The Effects of Light Intensity, Inoculum Size, and Cell Immobilisation on the Treatment of Sago Effluent with *Rhodopseudomonas palustris* Strain B1

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Abstract A study was carried out to determine a suitable light intensity and inoculum size for the growth of *Rhodopseudomonas palustris* strain B1. The pollution reduction of sago effluent using free and immobilised *R. palustris* cells was also evaluated. The growth rate in glutamate-malate medium was highest at 4 klux compared to 2.5 and 3 klux. The optimal inoculum size was 10% (v/v). Both the COD and BOD of the sago effluent were reduced by 67% after three days of treatment. The difference in biomass production or BOD and COD removal with higher inoculum sizes of 15 and 20% was minimal. This could be attributed to limited nutrient availability in the substrate. The use of immobilised cells of *R. palustris* reduced the pollution load 10% less compared to pollution reduction by free cells. Hence, there was no significant difference in using free or immobilised cells for the treatment of sago effluent.

Keywords: *Rhodopseudomonas palustris*, sago starch processing effluent, COD reduction, BOD reduction, agar immobilisation

INTRODUCTION

Phototrophic bacteria contribute to the purification of polluted water in nature as they have the ability to photometabolize many organic substances [1-3]. The possibility of using *Rhodopseudomonas palustris* for the treatment of sago starch processing wastewater (sago effluent) has been studied [4]. After starch extraction, sago wastewater contained about 5.4 g glucose/L, 0.11 g/L total nitrogen, and 0.67 g/L crude protein. The total solids and suspended solids were about 7.0 and 2.8 g/L respectively. *R. palustris* strain B1 had been observed to photoassimilate starch and produce biomass 4-fold higher when compared to biomass production in defined organic carbon sources [4,5]. Further research had shown that the purple non-sulphur bacteria can grow under anaerobic condition when sufficient light was present [4,5].

The aims of this study were (a) to optimize light intensity and inoculum for growth of *R. palustris* strain B1 and (b) to evaluate both immobilised and free cells *R. palustris* strain B1 in the reduction of pollution load of sago effluent.

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MATERIALS AND METHOD

Test Strain

The purple non-sulphur phototrophic bacteria, *R. palustris* strain B1 was obtained from the culture collection at the Institute of Postgraduate Studies, University of Malaya. The master stock cultures were maintained as stab cultures on glutamate-malate (GM) medium solidified with 1.5% (w/v) agar. The stab cultures were incubated at 30 ± 2°C for 48 h, then topped with sterile paraffin oil (to maintain microaerophilic conditions) and stored at 4 ± 2°C [6].

Inoculum Preparation

The stab culture of *R. palustris* strain B1 was revived in 2 mL of GM liquid medium in 5-mL Bijou bottles, incubated at 30 ± 2°C for 48 h in continuous illumination of 2.5 klux and checked for purity. The required inoculum volume was prepared in GM medium and the culture concentration was standardized to an optical density at 660 nm (OD_{660nm}) of 0.3. A 10% (v/v) culture was used as inoculum unless otherwise stated.

Culture Media

GM Medium

Liquid GM medium was prepared and 450 mL was dispensed in each of several 500-mL Schott bottles or 20

mL in 25-mL Bijou bottles. The bottles were sterilized at 15 psi and 121°C for 15 min, and then cooled before use.

Sago Starch Processing Wastewater

The sago decanter wastewater collected from a factory in Batu Pahat, Johore was stored in plastic containers at −20°C. Prior to use the effluent was thawed at 4°C and brought to 28 ± 2°C. The effluent was then analysed for Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), pH, total suspended solids (TSS), and turbidity using standard methods [10].

For the growth and pollution load reduction studies the sago decanter wastewater was settled for 24 h at 10°C to remove as much of the suspended solids as possible. The clear portion of the decanter wastewater was then carefully decanted and henceforth referred as sago effluent. Prior to use, the pH of the unsterilised sago effluent as substrate for growth of *R. palustris* strain B1, was adjusted from the initial pH of 3.46~3.58 to 5.5~6.0 using 1 M NaOH solution.

Culture Conditions

Optical Density Determination

The cell mass concentration was ascertained by measuring the optical density at 660 nm with a Shimadzu UV-160A spectrophotometer. A calibration graph of cell dry weight (g/L) against optical density at 660 nm (OD_{660nm}) with the GMM blank used for 0 cell dry weight was constructed. The linear correlation facilitated the determination of cell mass for a given value of optical density (Fig. 1).

