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Isolation and growth of the phototrophic bacterium *Rhodopseudomonas palustris* strain B1 in sago-starch-processing wastewater

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An indigenous strain of the purple non-sulphur phototrophic bacterium, *Rhodopseudomonas palustris strain* B1, was selected for the utilization and treatment of wastewater from a sago-starch-processing decanter. Growth of Strain B1 under anaerobic–light conditions in the carbohydrate-rich effluent was optimized by using 50% (v/v) effluent diluted in a basal minimal mineral medium with the addition to 0.1% (w/v) yeast extract. The optimum level of nitrogen source supplement, ammonium sulphate, was 1.0 g/l. Highest cell mass concentration was achieved by using tungsten lamps as the light source with a light intensity of 4 klux. Under these optimal conditions, a maximum biomass of about 2.5 g dry cell/l with a pigment content of about 1.1 mg carotenoid/g dry weight cell was achieved after 96 h of anaerobic cultivation. There was a 77% reduction in the chemical oxygen demand (COD) of the effluent. A cell yield of about 0.59 g dry weight cell/g COD was obtained.

Key words: Phototrophic bacteria, purple nonsulphur bacteria, *Rhodopseudomonas palustris,* single cell protein, wastewater bioremediation.

In 1990, Sarawak, East Malaysia produced about 45,000 tonnes of sago starch from the sago palm, *Metroxylon* sp. (Alang *et al*. 1993). The sago industry produces different types of wastes such as bark, pith residue, known locally as 'hampas', and wastewater (Chew & Shim 1993). The bark is either burned on site or used as platforms around the factory. Sago 'hampas' is used as a dietary fibre or cheap carbohydrate source in animal feed. It is also used as raw material in the paper and particleboard industry and as substrate in mushroom cultivation (Shim 1992; Vikineswary *et al*. 1994). It is estimated that 30–50 m^3 of water is used to extract about one tonne of sago starch (Chew & Shim 1993). Wastewater with high chemical oxygen demand (COD), contributed by the presence of 'hampas' and uncovered starch, is usually discharged indiscriminately into nearby rivers. This may cause severe devastation of river life unless effective treatment systems are developed. One possible utilization method is bioconversion of this carbohydrate-rich wastewater into single cell protein (SCP) and value-added metabolites (Vikineswary *et al*. 1994).

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Purple non-sulphur phototrophic bacteria are widely distributed in nature especially in water source polluted by organic matter. They play a major role in purifying the environment because they combine photosynthesis with the ability to photometabolize many organic substances (Kobayashi 1982). One advantage of using these bacteria in bioremediation of agroindustrial wastewaters is that they can grow directly in high organic load wastewater. Besides reducing the COD of the wastewater, this process produces bacterial biomass as potential feed supplement in the aquaculture and poultry industries (Sasaki *et al.* 1991). Previous studies have shown that cultivation of phototrophic bacteria in municipal and agricultural wastes produced protein-rich biomass (about 65% crude protein) with balanced essential amino acids, carotenoid pigments, cellular vitamin B_{12} and folic acid (Sasaki *et al.* 1981; Noparatnaraporn *et al*. 1983). Juvenile fish fed with phototrophic bacteria cells showed a significant increase in weight and survival rate compared with those fed the conventional diet (Kobayashi & Tchan 1973). Supplementation of carotenoids into animal feedstock intensified the colour of chicken egg yolk and the skin of carp and prawn (Noparatnaraporn & Nagai 1986). It has also been reported that *Rhodobacter capsulatus*, a

phototrophic bacterium, produces an antiviral substance against some fish viruses (cited in Hirotani *et al.* 1991).

This paper reports the possibility of using purple non-sulphur phototrophic bacteria in the utilization and treatment of sago-starch-processing wastewater. Some initial results on isolation and selection of potential indigenous strains and the effects of culture conditions on growth of the bacteria in the wastewater medium are presented.

