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*Full Length Research Paper*

# The use of the purple non sulfur bacterium isolate P1 and fermented pineapple extract to treat latex rubber sheet wastewater for possible use as irrigation water

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**A central composite design using two variables (concentrations of isolate P1 and Fermented Pineapple Extract (FPE) each at three levels was used to study their effects on the treatment efficiency of latex rubber sheet wastewater under microaerobic-light conditions. The optimum combination over a 72 h period consisted of 3% P1 and 0.13% FPE and resulted in the removal of 80% Chemical Oxygen Demand (COD), 82% Suspended Solids (SS) and 85% Un-ionized Hydrogen Sulfide: $H_2S$ in wastewater (UHS). The selected experimental condition was then verified by varying the retention times. A 96 h retention time gave the highest treatment efficiency with a 92% reduction of COD, 87% SS and 83% UHS and the effluent met both standards for industrial effluent discharge and crop irrigation. This effluent in its undiluted and diluted between 1: 25-1: 200 showed no phytotoxicity and also stimulated rice seed germination based on a germination index when compared with distilled water. Addition of 3% P1 into the raw wastewater either alone or in combination with 0.13% FPE yielded an effluent that passed the standard guidelines within 72 h, while with FPE alone a 96 h retention time was required. Based on morphological, physiological and biochemical properties, the isolate P1 was identified as *Rhodopseudomonas palustris*.**

**Key words:** Fermented plant extracts, hydrogen sulfide, latex rubber wastewater, purple nonsulfur bacteria, response surface method, wastewater treatment.

## INTRODUCTION

Many cooperative rubber sheet factories (CRSFs) exist throughout the Southern and Eastern parts of Thailand (Chaiprapat and Sdoodee, 2007) . Wastewater from the CRSFs consists of both organic and inorganic matters that originate from natural rubber latex and from chemicals used during their processing, including ammonia, formic acid, sodium metabisulfite and sodium sulfide (Kantachote et al., 2005). Lagoons or oxidation ponds are commonly used to treat wastewater from the CRSFs as they are inexpensive to construct and operate. However, these treatment systems require hydraulic retention times of over 20 days to achieve biochemical oxygen demand (BOD) removal efficiency above 80% (Chaiprapat and Sdoodee, 2007) and also cause a major problem by producing hydrogen sulfide ($H_2S$: rotten-egg odor). $H_2S$ not only gives an unpleasant smell but is also dangerous to human's health (Khanal and Huang, 2003).

Anoxygenic phototrophic bacteria, particularly the

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**Abbreviations: BOD,** Biochemical oxygen demand; **COD,** chemical oxygen demand; **CRSFs,** cooperative rubber sheet factories; **DS,** dissolved sulfide; **EC,** electrical conductivity; **EM,** effective microorganisms; **FPE,** fermented pineapple extract; **FPEs,** fermented plant extracts; **GI,** germination index; **HPC,** heterotrophic plate count; **LAB,** lactic acid bacteria; **PNB,** purple nonsulfur bacteria; **RAW,** raw latex rubber sheet wastewater; **RRE,** relative root elongation; **RSG,** relative seed germination; **RSM,** response surface methodology; **SCOD,** soluble chemical oxygen demand; **SS,** suspended solids; **TCOD,** total chemical oxygen demand; **TDS,** total dissolved solids; **TS,** total sulfide; **UHS,** unionized hydrogen sulfide.

Purple Nonsulfur Bacteria (PNB) are widely distributed in aquatic environments and wastewaters (Okubo et al., 2006; Hoogewerf et al., 2003; Zhu et al., 2002). The PNB are attractive organisms for wastewater treatment due to the fact that they can grow both photoautotrophically and photoheterotrophically under anaerobic- light or micro aerobic-light conditions (Imhoff, 2005). In addition, many grow anaerobically in the dark using fermentation processes while others can grow aerobically in darkness using respiration (Imhoff, 2005). It is also of interest that PNB have commercial value as a source of single cell protein for supplementing feed stock or as a biofertilizer (Koh and Song, 2007; Kantachote et al., 2005; Sasikala and Ramana, 1995). Among the PNB, members of the *Rhodopseudomonas*, *Rhodobacter* and *Rhodospirillum genera* are known to eliminate the $H_2S$ odor nuisance from the facultative ponds of waste stabilization ponds by oxidizing it to sulfur or sulfate (Kim et al., 2004; Tadesse et al., 2003; Veenstra et al., 1995).

The technology of using effective microorganisms (EM) was developed during the 1970's by Prof. Teruo Higa, University of Ryukyus, Okinawa, Japan *(*Higa and Chinen, 1998). EM have been used by many Thai people for the following purposes; agriculture, livestock and wastewater treatment. Recently, due to the expense of the EM stock, the use of EM has to a large extent been replaced by normal flora derived from indigenous plants in the form of fermented plant extracts (FPEs), and in addition to plant and vegetable products, other agricultural wastes including animal waste has also been used. The basis of using FPEs to treat wastewater is that they contain various organic acids such as lactic acid and acetic acid, and also beneficial microbes like EM (Kantachote et al., 2009; Prachyakij et al., 2008). FPEs have been used in local communities to treat wastewater such as domestic wastewater, some were successful but some had failed without any scientific explanation. Additionally, there is no published work on combining PNB and FPEs for treating wastewater. In such cases, the FPEs might provide EM or substrates to stimulate the growth of natural PNB as they in turn increase their ability to metabolize organic components, and possibly $H_2S$, in the wastewater.

Effluents from many kinds of treatment systems have been investigated for use in agriculture to improve crop production, increasing water reuse and preventing water pollution (Nagda et al., 2006; Lubello et al., 2004) . The evaluation of any effluent for its suitability for land applications commonly requires a bioassay for its toxicity (Fuentes et al., 2004). Seed germination and root elongation tests have been recommended as techniques for investigating phytotoxicity (Courtney and Mullen, 2009; Hoekstra et al., 2002). Hence, the aims of this work were to investigate the use of a selected strain of PNB (P1), previously involved in treating CRSFs, an isolate P1, and an FPE produced from a fermented pineapple extract, for treating rubber sheet wastewater and also to provide more scientific information on the use of FPEs in wastewater treatment. The effluent's phytotoxicity was determined by seed germination and root elongation tests. The isolate P1 was also identified.

