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Bioaugmentation of latex rubber sheet wastewater treatment with stimulated indigenous purple nonsulfur bacteria by fermented pineapple extract

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ABSTRACT

Background: Treating latex rubber sheet wastewater often leads to the generation of a rotten-egg odor from toxic H_2S. To increase the treatment efficiency and eliminate H_2S, purple nonsulfur bacteria (PNSB), prepared by supplementing non-sterile rubber sheet wastewater (RAW) with fermented pineapple extract (FPE), were used to treat this wastewater under microaerobic light conditions. The following 3 independent variables: chemical oxygen demand (COD), initial pH and FPE dose were investigated using the Box–Behnken design to find optimal conditions for stimulating the growth of indigenous PNSB (PNSBsi).
Results: The addition of 2.0% FPE into RAW, which had a COD of 2000 mg L^{-1} and an initial pH of 7.0, significantly decreased oxidation reduction potential (ORP) value and stimulated PNSBsi to reach a maximum of 7.8 log cfu mL^{-1} within 2 d. Consequently, these PNSBsi, used as inoculants, were investigated for their ability to treat the wastewater under microaerobic light conditions. A central composite design was used to determine the optimal conditions for the wastewater treatment. These proved to be 7% PNSBsi, 0.8% FPE and 4 d retention time and this combination resulted in a reduction of 91% for COD, 75% for suspended solids, 61% for total sulfide while H_2S was not detected. Results of abiotic control and treatment sets indicated that H_2S was produced by heterotrophic bacteria and it was then effectively deactivated by PNSBsi.
Conclusions: The stimulation of PNSB growth by FPE under light condition was to lower ORP, and PNSBsi proved to be effective for treating the wastewater.

1. Introduction

Since their founding in years 1994 and 1995 by the central government of Thailand, many of these small cooperative rubber sheet factories (CRSFs) are still functioning throughout Thailand [1]. One serious problem arising from the treatment of their wastewater is the associated noxious smell of rotten-egg gas (hydrogen sulfide, H_2S). Open lagoons or natural oxidation ponds used for this treatment now are standard in these factories [1,2]. It is well recognized that H_2S is a toxic gas which at high level is dangerous to human health and at low level causes nuisance odor [3,4]. Devising a means to solve this problem by removing H_2S requires the cooperative efforts of engineers and microbiologists. It is well recognized that phototrophic bacteria, especially purple nonsulfur bacteria (PNSB) are well capable of treating wastewaters due to their versatile metabolic pathways. They have a capability to grow as both photoautotrophs and photoheterotrophs under conditions of anaerobic or microaerobic-light, and chemoheterotrophs under conditions of anaerobic or aerobic-dark [5,6,7,8]. Some members of the PNSB such as *Rhodopseudomonas*, *Rhodobacter* and *Rhodospirillum* are known to reduce the H_2S odor from facultative waste stabilization ponds [9,10].

In our previous work, a combination of fermented pineapple extract (FPE) as growth stimulator and a selected purple nonsulfur bacterium *Rhodopseudomonas palustris* P1 showed a high efficiency for treating latex rubber sheet wastewater to meet the Thai standard guidelines within only 3 d. It was also found that the mix ratio of bacterial culture as inoculum had a great influence during the reaction period [2]. It is, however, difficult for CRSFs to have an access to the functional PNSB

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inoculants to treat their wastewater. It was also observed that FPE, when added at an optimal amount, *i.e.* 0.13%, facilitated the treatment of non sterile latex rubber sheet wastewater (RAW). This relatively small amount of FPE stimulated the growth of native/indigenous PNSB under conditions of anaerobic or microaerobic-light by providing simple substrates such as acetate and lactate that were easily consumed by PNSB [2,11,12]. This stressed the feasibility of preparing the PNSB inoculant culture with the help of a simple and easy to find FPE. However, successful bioaugmentation depends on the use of the effective inoculants, either single culture or consortium, which are usually well adapted to each individual wastewater. This is mainly related to the survival and degrading ability of microorganisms introduced to a wastewater system as dictated by various biotic and abiotic factors within the system. Therefore, autochthonous bioaugmentation is attractive to use for the enhancement of efficiency of wastewater system due to the fact that foreign microbes when used as inocula, are unable to avoid competition with normal flora.

The amount of added FPE into the wastewater medium decreases initial pH and increases chemical oxygen demand (COD) value, thereby optimum initial pH and COD values should be investigated as these are the key factors for stimulating growth of indigenous or native PNSB (PNSBsi) to make good inocula. The response surface methodology (RSM) which is a collection of mathematical and statistical techniques for the modeling and analysis of multivariate problems has been used to optimize the desired outputs [13]. This methodology is more practical as it arises from experimental methodology that includes interactive effects among the variables and eventually, it depicts the overall effects of the parameters in the process [14]. In the process of the wastewater treatments, RSM was reportedly effective in evaluating the interactive effects of operating parameters [15,16].

The aims of this study were firstly to stimulate indigenous PNSB (PNSBsi) in a RAW lagoon with FPE for re-inoculation in the treatment of rubber sheet wastewater, and secondly to determine the optimal values of the factors affecting the growth of indigenous PNSB and their efficiency to treat the rubber sheet wastewater.

2. Materials and methods

2.1. Rubber sheet wastewater medium

Rubber sheet wastewater was collected from a lagoon of a CRSF at Pichit suburb, Songkhla Province, Thailand. The collected wastewater was filtered through cheesecloth into a 25 liter non-transparent plastic tank and stored in a cold room at 6 ± 2°C until use. The wastewater without autoclaving was supplemented with 0.05% NH_4Cl as a supplementary nitrogen source to allow a reasonable growth of PNSB (based on our preliminary work). This medium was named RAW since the non-sterile rubber sheet wastewater was used.

