a competitive ALAD inhibitor as previously mentioned (Saikeur et al., 2009; Kars and Ceylan, 2013). Unfortunately, this increases the cost of ALA production. Wood vinegar (WV) is a byproduct from charcoal production and contains 12–17 mM levulinic acid (Matsushita et al., 2002). Hence, it would be attractive to try to use WV as a levulinic acid source for producing ALA by PNSB with an economical cost, and our work described here is the first known to investigate this possibility. According to the above information, the objectives of this study were to investigate the optimal conditions for ALA production in RSW by our ALA producer *R. palustris* strains (Nunkaew et al., 2015) and the use of WV containing levulinic acid to enhance the ALA content. This might lead to the possibility to use RSW effluent containing ALA for agricultural purposes like irrigation water to ameliorate rice growth in saline soil.

2. Materials and methods

2.1. Preparation of latex rubber sheet wastewater

Latex rubber sheet wastewater was collected from a lagoon pond of a CRSF at the Phichit suburb in Songkhla Province, Thailand. The wastewater was collected as a composite sample at a deep level roughly 20–100 cm from the surface at various positions of the pond for obtaining a representative sample and pouring them into a 25 L plastic tank. The collected wastewater was filtered using a cheesecloth to remove solid particles, and directly used as the raw wastewater medium without supplementing with any nutrients, and with no autoclaving (RSW).

2.2. ALA producing PNSB inocula

The selected *R. palustris* strains (TN114 and PP803) from our previous study (Nunkaew et al., 2015) were used for this study

due to their ability to release high amounts of ALA into a Glutamate–Acetate (GA) medium. Their culture supernatants containing ALA can encourage rice growth in hydroponics under salt stress conditions (Nunkaew et al., 2014). Each strain was grown in GA broth by inoculating one loopful into a screw cap test tube (150 × 15 mm: 20 ml) containing 18 ml GA medium, leaving a small space on the top of the medium to achieve microaerobic conditions and incubated under light condition (3500 lux) for 48 h (Nunkaew et al., 2014). The reason for using GA broth was due to that acetate (a short chain fatty acid), a major carbon source, is a preferable substrate for PNSB (Okubo et al., 2006), and the PNSB used in this study were proven strains that can utilize acetate (Nunkaew et al., 2015) and also various organics in RSW as a carbon source (Kornochalert et al., 2014a,b). The PNSB biomass was harvested by centrifugation at 2340*g* for 15 min and washed twice with sterile tap water. PNSB cell suspension of each culture was prepared by adjusting to an optical density (OD_{660}) of 0.5 by diluting with sterile RSW for obtaining a cell density of approximately 10^8 cells/ml; and this was used as the inoculum for producing ALA in RSW. Cell numbers in the inoculum were confirmed by counting viable PNSB cells using the spread plate technique and incubated in an anaerobic jar under the light for 96 h.

2.3. Analytical methods used

Values of pH were measured using a pH meter. An oxidation-reduction potential (ORP or redox) probe was used to measure the ORP values that were recorded after obtaining a constant value. The amount of ALA in the RSW was determined using HPLC with a RF-10AXL fluorescence detector, following the method as described by Tangprasittipap et al. (2007). Briefly, 50 µl of culture supernatant was mixed with 3.5 ml of acetylacetone/ethanol/water (15:10:75, v:v:v) containing 0.4% NaCl and 450 µl aqueous formalin (8.5% v/v), and placed at 100 °C for 30 min. The HPLC conditions were set as follows: Inertsil ODS-3 column (5 µm, 250 × 150 mm) (GL Science Inc., Tokyo, Japan) at 40 °C with methanol mixed in 2.5% (v/v) acetic acid in a ratio of 60:40 (v/v) as a mobile phase at a flow rate of 0.2 ml/min. The eluted sample was monitored at the excitation and emission wavelengths of 363 and 473 nm. The extracellular ALA concentration was calculated from the peak area, using 99.9% ALA–HCl as an authentic standard.