Materials and Methods

Isolation of Phototrophic Bacteria

Twenty-five samples of wastewater were collected from wastewater ponds and surrounding waterways in a starch noodle (*bihun*) factory near Butterworth, Penang, West Malaysia. The main ingredients used in the preparation of the noodles are rice and sago starch.

Malate-yeast extract (MYE) medium at pH 7.0 was used for the enrichment and isolation of purple non-sulphur phototrophic bacteria. One ml of the wastewater sample was inoculated into about 20 ml of sterile medium in 25 ml screw-capped clear bottles. The bottles were filled with sterile medium almost to the brim to maintain anaerobic conditions. The bottles were then incubated at a temperature of 30 ± 2 °C and under continuous illumination of 2000 lux with fluorescent lamps. Reddish or brownish-red bacterial growth developed after about 2 weeks of incubation. After two or three transfers into fresh MYE medium, the isolates were inoculated into a modified mineral medium (MM) with 2.0 g citrate/l as the sole carbon source (Sawada & Rogers 1977). Considering the starchy nature of the noodle factory wastewater, citrate was used to selectively enrich the starch-utilizing species *Rubrivivax gelatinosus* (Truper & Imhoff 1991). Purification was done by repeated streaking of reddish culture broth onto MM agar plates and incubating under similar culture conditions in anaerobic jars. Purified cultures were stored in MM agar stabs topped with sterile paraffin oil at 4 °C.

Preparation of Sago Effluent Medium

Sago-starch-processing decanter wastewater was collected from a factory near Batu Pahat, Johor, West Malaysia. Suspended solids were partially removed from the wastewater by settling the effluent for 1 h and the supernatant was collected. Sago effluent medium for strain selection consisted of 50% (v/v) of the supernatant. To ensure that bacterial growth was due to their ability to utilize organic substances from the wastewater, a basal minimal mineral (BM) medium containing (g/l) K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5 and NaCl, 3.0 was used to dilute the wastewater. The medium was adjusted to pH 7.0 and dispensed into 25 ml bottles prior to autoclaving at 121 °C for 15 min.

Selection and Identification of Potential Bacterial Strain

All isolated strains were screened for the ability to grow in MM medium with 2.0 g/l sago starch as the sole carbon source. The medium was adjusted to pH 7.0 and dispensed into 25 ml bottles prior to autoclaving. For all isolated tested, a 10% (v/v) inoculum of each isolated was inoculated into triplicate bottles. Culture bottles were incubated at 30 ± 2 °C for 7 days under anaerobic–light conditions. Growth was evaluated by measuring the optical density of the culture medium at 660 nm. Potential isolates were selected by comparing with growth responses in MM medium with citrate as the carbon source. Selected isolates were further screened for their ability to grow in the sago effluent medium. A 10% inoculum was used. A potential strain was selected by comparing the OD_{660} after 7 days of incubation in the effluent medium with the OD in BM medium with and without citrate as the sole carbon source.

Identification of the strain was based on morphological characteristics and photopigment analysis. The colour of cell suspensions, 'slime' formation, cell shape and size were observed in a 1-week-old culture. Gram staining was performed and the motility was observed by the hanging drop method (Santos *et al.* 1989). The *in vivo* absorption spectrum of photosynthetic pigments was determined by the method of Sojka *et al.* (1970).

Growth Optimization Studies

The selected strain was cultivated in sago effluent medium under anaerobic–light conditions to study the effect of different growth parameters tested include: effluent concentration and basal minerals (BM) supplementation, yeast extract supplementation, $(NH_4)_2SO_4$ supplementation, light source and light intensity. A 10% (v/v) inoculum of the bacterial culture in the exponential phase of growth with a cell density of OD_{660} 0.3 was used in all tests. Optimum levels of growth parameters were selected based on cell mass concentrations. Details of the experimental treatments are presented in Table 1.