2.2. Monitoring parameters

The methods used in this study are described in the Standard Methods [17]. All effluent samples including RAW were placed in a cold room for 2 h to allow sedimentation. The supernatant (clear liquid near the water surface) was sampled for the measurement of settleable COD by the dichromate reflux method. Sulfide was measured in 3 forms, as total sulfide (TtS), dissolved sulfide (DsS) and un-ionized hydrogen sulfide (UHS: H_2S) using the iodometric method, while sulfate was measured using the turbidimetric method. A portable multi gas detector (MX 2100, Oldham, France) was used to measure H_2S in headspace of the treatment bottles. In this work, volatile fatty acids (VFAs) were analyzed by distillation method. A pH meter (Seven multi, Metller Toledo, USA) was used to measure pH and electrical conductivity (EC). The oxidation–reduction potential (ORP, Eh) probe (La Motte, USA) was used to measure the Eh values and the data were recorded after obtaining a constant value. The probe was checked frequently in a quinhydrone buffer solution following the method described by the manufacturer. Total acidity was determined by a titration method and presented as a 'lactic acid amount'. The actual amounts of lactic and acetic acids were determined using gas chromatography according to the method of Yang and Choong [18]. Viable cells count of PNSB was performed on GM (glutamate–malate agar) and incubated in anaerobic light conditions for 5 d [5] whereas yeasts, heterotrophic plate count (HPC) and lactic acid bacteria (LAB) were enumerated on potato dextrose agar (PDA), plate count agar (PCA) and de Man Rogosa and Sharp (MRS) agar for 3 d. Both LAB and yeasts were counted because these microbial groups play important roles in the production of the FPE.

2.3. Fermented pineapple extract

FPE was produced in our laboratory according to the method described by Kantachote et al. [19]. The fermentation process lasted for 2 months. At that time, it composed of 1.90% total acidity, 0.58% lactic acid and 0.15% acetic acid with a pH of 3.61 and an EC value of 3.51 mS cm^{-1}. In addition to the nutrients, the population of HPC, yeasts and LAB were estimated in the region of 10^6 cfu mL^{-1} for each group. FPE was kept in a cold room until used.

2.4. Experimental design for studying the stimulation of indigenous PNSB from a lagoon with FPE to make an inoculum for treating RAW

RSM using the Box–Behnken Design (BBD) was chosen because relatively fewer experimental combinations of the variables were required to estimate potentially complex response functions with an acceptable reliability [20]. The BBD was used to optimize three input independent variables on the response of the amount of PNSBsi in RAW. The significant variables: initial COD, initial pH and amount of FPE were selected as the critical independent variables and designated as X_1, X_2 and X_3, respectively. The low, middle and high levels of each variable were coded as -1, 0, +1, respectively (Table 1) with the design matrix of a 17-trial experiment, established using a Design Expert 6 software (Stat Ease Inc. Minneapolis, USA). Variations on the values of each independent variable were designed according to the results of our preliminary work. The behavior of the system was explained by the following quadratic equation model.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad \text{[Equation 1]}$$

where Y is the predicted response; β_0 is the intercept; β_1, β_2 and β_3 are the linear coefficients, β_{11}, β_{22} and β_{33} are the square coefficients and β_{12}, β_{13} and β_{23} are the interaction coefficients. To predict the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variables and the response values (dependent variables; PNSBsi and ORP). The optimal conditions for stimulating PNSB (PNSBsi) were obtained by solving the regression equations and also by analyzing overlay interaction plots.

The experimental runs were performed in 120 mL serum glass bottles, containing 100 mL of RAW medium per bottle to achieve microaerobic conditions and all serum bottles were incubated under tungsten light conditions of 3500 lx for 2 d. A Denki light meter (model DK-211) was used to measure light intensity. Three replicates were set for each experimental run. The following parameters: PNSB, COD, pH and ORP, were monitored at the start and at the end of the experiment (2 d incubation). The ORP was also monitored as a dependent variable as it might help explain the role of FPE on stimulating PNSB growth. To validate the optimum conditions of the 3 independent variables, confirmatory experiments were carried out under microaerobic light conditions. To determine the effect of FPE on PNSBsi growth in RAW, there were 2 sets of verified tests, one with sterile and one with non-sterile FPE, beside a control set without FPE

Table 1
The BBD matrix for the real variables along with the actual and predicted responses of the ORP and stimulated indigenous PNSBsi values in RAW under microaerobic light conditions for 2 d after adding FPE.

Run no.	Code of variables and levels			ORP (mV)		PNSBsi (log cfu mL^{-1})	
	X_1	X_2	X_3	Actual	Predicted	Actual	Predicted
1	1500	8	2	-188.5	-186.4	7.09	7.25
2	2000	8	1.5	-203.9	-205.12	7.53	7.52
3	2000	7	2	-230.7	-228.2	7.79	7.8
4	2000	7	2	-226.7	-228.2	7.79	7.8
5	2500	7	2.5	-205.3	-204.45	7.53	7.67
6	2500	7	1.5	-190.7	-190.13	7.2	7.2
7	2500	8	2	-188.9	-188.22	6.94	6.95
8	1500	7	1.5	-177.1	-177.95	7.19	7.05
9	2000	8	2.5	-206.9	-208.46	7.46	7.3
10	1500	7	2.5	-173	-173.5	7.1	7.1
11	2000	7	2	-227.3	-228.2	7.83	7.8
12	2000	7	2	-228.6	-228.2	7.8	7.8
13	2500	6	2	-198.7	-200.8	6.94	6.78
14	2000	7	2	-227.7	-228.2	7.81	7.8
15	1500	6	2	-158.8	-159.48	5.79	5.78
16	2000	6	1.5	-197.9	-196.34	6.06	6.21
17	2000	6	2.5	-204.1	-202.88	6.94	6.96

X_1: initial chemical oxygen demand, COD (mg L^{-1}), X_2: initial pH; X_3: FPE (%, V/V).

addition. Parameters: pH, EC, ORP, VFAs, HPC, LAB and PNSBsi, were monitored at the beginning and end of the 2-d incubation. These experiments were conducted in triplicate and ANOVA (Tukey HSD post-hoc test) was used to analyze data in this verification test with the significance level of 0.05.

2.5. Experimental design for studying the treatment of rubber sheet wastewater using stimulated PNSB inoculum and FPE

To determine the optimum conditions for the treatment of CRSF wastewater, the key factors most likely to affect the efficiency were investigated: PNSBsi numbers (X_1), FPE concentrations (X_2) and retention times (RT) (X_3). To fit a second order response surface, central composite design (CCD) is used. The levels of these 3 independent variables (X_1, X_2 and X_3) were studied at 5 coded levels; -1.682, -1, 0, +1 and +1.682 establishing the design matrix for a 20 trial experiment. Variable levels were selected based on the results obtained from preliminary work. Design Expert (version 6, Stat Ease Inc. Minneapolis, USA) was used for experimental design and statistical analysis. The quadratic model equation [Equation 1] was also used to explain the behavior of the system.