2.4. Optimization of ALA production in RSW by PNSB strains

A Box–Behnken Design (BBD) was used to determine the optimum conditions for ALA production in RSW by the three independent variables; pH (X_1), inoculum size (X_2), and initial COD (X_3) at 3 levels (Table 1). In our previous works with other strains of PNSB as inoculants, it was found that inoculum sizes in a range of 3–10% showed a high efficiency to reduce COD; however, a low ALA production resulted at 3–4% inoculum sizes (unpublished data). Based on the preliminary works and ALA production cost, inoculum sizes were designed from 5.00% to 7.00% while initial pH (5.50–7.50) and COD levels (2000–4000 mg/L) were designed by considering the optimal growth range of PNSB and the most likely variation of COD levels in RSW. In order to describe the nature of the response surface methodology in the experimental region, BBD was entered into the Design-Expert Software Trial Version (Stat-Ease, Minneapolis, MN, USA). This represents the design matrix of a 17 set experiment and the experimental sequence was randomized in order to minimize the effect of light intensity and temperature by the distance from the tungsten light source, and each run was conducted in triplicate. For predicting the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variables and the response (ALA content). The optimum conditions for ALA production in RSW by PNSB were obtained by solving the regression equations and also by analyzing the response surface contour plots using the same software. The quality of the fit of the model equations was expressed by the coefficient of determination R^2.

In order to correlate the relationship between the variables and the response, a quadratic polynomial equation was used for fitting such as that in Eq. (1)

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

where Y_i is the dependent variable, b_0 is the intercept, b_1 to b_{33} are the regression coefficient and X_1, X_2, X_3 are the three independent variables.

All runs were conducted in 120 ml serum bottles containing 80 ml RSW in each bottle and incubated under microaerobic-light conditions as previously described for 96 h. RSW with undiluted and tap water diluted samples required to meet the design of the BBD were used. The inoculum used was a single culture of TN114 or PP803 and a mixed culture between both strains at a ratio of 1:1. The following parameters; pH, ORP, extracellular ALA, COD and viable PNSB cells were monitored at the start and the end of experiment at 96 h. The viable PNSB cells at the start point were calculated from PNSB population in the inoculums at 5.00%, 6.00% and 7.00% as previously described, while the viable PNSB cell counts at 96 h were performed only in the experiments that produced maximal ALA contents. Viable PNSB cells were counted using the spread plate technique and incubated in an anaerobic jar under the light for 96 h. The optimal conditions for TN114, PP803 and a mixed culture were then used to confirm them in a verification test, and uninoculated PNSB was also included as a control set as native populations of PNSB developed. All parameters as previously mentioned were also determined in the verified test.

2.5. Effect of wood vinegar (WV) on ALA production by PNSB in optimized RSW

After the optimum conditions were determined, verified and shown to be the same for each of the PNSB; these optimal conditions obtained from a previous experiment were used to study the effect of WV as a cheap source of levulinic acid on ALA production in each optimized RSW. Based on our preliminary work, varying concentrations of WV at 0.00%, 0.31%, 0.63%, 1.25% and 2.50% (v/v) were fed to the PNSB when they were in their mid log phase of growth in order to provide levulinic acid to suppress the activity of ALAD and increase ALA levels (Chung et al., 2005). The parameters for monitoring were determined after 72 h incubation in the same way as for the previous experiment. The compositions of WV including levulinic acid content was determined by HP 6890

Table 1 Effects of the PNSB inoculum size, initial pH and initial chemical oxygen demand (COD) on ALA production in RSW under microaerobic light conditions for 96 h.

Run no.	pH, X_1	Inoculum, X_2 (% v/v)	COD, X_3 (mg/L)	ALA (μM) TN114		ALA (μM) PP803		ALA (μM) Mixed culture (1:1)	
				Actual	Predicted	Actual	Predicted	Actual	Predicted
1	5.50	5.00	3000	0.75	1.25	1.87	1.93	1.13	0.67
2	7.50	5.00	3000	8.65	10.57	16.24	16.39	16.23	16.32
3	5.50	7.00	3000	1.91	0.01	7.09	6.94	0.87	0.78
4	7.50	7.00	3000	24.64	24.14	24.72	24.66	24.29	24.75
5	5.50	6.00	2000	0.46	2.55	0.78	1.16	2.24	2.72
6	7.50	6.00	2000	31.11	31.78	11.45	11.74	13.18	13.11
7	5.50	6.00	4000	1.46	0.79	2.76	2.76	2.03	2.10
8	7.50	6.00	4000	7.10	5.01	24.46	24.08	31.82	31.34
9	6.50	5.00	2000	31.26	28.67	2.90	2.46	2.16	2.14
10	6.50	7.00	2000	23.26	23.21	5.32	5.26	9.70	9.31
11	6.50	5.00	4000	2.61	2.78	5.03	5.26	13.45	13.84
12	6.50	7.00	4000	17.97	20.56	15.49	15.93	15.21	15.23
13	6.50	6.00	3000	25.53	26.17	18.73	18.46	18.72	18.73
14	6.50	6.00	3000	26.53	26.17	18.64	18.46	18.96	18.73
15	6.50	6.00	3000	23.97	26.17	18.44	18.46	18.83	18.73
16	6.50	6.00	3000	27.66	26.17	18.15	18.46	18.14	18.73
17	6.50	6.00	3000	27.14	26.17	18.36	18.46	19.02	18.73