## MATERIALS AND METHODS

### Analytical methods

The following standard methods used in this study are described in APHA, AWWA and WPCF (1998). $BOD_5$ (5 days) was determined by the azide modification method while COD was determined by the dichromate reflux method. Sulfide was measured in 3 forms as Total Sulfide (TS), Dissolved Sulfide (DS) and un-ionized hydrogen sulfide (UHS: $H_2S$ in wastewater) using an iodometric method, whereas sulfate was measured using a turbidimetric method. $H_2S$ in the air space of the treatment bottles was measured using a portable multi gas detector (MX 2100, Oldham, France). Suspended Solids (SS) and Total Dissolved Solids (TDS) were determined after filtration using a standard glass fiber filter; the residue retained on the filter was dried to a constant weight at 103 - 105°C for SS while the filtrate was dried to a constant weight at 180°C for TDS. A pH meter (Seven multi, Metller Toledo, USA) was used to determine the pH while an Electrical Conductivity (EC) meter (Seven multi, Metller Toledo, USA) was used to measure EC. Total acidity presented as lactic acid was determined by a titration method, whereas the actual amounts of lactic acid and acetic acid were determined using gas chromatography according to the method of Yang and Choong (2001). The following elements, Mn, Cu, Zn, Pb and Cd were measured using inductively coupled plasma-optical emission spectroscopy (4300 DV, Perkin-Elmer, Germany).

### Raw rubber sheet wastewater medium

Wastewater was collected from a lagoon pond of a CRSF at Ban Yung Thong in the Hat Yai district, Songkhla province, Thailand. The wastewater was collected as a composite sample for obtaining a representative sample at a deep level roughly 20 - 100 cm from the surface at various positions of the pond and pouring into a 30 liter plastic tank until nearly full to prevent aerobic degradation. Light cannot penetrate to the plastic tank water content as it is made from non translucent plastic to prevent photosynthesis and photooxidation. The collected wastewater was filtered through cheesecloth and used as the raw wastewater medium without supplementing with any nutrients (RAW) and no autoclaving. RAW used for the Response Surface Method (RSM) had the following composition (mg/l); 789 TCOD (total COD), 48.15 SS, 9.11 TS and 3.94 UHS. A different batch of RAW was used in order to confirm the results derived from the RSM analysis and this batch contained (mg/l), 1457 TCOD, 48 SS, 12.22 TS and 3.97 UHS. RAW was stored in a cold room at 6 ± 2°C while not in use.

### Fermented pineapple extract

Pineapple was selected as the raw material for producing the fermented plant extract due to the large quantities that are discarded from the coring process in a pineapple canning factory. FPE was produced in our laboratory following the previous work (Kantachote et al., 2009). The fermentation process was stopped after 2 months and was stored in a cold room until used. The FPE contained (in mg/l); 17400 total acidity, 5300 lactic acid, and 1400 acetic acid while the pH and EC were 3.61 and 3.51 mS/cm, respectively. Only lactic and acetic acids were determined due to both being the main products of the fermentation process (Prachyakij et al., 2008).

**Table 1.** Central composite design along with the actual and predicted values of COD, SS and UHS after treatment by different combinations of inoculant P1 and FPE under microaerobic-light conditions for 72 h.

| Run | FPE ($x_1$) | Inocula ($x_2$) | COD (mg/l) | | SS (mg/l) | | UHS: $H_2S$ (mg/l) | |
|---|---|---|---|---|---|---|---|---|
| Number | Ratio (v/v) | % (v/v) | Actual | Predicted | Actual | Predicted | Actual | Predicted |
| 1 | 1:500 | 2.0 | 267.80 | 264.31 | 18.33 | 19.47 | 1.17 | 1.17 |
| 2 | 1:1500 | 2.5 | 205.40 | 200.68 | 13.33 | 14.39 | 0.75 | 0.69 |
| 3 | 1:750 | 2.5 | 171.60 | 171.06 | 11.67 | 12.36 | 0.68 | 0.67 |
| 4 | 1:750 | 2.0 | 189.80 | 194.61 | 16.67 | 15.50 | 0.86 | 0.83 |
| 5 | 1:750 | 2.5 | 176.80 | 171.06 | 13.33 | 12.36 | 0.62 | 0.67 |
| 6 | 1:1500 | 3.0 | 176.80 | 182.84 | 13.33 | 12.25 | 0.64 | 0.66 |
| 7 | 1:1500 | 2.0 | 221.00 | 219.68 | 16.67 | 16.70 | 0.76 | 0.78 |
| 8 | 1:750 | 2.5 | 176.80 | 171.06 | 11.67 | 12.36 | 0.66 | 0.67 |
| 9 | 1:750 | 2.5 | 161.20 | 171.06 | 13.33 | 12.36 | 0.68 | 0.67 |
| 10 | 1:500 | 3.0 | 205.40 | 209.28 | 11.67 | 11.70 | 0.80 | 0.81 |
| 11 | 1:750 | 3.0 | 158.60 | 148.68 | 8.33 | 9.39 | 0.61 | 0.58 |
| | | | (79.9) | (81.2) | (82.7) | (80.4) | (84.5) | (85.2) |
| 12 | 1:500 | 2.5 | 236.60 | 236.21 | 16.67 | 15.50 | 0.96 | 0.96 |
| 13 | 1:750 | 2.5 | 163.80 | 171.06 | 11.67 | 12.36 | 0.66 | 0.67 |
| $R^2$ | | | 0.9670 | | 0.8850 | | 0.9673 | |
| Adjusted $R^2$ | | | 0.9434 | | 0.8029 | | 0.9440 | |

Numbers in brackets are reduction percentages in the run number 11.

**Inoculum preparation**

The isolate P1, previously isolated and selected in our laboratory, was used because of its remarkable ability to consume $H_2S$. One loopful of P1 from a stab culture was inoculated into a screw cap test tube (20 x 150 mm: 30 ml) containing 28 ml G-5 broth (Ormerod et al., 1961) leaving a small space on the top of the medium to provide microaerobic conditions and the culture was incubated with a light intensity of 3500 lux, generated by a 100 watt incandescent lamp for 48 h. A cell suspension of the isolate P1 was adjusted to an Optical Density (OD) of 0.5 at 660 nm using sterile distilled water as diluent and an inoculum of 10% was added to the sterile RAW medium, pH 7 under the same growth conditions. After that, the culture broth was adjusted to obtain 0.5 $OD_{660\ nm}$ by sterile RAW medium and this containing 4.0 x $10^7$ cfu/ml (7.6 log cfu/ml). A known volume of culture broth was centrifuged at 6000 rpm (Sorvall RC 5C Plus, Du-pont, Delaware, USA) for 15 min and the cell pellet was washed twice by 0.85% NaCl prior to use as an inoculum for treating RAW as described in the section "Experimental design".

**Microbial counts**

In order to know the role of bacteria in wastewater treatment, the following bacterial groups; Heterotrophic Plate Count (HPC), Lactic Acid Bacteria (LAB) and PNB were enumerated on Plate Count Agar, de Man Rogosa and Sharp (MRS) and G5 medium, respectively, by the pour plate method. Plate counts for HPC and LAB were incubated at 30°C for 48 h while PNB plates were incubated in anaerobic jars under light conditions as described above. LAB was also counted because this bacterial group plays an important role in producing the FPEs.

