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Biological Characterization of *Rhodomicrobium vannielii* Isolated from a Hot Spring at Gadek, Malacca, Malaysia

Ainon, H.[1*], Tan, C. J.[2] and Vikineswary, S.[2]

[1]School of Biosciences and Biotechnology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor Darul Ehsan, Malaysia.
[2]Institute of Biological Sciences, University of Malaya,
59100 Kuala Lumpur, Federal Territory, Malaysia.
E-mail: antara@pkrisc.cc.ukm.my

ABSTRACT

A purple nonsulfur anoxygenic phototrophic bacterium, identified as *Rhodomicrobium vannielii*, was isolated from water sample of a hot spring using glutamate-malate medium (GMM) and Pfennig's M2 medium. The cells were motile, Gram negative, ovoid to spherical in shape and did not form intracellular sulfur globules. The isolate viewed under transmission electron microscope showed budding filament formation, which is a characteristic of *Rm. vannielii*. The isolate produced red pigment in both media. The dominant photosynthetic pigment is bacteriochlorophyll *a* and carotenoids of lycopene and rhodopin. The growth of *Rm. vannielii* was better in anaerobic-light condition compared to growth in aerobic-dark. Optimum carotenoid production was achieved in 24 hours culture in GMM (pH 7.0) without yeast-extract and incubated in anaerobic-light condition at light intensity of 2000 lux.

Key words: Purple nonsulfur bacteria, hot spring, *Rhodomicrobium vannielii*, carotenoids.

INTRODUCTION

Phototrophic bacteria are ubiquitous and have been reported from many terrestrial and marine ecosystems (Tan *et al.,* 1999). They are a great interest to study because they can use light as an energy source and carbon dioxide as a carbon source. Further, phototrophic bacteria have been found in hot springs at higher temperatures of 45°C (Gorlenko *et al.,* 1985). With these characters, phototrophic bacteria may have potential application in bioremediation and anaerobic treatment under high temperature conditions. *Chloroflexus aurantiacus,* a green filamentous phototrophic bacterium which grew at temperatures from 50°C to 60°C was first isolated by Pierson and Castenholz (1974) from hot springs in United States, Guatemala, Iceland and New Zealand followed by isolation from Japanase hot springs (Hanada *et al.,* 1995). *Chromatium tepidium,* a purple sulfur bacterium was isolated from hot spring at temperature 40-60°C (Madigan, 1986). Other species which include the purple nonsulfur bacterium, *Rhodospirillum centenum* (Flavinger *et al.,* 1989), *Rhodopseudomonas cryptolactis* (Stadwald-Demchick *et al.,* 1990); green sulfur bacterium, *Chlorobium tepidum* (Wahlund *et al.,* 1991) and purple sulfur bacterium, *Rhodobium marinum* (Kompantseva and Panteleeva, 1997) have been isolated and characterized.

The aims of this study were to isolate species of anoxygenic thermophilic phototrophic bacteria from Gadek Hot Spring water samples and also to study the growth profiles and pigment production by *Rm. vannielii* under varying physico-chemical parameters.

***Corresponding author**

MATERIALS AND METHODS

Sampling

Water samples were collected in 1L sterile Schott bottles at depth of 0.8-1.4 m from the water surface using an extendable metal sampler from Gadek Hot Spring in Malacca, Malaysia. The water samples were stored in ice during transportation. The temperature and pH of the water samples were measured *in situ*.

Isolation of phototrophic bacteria

Phototrophic bacteria in the water samples were isolated on two media: (i) glutamate-malate medium (GMM): sodium-L-glutamate, 2.0 g; DL-malic acid, 3.0 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $(NH_4)_2HPO_4$, 1.0 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.6 g; $MnSO_4.5H_2O$ (1 mg/ml), 1.3 ml; $CoCl_2.6H_2O$ (1 mg/ml), 1.0 ml; ferric citrate (10 mg/ml in 30% NH_4OH), 0.25 ml; nicotinic acid (1 mg/ml), 1.0 ml; thiamine-HCl (1 mg/ml), 1.0 ml; biotin (100 µg/ml), 1.0 ml; yeast-extract, 1.0 g; distilled water added to 1L and (ii) Pfennig's M2 medium (Biebl and Pfennig, 1978). The pH of both media was adjusted to 6.8 for isolation of green sulfur bacteria and 7.3 for isolation of purple sulfur bacteria using 0.1 M HCl and 0.1 M NaOH, respectively.

Five ml of water samples were added to 20 ml of sterile GMM and M2 in the 25 ml screw-capped bottles completely filled to the brim to maintain anaerobic condition. All inoculated bottles were incubated at 2000 lux light intensity of continuous illumination from 60 W (Morries) tungsten lamps at temperature 38-40°C. In the M2 isolation medium, 0.25 ml - 0.5 ml neutralized Na_2S

(150 mM) was added after disappearance of elemental sulfur (after 4 days of incubation) to stabilize the growth of purple/green sulfur bacteria. Water samples were also added to 20 ml of GMM in 100 ml conical flask and incubated in aerobic dark condition at 100 rpm.