The optimum wastewater treatment conditions by PNSBsi were obtained by solving the regression equations and also by analyzing the response surface overlay contour plots. The quality of the fit of the model equations was expressed by the coefficient of determination, R^2, while regression coefficients were used to generate a contour map of the regression model. All experimental runs were conducted in 120 mL serum glass bottles and incubated in microaerobic light conditions previously described, and the following parameters were determined: COD, pH, PNSB, suspended solids (SS) and TtS. The effectiveness of the treatments was evaluated from the response values (COD, SS and TtS).

The results from the CCD were selected for obtaining the optimal percentage of FPE, inoculum size of PNSBsi and RT. The optimum conditions based on calculation from the CCD were confirmed. In order to explain the roles of PNSBsi, FPE, and a combination of starter cultures plus FPE, the experimental design was as follows: RAW (native control), RAW with optimal amount of FPE, RAW with optimal dose of PNSBsi, RAW with optimal amounts of both FPE and starter PNSBsi, and the other 4 sterile sets of each corresponding treatment were also conducted in the same way. After 4 d, the efficiency was assessed by the loss of COD, SS, TDS, sulfate ion (SO_4^{2-}), TtS, DsS, UHS (H_2S in wastewater) and H_2S in the head space. The amounts of HPC, LAB and PNSB were also counted. All data in this experiment were analyzed by ANOVA (Tukey HSD post-hoc test) with a significance level of 0.05.

3. Results

3.1. Stimulation of indigenous PNSB from a lagoon with FPE for use as inoculums

The experimental results of BBD were analyzed by regression analysis consisting of the linear, quadratic and interaction effects which generated the following regression equations with increasing

Table 2
ANOVA of the quadratic models for the values of stimulated indigenous PNSBsi and ORP in RAW incubated under microaerobic light conditions for 2 d after adding FPE.

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*	R^2
PNSBsi value						
Model	5.2744	9	0.5860	24.1706	0.0002	0.9688
Residual	0.1697	7	0.0242			
Lack of fit	0.1411	3	0.0470	6.5775	0.0502	Not significant
Pure error	0.0286	4	0.0072			
Corrected total	5.4441	16				
ORP value						
Model	7296.50	9	810.72	197.89	<0.0001	0.9961
Residual	28.68	7	4.10			
Lack of fit	19.05	3	6.35	2.64	0.1858	Not significant
Pure error	9.62	4	2.41			
Corrected total	7325.18	16				

PNSBsi and reducing ORP values as a function of COD (X_1), pH (X_2), and FPE (X_3).

$$Y_{PNSBsi} = -51.48 + 0.01X_1 + 12.02X_2 + 4.46X_3 - \left(1.64 \times 10^{-6}\right)X_1^{\,2} - 0.67X_2^{\,2} - \left(6.51 \times 10^{-4}\right)X_1X_2 - 0.48X_2X_3 \quad \text{[Equation 2]}$$

$$Y_{ORP} = 1421.52 - 0.61X_1 - 240.77X_2 - 156.34X_3 + \left(1.22 \times 10^{-4}\right)X_1^{\,2} + 13.89X_2^{\,2} + 44.43X_3^{\,2} + 0.019X_1X_2 - 0.018X_1X_3 \quad \text{[Equation 3]}$$

Population of PNSBsi (Y_{PNSBsi}) and value of ORP (Y_{ORP}) at specific combination of three independent variables could be predicted by substituting the corresponding values of each variable in [Equation 2 or Equation 3], respectively. The predicted values from both equations for PNSBsi and ORP of each experimental run are presented in Table 1. ANOVA for the response surface model is summarized in Table 2. The statistical significance of model equation was evaluated by *F*-test. As the greater *F*-value indicates that the factors explain adequately the data variation about its mean and the estimated factors are real. In this study, *F*-values of 24.17 for PNSBsi model and 197.89 for ORP model, signify that the models were significant (Table 2). ANOVA at the low probability value ($P > F$) also demonstrated that the quadratic model of PNSBsi value ($P > F = 0.0002$) and ORP value ($P > F < 0.0001$) were significant. However, a lack of fit *F*-values of 6.5775 and 2.64 of these models suggested that it was not significantly relative to the pure error. Non-significant lack of fit would be appropriate for this experiment. These are 5% and 19% chances that a lack of fit *F*-value could occur due to the experimental errors in PNSBsi and ORP models, respectively. Based on these results, PNSBsi model was chosen to produce the response surface contour plots for the determination of optimal conditions. In addition, the coefficients of variation (R^2) were 0.9688 for PNSBsi population and 0.9961 for ORP value indicating a high correlation between the observed and predicted values from models [Equation 2 and Equation 3]. Thus, both equations could be used for predicting the amount of PNSBsi population and ORP value under conditions varied with only three independent variables in the experimental range.

The regression coefficient in the response surface model for the linear, quadratic and interaction effects of the variables are presented along with the *P*-value in Table 3. The linear effect of COD ($P < 0.05$), pH ($P < 0.01$) and FPE ($P < 0.05$) was statistically significant for increasing PNSBsi population, whilst, the linear effect of COD ($P < 0.01$), pH ($P < 0.01$) and FPE ($P < 0.05$) was also statistically significant for reducing ORP value. Both regression equations show significant

Table 3
Estimated regression coefficient and corresponding *P*-value for the values of stimulated indigenous PNSBsi and ORP in RAW incubated under microaerobic light conditions for 2 d after adding FPE.

Term	PNSBsi value		ORP value	
	Coefficient	*P*-value	Coefficient	*P*-value
Constant	-51.47809	0.0002**	1421.52	<0.0001**
COD (X_1)	0.010655	0.0141*	-0.61	<0.0001**
pH (X_2)	12.02	0.0001**	-240.77	0.0015**
FPE (X_3)	4.46422	0.0487*	-156.34	0.0107*
COD × COD	-1.64E-06	0.0010**	0.00	<0.0001**
pH × pH	-0.66787	<0.0001**	13.89	<0.0001**
FPE × FPE	-0.42108	0.2079	44.43	<0.0001**
COD × pH	-6.51E-04	0.0041**	0.02	<0.0001**
COD × FPE	4.17E-04	0.2228	-0.02	0.0024**
pH × FPE	-0.47868	0.0180*	1.60	0.4552

* $P < 0.05$.
** $P < 0.01$.