Effect of Light Intensity on the Growth of R. palustris *strain B1 in GM Medium*

Triplicate bottles of GM medium were inoculated with a 10% (v/v) inoculum, filled to the brim of bottles to maintain anaerobic condition and incubated at 30 ± 2°C under varying light intensities of 2.5, 3, and 4 klux. The continuous illumination was provided by 60-W tungsten bulbs. OD_{660nm} measurements for the inoculated samples were taken once each day for five days.

Effect of Inoculum Size on Growth of R. palustris *strain B1 in Sago Effluent*

The supernatant of sago effluent was inoculated with varying inoculum sizes of 0 (control), 10, 15, and 20% (v/v) for 48 h culture of *R. palustris* as free cells. Triplicates were prepared for each inoculum studied and the bottles were incubated in anaerobic-light conditions at 30 ± 2°C and 4 klux. Over a three-day period, samples were taken at 24-h intervals and centrifuged for 20 min. The supernatant was analysed for BOD and COD. pH and OD_{660nm} were also measured for each set.

Immobilisation of *R. palustris* Cells in Agar and its Effect on Pollution Load Reduction

The optimal inoculum size for sago effluent treatment was determined with free cell culture as compared to immobilised cell culture in the same medium. *R. palustris* strain B1 cells were immobilized in agar (Difco Bacteriological Agar).

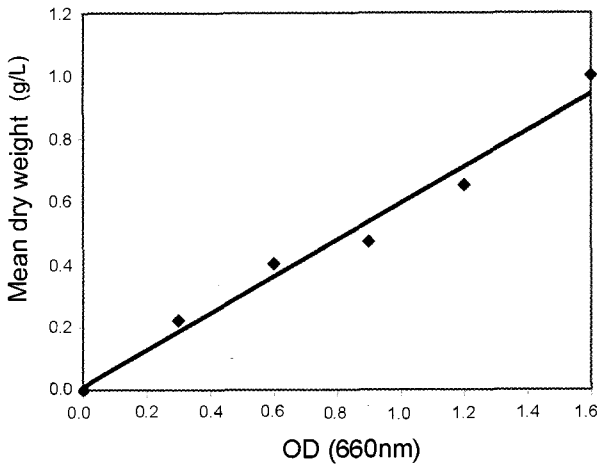

Fig. 1. Standard curve of B1 dry cell mass against OD 660 nm.

Eighty mg of bacterial biomass of strain B1 was harvested by centrifuging the liquid culture at 4,000 rpm for 45 min. The cells were resuspended in 5 mL of sterile phosphate buffer (pH 7.0) and then thoroughly mixed with 20 mL of sterile agar solution to give a final agar concentration of 1% (w/v) agar [9]. The selected agar concentration had been reported to have a good equilibrium between a good mechanical strength and a high cell retention capacity [7]. The mixture maintained at 43 ± 2°C was pumped through a syringe into a beaker containing sterile ice-cold distilled water covered with a 2- to 3-mm layer of sterile paraffin oil. The agar solution immediately solidified into spheres as polymerization occurred in the polar paraffin layer. The beads were washed thrice with sterile distilled water.

For each treatment triplicate bottles were prepared and incubated at 30 ± 2°C and at 4 klux under continuous anaerobic-light static culture system. Samples were taken at regular intervals over a three-day period, centrifuged for 20 min and the supernatants were analysed for reduction in BOD and COD.

RESULTS AND DISCUSSION

Proximate Analysis of Sago Effluent

The characteristic of sago effluent is summarized in Table 1. The supernatant of sago starch processing effluent was faint brownish in colour with bearable odour. However, it still could not be released into the watercourse as the effluent quality did not comply with EQA 1974 (Malaysia, Department of Environment).