series gas chromatographs (Hewlett–Packard, Palo Alto, CA) with a flame ionization detector (FID) following the method of Chang et al. (2007) with an FFAP capillary column (30 m × 0.32 mm × 0.33 μm) using a linear temperature program of 15 °C/min (initial 90 °C, final 210 °C, injector 240 °C, detector 250 °C).

2.6. Statistical analysis

All experiments in this study were carried out in triplicate. Data are presented as means with their standard deviations. A one way ANOVA was used to analyze data by considering the significance at a P-value < 0.05; and statistical differences of mean comparisons were performed by Duncan's multiple range test.

3. Results

3.1. Optimization of ALA production in RSW by PNSB strains

From the BBD experiment, runs no. 6, 4 and 8 produced the highest ALA content of 31.11, 24.72 and 31.82 μM for TN114, PP803 and a mixed culture, respectively (Table 1). The conditions of runs no. 6, 4 and 8 for initial pH, % inoculum size and initial COD in mg/L were as follows: 7.50, 6.00 and 2000; 7.50, 7.00 and 3000, and 7.50, 6.00 and 4000, respectively. The optimal inoculum sizes in a range of 6.00–7.00% provided initial cell density roughly 10^6 CFU/ml. The predicted highest values of ALA in runs no. 6, 4 and 8 were 31.78, 24.66 and 31.34 μM, respectively. Both the actual and predicted values of the ALA content in runs no. 6, 4 and 8 were very close. Hence, these runs were considered to provide the optimal conditions for ALA production of each PNSB strain and the mixed culture; and this was supported by calculations from the models such as the optimal condition for a mixed culture set was initial pH 7.50%, 6.08% inoculum size and a 3999.9 mg/L initial COD.

Design Expert software was used to analyze the relationship of the variables to the response using the regression model with the significance level $\alpha = 0.05$. The P-value is a tool for evaluating the significance, and thus the quadratic models were appropriate by considering the P-value ($P < 0.5$), lack of fit ($P \geqslant 0.05$) and the test statistics (Std. Dev PRESS lower, and higher R^2 and adjusted R^2) combination. The F-value was high and P-value was low, which indicated that the model was good (Table 2). For ALA production by PNSB, the P-value for X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, X_1X_3 and X_2X_3 were less than 0.05 and can be found in the following equations:

$$\text{ALA by TN114} = 26.17 + 8.37X_1 + 3.08X_2 + 7.13X_3 - 12.98X_1^2 - 4.21X_2^2 - 3.16X_3^2 + 3.71X_1X_2 - 6.25X_1X_3 + 5.81X_2X_3 \quad (2)$$

$$\text{ALA by PP803} = 18.46 + 8.05X_1 + 3.32X_2 + 3.41X_3 - 1.65X_1^2 - 4.33X_2^2 - 6.95X_3^2 + 0.82X_1X_2 + 2.76X_1X_3 + 2.01X_2X_3 \quad (3)$$

$$\text{ALA by mixed culture} = 18.73 + 9.91X_1 + 2.14X_2 + 4.40X_3 - 2.96X_1^2 - 5.15X_2^2 - 3.46X_3^2 + 2.08X_1X_2 + 4.71X_1X_3 - 1.44X_2X_3 \quad (4)$$

The soundness of the regression models can be checked by the determination coefficient, R^2, and the adjusted R^2. The value of the adjusted R^2 indicated that the total variation at 96.09%, 99.74% and 99.70% for ALA production by TN114, PP803 and a mixed culture, respectively (Table 2). These values were attributed to the independent variables and about 3.91%, 0.26% and 0.30% of the total variation could not be explained by the modals. The P values of a lack-of-fit (0.0816, 0.0533 and 0.1397) for the regression equations indicated that each modal was adequate for predicting the ALA yield under any combination of the variables (Table 2).