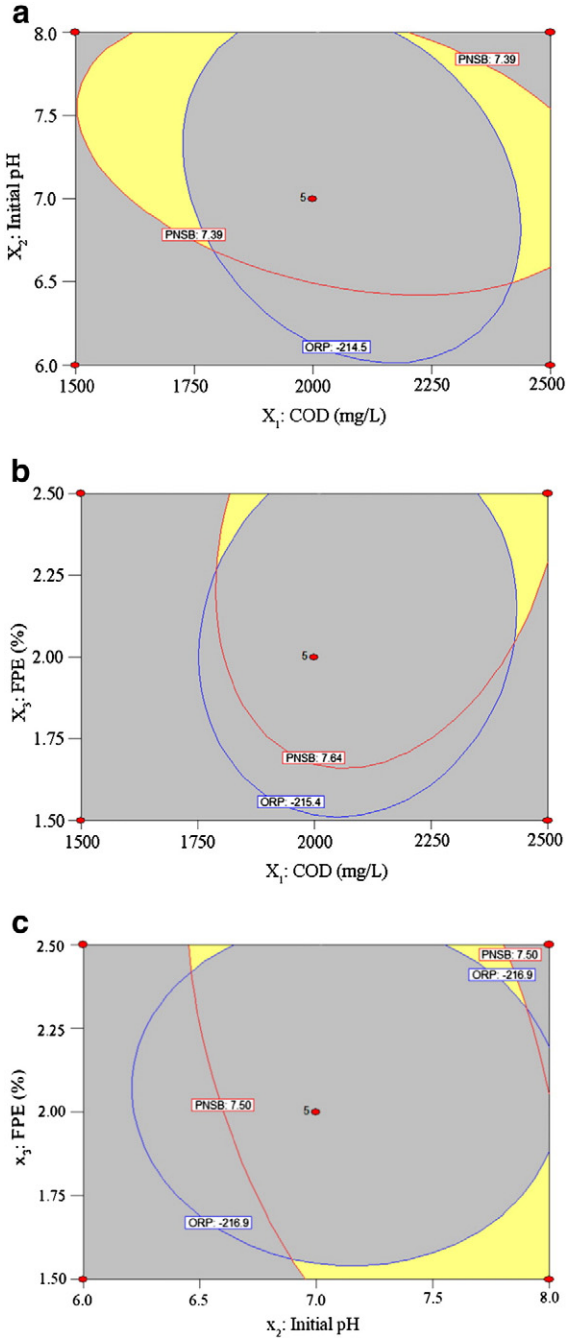

Fig. 1. The overlay interaction plots of two variables: chemical oxygen demand (COD) and initial pH (a), COD and fermented pineapple extract (FPE) (b), initial pH and FPE (c) on oxidation–reduction potential (ORP) value and stimulating purple nonsulfur bacteria (PNSB) growth in non-sterile rubber sheet wastewater (RAW) under microaerobic light conditions over 2 d incubation.

effects of interacting relationships between the COD *vs.* initial pH on the PNSBsi population ($P < 0.01$) and the ORP values ($P < 0.01$). However, a significant effect of interacting relationships between the initial pH *versus* the FPE concentration was observed only on the PNSB population ($P < 0.05$) while a significant effect of the interacting relationship between the COD *versus* the FPE concentrations was found only on the ORP ($P < 0.01$).

The interacting relationship between 2 factors using the overlaid contour plot is presented by designation of another factor as zero level or mid-point. A 2% FPE was designed for the consideration of the interacting relationship between the COD and the initial pH on PNSBsi

Table 4
Verification experiments of the stimulation of PNSB by adding sterile FPE and non sterile FPE to RAW and incubating under microaerobic-light conditions for 2 d.

Parameters	Treatments			
	RAW, 2000 mg L^{-1} COD, pH 7, t = 0	Control No addition of FPE	Verify test (2.0% FPE)	Verify test (Sterilized 2.0% FPE)
pH	7.0 ± 0.0[a]	7.5 ± 0.0[c]	7.2 ± 0.0[b]	7.2 ± 0.0[b]
EC (mS cm^{-1})	2.69 ± 0.05[b]	2.47 ± 0.09[a]	2.78 ± 0.05[b]	2.97 ± 0.06[c]
ORP (mV)	73 ± 1.1[d]	-78 ± 1.7[c]	-201 ± 0.4[a]	-163 ± 0.1[b]
VFAs (mg L^{-1})	53 ± 6[d]	47 ± 6[c]	23 ± 6[a]	37 ± 6[b]
HPC (log cfu mL^{-1})	5.42 ± 0.07[a]	5.88 ± 0.08[c]	5.27 ± 0.09[a]	5.65 ± 0.08[b]
LAB (log cfu mL^{-1})	0	0	0	0
PNSBsi (log cfu mL^{-1})	0[a]	0[a]	8.13 ± 0.06[c]	7.69 ± 0.07[b]

Different lowercase letters in each row indicate significant differences ($P < 0.05$).
EC: electrical conductivity; FPE: fermented pineapple extract; HPC: heterotrophic plate count; LAB: lactic acid bacteria; ORP: oxidation reduction potential; PNSB: purple nonsulfur bacteria; PNSBsi: stimulated indigenous purple nonsulfur bacteria; RAW: non sterile rubber sheet wastewater; VFA: volatile fatty acids.

and ORP. It was found that the increase of the PNSBsi population with the decrease of ORP value relied on the following ranges of the examined variables: COD 1750–2400 mg L^{-1} and initial pH 6.5–8.0 (Fig. 1a). In a similar manner, the initial pH of 7 was used to consider the effect of COD and FPE showing that PNSBsi increased with the decrease of ORP when the COD was in the range of 1800–2400 mg L^{-1} and FPE was between 1.65 and 2.5% (Fig. 1b). When the COD was around 2000 mg L^{-1}, an increase of the PNSBsi with the decrease of ORP was observed at an initial pH and FPE in a range of 6.5–8.0 and 1.51–2.50% (Fig. 1c), respectively. The overlaid contour plot (Fig. 1) was also used to indicate the region where the optimization routine was searched for optimal points. The optimum conditions determined were COD concentration 2103 (mg L^{-1}); initial pH 7.0 and FPE 2.0%. The predicted response of PNSBsi was 7.82 log cfu mL^{-1} while the actual value was between 7.79 and 7.83 log cfu mL^{-1} (run numbers 3, 4, 11, 12 and 14) (Table 1). These results were considered to be adequate and acceptable as the desirability obtained was 0.999. Hence, the optimal conditions for stimulating PNSB were 2000 mg L^{-1} COD, pH 7 and 2.0% FPE, and were therefore further verified, whilst the actual ORP values for those run numbers were in a range of -227 mV to -231 mV with the predicted value of -228 mV.