Effect of Light Intensity on Growth of *R. palustris* in GM Medium

R. palustris strain B1 grown in GM medium under an-

Table 1. Proximate analysis of sago starch processing effluent

Parameter	Sago effluent (4 samples)
pH	3.46~3.58
BOD (mg/L)	900~1,300
COD (mg/L)	780~1,250
TSS (mg/L)	19~25
Turbidity (NTU)	11~14

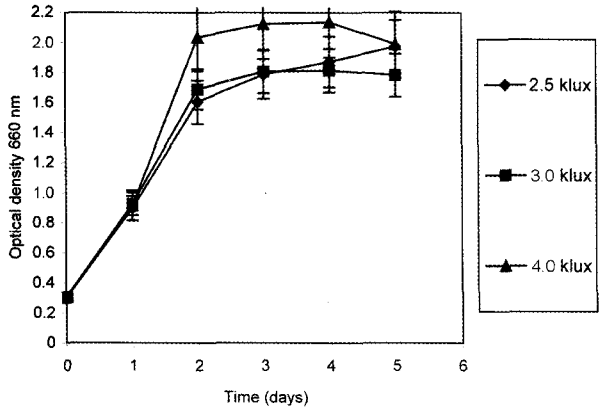

Fig. 2. The effect of light intensity on growth of *R. palustris* strain B1 in GM medium at 30 ± 2°C.

aerobic-light condition changed from a faint pink to reddish-brown after 48 h of incubation at 30 ± 2°C. The rate of growth at the different light intensities based on optical density measurements at 660 nm is reflected in Fig. 2, and the corresponding rate of cell mass production (conversion from OD_{660nm} to cell mass using the calibration graph) is given in Table 2. The growth profiles for 2.5 and 3 klux almost overlap, with growth rates of 0.421 and 0.460 g/day, respectively after 48 h of incubation. At 4 klux, however, the cell growth rate of 0.648 g/day on the second day is 40~54% higher than the rates at lower light intensities. At all light intensities, further increment in OD_{660nm} beyond the 48 h of growth is very minimal. Thus, at the light intensity of 4 klux, it may be recommended that a retention time of 48 h was sufficient to provide required treatment efficiency.

In anaerobic condition, phototrophic bacteria depend on light as their energy source to carry out photosynthesis. At a higher light intensity the bacteria obtained more energy to assimilate the carbon sources in the GM medium, and consequently the bacteria multiplied rapidly in a short period. The higher rate of cell production also implied that more carbon source was being degraded and thus the treatment process could be accelerated. However, it was reported that there was no increase in bacterial growth rate at light intensities exceeding 5 klux [5].

Effect of Inoculum Size on Growth of *R. palustris* in Sago Effluent

Biomass production during the growth of *R. palustris*

Table 2. The effect of varying light intensities on the growth rate of *R. palustris* strain B1 in GM medium incubated at 30 ± 2°C

	Growth rate (dx/dt) day^{-1} (mean of three replicate values)		
Time (h)	2.5 klux	3 klux	4 klux
24	0.233 ± 0.210	0.251 ± 0.020	0.227 ± 0.025
48	0.421 ± 0.038	0.460 ± 0.037	0.648 ± 0.071
72	0.097 ± 0.009	0.056 ± 0.004	0.011 ± 0.001
96	0.055 ± 0.005	0.019 ± 0.002	0.005 ± 0.0006
120	0.075 ± 0.007	0.037 ± 0.003	0.021 ± 0.002

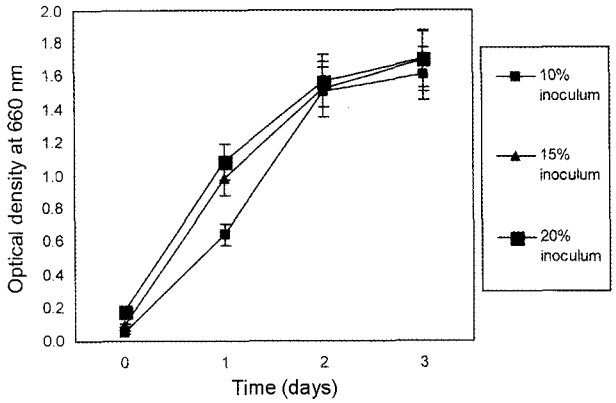

Fig. 3. The effect of varying inoculum sizes on growth of *R. palustris* strain B1 in sago effluent medium under continuous illumination of 4 klux and anaerobic condition at 30 ± 2°C.

in sago effluent at varying inoculum sizes is given in Table 3 and Fig. 3. Over the first 24 h, the growth rate of 10% inoculum lagged behind with an increase of only 0.355 g/L of cell mass concentration compared to 0.532 and 0.539 g/L for the 15 and 20% inoculum sizes, respectively. On the second day however, the rate at 10% size had increased by more than 60% compared to the higher inoculum sizes, as shown by the increased slope of the plot for 10% in Fig. 2. By the third day, there was minimal difference in the rate of biomass produced (less then 0.06 g/L) with the three different inoculum sizes studied. This is attributed to limited nutrient in the sago effluent for the cells. The maximum biomass of 1.03 g/L was obtained from after 72 h of growth using 20% (v/v) inoculum.