The growth profile of the selected strain was later studied under optimal conditions obtained from the growth optimization studies. The sago effluent medium was prepared from the whole (unsettled) wastewater, using the most suitable effluent concentration, basal minerals supplementation and suitable yeast extract and $(NH_4)_2SO_4$ concentrations. A 10% inoculum was introduced into Schott bottles containing 100 ml of the effluent medium. Growth was studied at 30 ± 2 °C under anaerobic–light conditions. Triplicate bottles were analysed on days 0, 2, 4, 6, and 8 for culture pH, dry weight of biomass, total carotenoids, chemical oxygen demand (COD) and total carbohydrate.

Analysis

The cell mass concentration was determined by measuring optical density at 660 nm and converted to cell dry weight using a calibration curve. Dry weight of biomass in whole effluent medium was determined by harvesting the cells at 7000 rev/min for 20 min and the biomass was dried in the oven at 105 °C for 24 h. Carotenoid was extracted with acetone/methanol (7:2 v/v) and measured spectrophotometrically (Sojka *et al.* 1970). The COD was determined by the method described in *Standard Methods for the Examination of Water and Wastewaters* (APHA, AWWA & WPCF 1989). Total carbohydrate was determined by the anthrone method of Herbert *et al.* (1971). Light intensity was measured with a lux meter. Analysis of variance was done to test the significance of effluent concentration, mineral, yeast extract and $(NH_4)_2SO_4$ supplementation, light source and light intensity on the growth of the bacteria.

Results and Discussion

Isolation, Screening and Selection of Potential Isolates

Of the 25 wastewater samples collected from the starch noodle factory, 10 strains of purple non-sulphur

Table 1. Experimental treatments to select optimal growth parameters in sago effluent medium.

Parameters	Effect of effluent concentration and BM supplementation		Effect of yeast extract supplementation	Effect of $(NH_4)_2SO_4$ supplementation	Effect of light source	Effect of light intensity
Effluent concentration (%, v/v)	25%	50%	50%	50%	50%	50%
Mineral supplementation (BM)	+, –	+, –	+	+	+	+
Yeast extract concentration (%, w/v)	0	0	0, 0.01, 0.1	0.1	0.1	0.1
$(NH_4)_2SO_4$ concentration (g/l)	1	1	1	1, 2, 3, 4, 5, 10, 15	1	1
Light source	F	F	F	F	F, T	T
Light intensity (klux)	2	2	2	2	2	2, 4, 6
No. of samples analysed for each treatment	3		3	3	4	4
OD_{660} read on day	0, 2, 4, 6, 8		0, 2, 4, 6, 8	6	6	6

(+) Effluent diluted in basal minerals medium; (–) effluent diluted in distilled water; (F) fluorescent lamp; (T) tungsten lamp.

phototrophic bacteria were isolated. The isolates showed good growth in synthetic medium with malate and citrate as the sole carbon source. Four of the isolates, B1, B10, B14 and B17, grew well in synthetic medium with cooked sago starch as the sole carbon source. Secondary screening was done later to select one potential isolate for biomass production in the sago decanter wastewater. After 7 days of anaerobic–light cultivation in the sago effluent medium, isolate B1 was selected for its ability of fast growth and efficient cell mass production in the wastewater.

Identification of Strain B1

Cultures of isolate B1 grown in liquid medium under anaerobic–light conditions were reddish-brown. They did not produce extracellular 'slime' in the growth medium. On agar plates, they formed small red-brown colonies. The isolate was Gram negative and showed rod to ovoid shaped cells, 0.5–0.7 μm wide and 2.0–2.5 μm long. Examination under phase contrast microscopy showed motile rods with some of them having opaque polar swellings giving the characteristic dumbbell shape. Cultures also had a tendency to form rossette-like clusters of cells in the medium. Morphological studies indicated that strain B1 resembled the type strain *Rhodopseudomonas palustris* (Hiraishi *et al*. 1992; Fujii *et al*. 1993).