**Experimental design**

To reduce cost of the inoculants isolate P1, FPE was used to stimulate the bacterial growth for treating RAW. Based on our preliminary work, the use of the ratios of FPE to RAW (v/v) between 1: 300 and 1: 1000 promoted growth of PNB like the isolate P1. Additionally, the isolate P1 at an inoculum size of 3.5% (v/v) over 96 h gave the best treatment efficiency by removal of 86% COD, 59% SS and 50% UHS. Hence, varying concentrations of FPE and the isolate P1 were evaluated in optimal conditions using the FPE to stimulate the growth of the isolate P1. For example, the concentration of the isolate P1 was designed in a range of 2.0 - 3.0% because the growth of the isolate P1 should be stimulated by FPE to achieve a cell density of 3.5% inoculum size. RSM has been applied to use for investigating process variables with fewer experimental trials compared to the study of one variable at a time and the Central Composite Design (CCD) is commonly used in the optimal vicinity to locate the true optimum values of the multiple variables (Khuri and Cornell, 1987). Hence, this statistical technique was applied in this study to investigate the optimum combination of inoculant P1 and FPE under different levels of FPE ($x_1$) and inoculants P1 ($x_2$).

The levels of the two independent variables ($x_1$ and $x_2$) were studied at three levels, coded -1, 0, +1 for low ($x_1$, 1:1500 and $x_2$, 2.0%), middle ($x_1$, 1:750 and $x_2$, 2.5%) and high concentrations ($x_1$, 1:500 and $x_2$, 3.0%), respectively. It is well recognized that the key parameters normally used to indicate efficiency of the wastewater treatment are COD and SS (Bitton, 2005) and in this study we also evaluated UHS removal because this is a problem in a lagoon system of CRSFs. Hence, the following parameters; COD, SS and UHS were chosen as dependent variables (response values) to evaluate treatment efficiency of RAW. Table 1 represents the design matrix of a 13-trial experiment. For predicting the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variables and the response. For the two factors, the following equation was used.

$$y = {}_0 + {}_1X_1 + {}_2X_2 + {}_{11}X_1^2 + {}_{22}X_2^2 + {}_{12}X_1X_2 \quad (1)$$

Where y is the predicted response, $_0$ model constant; $x_1$ and $x_2$ are independent variables, $_1$ and $_2$ are linear coefficients; $_{11}$ and $_{22}$ are the quadratic coefficients and $_{12}$ is a cross product coefficient. Design-Expert Software, Stat-Ease, USA, was used to design the experiments, analyze the data and build a quadratic model. The optimum treatment conditions for removal of COD, SS and UHS 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 were expressed by the coefficient of determination, $R^2$.

The above experimental runs were performed in 370 ml glass, flat bottles, containing 300 ml of RAW medium per bottle to achieve microaerobic condition and all bottles were incubated under light condition of 3500 lux for 72 h. The temperature was 33 ± 2 due to the experiment being carried out in an air conditioner control room. Each run was performed in three replicates. The following parameters, pH, COD, SS, TDS, TS, DS, $H_2S$ (both in the air space: $H_2$ S and wastewater: UHS), HPC, LAB and PNB were monitored at the start and at the end of the experiments (72 h). However, only 3 experimental runs were chosen for bacterial counting by consideration from their COD removal. At the end of the experiment, soluble COD (SCOD) was determined on the supernatant of a centrifuged sample (8000 rpm, 15 min). Therefore, the percentage of COD removal in this study and the next experiment was calculated as follows:

% COD removal = ((TCOD – SCOD)/TCOD) x 100

#### Verification test

The selected experimental conditions were verified by varying the retention times (duration of reaction), of 72, 96 and 120 h. RAW without addition of the inoculant P1 and the FPE served as a control set. All parameters as mentioned above were measured according to retention times and at the start of the experiment (t=0) and the following heavy metals; Cu, Mn, Zn, Pb and Cd were monitored at the start and after 96 h of retention time. In addition, BOD was also determined in the verification test at t = 0 and 96 h. Experiments in this study were conducted in triplicate and mean values and Standard Deviations (SD) were calculated.

#### Phytotoxicity test

A bioassay using seed germination is one of the most popular techniques used to assess phytotoxicity (Kapanen and Itavaara, 2001). A rice (*Oryzae* sp.) seed germination test was conducted to investigate the toxicity of the effluent following the methods of Wong et al. (2001). Briefly, 20 ml of the effluent from the verification tests (1:750 = 0.134% FPE and 3% inoculant P1) after 96 h was centrifuged at 10000 rpm (Sorvall RC 5C Plus, Du-pont, Delaware, USA) for 15 min followed by filtration through a sterile 0.45 µm cellulose filter. Five ml of filtrate was diluted in the range of 25 - 1000 then each was poured into a 9 cm diameter Petri dish containing a Whatman no. 1 filter paper. Ten rice seeds after overnight soaking in distilled water were distributed evenly on the filter paper and the Petri dishes were incubated in the dark at room temperature (28 ± 3°C) for 120 h. Distilled Water (DW) was used as a control set for the testing of seed germination (Hoekstra et al., 2002; Fuentes et al., 2004). This experiment was conducted in three replicates. The seed germination bioassay was evaluated by computing the Germination Index (GI), a factor derived from the Relative Seed Germination (RSG) and the Relative Root Elongation (RRE). These parameters were calculated as follows.

RSG (%) = ((number of seeds germinated in effluent) / number of seeds germinated in control) x 100

RRE (%) = ((mean root length in effluent) / mean root length in control) x 100

GI (%) = RSG x RRE / 100

One-way analysis of variance was used followed by the Duncan' multiple comparison test to analyze significant differences between treatments using the SPSS version 10 for Windows.

#### Bacterial identification

The isolate P1 was identified according to Bergey's Manual of Systematic Bacteriology 2nd edition, vol. 2 part C (Imhoff, 2005). Briefly, requirement for vitamins, utilization of organic substrates, sulfide and thiosulfate, whole cell pigment scans were investigated. In addition, optimal growth conditions for pH and temperature were determined due to the aim to use this bacterium as an inoculant for rubber sheet wastewater treatment. All identification methods in this study were previously described by Kantachote et al. (2005). Internal photosynthetic membranes were investigated using a transmission electron microscope; JEM-2010, JEOL, Japan.

## RESULTS AND DISCUSSION

### Efficiency of wastewater treatment using a combination of isolate P1 and FPE

Isolate P1 and the FPE were used to treat raw rubber sheet wastewater by varying their amounts as determined by the CCD. The efficiency of wastewater treatment under microaerobic-light conditions after 72 h of incubation, in each experimental run is shown by the actual and predicted values (Table 1). Data obtained from the experiments (Table 1) were analyzed by linear multiple regression using Design Expert software. The center point in the design was repeated five times for estimation of possible errors (Table 1). As shown in Table 1, the prediction, for run no. 11 gave optimal values of COD, SS and UHS in (mg/l) of 148.68, 9.39 and 0.58 respectively (corresponding to a reduction percentages of 81.2, 80.4 and 85.2 respectively) while the actual values of COD, SS and UHS as (mg/l) were 158.60, 8.33 and 0.61 (corresponding to a reduction percentage of 79.9, 82.7 and 84.5). The application of the response surface method provided the following regression equation 2 for determination of COD.

$$y\ (COD) = 410.09 - 2251.53\ x_1 - 39.32\ x_2 + 10714.63\ x_1^2 \quad (2)$$

where the variables taking their coded values, represent COD value (y) as a function of the FPE ($x_1$) and the inoculant P1 ($x_2$).