Prior to treatment, the initial pH of sago effluent medium was adjusted to 5.5~6.0. This was the optimum pH for growth of *R. palustris* strain B1 in sago effluent [5]. The pH change in the system was minimal (5.8~6.1) after 72 h of treatment and may be due to products of degradation that are acidic in nature [5].

Effect of Inoculum Size on BOD and COD Reduction of Sago Effluent Medium

Fig. 4 gives the COD and BOD reduction for sago effluent at the varying inoculum sizes of *R. palustris* tested. In control bottles (strain B1 was not added), there was a

Table 3. Cell mass concentration of *R. palustris* strain B1 in the sago effluent medium, under 4 klux light-anaerobic system at 30 ± 2°C

Day	Cell mass concentration, g dry weight/L (mean of three values)		
	10% inoculum	15% inoculum	20% inoculum
0	0.043 ± 0.004	0.068 ± 0.007	0.121 ± 0.012
1	0.398 ± 0.039	0.600 ± 0.066	0.660 ± 0.066
2	0.900 ± 0.090	0.921 ± 0.101	0.945 ± 0.095
3	0.965 ± 0.097	1.022 ± 0.112	1.029 ± 0.103

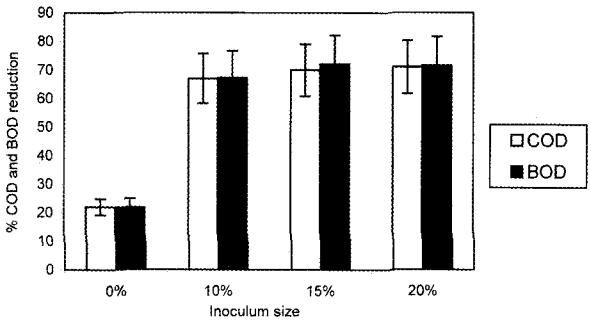

Fig. 4. The effect of varying inoculum sizes of *R. palustris* strain B1 on COD and BOD reduction of sago effluent medium after 72 h at 30 ± 2°C and anaerobic condition with 4 klux continuous illumination.

22.0% COD reduction and 21.8% BOD reduction. This reduction may be attributed to microorganisms present in the unsterilised medium that were able to assimilate the organic loading. But the results clearly indicated that *R. palustris* strain B1 had enhanced the COD and BOD reduction of sago effluent after 72 h at 4 klux light intensity by 67.3% removal of BOD and 67.0% removal of COD. These BOD and COD reductions were about 3-fold higher than in the control bottles. Further, a small inoculum size of 10% (v/v) inoculum was observed to be sufficient for the primary treatment of sago effluent. However, further studies on the treatment efficiencies are required before this can be recommended for application.

A 60.4% COD removal of sterile sago effluent inoculated with a 10% inoculum after 72 h incubation has been reported [5]. The increased efficiency in this study may be due to the natural microorganisms in non-sterile sago effluent medium, which played a role in the reduction of BOD and COD as evident from the result of the control (0% inoculum) sample.

Effect of Immobilisation of *R. palustris* in Agar on BOD and COD Reduction

Beads of immobilised *R. palustris* were rigid and spherical shape. The size of beads could be controlled by applying different pressure to the syringe. When the immobilised cells of *R. palustris* strain B1 were inoculated into sago effluent medium with 4 klux illumination at 30 ± 2°C, the beads gradually turned to red. This was because the cells grew up to the surface of the beads in order to be in direct contact with the substrate [8].