Table 2 ANOVA analysis of the quadratic models for ALA production by TN114, PP803 and a mixed culture (1:1) in RSW under microaerobic light conditions for 96 h.

Source	Degree of freedom	Sum of squares	Mean squares	*F*-value	*P*-value	R^2	*Adj*-R^2
TN114							
Model	9	2271.32	252.37	44.73	< 0.0001	0.9829	0.9609
Residual	7	39.49	5.64				
Lack of fit	3	30.93	10.31	4.81	0.0816		
Pure error	4	8.57	2.14				
Corrected total	16	2310.81					
PP803							
Model	9	1066.99	118.55	681.69	< 0.0001	0.9989	0.9974
Residual	7	1.22	0.17				
Lack of fit	3	1.01	0.34	6.33	0.0533		
Pure error	4	0.21	0.053				
Corrected total	16	1068.21					
Mixed culture							
Model	9	1311.47	145.72	593.39	< 0.0001	0.9987	0.9970
Residual	7	1.72	0.25				
Lack of fit	3	1.22	0.41	3.30	0.1397		
Pure error	4	0.5	0.12				
Corrected total	16	1313.19					

The quadratic equation of regression for the ALA response was represented by a 3D surface and contour plot as shown in Fig. 1, and to analyze the interacting effects of three variables. This figure shows that initial pH had a higher influence on ALA production (Fig. 1a, b, d, e, g, h) than the % PNSB inoculums and the initial COD values (Fig. 1c, f, i). Moreover, a strong positive interaction between initial pH and initial COD were observed for PP803 and a mixed culture sets (Fig. 1e, h), but it produced a negative interaction in the TN114 set (Fig. 1b).

Based on the results in Table 1, the optimum conditions as shown in runs no. 6, 4 and 8 were confirmed in RSW under microaerobic-light conditions for 96 h. ALA production in RSW by TN114, PP803 and a mixed culture in a verified test (31.55 ± 1.57, 24.44 ± 1.22, 31.30 ± 1.56 μM) was approximately the same with the optimal conditions from the models, while a control (uninoculated set) had only 0.02 ± 0.01 μM ALA (Table 3). It should be noted that experimental values obtained were in good agreement with the values predicted from the models, with relatively small error between the predicted and the actual values of only 1.66%, 1.33% and 0.50% for TN114, PP803 and a mixed culture, respectively. Hence, it indicated that model fitness to predict the ALA production was good as the error was less than 10%. Verification test also shows that at the end of the experiment (96 h), ORP values in the PNSB sets were between −204 and −239 mV, but in the uninoculated set (control) it was only −106 mV (Table 3). The mixed culture was the suitable set to remove COD (75.33%, 4000 mg/L initial COD) followed by PP803 (70.65%, 3000 mg/L initial COD), TN114 (62.63%, 2000 mg/L initial COD) and the control (58.10%, 2000 mg/L initial COD), and this corresponded to the numbers of PNSB (8.4, 8.3, 7.4 and 5.8 log CFU/ml), respectively (Table 3).

3.2. Effect of wood vinegar (WV) on ALA production by PNSB in optimized RSW

Analysis of wood vinegar used in this study found levulinic acid at 9.47 mM with pH 3.00 while methanol and acetic acid are the major composition (see details Table 4). Fig. 2 shows that a 1.25% WV (v/v) containing levulinic acid of 118 μM in the optimized RSW after 72 h incubation produced the highest ALA content (130.84, 89.61 μM) for the mixed culture and the PP803 sets, respectively. In contrast, the amount of WV was only 0.63% (59 μM levulinic acid) in the TN114 set giving the maximum ALA content (128.49 μM). At the optimum amount of WV in each set, the final pH was in a range of 6.70–6.80 and this increased PNSB growth at 72 h incubation from 6.70, 6.90 and 7.10 log CFU/ml in control sets (no WV) to 8.10, 8.50 and 8.61 log CFU/ml for TN114, PP803 and a mixed culture, respectively. However, the highest WV concentration used (2.50% v/v) gave a negative effect on ALA production and the final pH value decreased roughly 1.0 unit compared with other treatments. The result showed that the pH value, viable PNSB cells and the levulinic acid content in WV are important keys to enhance ALA production by PNSB.