The least COD value obtained from the prediction was 148.68 mg/l (run no. 11) (Table 1). The statistical significance of the Equation (2) was checked by the

**Table 2.** ANOVA for response surface quadratic model for chemical oxygen demand (COD).

| A: Analysis of variance table | | | | | |
|---|---|---|---|---|---|
| **Source** | **Degree of freedom** | **Sum of squares** | **Mean square** | **F value** | **p value** |
| Model | 5 | 12464.09 | 2492.82 | 40.99 | < 0.0001 |
| Residual | 7 | 425.67 | 60.81 | | |
| Lack of fit | 3 | 214.75 | 71.58 | 1.36 | 0.3753 |
| Pure error | 4 | 210.91 | 52.73 | | |
| Corrected total | 12 | 12889.76 | | | |

| B: Coefficients of the model | | | | |
|---|---|---|---|---|
| **Variable** | **Coefficient** | **Standard error** | **F value** | **p value** |
| $\beta_0$ | 171.06 | 3.24 | 40.99 | < 0.001 |
| $\beta_1 x_1$ | 17.77 | 3.18 | 31.15 | 0.0008 |
| $\beta_2 x_2$ | -22.97 | 3.18 | 52.04 | 0.0002 |
| $\beta_{11} x_1^2$ | 47.38 | 4.69 | 101.97 | < 0.0001 |
| $\beta_{22} x_2^2$ | 0.58 | 4.69 | 0.02 | 0.9047 |
| $\beta_{12} x_1 x_2$ | -4.55 | 3.90 | 1.36 | 0.2814 |

$R^2$ = 0.9670, Adjusted $R^2$ = 0.9434.

**Table 3.** ANOVA for response surface quadratic model for suspended solids (SS).

| A: Analysis of variance table | | | | | |
|---|---|---|---|---|---|
| **Source** | **Degree of freedom** | **Sum of squares** | **Mean square** | **F value** | **p value** |
| Model | 5 | 82.83 | 16.57 | 10.78 | 0.0035 |
| Residual | 7 | 10.76 | 1.54 | | |
| Lack of fit | 3 | 7.43 | 2.48 | 2.97 | 0.1601 |
| Pure error | 4 | 3.33 | 0.83 | | |
| Corrected total | 12 | 93.59 | | | |

| B: Coefficients of the model | | | | |
|---|---|---|---|---|
| **Variable** | **Coefficient** | **Standard error** | **F value** | **p value** |
| $\beta_0$ | 12.36 | 0.51 | 10.78 | 0.0035 |
| $\beta_1 x_1$ | 0.56 | 0.51 | 1.20 | 0.3087 |
| $\beta_2 x_2$ | -3.06 | 0.51 | 36.44 | 0.0005 |
| $\beta_{11} x_1^2$ | 2.59 | 0.75 | 12.02 | 0.0105 |
| $\beta_{22} x_2^2$ | 0.09 | 0.75 | 0.01 | 0.9112 |
| $\beta_{12} x_1 x_2$ | -0.83 | 0.62 | 1.81 | 0.2208 |

$R^2$ = 0.8850, Adjusted $R^2$ = 0.8029.

*F*-test, and the analyses of variance (ANOVA) for a response surface linear model are summarized in Table 2A. The ANOVA of the linearity of the Equation (2) states that the model significance has a p-value of < 0.0001. The fitness of the model can be checked by the determination coefficient $R^2$ (0.9670) and the value of the adjusted $R^2$ (0.9434) for Equation (2) indicates that 94% of the total variation for COD is attributed to the independent variables and only about 6% of the total variation cannot be explained by the model. The value of the lack-of-fit was not significant (p = 0.3753); a non significant lack of fit is good. The coefficient estimates of Equation (2) and the corresponding p-value are shown in Table 2B. The p-value of variables $_1$ ($x_1$) and $_2$ ($x_2$) were 0.0008 and 0.0002 whereas the p-value of variable $_{11}$ ($x_1^2$) was < 0.0001. Results show that the amount of inoculant P1 ($x_2$) had a big effect on the reduction of the COD value whereas the amount of FPE ($x_1$) produced a big increase in the COD value. In the above analysis the model for SS determination is based on Equation (3) and indicates that the total variation of 80% for SS is attributed to the independent variables and about 20% of the total variation cannot be explained by the model, the adjusted $R^2$ = 0.8029 (Table 3A).

**Table 4.** ANOVA for response surface quadratic model for unionized hydrogen sulfide (UHS: $H_2S$).

**A: Analysis of variance table**

| Source | Degree of freedom | Sum of squares | Mean square | F value | p value |
|---|---|---|---|---|---|
| Model | 5 | 0.301 | 0.0602 | 41.46 | < 0.0001 |
| Residual | 7 | 0.010 | 0.0015 | | |
| Lack of fit | 3 | 0.007 | 0.0024 | 3.26 | 0.1417 |
| Pure error | 4 | 0.003 | 0.0007 | | |
| Corrected total | 12 | 0.311 | | | |

**B: Coefficients of the model**

| Variable | Coefficient | Standard error | F value | p value |
|---|---|---|---|---|
| $\beta_0$ | 0.67 | 0.016 | 41.46 | < 0.001 |
| $\beta_1 x_1$ | 0.13 | 0.016 | 72.64 | < 0.0001 |
| $\beta_2 x_2$ | -0.12 | 0.016 | 62.02 | 0.0001 |
| $\beta_{11} x_1^2$ | 0.15 | 0.023 | 44.15 | 0.0003 |
| $\beta_{22} x_2^2$ | 0.03 | 0.023 | 1.88 | 0.2123 |
| $\beta_{12} x_1 x_2$ | -0.06 | 0.019 | 10.66 | 0.0138 |

$R^2$ = 0.9673, Adjusted $R^2$ = 0.9440.

$$y\,(SS) = 30.73 - 4.49\,x_2 + 584.82\,x_1^2$$

The coefficient estimates of Equation (3) and the corresponding *p*-values are shown in Table 3B. The p-value of variable $_2$ ($x_2$) was 0.0005, whereas the p-value of $_{11}$ ($x_1^2$) was 0.0105. Results show that the inoculant P1 ($x_2$) gave a decrease in SS value while the FPE ($x_1$) produced a big increase in SS value. The model for UHS determination is based on Equation (4) and this equation applies to 94% of the total variation of $H_2S$ in wastewater (UHS) and only 6% of the total variation cannot be explained by the model, adjusted $R^2$ = 0.9440 (Table 4A).