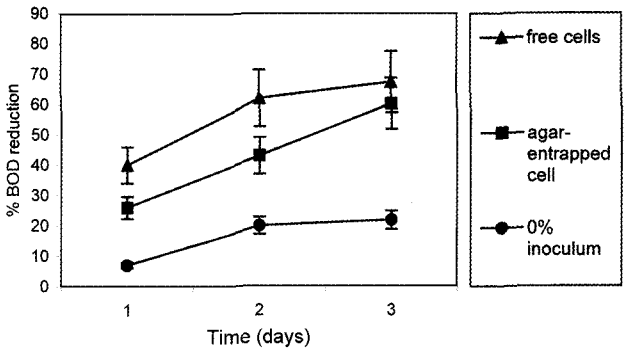

Fig. 5. The comparison of BOD reduction of sago starch processing effluent by free and agar-trapped cells of *R. palustris* strain B1 incubated at 30 ± 2°C in an anaerobic-light (4 klux) system.

Analysis of sago effluent medium after 72 h of treatment revealed that the free and immobilised cells of *R. palustris* strain B1 had similar BOD and COD removal patterns. The results are shown in Figs. 5 and 6. The free cells contributed to a higher BOD and COD reduction compared to the immobilised cells. The highest BOD removal of 67.3% and COD removal of 67.0% by free cells of *R. palustris* was after three days treatment while the immobilised cells recorded only 60.2% of BOD removal and 61.9% of COD removal after the same treatment time. Although there were only 7.1 and 5.1% difference of BOD and COD removal, respectively, the free cell system would be currently preferred, in consideration of the costs of cell immobilisation.

As with the freely suspended cells the treatment efficiency in this study was higher compared to a previous study [5] for a similar system. This may be due to the non-sterile settled sago effluent medium used in this study. The other microorganisms present may have also degraded the carbon sources in the effluent. However, if the immobilised cells were used in unsettled sago effluent treatment, a COD removal of 79.5% could be achieved after three days treatment [5].

The reduced efficiency of immobilised cells compared to freely suspended cells can be due to a few factors. Firstly, the immobilised cells may require more time to get acclimatized to the environment. The diffusion limitation of substrate and carbon dioxide through the entrap ment could be another factor that impaired the reactions to remove BOD and COD. Free microorganisms are able to get carbon dioxide and substrate directly from the surrounding atmosphere or from the liquid medium [8]. Another possible reason for reduced efficiency of immobilised cells was its static condition in the treatment system. The immobilised beads settled to the bottom, mixing was inadequate as the culture was only gently shaken once every 24 h and surface area of contact between the beads and medium was not maximized, thus nutrients in the sago effluent medium was not completely available to

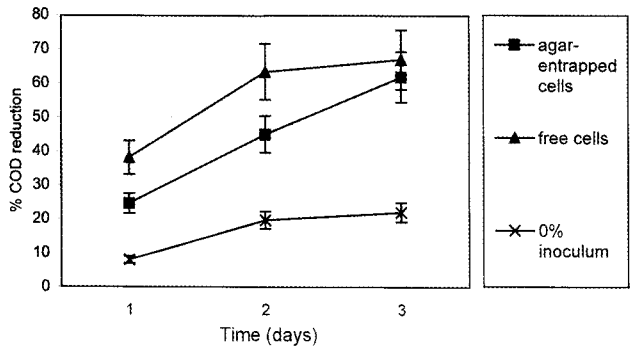

Fig. 6. The comparison of COD reduction of sago effluent by free and agar-trapped cells of *R. palustris* strain B1 incubated at 30 ± 2°C in an anaerobic-light (4 klux) system.

the immobilised cells.

CONCLUSION

The experiments with GM medium indicated that an optimal light intensity of 4 klux for the growth of *R. palustris* strain B1 and this condition was used in the study of pollution reduction in sago mill effluent. The results showed that non-sterile sago effluent undergoes some extent of degradation even without inoculation of the bacteria. Varying the inoculum size from 10 to 15 and 20% had no significant effect on biomass production or BOD and COD removal. This is attributed to the limited nutrient availability in the substrate. After 72 h of culture the 10% inoculum size demonstrated cell growth and pollution reduction sufficiently comparable to the higher inoculum sizes.

At the conditions of the experimental work in this study, the free cells system performed better than immobilised cells in the treatment of sago effluent, though the difference is less than 10%. Presently, time and cost factors do not favour the immobilised cells to be used in this treatment system. However, the low 10% difference in pollution reduction compared to freely suspended cells, indicate the potential of immobilised systems. Further work on the optimization of immobilisation and the system's operation may well make the system more feasible for practical application, particularly in consideration with the advantages of immobilisation such as the possibility of cell reuse and reduced steps for cells separation after treatment.

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