4. Discussion

The BBD experiment showed that the initial pH was the most influential factor for optimum ALA production under microaerobic-light conditions (Fig. 1). ALA synthase (ALAS) and ALA dehydratase (ALAD) are key enzymes for the accumulation of ALA (Heinemann et al., 2008; Liu et al., 2005). In photosynthetic bacteria, high ALAS activity and moderate inhibition of ALAD occurred roughly at pH 7.0 (Sasaki et al., 1987). However, it had been reported that the optimal pH for ALA production by *Rhodopseudomonas* sp. L-1 was 7.5, and this strain was able to produce ALA in 4 kinds of wastewater tested such as monosodium glutamate and citric acid wastewaters, but removal of COD was low when levulinic acid, glycine and succinate were added to increase ALA production (Liu et al., 2005). In our study, an increase of the pH increased the ALA content in RSW under microaerobic-light conditions by both strains of *R. palustris* including the mixed culture and all had the same optimal pH at 7.5 (Fig. 1 and Table 1). The pH values of the cultures in all run numbers

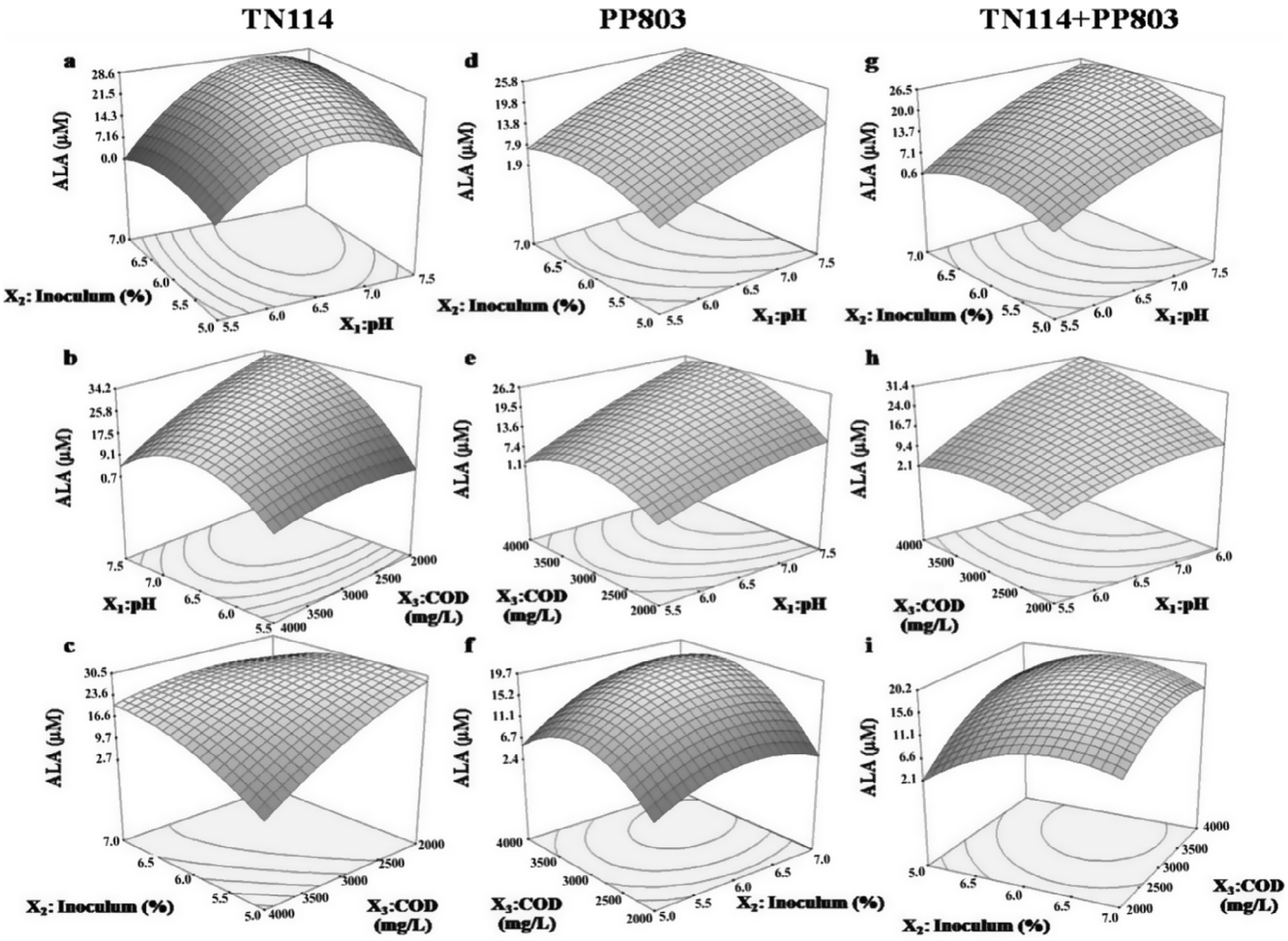

Figure 1 Three-dimensional response surfaces illustrating the values of ALA production in RSW by *R. palustris* under microaerobic-light conditions for 96 h; (a–c) for TN114; (d–f) for PP803, and (g–i) for a mixed culture as functions of initial pH, PNSB inoculum, and initial COD. Each graph displays the interactive effects of two variables while the third variable was fixed at its central level as shown in Table 1.

changed at the end of the experiment (96 h) due to the metabolism of the cultures. The final pH values after 96 h incubation were between 5.32–8.41, 5.40–8.63 and 5.41–8.42 for sets of TN114, PP803 and a mixed culture, respectively. It should be noted that maximal values of the final pH were observed in the optimal conditions for strains TN114 (8.41), PP803 (8.63) and a mixed culture (8.43). This might be that higher PNSB populations found in the optimal conditions of strains TN114, PP803 and a mixed culture as 7.30, 8.41 and 8.52 log CFU/ml, respectively when compared with other runs.