$$y\,(UHS) = 1.80 - 2.53\,x_1 - 0.62\,x_2 + 34.45\,x_1^2 - 1.87\,x_1 x_2 \quad (4)$$

In a similar way, Table 4B demonstrates that the inoculant P1 ($x_2$) decreased the UHS value ( $_2$: $x_2$, *p*-value = 0.0001) and the FPE ($x_1$) produced an increase in the UHS value ( $_1$: $x_1$, *p*-value <0.0001; $_{11}$: $x_1^2$, p-value = 0.0003). The three dimensional response surfaces were generated by the Design Expert (6.0.5) program to study directly the interactions between the two factors tested and to visualize the combined effects of factors on reduction of COD SS and UHS (Figure 1, a1, b1, c1). In the investigated area, the removal of COD, SS and UHS were affected by the inoculant P1 and FPE. Especially, the inoculant P1 played an important role in COD, SS and UHS removal (p = 0.0002, 0.0005 and 0.0001, respectively).

The elliptical shapes of the contour plots, also show if the mutual interactions between the variables were significant or not. Hence, the elliptical contour plots shown in Figure 1 indicate that the mutual interactions (3) between the effect of inoculant P1 and FPE on UHS is significant (p = 0.0138) but on the COD (p = 0.2814) and SS (p = 0.2208) are not significant. However, the plots and contours showed that optimal conditions for the highest removal of COD and SS were in this range. Figure 1 also shows the removal of COD, SS and UHS were remarkably decreased when the inoculant P1 value was increased but the FPE value decreased.

Graphical representations of the response surfaces are presented in Figure 1 to explain visually the effects of the inoculant P1 and the FPE concentrations on the values for COD, SS and UHS. The contour curve in Figure 1 (a2) shows that a decrease of COD value occurred with the inoculant P1 cell density roughly between 2.56- 3% and a concentration of the FPE roughly between 0.084-0.164% gave the lowest value for COD (167 mg/l). The effect of the inoculant P1 on the SS value was similar to its effect on the COD value with the lowest value (11 mg/l) being found at about 2.78- 3% inoculum size for the isolate P1 (Figure 1 (b2)). However, a decrease of SS values was also dependent on the FPE concentration at roughly 0.084 - 0.190%. The contour curve in Figure 1 (c2) shows that the lowest UHS value (0.67 mg/l) was found at the concentrations of inoculum size for the isolate P1 at roughly 2.4 - 3% and FPE at 0.067 - 0.170%. Consequently the optimal levels for the two factors as obtained from the minimum point of the polynomial model were estimated using the Design Expert software. From this the concentrations of the inoculant P1, at 3% (initial cell density = 6.08 log cfu/ml) and the FPE at 1: 730 ( 0.137%) gave predicted values (mg/l) for COD (150), SS (9) and UHS (0.59) with the desirability ($R^2$) being 0.9640. Based on the experimental results from run no. 11 (Table 1) the actual results were

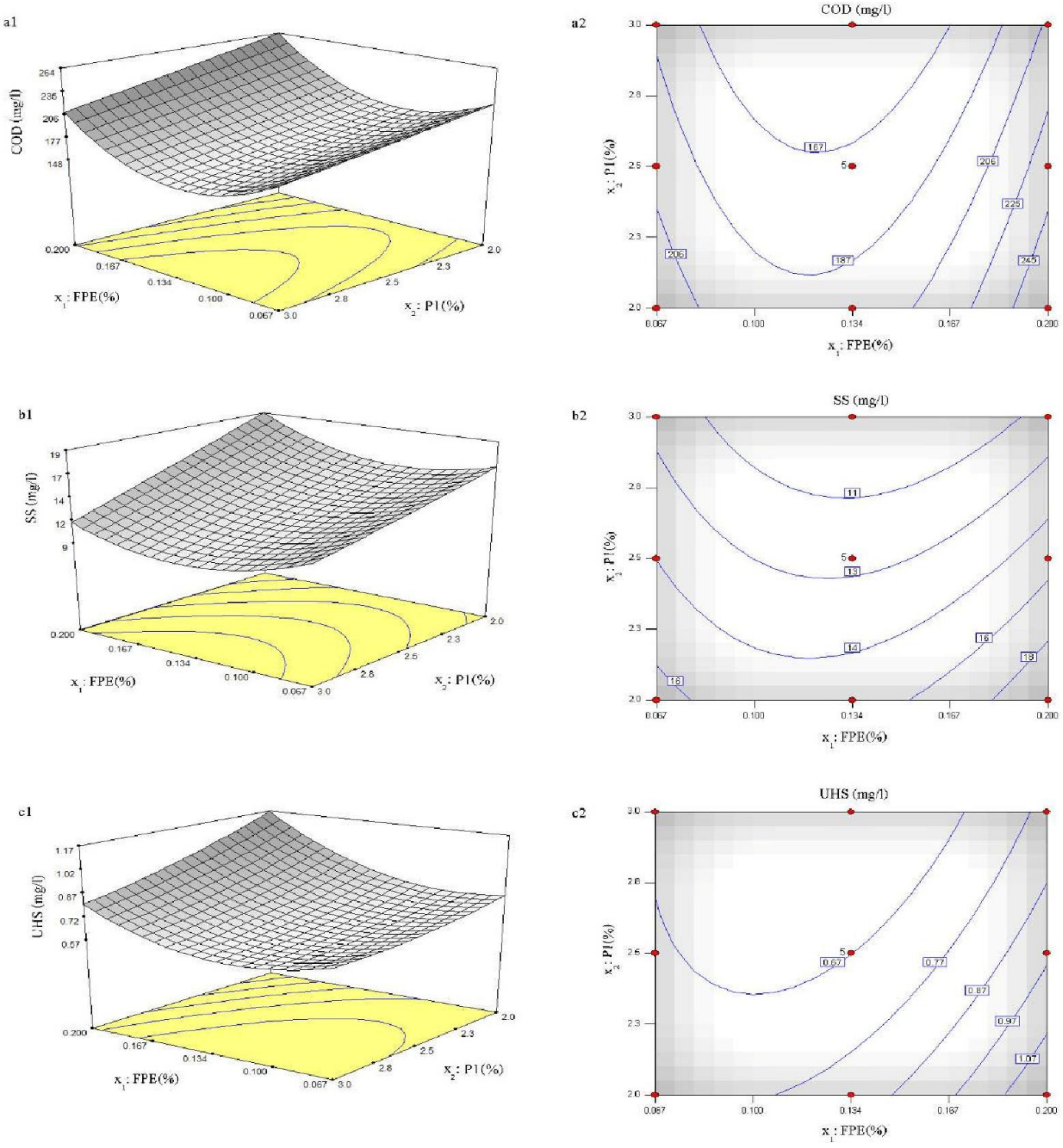


**Figure 1.** Three-dimensional plots and corresponding contour plots of the effect of two variables on removal of COD (a1, a2), SS (b1, b2) and UHS: $H_2S$ (c1, c2) showing the interactions between concentrations of fermented pineapple extract (FPE) and inoculant P1 (P1).