Results of the verification experiments under microaerobic light conditions over 2 d test period with sterile and non-sterile FPE are shown in Table 4. Under the optimized conditions as calculation, significant differences were found for the amounts of HPC and PNSBsi, *i.e.* in a non-sterile FPE set, PNSBsi were 8.13 log cfu mL^{-1} and 7.69 log cfu mL^{-1} in a sterilized FPE set and no PNSB were detected in the control set. The lowest amount of the HPC was observed in a non-sterile FPE set which had the highest amount of PNSBsi. In contrast, there was no significant difference found in both verification sets for the following parameters: pH and LAB with the exception of ORP, VFAs and EC. The values of pH, VFAs and ORP including HPC in the control set were significantly higher than those found in both verification sets. However, no population of LAB was detected in any set.

3.2. Treatment of rubber sheet wastewater using stimulated PNSB and FPE

Results of CCD experiment are shown in Table 5 along with experimental and predicted values using RSM. COD, SS, and TtS were used as key responses to evaluate the efficiency of the treatment and the data obtained from all runs were analyzed by fitted regression models. The fitted regression models are as follows.

$$Y_{COD} = 4372.87 - 145.87X_1 - 957.70X_3 - 127.72X_1X_2 + 26.27X_1X_3 + 7.18X_1^2 + 2863.12X_2^2 + 84.17X_3^2 \quad \text{[Equation 4]}$$

$$Y_{SS} = 102.05 - 2.11X_1 - 21.61X_3 + 0.52X_1X_3 + 1.41X_3^2 \quad \text{[Equation 5]}$$

Table 5
The central composite design for treating non sterile RAW with initial values in mg L^{-1} of 2024 COD, 57 SS and 13.56 TtS by PNSBsi inoculum under microaerobic light conditions.

Run no.	% PNSBsi	% FPE	RT (d)	COD (mg L^{-1})		SS (mg L^{-1})		TtS (mg L^{-1})	
	(X_1)	(X_2)	(X_3)	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	2	0.75	4	770	953	32	38	8.67	8.98
2	4	0.625	5	673	596	30	25	8.67	8.36
3	8	0.625	3	724	825	32	33	8.89	8.88
4	4	0.875	3	1061	914	40	35	9.56	9.3
5	8	0.875	3	472	503	17	17	7.11	7.15
6	8	0.875	5	537	676	25	24	8	7.8
7[a]	6	0.75	4	288 (16)	289	24 (1.15)	24	6.89 (0.26)	6.77
8	6	0.5	4	660	797	27	30	8.44	8.63
9	6	0.1	4	867	794	23	25	7.78	7.97
10	10	0.75	4	394	275	15	14	7.33	7.39
11	4	0.625	3	1578	1393	52	49	9.33	9.27
12	8	0.625	5	976	868	28	26	8.44	8.3
13	6	0.75	2	1533	1504	48	48	8.89	8.85
14	4	0.875	5	1520	1582	47	45	9.11	8.99
15	6	0.75	6	886	979	27	33	8.22	8.63
R^2				0.9369		0.8947		0.9352	
Adj. R^2				0.8801		0.8000		0.8769	

[a] The experiment was repeated 6 times and the responses represented average values with their standard deviation in parenthesis.

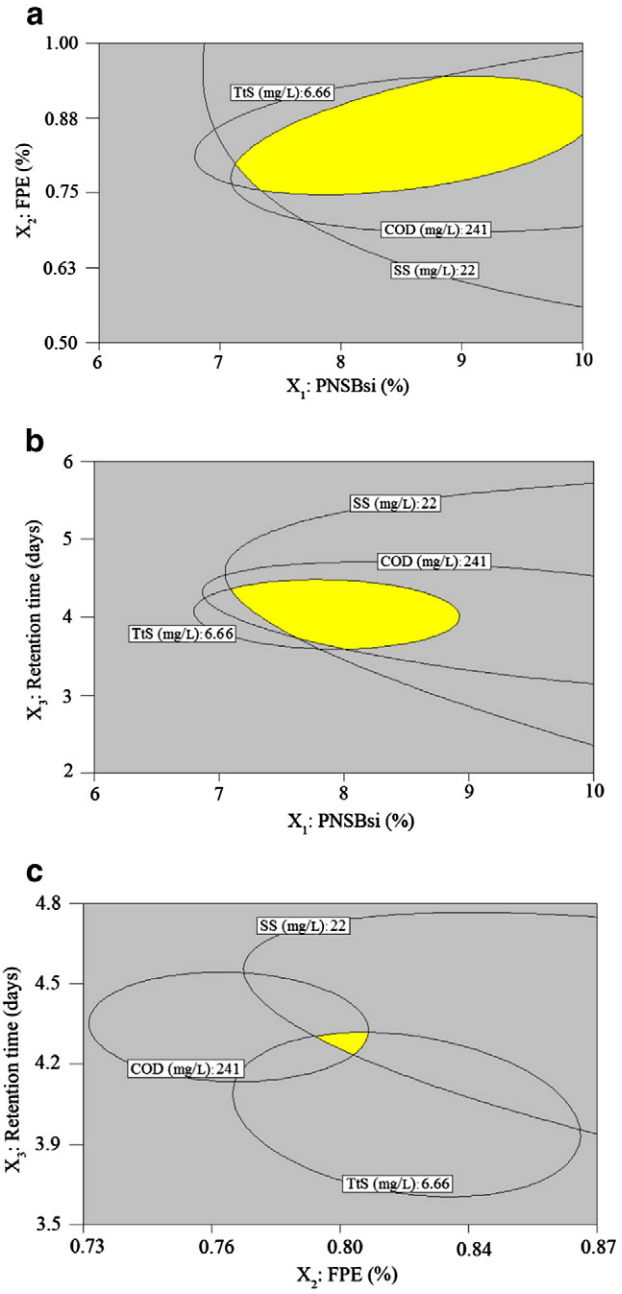

Fig. 2. The overlay interaction plots of two variables: stimulated indigenous purple non sulfur bacteria (PNSBsi) and fermented pineapple extract (FPE) (a), PNSBsi and retention time (RT) (b), FPE and RT (c) on the efficiency to treat non sterile rubber sheet wastewater under microaerobic light conditions.