The *in vivo* absorption spectrum of strain B1 is given in Figure 1. Absorption maxima at 378, 592, 806 and 863 nm indicated the presence of bacteriochlorophyll *a* (Dow 1982). Peaks at 471, 498 and 530 nm indicated the presence of carotenoids of the normal spirilloxanthin series (Akiba *et al*. 1983). The peaks of carotenoids in strain B1 were similar to that of *R. palustris* reported in literature (Santos *et al*. 1989). Strain B1 was confirmed as being *R. Palustris* at the centro di Studio di Microorganismi Autotrofi del CNR e, Firenze (Italy). Contrary to known *R. palustris* strains (Imhoff & Truper 1976), strain B1 grew very well on citrate as the sole carbon source.

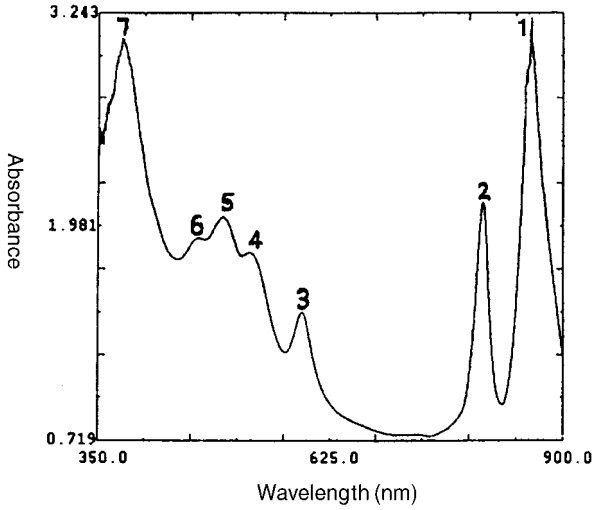

Figure 1. Absorption spectrum of whole cell suspensions of isolated strain B1. Peak 1 = 863 nm; peak 2 = 806 nm; peak 3 = 591.5 nm; peak 4 = 529.5 nm; peak 5 = 498 nm; peak 6 = 470.5 nm; peak 7 = 378 nm.

Therefore, the isolated strain is referred to as *R. palustris* strain B1. This strain has been deposited in the culture collection at Shri AMM Murugappa Chettiar Research Centre, Photosynthesis and Energy Division, Madras, India.

Effects of Effluent Concentration and Basal Minerals Supplementation

Figure 2 shows the effects of sago effluent concentration and basal minerals supplementation on the increase in cell dry weight during growth of strain B1. After 8 days of incubation, about 0.11 g dry cell/l was obtained in 25% effluent (v/v in water) and 0.05 g dry cell/l was obtained in 50% effluent (v/v in water). When growth of strain B1 in effluent diluted in water was compared with growth in effluent diluted in basal mineral (BM) medium, cell mass production showed significant improvement in the latter. Highest cell dry weight of

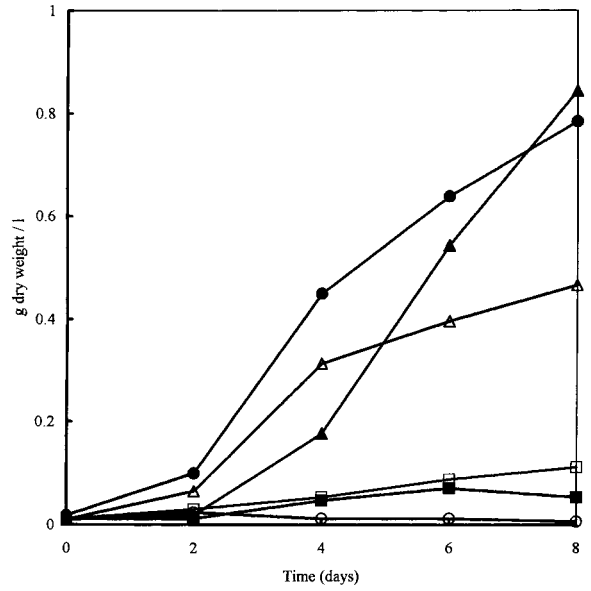

Figure 2. Effects of effluent concentration and mineral supplementation on growth of strain B1 in sago effluent medium. BM medium without carbon source (○), BM medium with carbon source (●), 25% effluent diluted in water (□), 50% effluent diluted in water (■), 25% effluent diluted in BM medium (△) and 50% effluent diluted in BM medium (▲).