After 3 to 4 days, pink to red pigmentation appeared in the medium and one loopfull of the inoculum was streaked onto GMM and M2 agar. Agar plates were incubated in anaerobic jar with H_2 and CO_2 atmosphere (AnaeroGen, OXOID) at 2000 lux for 3-4 days at 38°C. Pure colonies were obtained by repeated streaking on GMM or M2 agar plates.

Identification

The isolate was identified morphologically by observing the development of color of bacterial suspension in the medium, formation of slime and Gram staining**.** Motility was observed by hanging-drop method. The size and shape of the cells were determined by phase contrast microscopy. The present of flagellum and intra-cytoplasmic membrane structure or chlorosome were determined by transmission electron microscope (Philips CM-12) by negative staining using phosphotungstic acid.

The presence of intracellular sulfur globules was determined by incubating the cells with neutral sodium sulfide solution (3.6% w/v) on a microscopic slide (Truper and Pfennig, 1981). The slides were irradiated with tungsten lamp (Morries) for 20 minutes and the intracellular sulfur globules formation was observed under light microscope.

Photopigment analysis

The *in vivo* absorption spectrum of isolate at exponential phase of growth (24 hours incubation) was measured by Bovine Serum Albumen method (BSA) (Sojka *et al.,* 1970) to determine the bacteriochlorophyll and carotenoid content.

Determination of growth profile and characterization of the isolate

Preparation of standardized culture

A single colony of the isolate was inoculated in 25 ml GMM in Universal bottle and incubated anaerobically in the light (2000 lux light intensity) for 4 days. Bacterial cells were harvested by centrifugation at 6000 rpm, 4°C (Sorvall RC2B) for 30 minutes. The pellet was suspended with sterile normal saline solution (0.85% NaCl) and the optical density of the cell was adjusted to 0.5 at 660 nm wavelength using Shimadzu UV-120-01 spectrophotometer. Then, 10% (v/v) of this standardized culture was used in the subsequent tests.

Growth profile and characterization of the isolate

2 ml of the standardized culture was inoculated into 10 bottles each containing 18 ml GMM pH 7.3. All the bottles were incubated anaerobic in the light with continuous illumination at 2000 lux. After 24, 48, 96 and 120 hours, two bottles were selected randomly and the cell growth was determined by measuring the optical density at 660 nm. Total carotenoid content was extracted by Sojka *et al.,* (1970) method and quantified using formula by Jensen and Jensen (1971).

For aerobic dark growth, the same amount of medium was placed in 100 ml conical flask and incubated in aerobic dark at 100 rpm at 38°C. The dry weight of the cell was determined as described by Sawada and Rogers (1977).

Optimization of growth of the isolate

In the subsequent studies, 2 ml of the standardized culture (prepared as above) was added into 18 ml of GMM in completely filled screw-capped bottles. Each tests were done in duplicate and total carotenoid content was measured and used as growth parameter.

Effect of different light intensities on growth and pigment production

The standardized cultures in GMM (pH 7.3) were incubated anaerobically at light intensities of 1000, 2000, 3000 and 4000 lux and assayed for total carotenoid content after 24, 48, 72 and 96 hours of incubation.

Effect of different pH on growth and pigment production

GMM at varying pH of 6.0, 6.5, 7.0, 7.5 and 8.0 was inoculated with standardized culture and incubated anaerobically under continuous illumination at light intensity of 2000 lux. The pH was adjusted using 1M HCl or 1M NaOH. Total carotenoid content was determined after 24 hours of incubation.

Effect of different concentration of yeast-extract on growth and pigment production

GMM (pH 7.0) with 0, 0.01, 0.1, 1.0 and 2.5% (w/v) of yeast-extract were inoculated with standardized culture and incubated anaerobically at light intensity of 2000 lux. Total carotenoid content and dry weight of cells were determined after 24 and 48 hours of incubation.

Effect of different carbon sources on growth and pigment production

Malate-yeast extract (MYE) medium (Dow 1982) pH 7.0 (without NaCl) was used as basal medium and different carbon sources: xylose, maltose, glucose, galactose, mannose, fructose, sucrose, malate, lactate, sorbitol, mannitol, pyruvate, succinate, acetate, citrate, benzoate and tartrate; each at 1% (w/v) was added separately to

MYE. 2 ml of the standardized culture was added to 18 ml of carbohydrate-incorporated MYE in McCartney bottles and incubated anaerobically at light intensity of 2000 lux for 24 to 48 hours. In all growth response tests, growth was monitored turbidometrically at 660 nm with spectrophotometer (Shimadzu UV-160A). Growth of the cultures that give optical density reading > 0.2 were chosen for the subsequent study.