It was of interest that the strains we used still had the ability to remove COD in the range of 63–75% under optimal conditions for ALA production as only 58% COD was reduced in the control set without PNSB inoculation (Table 3). This was confirmed with all equations from the BBD experiment that the highest interactive influence was between the initial pH and the COD. These results were related to the higher number of PNSB being present in all inoculated sets compared to the control set by roughly 2–3 log CFU/ml after a 96 h treatment (Table 3). The PNSB population found in the control set was due to the stimulation of the growth of indigenous PNSB under microaerobic light conditions.

The results confirmed that both our strains were in competition with other microbes including indigenous PNSB in the non sterile wastewater (Table 3). In addition, this was also supported by the evidence of elevated ALA contents in the inoculated treatments (24.42–31.62 μM) that were significantly higher than that found in the control at only 0.02 μM (Table 3). A remarkable increase of ALA content in the inoculated treatments proved that our PNSB strains as ALA producers were able outcompete the indigenous PNSB. This is in agreement with the work of Kornochalert et al. (2014b) who reported that a 2% or 3% *R. palustris* P1 inoculum added to RSW was enough for it to compete effectively against other microbes and become the most dominant microbe in the RSW. In the present work, no molecular work has been done to discriminate inoculants (TN114 and PP803) from the indigenous PNSB. However, the results proved that the selected PNSB strains were screened as effective ALA producers. The concurrent significant increase in ALA concentration and COD removal (63–75% in inoculated treatments vs. 58% in the control) with PNSB viable cells (CFU/ml) in the inoculated sets was inferred to the effects of increase in the population of the inoculants as shown in Table 3.

Table 3 Verification test of ALA production in RSW by single or a mixed culture of *R. palustris* under microaerobic light conditions after 96 h incubation.