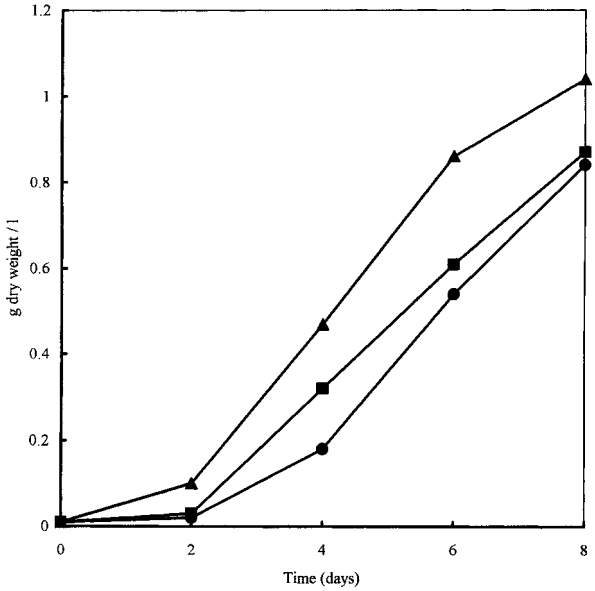

Figure 3. Effect of yeast extract supplementation on the growth of strain B1 in 50% effluent diluted in BM medium. 0% Yeast extract (●), 0.01% yeast extract (■) and 0.1% yeast extract (▲).

about 0.84 g/l was achieved in 50% effluent (v/v in BM medium) after 8 days of incubation.

Enrichment of growth medium with basal minerals is necessary so as not to limit growth of the phototrophic bacteria (Noparatnaraporn *et al.* 1986). Potassium and sodium phosphates are needed for the formation of organic phosphorus compounds such as nucleic acids and lipids. Magnesium and sodium are also essential for the synthesis of bacteriochlorophyll and the photosynthetic apparatus (Imhoff 1982). Therefore, growth of strain B1 in sago effluent medium is improved by the addition of these minerals. The results also showed that growth in 50% effluent with BM supplementation achieved higher cell mass over a longer period of incubation, compared with 25% effluent with BM supplementation. Preliminary studies had shown poor growth of strain B1 in undiluted sago effluent medium (Getha 1995). These results support the observations made by Earle *et al.* (1984) where little bacterial growth was recorded at either extreme concentrations of waste solids. Poor growth at lower and higher effluent concentrations may be due to the insufficient nutrient levels and increased biological oxygen demand, respectively.

Effects of Yeast Extract and $(NH_4)_2SO_4$ Supplementation

Figure 3 shows the effect of yeast extract supplementation on cell mass formation of strain B1. Growth was significantly stimulated when 0.1% (w/v) yeast extract was added into the 50% effluent medium with minerals supplementation. Yeast extract provides additional nitrogenous and carbon compounds and vitamins required for cellular biosynthesis. It contains about 5% amino acid nitrogen and trace metals such as iron and copper (Sawada & Rogers 1977). From the growth study with different levels of yeast extract (Figure 3), it was evident that addition of 0.1% yeast extract reduced the initial lag period. Since it was reported that 0.1% yeast extract can replace essential vitamins such as biotin and *p*-aminobenzoic acid as the growth factor for *R. palustris* (Pfennig 1974; Fujii *et al.* 1993), this may minimize growth instabilities of strain B1 in the effluent medium. In large-scale effluent treatment processes using phototrophic bacteria, growth factors like yeast extract are supplied from cellular metabolic products or autolysed cells.