very close to the predicted values for the best treatment efficiency as calculated above, thereby a combination of the inoculants P1 and the FPE at concentrations of 3% and 1: 750 (0.134%), respectively was selected to verify the findings using a different batch of wastewater. This would confirm that these results were widely applicable. The results of the CCD experiments for studying the effects of combinations of two independent variables on

wastewater efficiency show good agreement between the model predictions and the experimental data. Based on the removal efficiencies of COD, SS and UHS, it was found that the inoculant P1 had the biggest influence while the effect of FPE was minor (Table 1 and Figure 1). Each experiment produced the same 50% reduction in the level of $H_2S$ in the air space of the bioreactors from 2 mg/l to 1 mg/l (data not shown). One explanation for this could be that the pH values changed from 7.03 (t = 0) to 7.62 - 7.83. The higher alkaline conditions convert sulfide into its $HS^-$ form (Markl, 1999). Normally PNB growing in rubber sheet wastewater increases the pH of the effluent (Kantachote et al., 2005). An increase of the pH of effluents was also found in this study; however, FPE added at a concentration of 1:500 decreased the pH to be between 7.62 - 7.64 while at a lower concentration 1:750 and 1: 1500 the pH was between 7.75 - 7.83. Undiluted FPE had a 17400 mg/l total acidity (5300 mg/l lactic acid, 1400 mg/l acetic acid with others), so the addition of FPE in the combination tests produced only a very small increase in acidity to the wastewater.

Hence, the reduction of pH due to the FPE may be indirect with the constituent organic acids and other nutrients acting as substrates for HPC to produce acids such as formic acid and acetic acid by fermentation (Bitton, 2005). PNB rapidly utilize these short chain fatty acids by photo-fermentation and the pH rises (Kim et al., 2006). It is well recognized that PNB are highly efficient at metabolizing organic acids and short chain fatty acids to produce $H_2$ and $CO_2$ (Tao et al., 2007; Okubo et al., 2005). During the 72 h of the experiments (run numbers 4, 9 and 11) HPC increased from 6 log cfu/ml at zero time to 7.4-7.8 log cfu/ml while the amount of PNB increased from 5.0 - 6.0 log cfu/ml to 7.4 - 8.9 log cfu/ml (data not shown). Results indicate that PNB could compete the HPC with supporting of FPE addition. Additionally, photosynthetic reduction of UHS also occurred as the $H_2S$ levels decreased with an increase of the inoculant P1. Isolate P1 does use UHS as an electron donor for photosynthesis (Table 6) and this is confirmed by the measured increase of sulfate from 3.9 mg/l at zero time to up to 5.5 mg/l after 72 h. However, in experimental run no. 1 the final sulfate was only 1.0 mg/l. In this case it was assumed that sulfate decreased with increasing amounts of inoculant P1 due to sulfate assimilation by this bacterium (Table 6).

The combinating of a 3% P1 inoculant and a 0.134% FPE (run no. 11) was the best combination set. This could be because these proportions of the two main culture additions provided the best balance of HPC and PNB cultures. This allowed for the minimum amount of sulfate and UHS and reduced COD, SS, UHS and $H_2S$ with the highest efficiency. However, the remaining amounts of both sulfides, particularly $H_2S$ in air (50% of the original) was still significant. Perphaps a longer retention time may be required to completely remove $H_2S$ in the air and eliminate unpleasant smells.

**Verification test for efficient wastewater treatment**

A verification test was conducted using a different RAW sample and extended retention times. Conditions included additions of 0.134% FPE, 3% P1 and a mixture of 0.134% FPE and 3% P1 and results were compared with run no. 11. With the conditions of run no. 11 after 72 h retention, reduction of COD was 84.0% compared with 79.9% in the run no. 11. On the other hand, reductions of SS (79.0%) and UHS (78.0%) in the verifying test were lower than actual values for the run no. 11 (82.7% SS and 84.5% UHS) (Tables 1 and 5). These differences were acceptable that is, < 10%. When the retention times were increased treatment efficiencies increased up to 96 h then no further change was found after 120 h. The results after 72 and 96 h are shown in Table 5. Based on the removal percentages of SS, TDS, COD, sulfate, UHS, $H_2S$, TS and DS over 96 h the treatment efficiency of wastewater in the verification efficiency test was in order of verified set, > 3% inoculum P1 set, > 0.134% FPE set, > control set. Results in this study confirmed that the inoculant P1 had the biggest beneficial effect on the efficiency of RAW treatment and the results of the verification test confirmed the most effective operating conditions and produced the highest numbers of PNB (8.7 log cfu/ml). SS reduction in the verified set was greater than the control set. This should be governed by PNB and HPC as LAB were not detection throughout the experiment. Hence, the growth yield ($Y_{biomass/substrate}$, Yb/s) could be used to explain the above results in that normally HPC (ex. Yb/s 0.4-0.6) (Heijnen and Roels, 1981) have a higher growth yield than PNB (ex. Yb/s 0.28) (He et al., 2010). It means that HPC consumed 100 g of substrate or COD and produced 40 - 60 g biomass while PNB produce only 28 g biomass from 100 g COD.

Addition of the FPE to the strain P1 did have a synergistic action with strain P1 on the treatment process as previously mentioned. Results indicated that under microaerobic-light conditions native PNB did grow in the control set (6.1 log cfu/ml) and the FPE at 0.134% concentration did stimulate growth of the native PNB (7.5 log cfu/ml). In contrast, addition of the FPE had no effect on the numbers of HPC (Table 5). However, addition of the inoculant P1 suppressed the growth of HPC as numbers were reduced from 7.4 log cfu/ml to 6.4 and 6.9 log cfu/ml in sets after adding the inoculant P1. As FPE contains LAB it was expected that LAB would be present in RAW after FPE addition. However, no LAB were detected in any of the treatment sets. This implies that RAW, incubated under microaerobic-light conditions, did not support the growth of LAB from such a low inoculum size present in the FPE. Addition of FPE by itself at a low concentration (0.134%) into RAW is an appropriate technology because it can be easily produced from agricultural waste. However, a significant decrease in the COD, SS and UHS levels observed in the FPE set above was due to native PNB growing under microaerobic-light

**Table 5.** Characteristics of effluents after treatments by FPE, inoculant P1 (P1) and a combination of inoculant P1 and FPE after 72 and 96 h in the verification test.