$$Y_{TtS} = 17.44 - 0.27X_1 - 0.36X_1X_2 + 0.61X_2X_3 + 0.03{X_1}^2 + 8.65{X_2}^2 + 0.17{X_3}^2 \quad \text{[Equation 6]}$$

The models for the removals of COD, SS and TtS (Y_{COD}, Y_{SS} and Y_{TtS}) were significant by the *F*-test. The ANOVA for the quadratic models were highly significant as all had low *p*-value ($P < 0.0001$, 0.0008, < 0.0001 for COD, SS and TtS, respectively). The goodness of fit for COD, SS and TtS was confirmed by R^2 for COD, SS and TtS of 0.9369, 0.8947 and 0.9352, respectively, suggesting that only 6.31%, 10.53% and 6.48% of the total variation could not be explained by each respective model. In this study, the adj. R^2 values for COD, SS and TtS were 0.8801, 0.8000 and 0.8769, respectively, which are close to R^2 values. This is due to the fact that if there are many terms in the model and the sample is not very large, the adjusted R^2 might be noticeably smaller than the R^2. Based on the above results, the COD multiple regression model was selected to build the surface overlay contour plots and to derive the optimal condition for verification test.

Considering [Equation 4 and Equation 5], RT had a significant effect on reducing the COD and SS values ($P < 0.05$), and it also implies that the growth of the PNSBsi is dependent on the RT. Consequently, the PNSBsi had a significant influence on reducing the TtS [Equation 6]. The regression [Equation 4] shows the significant effects of interacting relationships between PNSBsi *versus* FPE and PNSBsi *versus* RT on the COD value. However, [Equation 5] shows that the interaction between PNSBsi *vs.* RT had a substantial impact on the SS value, whilst the TtS value was sensitive to the interaction between PNSBsi *vs.* FPE and FPE *vs.* RT [Equation 6].

Overlaid contour plots were used to present interacting relationships between 2 factors by providing another factor at a mid-point for the determination of optimum conditions. The interacting relationship between PNSBsi and FPE when the treatment was 4 d had decreasing values of COD, SS and TtS, when the levels of PNSBsi and FPE were in a range of 7.2–10% and 0.75–0.94%, respectively (Fig. 2a). When 0.75% FPE was the midpoint, the interacting relationship between PNSBsi and RT showed that when both factors were in a range of 7.2–8.9% and 3.7–4.3 d, there was a significant decrease of COD, SS and TtS values (Fig. 2b). The interacting relationship between FPE and RT when PNSBsi was 6% showed that values of COD, SS and TtS decreased when the FPE and RT levels were 0.79–0.81% and 4.2–4.3 d (Fig. 2c). The overlaid contour plot (Fig. 2) was also used to indicate the region where the optimization routine was found. The optimum conditions were determined as 7% PNSBsi population, 0.81% FPE and 4.22 d for the RT. The predicted responses of COD, SS and TtS were 241, 22 and 6.66 mg L^{-1} (corresponded to 88, 61 and 51%, respectively) and these were close to actual values of run number 7 as COD, SS and TtS were 288, 24 and 6.89 mg L^{-1} with the conditions of 6% PNSBsi, 0.75% FPE and 4 d (Table 5). The results were considered to be adequate and acceptable as the desirability was 0.910. However, for practical work, the optimal conditions were adjusted to 7% PNSBsi, 0.8% FPE and 4 d of RT with little changes of FPE and RT in the verification test.

3.3. The role of FPE and PNSBsi to treat rubber sheet wastewater

The wastewater treatment efficiencies of FPE, PNSBsi and combination of FPE and PNSBsi (verification test) in a non-sterile set under microaerobic light conditions over 4 d are shown in Table 6. A native control set without the addition of FPE and PNSBsi produced the least efficient reduction of COD, SS and TtS levels and in this set PNSB were not detected. However, the addition of 0.8% FPE stimulated PNSB growth to 7.3 log cfu mL^{-1} and provided a higher efficiency for the reduction of COD than the native control. On the other hand, with the addition of only 7% PNSBsi, 8.11 log cfu mL^{-1} were achieved, with the reduction percentages of 85 for COD, 63 for SS and 52 for TtS. The best treatment efficiency was observed in a verification test with the addition of both 0.8% FPE and 7% PNSBsi as the reduction percentages were then 91 for COD, 75 for SS and 61 for TtS. However, H_2S was not detected in any set except that RAW had an initial H_2S level of 10 mg L^{-1}. The highest amount of HPC was found in the native control set followed by a FPE set and the least in sets with PNSBsi. No detection of LAB was observed in any set. According to the results above, the verification test produced significantly higher efficiency than the predicted values derived from CCD experiment for COD (88%), SS (61%) and TtS (51%).

Results of a parallel sterile set to treat sterile RAW over 4 d are presented in Table 7. The abiotic control showed little degradation in RAW; however, with the addition of 0.8% FPE, the amount of HPC was 4.51 log cfu mL^{-1}. Therefore, higher efficiencies for reducing COD, SS and TtS were found, but the most marked decrease of these

Table 6
Characteristics of the effluents from the non sterile sets after treatment by FPE, PNSBsi inoculants and results of the verification test.