Addition of varying concentrations of $(NH_4)_2SO_4$, 1.0–5.0 g/l, to the effluent medium did not show any significant differences in cell dry weight after 6 days of growth. An average cell dry weight of about 0.61 g/l was obtained (Table 2). Further increase in the salt concentration to 10.0 and 15.0 g/l caused a significant decrease in cell dry weight of about 16% and 85%, respectively. It is presumed that higher levels of $(NH_4)_2SO_4$ inhibited the growth of strain B1. Addition of about 1.0 g/l $(NH_4)_2SO_4$ with the other basal minerals is therefore sufficient to obtain good growth in the effluent medium. For industrial uses, nitrogen sources such as ammonium salts which are readily available at low cost and readily assimilated for growth of phototrophic bacteria, are preferred (Miyake *et al. 1982;* Demchick *et al.* 1990).

Table 2. Effect of $(NH_4)_2SO_4$ concentration on the growth of strain B1 in sago effluent medium.

$(NH_4)_2SO_4$ (g/l)	Cell dry weight after 6 days growth (g/l)
1.0	0.60
2.0	0.60
3.0	0.61
4.0	0.61
5.0	0.60
10.0	0.51
15.0	0.09

Effects of Light Source and Light Intensity

When two different light sources, fluorescent and tungsten lamps, with the same intensity of 2 klux were tested, cell mass production showed improvement under the latter. Under the fluorescent light, cell dry weight after 6 days of growth was about 0.63 g/l compared with 0.79 g/l under the tungsten light. Highest cell dry weight of about 0.89 g/l was achieved using tungsten lamps at a light intensity of 4 klux. At a higher intensity of 6 klux, lower cell dry weight of about 0.84 g/l was recorded.

Phototrophic bacteria require light for energy generation during photoheterotrophic growth under anaerobic conditions (Sasaki *et al.* 1987). The tungsten lamps are more suitable for growth because they generate emissions in the near infra-red (IR) region of 800–900 nm. These wavelengths of light are effectively absorbed by bacteriochlorophyll in the purple non-sulphur phototrophic bacteria. Moreover, tungsten lamps can supply much higher light intensities. With the increase in light intensity, bacterial growth increased until cells in the medium were saturated with light (Kim *et al.* 1982). No further increase in growth occured after the saturating light intensity. At very high intensities, depression in the synthesis of photopigments could also occur (Firsow & Drews 1977). This would reduce the ability of photoheterotrophic growth in the bacteria.

Growth Profile of Strain B1 in Sago Effluent Medium

The growth profile of *R. palustris* strain B1 in the whole decanter wastewater medium under optimal growth conditions is shown in Figure 4. A maximum biomass of about 2.5 g dry weight/l was obtained after 4 days. Initial biomass in the sago effluent medium consisted mostly of starch residue solids present in the carbohydrate-rich wastewater. The pH fell as growth proceeded. Purple non-sulphur phototrophic bacteria produce organic acid compounds during photoheterotrophic growth on carbohydrate or sugars (Mangels *et al.* 1986). It may be possible that strain B1 metabolized soluble starch molecules released from gelatinized starch residues during growth in the sago effluent medium. Figure 4 shows that strain B1 efficiently utilized the total carbohydrates causing a sharp decrease in 2 days. About 81% of the total carbohydrates was removed from the effluent medium after 4 days. The rate of COD removal by strain B1 was also very rapid over the first 2 days. After 4 days of cultivation, about 76.8% COD was removed.