Parameter Optimal conditions	Control pH 7.50, uninoculated, 2000 mg/L	TN114 pH 7.50, 6% inoculum size, 2000 mg/L	PP803 pH 7.50, 7% inoculum size, 3000 mg/L	Mixed culture (1:1) pH 7.50, 6% inoculum size, 4000 mg/L
ORP (mV)	−106 ± 4.43	−204 ± 3.51	−239 ± 4.32	−237 ± 2.54
pH	8.88 ± 0.02	8.34 ± 0.01	8.59 ± 0.02	8.39 ± 0.01
COD (mg/L)	838 ± 5.43	748 ± 4.45	881 ± 3.22	987 ± 4.33
COD removal (%)	58.10	62.63	70.65	75.33
Initial PNSB (log CFU/ml)	0	6.08 ± 0.10	6.15 ± 0.10	6.10 ± 0.11
PNSB (log CFU/ml)	5.80 ± 0.11	7.40 ± 0.10	8.30 ± 0.10	8.40 ± 0.11
ALA (μM)	0.02 ± 0.01	31.62 ± 1.57	24.42 ± 1.22	31.33 ± 1.56

Each value represents a mean value ± standard deviation of three determinations.

Table 4 Major and minor compositions of wood vinegar (WV) used in this study.

Wood vinegar composition	Concentration (mM)
Methanol	478.62 ± 5.54
Acetic acid	610.99 ± 9.44
Propionic acid	30.37 ± 2.43
Butyric acid	12.37 ± 1.07
Valeric acid	0.58 ± 0.02
Levulinic acid	9.47 ± 0.34
Phenol	15.94 ± 1.01

Each value represents a mean value ± standard deviation of three determinations.

In this study the mixed culture gave the best ALA production with an initial COD (4000 mg/L) that was higher than that for the single cultures, and the normal characteristic of RSW for the initial COD and pH was approximately 4000 mg/L and 5.24, respectively (Chaiprapat and Sdoodee, 2007). This meant that the wastewater can be directly used without dilution for adjustment of the initial COD as the only requirement was to adjust the pH by adding ash from the firewood of the rubber smoking process. In addition, the mixed culture produced the maximal COD removal at 75% (Table 3) yielding the effluent of 987 mg/L COD. For an effective concentration of ALA to stimulate rice growth (our unpublished data), the treated RSW with WV additive should be diluted roughly 50 times. This fact indicates that the partially treated effluent could effectively be used for agricultural irrigation purpose, and this is supported by our previous work that undiluted RSW by *R. palustris* P1 or its dilution between 1:25 and 1:200 stimulated rice seed germination (Kantachote et al., 2010). The strains we used as a mixed culture were proven to be a better alternative for both ALA production and COD reduction. The process described could be a good approach for farmers to add value to their wastewater treatment as sources of ALA and also biofertilizers for agriculture use. This is due to the fact that *R. palustris* is able to fix N_2 and both PNSB strains, particularly PP803 could reduce methane emissions from anaerobically organic degradation (Nunkaew et al., 2014, 2015). This would lead to the reduction of global warming when the effluent containing mixed PNSB is being used in paddy fields.

It has long been known that levulinic acid is an inhibitor of ALAD in the C_4 pathway and this inhibitor was added into the culture medium for enhancing ALA accumulation (Miyachi et al., 1998; Saikeur et al., 2009). We are the first research group to investigate the possible use of WV as a low cost levulinic acid, and it was very effective in enhancing ALA production when it was applied at only 0.63% and 1.25% by providing levulinic acid at concentrations of 59 μM for TN114, and 118 μM for PP803 and for the mixed culture. By adding the optimal amounts of WV into the RSW treatment by the PNSB sets under their optimum conditions the ALA content was increased by 3.7–4.2 fold (89.61–130.84 μM) compared to their controls without the addition of WV (Fig. 2). It was of interest, that in this study the amount of levulinic acid used was much lower than in previous studies and it was not a pure compound. Saikeur et al. (2009) reported that *R. palustris* KG31 grown with a volatile fatty acid medium supplemented with 10 mM pure levulinic acid under microaerobic-light conditions produced a maximum amount of ALA of 134.47 μM. These results also stated that a cost analysis revealed that ALA could reduce costs by 31 times on the basis of the amount of levulinic acid used. Recently, *Rhodobacter sphaeroides* OU 001 was used to produce ALA in waste barley hydrolysate by adding 30 mM pure levulinic acid; and this resulted in an increase of the ALA content up to 67.40 μM (Kars and Ceylan, 2013).