Parameter	Non sterile	Control	Addition	Addition	Verification test,
mg L^{-1}[a]	RAW	Native control	0.8% FPE	7% PNSBsi	0.8% FPE + 7% PNSBsi
	T = 0	D 4	D 4	D 4	D 4
pH	7.03 ± 0.01[a]	7.46 ± 0.03[d]	7.31 ± 0.03[b]	7.39 ± 0.01[c]	7.35 ± 0.02[bc]
COD	2722 ± 11[e]	1196 ± 11[d] (56)	1033 ± 11[c] (62)	414 ± 11[b] (85)	239 ± 11[a] (91)
SS	43 ± 3[d]	32 ± 3[c] (26)	29 ± 5[c] (33)	16 ± 6[b] (63)	11 ± 3[a] (75)
TDS	547 ± 12[c]	464 ± 12[b]	433 ± 42[ab]	367 ± 31[a]	380 ± 20[a]
Sulfate	5.1 ± 0.2[d]	3.9 ± 0.1[c]	2.7 ± 0.1[b]	2.1 ± 0.1[a]	2.0 ± 0.2[a]
TtS	13.78 ± 0.38[d]	8.44 ± 0.38[c] (39)	7.67 ± 0.67[bc] (44)	6.65 ± 0.77[ab] (52)	5.33 ± 0.67[a] (61)
DsS	12.89 ± 0.38[d]	10.22 ± 0.38[c]	6.22 ± 0.38[b]	5.78 ± 0.38[ab]	5.11 ± 0.38[a]
UHS	5.67 ± 0.17[d]	2.45 ± 0.09[c]	1.74 ± 0.11[b]	1.39 ± 0.09[a]	1.23 ± 0.09[a]
H_2S	10 ± 2[b]	0[a]	0[a]	0[a]	0[a]
HPC	8.3 ± 0[d]	7.83 ± 0.1[c]	7.63 ± 0.1[b]	7.09 ± 0[a]	7.13 ± 0[a]
LAB	0	0	0	0	0
PNSB	0[a]	0[a]	7.3 ± 0[b]	8.11 ± 0[c]	8.39 ± 0[d]

Numbers in parentheses are reduction percentages.
Different letters in each row indicate the significant differences ($P < 0.05$).
TDS: total dissolved solids; TtS: total sulfide; DsS: dissolved sulfide; UHS: unionized hydrogen sulfide (H_2S in wastewater); HPC: heterotrophic plate count; LAB: lactic acid bacteria; PNSB: purple nonsulfur bacteria.
[a] Unless otherwise stated and unit for microbial population is log cfu mL^{-1}.

levels were found in sets of verified and only addition of PNSBsi. A 7% PNSBsi inoculum provided PNSBsi (7.93 log cfu mL^{-1}) and HPC (4.19 log cfu mL^{-1}), and this effectively reduced COD 84%, SS 51% and TtS 49%. There were significant differences for the amounts of HPC and PNSBsi between the PNSBsi set and a verified set. Therefore, the verifying set produced the highest efficiency by reducing COD, SS, and TtS at 88%, 70% and 56%, respectively. The results of H_2S in this study indicated that this gas was produced by HPC from 4 mg L^{-1} to 15 mg L^{-1} in a FPE set and it was completely removed by PNSBsi. However, a loss of H_2S was also observed in an abiotic control.

4. Discussion

4.1. The role of FPE for stimulating PNSB growth

It is well recognized that PNSB prefer to grow under a low oxygen tension with light conditions [21] and in this study it was confirmed that the lowest ORP (-228 mV) strongly promoted the growth of PNSB (Table 1). This result was supported by Izu et al. [21] who reported that maximum PNSB ratios of up to 80% of the total microbes were obtained using non-aeration conditions with ORP values of less than -200 mV. The ORP in the sediments of paddy fields is in the range of -200 to -250 mV was found as the most suitable condition for enhancing PNSB growth [22]. COD, initial pH and FPE are independent variables each, having significant influences on the ORP value (Table 3). This is because both COD and FPE factors are a source of nutrient and optimal pH that could stimulate the growth of normal flora and the consequent depletion of oxygen (ORP decreased). Hence, anaerobic conditions under light stimulated indigenous PNSB growth so they became the dominant organism. No PNSB were detected in a control set, without FPE, while under light conditions, 2.0% FPE had enough nutrients to stimulate PNSB growth (Table 4). This was because the FPE consisted of 1.90% organic acids and other nutrients with an EC value of 3.51 mS cm^{-1}. In contrast, lower nutrients in the control set without added FPE in the light was enough to support some algal growth. However, why would FPE stimulate PNSB but have little effect on the HPC? It is possible that the VFAs in FPE in both verified sets were used preferentially as electron donors for the photosynthesis in the partially anaerobic light conditions as a lower amount of VFAs was found in both sets when compared with the control set (Table 4). It was found that LAB was not detected in any set (Table 4). This might be a result of very low number of LAB in the stored FPE since they could not survive the high competition for growth substrates from the heterotrophs and PNSB in the RAW under light conditions.

According to BBD, the optimal conditions for promoting PNSB growth were a COD of 2000 mg L^{-1}, initial pH 7 and 2% FPE (Fig. 1 and Table 4). This can be explained by the fact that PNSB are photoorganotrophs therefore they prefer to grow in a condition that has a relatively high

Table 7
Characteristics of effluents from sterile sets after treatment by FPE, PNSBsi inoculum and the verification test.

Parameter	Sterile	Abiotic	Addition	Addition	Verification test,
mg L^{-1}[a]	RAW	Control	0.8% FPE	7% PNSBsi	0.8% FPE + 7% PNSBsi
	T = 0	D 4	D 4	D 4	D 4
pH	7.06 ± 0.01[c]	7.33 ± 0.02[d]	6.85 ± 0.02[a]	6.96 ± 0.02[b]	7.73 ± 0.02[e]
COD	2684 ± 11[e]	2632 ± 11[d] (2)	1558 ± 11[c] (42)	443 ± 11[b] (84)	326 ± 11[a] (88)
SS	37 ± 6[b]	35 ± 5[b] (3)	33 ± 3[b] (11)	18 ± 3[a] (51)	11 ± 5[a] (70)
TDS	467 ± 12[b]	459 ± 21[b]	447 ± 12[b]	440 ± 20[b]	387 ± 12[a]
Sulfate	4.8 ± 0.1[d]	4.7 ± 0.1[d]	2.5 ± 0.1[c]	2.1 ± 0.2[b]	1.9 ± 0.1[a]
TtS	13.11 ± 0.38[c]	12.65 ± 0.38[c] (3)	10.22 ± 0.38[b] (22)	6.67 ± 0.67[a] (49)	5.78 ± 0.38[a] (56)
DsS	12.44 ± 1.02[b]	12.22 ± 0.38[b]	7.78 ± 0.38[a]	7.33 ± 0.67[a]	6.44 ± 0.77[a]
UHS	4.73 ± 0.39[d]	3.42 ± 0.11[c]	4.13 ± 0.47[cd]	1.76 ± 0.16[b]	0.84 ± 0.10[a]
H_2S	4 ± 1[b]	0[a]	15 ± 3[c]	0[a]	0[a]
HPC (log cfu mL^{-1})	0[a]	0[a]	4.51 ± 0.03[d]	4.19 ± 0.03[c]	4.10 ± 0.06[b]
LAB (log cfu mL^{-1})	0	0	0	0	0
PNSB (log cfu mL^{-1})	0[a]	0[a]	0[a]	7.93 ± 0.05[b]	8.10 ± 0.02[c]