| Parameter | Percentage reduction | | | | | |
|---|---|---|---|---|---|---|
| mg/l[a] | [RAW] | Control | 0.134% FPE | 3% P1 | Verification test, 0.134% FPE + 3% P1 | |
| | t = 0 | 96 h | 96 h | 96 h | 96 h | 72 h |
| [pH] | 7.06 ± 0.01 | 7.52 ± 0.03 | 7.48 ± 0.02 | 7.49 ± 0.01 | 7.48 ± 0.02 | 7.51 ± 0.02 |
| BOD | nd | nd | nd | nd | 97 | nd |
| COD | 1457 | 58.1 | 73.4 | 81.0 | 92.2 | 84.0 (79.9) |
| SS | 48 | 31.9 | 42.6 | 75.0 | 87.3 | 79.0 (82.7) |
| TDS | 613 | 28.4 | 32.5 | 46.2 | 43.4 | 40.1 |
| Sulfate | 4.8 | 21.3 | 47.5 | 51.1 | 58.3 | 54.2 |
| TS | 12.22 | 43.9 | 47.6 | 60.0 | 70.7 | 65.9 |
| DS | 8.44 | 21.0 | 48.0 | 62.2 | 74.0 | 66.0 |
| UHS | 3.97 | 61.8 | 70.0 | 77.6 | 83 | 78.0 (84.5) |
| $H_2S$ | 3 | 100 | 100 | 100 | 100 | 100 |
| [HPC] (log cfu/ml) | 7.8 ± 0.1 | 7.4 ± 0.1 | 7.3 ± 0.1 | 6.4 ± 0 | 6.9 ± 0.1 | 7.0 ± 0 |
| [LAB] (log cfu/ml) | 0 | 0 | 0 | 0 | 0 | 0 |
| [PNB] (log cfu/ml) | 0 | 6.1 ± 0.1 | 7.5 ± 0.1 | 8.4 ± 0 | 8.7 ± 0 | 8.5 ± 0 |

[a]Unless otherwise stated, nd = not determined, numbers in parentheses are reduction percentages in the run no. 11 of CCD (Table 1). Data in brackets are given as means ± standard deviations (n = 3).

conditions. Thailand is located in the tropical zone with plenty of sunny and warm temperature to support the use of this appropriate technology. Our further work can focus on the study of the efficiency of FPE's operating under anaerobic-dark conditions. The composition of FPE was considered in order to explain the roles of FPE in wastewater treatment as organic acids are major components, followed by some remaining sugar and other soluble elements (Kantachote et al., 2009; Prachyakij et al., 2008). As previously mentioned the optimal level of the FPE in the present study was very low at 0.134%; however, trace compounds and elements might still provide conditions for microbes including PNB to proliferate and help to destroy either organic or inorganic matter, as found in this study.

Why was the optimum amount of FPE so low? We have some preliminary evidence that addition of 2 - 5% FPE into RAW produced a significant reduction in pH levels with increased values of COD, SS and UHS. High concentrations of the FPE under micro aerobic-light conditions completely inhibited growth of PNB and reduced the HPC; however, growth of LAB was stimulated (data not shown). When FPE addition was decreased to 0.5 - 1% it stimulated the growth of PNB and HPC while no LAB were detected in the effluents. However, these concentrations were still not suitable to treat wastewater because growth of HPC was higher than the PNB (data not shown).

In Thailand, EM and FPEs have been used to treat various types of wastewater such as domestic and industrial wastewaters; however, no scientific publication has confirmed their benefits. It is recognized that both EM and FPEs are added in the hope of extending the range of chemical pollutants in the wastewater that can be degraded. Banerji et al. (undated) indicated that introducing EM into wastewater treatment systems helped to reduce the unpleasant odoriferous by-products of this decomposition and also decreased the sludge volume. In contrast in Australia, Szymanski and Patterson (2003) reported that there were not sufficient changes to sludge volume or SS content to indicate a clear benefit from the use of EM in wastewater.

One principle of bioremediation, is to first attempt to stimulate or encourage native flora to destroy any pollutants in preference to augmentation (inoculation of microbes to destroy specific pollutants). In this study the use of augmentation with the right amount of FPE is an appropriate technology to use instead of using bioaugmentation with EM. The optimal amount of the FPE under micro aerobic light conditions stimulates native PNB to treat rubber sheet wastewater although the results showed that an increase in the numbers of inoculant P1 did produce a bigger improvement of the treatment process. This may be that the inoculant P1 reduced the $H_2S$ toxicity for other microbes in the wastewater treatment. This is in agreement with Wang et al. (2009) who reported that bioaugmentation with the nicotine degrading bacterium; strain HF-1, resulted in a higher efficiency to treat tobacco wastewater due nicotine toxicity being minimized for other organisms in the wastewater system. In order to achieve the same treatment efficiency as occurred by adding another batch

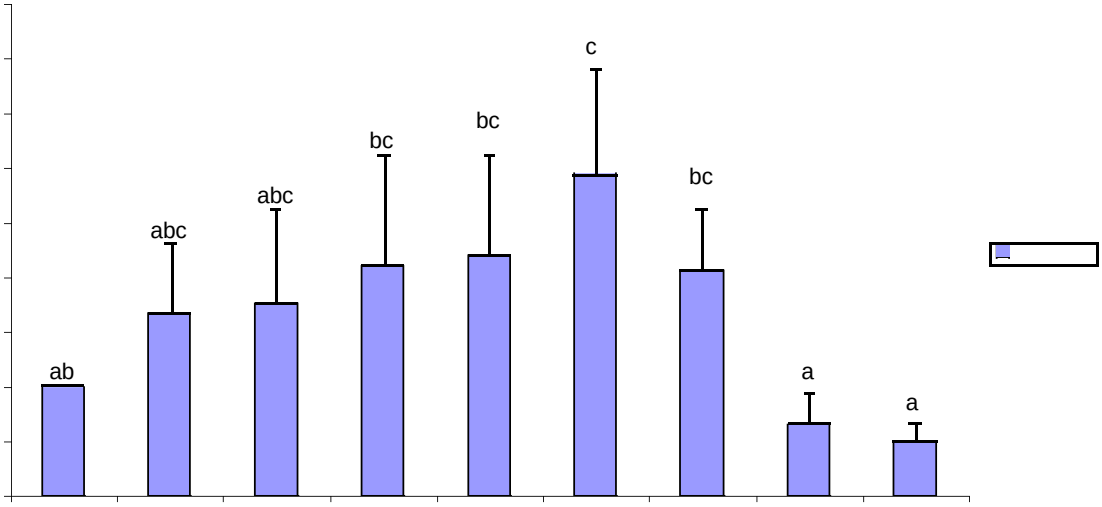


**Figure 2.** Percentage of germination index of rice seeds by varying concentrations of the effluent of rubber sheet wastewater, which was treated using 0.134% FPE and 3% inoculant P1 for 96 h retention time. Data are given as means ± standard deviations (n = 30, 3x10). Lowercase letters above bars, when different, indicate significant differences between means ($p < 0.05$). DW = distilled water, Undiluted = Undiluted effluent.

of P1 inoculant, one further addition of the FPE at a later time may have a similar effect on the natural PNB and avoid the need for addition of organisms such as the isolate P1. Hence, our future work will investigate that possibility.