Enhancement of ALA production by WV in this study may provide not only levulinic acid but also more suitable conditions for producing ALA as WV was added at the mid log phase. Normally, an increase of pH was found after PNSB growth in RSW (Kornochalert et al., 2014b), hence a small amount of added WV decreased the pH and promoted growth. This was supported by adding 2.5% WV, at a final pH of 5.51 after 72 h incubation compared to roughly pH 7.02 for sets with added optimal doses of WV (Fig. 2), but a lower pH strongly suppressed the ALA production by decreasing the activity of ALAS (Alber et al., 2006). Moreover, Table 4 shows that WV is a rich source of volatile fatty acids (VFAs); particularly acetic acid; and these VFAs can be used as electron donors for photosynthesis by our selected strains (Nunkaew et al., 2015). This is supported by the evidence that the amount of PNSB after adding WV was higher than that of the control sets (no WV) roughly 1.5 log cycle/ml. As both of our PNSB strains cannot use methanol (Nunkaew et al., 2015); however,

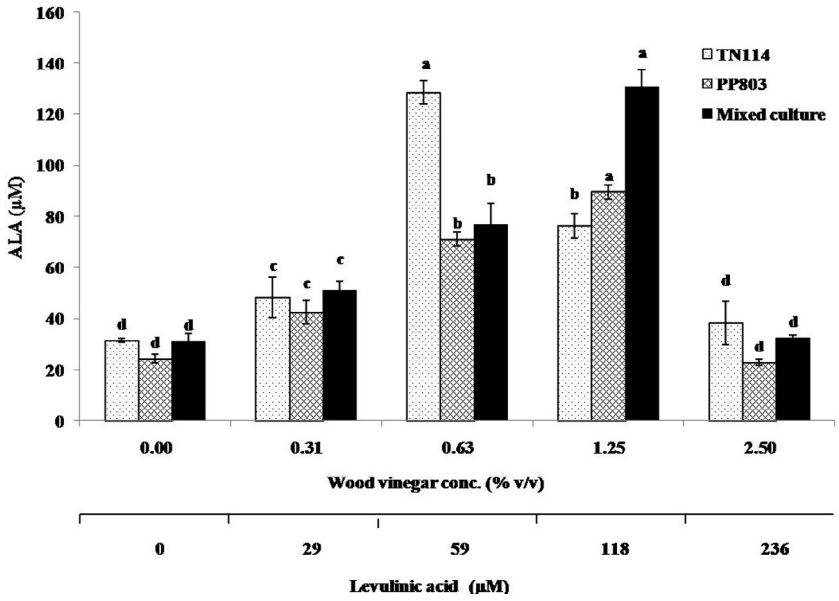

Figure 2 Effect of adding wood vinegar (v/v) at mid log phase on ALA production in RSW under optimized conditions for 72 h by *R. palustris* (TN114, PP803 and a mixed culture). Each value represents a mean value ± standard deviation ($n = 3$). Different lowercase letters above bars indicate significant differences at a $P < 0.05$.

it was found that they were able to resist methanol concentration found in WV addition treatments. These results proved that adding a little amount of WV in the optimized RSW is not only a source of levulinic acid to enhance ALA production but also a source of VFAs to stimulate the PNSB growth. Overall results in this study states that RSW after treatment by a mixed culture of *R. palustris* TN114 and PP803 (1:1) under microaerobic-light conditions produced the effluent containing ALA that is high enough to ameliorate rice growth in saline soil; thereby, this hypothesis is currently being investigated in green house experiments with expectation for its use in saline paddy fields.

5. Conclusions

The initial pH as slightly alkaline was the most effective variable for optimal ALA production by *R. palustris* strains used in RSW under microaerobic-light conditions, together with a little WV additive as a low cost source of levulinic acid that allowed a remarkable increase of ALA production. Therefore, this study has explored the appropriate technology that makes use of cheap local materials; RSW and WV, to produce a valuable ALA by our promising PNSB strains for use in agricultural purposes.

Acknowledgements

This work was financially supported by Thailand Research Fund – Thailand through the Royal Golden Jubilee Ph.D. Program; grant number PHD/0200/2551, and partly supported by Graduate School, Prince of Songkla University – Thailand. We also thank Dr. Brian Hodgson for assistance with the English.

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