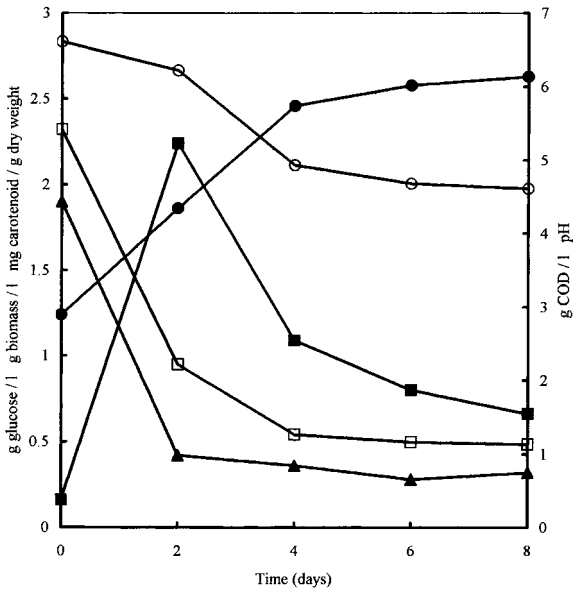

Figure 4. Growth profile and carotenoid contents of strain B1 in relation to certain physicochemical characteristics of the sago effluent medium, under anaerobic–light conditions. Biomass (●), pH (○), total carotenoid (■), chemical oxygen demand (□) and total carbohydrate (▲).

It is interesting to note that continous incubation periods lead to a decrease in the carotenoid content. After 4 days, the carotenoid content was about 1.09 mg/g dry weight. Several factors during growth could have been the inhibiting factor. In this study, growth was carried out in 100 ml bottles arranged in a static position with light sources at both sides. Without continuous mixing, poor heat dissipation within the culture medium was suspected to have an inhibiting effect on the cell pigments. When a continuous, low-speed mixing was provided by a rotary shaker operated at 130–140 rev/min, carotenoid content in the biomass increased to about 2.5 mg/g dry weight after 4 days (Getha 1995). Mechanical mixing which also prevents cell flocculation, could increase the conversion efficiency of carbon sources in the effluent medium (Kim *et al.* 1982). This could increase photopigment production in the bacteria to facilitate the high substrate consumption during photoheterotrophic growth.

Table 3 presents the growth characteristics of strain B1 and other phototrophic bacteria grown in waste medium. The cell mass yield of strain B1 of about 0.59 g/g COD falls in a range which is acceptable for cell mass production (Sasaki *et al.* 1991). The removal of about 77%

Table 3. Growth characteristics of phototrophic bacteria from various wastes.

Bacterial strain	Biomass (g/l)	Carotenoid (mg/g cell)	T_m (h)	COD removal (%)	Cell yield from starch (S), total sugar (TS) or COD (g biomass/g)	Waste medium	Growth conditions
R. gelatinosus R7 (2)	5.6	1.2	120	86.0	0.24 g/g COD	Tuna condensate + Shrimp blanching water	ANL, 3 klux, 30 °C
PSB VI (1)	4.7	NA	264	95.6	0.40 g/g Ts	Cassava starch processing (separator wastewater)	MAL, 3–5 klux, 35 °C
R. sphaeroides S (1)	3.7	NA	50	84.0	0.83 g/g COD	Mandarin orange peel	MAL, 3 klux, 30 °C
R. gelatinosus (3)	1.8	NA	120	NA	0.83 g/g S	Cassava starch medium	ANL, 25 klux, 30 °C
R. capsulatus (1)	0.9	NA	144	NA	NA	Cow dung (anaerobic digestion effluent)	ANL, 10 klux, 37 °C
R. palustris strain B1	2.5	1.1	96	77.0	0.59 g/g COD	Sago starch processing (decanter) wastewater	ANL, 4 klux, 30 °C

(1) Sasaki *et al.* 1991; (2) Prasertsan *et al.* 1993; (3) Noparatnaraporn *et al.* 1983. NA, Not analysed; T_m, time for maximum biomass; MAL, microaerobic–light; ANL, anaerobic–light.

COD indicates the advantages of using strain B1 for subsequent treatment of the sago decanter wastewater. Furthermore, effective COD removal and biomass production are achieved in a shorter period of time compared with the other anaerobic–light systems in Table 3. Another striking characteristic of strain B1 was its high carotenoid content during growth in the waste medium. Based on these promising results, further studies are needed to transfer the system from a small-scale laboratory study to an industrial scale. This opens a new avenue for the utilization of not only sago processing wastewater, but also other kinds of wastewaters readily available in Malaysia.

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