**The possible use of the effluent as irrigation water**

Standard guidelines for the use of treated wastewater set by the Industrial Factory Department and Irrigation Department, Thailand are (in mg/l; 120 - 400 COD, 20 BOD, 30 - 50 SS and 1 UHS, 5 Mn, 1-2 Cu, 5 Zn, 2 Pb and 0.03 Cd while the pH should be between 5.5 - 9.0 or 6.5 - 8.5). All our treatment sets under micro aerobic-light conditions passed all standard sets but with different quality effluents (Table 5). In sets that were treated with inoculant P1, effluents met both standard sets after 72 h of retention time while the FPE set by itself took a longer time (96 h) to pass with a lower quality of effluent. The average level of heavy metals in control sets and treatment sets were (in mg/l): 0.08 Mn, 0.03 Cu, 0.01 Zn, < 0.01 Pb and 0.001 Cd; all within acceptable levels for discharge. However, the control set did not pass standard guidelines due to the amounts of COD, SS and UHS that all exceeded the set standards.

A retention time of 96 h with the combination treatment (verified test) gave the most efficient treatment by reductions of 92.2% COD (97% $BOD_5$), 87% SS and 83% UHS and 100 $H_2S$ in air (corresponding to mg/l of: 114 COD, 18 BOD, 6 SS, 0.68 UHS and 0 $H_2S$ in air). Indeed in the present study, TCOD and SCOD were used to calculate the removal percentage of COD; however, in general SCOD still has residual degradable and non- or slowly biodegradable influent substrates; soluble microbial product, particularly the soluble fraction of the effluents from the anaerobic treatment systems (Barker et al., 1999). That is why the removal percentage in the form of BOD was higher than that in the form of COD.

Results in this study were similar with Kantachote et al. (2005) who reported that *Rhodopseudomonas* sp. DK6 with the same conditions took 96 h to reduce 90% of both COD and BOD in raw latex rubber sheet wastewater. However, strain DK6 cannot use $H_2S$. PNB have been used by other research workers in laboratory scale experiments that is, mixed cultures of *Rubrivivax gelatinosus* SS51 and SY40 were used with an optimal ratio of 14:7 and they reduced the COD of concentrated latex wastewater by 57% (Choorit et al., 2002). In another case *R. gelatinosus* was used to treat slaughterhouse wastewater under anaerobic light conditions for 10 days and reduced COD by 91% (Ponsano et al., 2008).

A phytotoxicity test and stimulating effects of the effluent obtained from the verification test, with 0.134% FPE and 3% inoculant P1 after 96 h, was assessed using the GI of rice seeds (Figure 2). The effluent at 1: 100 gave the best value for GI (295) while the GI values of undiluted effluent and the rest of the diluted effluents were not significantly different. Results showed that there was no phytotoxicity as the samples gave similar or larger GI values than for DW (Amaral et al., 2005). GI has been proven to be a very sensitive index of phytotoxicity

**Table 6.** Characteristics of the isolate P1 based on morphological, biochemical and physiological properties under anaerobic-light conditions.

| Characteristic | Isolate P1 | *R. palustris*[a] |
|---|---|---|
| Color of culture (anaerobic-light) | Red | Red |
| Color of culture (aerobic-dark) | White | White to pink |
| Cell shape | Short rod | Rod |
| Gram staining | Negative | Negative |
| Motility | Motile | Motile |
| Spectrum absorption (nm) | 333, 376, 590, 805 and 868 | 375, 468, 493, 520, 545, 589, 802 and 860 - 875 |
| Photosynthetic pigments | Bacteriochlorophyll *a* | Bacteriochlorophyll *a* |
| Internal membrane system | Lamellae | Lamellae |
| Biotin | No requirement | Some strains require |
| Nicotinic acid | No requirement | na |
| p-aminobenzoic acid | Requirement | Requirement |
| Thiamine hydrochloride | No requirement | na |
| pH (growth) | 5-8.5 | 5.5-8.5 |
| Optimum pH | 6.5-7.5 | 6.9 |
| Temperature (25-40ëC) | growth | na |
| Optimum temperature (ëC) | 30-35 | 30-37 |
| Acetate | + | + |
| Benzoate | + | + |
| Butyrate | + | + |
| Citrate | + | +/- |
| Ethanol | + | +/- |
| Formate | + | + |
| Fructose | - | +/- |
| Gelatin | - | - |
| Glucose | - | +/- |
| Glutamate | + | + |
| Glycerol | + | + |
| Lactate | + | + |
| Malate | + | + |
| Mannitol | + | +/- |
| Methanol | + | +/- |
| Propanol | + | + |
| Propionate | + | + |
| Pyruvate | + | + |
| Sorbitol | + | + |
| Succinate | + | + |
| Tartrate | - | - |
| Sulfide | + | + |
| Thiosulfate | + | + |
| Sulfate assimilation | + | + |

+ = utilized, - = not utilized, na = not applicable and a = Code from Imhoff (2005).

and the results showed that the effluent stimulated seed germination probably by providing nutrients. Assessment of the effluent was considered based on results in Table 5, heavy trace metal amounts and Figure 2. The overall assessment is that the rubber sheet effluent may be directly used as irrigation water without any requirement for dilution and this will be further investigated.

**Bacterial identification**

Table 6 shows the morphology and physiology of the isolate P1. This bacterium was a motile, gram negative, short rod, and multiplied by budding. Internal photosynthetic membranes, appeared as lamella, lying parallel to the cytoplasmic membrane. Growth under

anaerobic-phototrophic conditions produced cell suspensions that were red and the absorption spectra of a living cell suspension showed peaks for bacteriochlorophyll *a* (333, 376, 590, 805 and 868). Therefore, the isolate P1 was identified as *Rhodopseudomonas* sp. (Imhoff, 2005) . Under anaerobic conditions in the light, this bacterium was able to use various organic compounds and inorganic compounds (sulfide/thiosulfate) as electron donors and required only p-aminobenzoic acid as a growth factor (Table 6). The isolate P1 therefore had a closest relationship to *Rhodopseudomonas palustris* although some differences were observed that is, short rod shaped cells in the isolate P1 but rod shaped in the reference strain as shown in Table 6 (Imhoff, 2005).

## Conclusions

One of the new technologies being proposed to enhance rubber sheet wastewater treatment efficiency is the use of FPEs. In this work we demonstrate that a small amount of FPE stimulated the growth of native PNB including *R. palustris* P1 under microaerobic/anaerobic light conditions and this large increase in PNB produced an effluent quality that complied with the national standards for discharge into natural areas and for potential to use for irrigation purposes.


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