Different letters in each row indicate the significant differences ($P < 0.05$).
For identification of abbreviations see Table 6.
[a] Unless otherwise stated and numbers in parentheses are reduction percentages.

organic matter [2,5,10]. The 2.0% FPE addition in this study provided an optimal concentration of organic matter for proliferation of PNSB because a lower organic content would facilitate the growth of cyanobacteria as previously described in the control set. In contrast, too much organic matter may promote the growth of HPC and repress the growth of PNSB [2]. It is generally known that a neutral pH supports the growth of most microorganisms including PNSB [2,23]. Therefore, it was not surprising that PNSB became the dominant organism due to the provision of the most suitable conditions provided for the growth of photoheterotrophs under microaerobic light condition, which quickly switched to anaerobic conditions with the addition of FPE (ORP = -228 mV) (Table 1).

4.2. The role of PNSBsi for treating rubber sheet wastewater

Efficiency of the rubber sheet wastewater treatment is dependent on PNSBsi, FPE and RT. The results demonstrated that RT had a significant effect on the reduction of COD and SS values whereas the PNSBsi strongly reduced TtS [Equation 4, Equation 5, Equation 6]. RT is one of the major factors, which controls the degradation of carbonaceous wastes as it represents the length of time the microbial cells are in contact with the substrate, which directly dictates the efficiency of wastewater treatment [24]. In this case, it ensures that the PNSBsi had enough time to consume most of the substrate and nutrients over the RT of 4 d. There was a significant reduction of TtS caused by the PNSBsi because some members of the PNSB can use sulfide or other reduced forms of sulfur as an extra source of an electron donor to support photosynthesis [5,10]. COD value was also dependent on the interactive relationship between PNSBsi *vs.* FPE and PNSBsi *vs.* RT [Equation 4], this again illustrated that FPE stimulated PNSB growth as previously described.

The SS values were significantly governed by the interactive relationship between PNSBsi *vs.* RT as their biomass increased with time after consuming nutrients [Equation 5]. However, the PNSBsi gave a higher efficiency to reduce SS value than the HPC (Table 6 and Table 7). The reason is that PNSB have a growth yield ($Y_{biomass/substrate\ (COD)}$) in a range of 0.28–0.45 [25,26], while, usually, HPC growth yields are around 0.40–0.60 [27]. The interactive relationships between PNSBsi *vs.* FPE significantly decreased TtS while the FPE *vs.* RT interaction increased TtS [Equation 6]. This suggested that PNSBsi removed TtS while the FPE was involved with producing TtS as it supported the growth of the HPC. This corresponded with the results in Table 7. In contrast, H_2S was not detected in the abiotic control after 4 d, although it was observed at time zero at 4 mg L^{-1} (Table 7). This indicated that the loss of rotten gas odor may be caused by the change of pH from 7.06 to 7.33 because a higher pH promotes the conversion of sulfide into its HS^- and also some may be lost by precipitation with small amount of metal ions existed in the medium.

According to the overlaid contour plot, the adjusted optimal conditions for treating RAW were 7% PNSBsi, 0.8% FPE and 4 d treatment time, and this condition was verified with non-sterile and sterile FPE, including controls (Table 6 and Table 7). Results of the native control show that the addition of FPE stimulated PNSB growth which is consistent with the results shown in Table 4. As there was little change of most of the measured parameters in the abiotic control set (Table 7). The efficiency to treat RAW in the sets of non-sterile and sterile with no/addition of FPE/PNSBsi appeared to be governed by the microbes and the efficiencies were in the following order: the verified sets > PNSBsi > FPE > native control. As only 0.8% FPE was added into the sterile RAW, this allowed HPC growth to increase to 4.51 log cfu mL^{-1} (Table 7) and this could also treat RAW, although their activity was significantly lower than the treatments with the PNSBsi inoculum (Table 6 and Table 7). Addition of PNSBsi as inoculum also included some HPC population (Table 7). The removal efficiency to treat RAW was positively related to the additions of FPE and PNSBsi into RAW, although some normal flora present in the RAW may have involved (Table 6 and Table 7). The verified set in this study (Table 6) had higher efficiencies (91% COD, 75% SS and 61% TtS) to treat RAW that had an initial COD of 2722 mg L^{-1} than those predicted values (88% COD, 61% SS 51% TtS), with an initial COD of 2024 mg L^{-1} (Table 5). A possible reason for that is that the different batches of the wastewater used generally show high variations in both biotic and abiotic properties. The results in Table 7 showed that the sterile verified set had the removal efficiency of 88% for COD, 70% for SS and 56% for TtS, all of which were close to the predicted values. This means that biotic component in non-sterile verified set also supported RAW treatment.

The PNSBsi inocula not only easily adapted themselves to their original source (RAW) but they were also able to compete with the normal flora for treating RAW. This was evidenced by their higher efficiencies in the non-sterile condition than those in the sterile condition (Table 6 and Table 7). The results indicated that the use of autochthonous bioaugmentation (re-inoculation) for treating rubber sheet wastewater was a practical technique to be used by farmers who desire to use the treated RAW for the preparation of PNSB inoculum. The inoculum can be carried out onsite with a simple procedure of adding the appropriate amount of FPE into a portion of wastewater in a shallow pond nearby the plant under light condition. This application can sustain the need for inoculation and support a long term operation of this community level wastewater treatment ponds. However, the amount of UHS remained in the effluent must be solved and the use of selected PNSB strains might be possible to solve the problem, and this will be further studied.

5. Concluding remarks

This work showed that the role of FPE in the stimulation of PNSB growth under light condition was to lower ORP leading to the reducing environment in the culture. A new approach for the preparation of indigenous PNSBsi inoculum from rubber sheet wastewater with the addition of optimal amount of FPE under microaerobic/anaerobic light conditions was developed. In addition, autochthonous bioaugmentation using PNSBsi proved to be effective for treating the wastewater.

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Author contributions

Conceived and designed the experiments: DK, NK; Wrote the paper: DK, SC; Performed the experiment: NK; Analyzed the data: DK, NK, ST.

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