Effect of selected carbon sources on cell growth and pigment production

Standardize inoculum (2.5 ml) was added to 22.5 ml GMM in McCartney bottles with selected carbon sources at 1% (w/v) and 0.1% yeast extract, pH 7.0 and incubated anaerobically at light intensity of 2000 lux for 24 and 48 hours. Total carotenoid content and dry weight of the cells were determined.

Effect of succinate at different concentration on cell growth and pigment production

The carbon source that gave highest carotenoid content was selected for further tests. The isolate was grown in GMM (pH 7.0) with addition of 0.1% (w/v) yeast-extract and different concentration of succinate; 0.5%, 1.0%, 1.5% and 2.0% (w/v), and incubated anaerobically for 24, 48, 72 and 96 hours at 2000 lux. Carotenoid production and dry weight of the cells were determined after the end of incubation time.

RESULTS AND DISCUSSION

Physical characteristics of Gadek Hot spring

The water temperature at Gadek hot spring was between 50^{o}C to 58^{o}C and the pH values ranging from 6.8 to 8.0. The smell of sulfur is strong and on the surface of water there are layers of slimy matt of green and brownish material.

Isolation and characteristics of the isolate

The growth of anoxygenic phototrophic bacteria was indicated by the change in color of GMM from a transparent yellow to red after seven days incubation anaerobically at continuous light illumination. Growth in M2 medium was slower, with pink to light red color appearing after more than 10 days incubation in the same condition. This result showed that growth of the isolate was favored in GMM compared to M2 medium. This is because the incorporation of yeast extract in GMM at 1 g per liter is more than enough to increase the cell growth. Castenholz (1973) showed that the addition of 50 mg per liter of yeast extract together with bicarbonate in anaerobic light condition was enough to increase the dry weight of the bacterial cells to 0.3 mg per ml. This result showed that photoorganotrophic metabolism support faster growth for this isolate compared to photoautotrophic with sulphide, as the probable electron donor in M2 medium contained Na_2S and $Na_2S_2O_3$. Toxicity of H_2S to Rhodospirillaceae varied according to species (Brune 1989).

Growth of this isolate in aerobic-dark condition in shake-flask was the slowest, and light-red colour was visible after more than two weeks of incubation. This result showed that anaerobic-light condition promoted the growth of the isolate.

On agar plates the isolate formed small, round, smooth-surface red color colonies with diameters of 0.5-1.0 mm after five days of incubation in anaerobic-light condition on GMM. On the M2 medium the growth was slower and took more than seven days for the colonies to appear on the plates.

Identification of the isolate

By phase-contrast microscopy, cells were ovoid to spherical, 2.0-2.5 μm long and 1.2-1.5 μm wide. This isolate was Gram negative, motile and did not produce slime.

There was no intracellular sulfur globules formed when isolate was exposed to neutral sulfide solution, indicating a possible strain of purple non-sulfur bacteria. In transmission electron micrographs the cells were observed to be attached with filaments forming chains or aggregates of cells (Figure 1). This important observation of budding filament formation is the characteristics of *Rhodomicrobium vannielii*, the only species of this genus described (Imhoff and Truper, 1989) in Rhodospirillaceae of the purple nonsulfur bacteria family. Therefore, the isolate was presumptively identified as a strain of *Rm. vannielii.* A mature mother cell forms a filament and young daughter cell buds at the end of the filament. Mature buds, which remain attached to the filament, may form another filament at the opposite pole under certain conditions until a chain of cells is formed (Dworkin, 1992). The presence of *Rm. vannielii* in the hot spring and its ability to sustain growth in GMM reaching 40^{o}C might be due to its ability to form thermotolerant exospores when the nutrient level is below a certain limit (Dworkin, 1992). However, no exospores were observed in culture presumably due to the high nutrient level of GMM.

Species of purple sulfur bacteria and green sulfur bacteria were not isolated from the water samples. They were probably being outgrown by *Rm. vannielii* in the initial liquid culture or due to isolation technique or media selected which favored the enrichment and growth of *Rm. vannielii.*

Analysis of *Rm. vannielii* photopigment

The *in vivo* absorption spectrum of *Rm. vannielii* showed four peaks each at 379 nm, 591 nm, 806 nm and 873 nm, which corresponded to the presence of bacteriochlorophyll *a* (Imhoff and Truper, 1989) and carotenoid absorption maxima at 460 nm, 488 nm and 522 nm, indicating the presence of photopigment of lycopene and rhodopene as the major carotenoid content in *Rm. vannielii* (Imhoff & Truper, 1989).

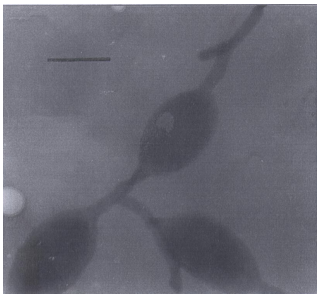

Figure 1: Transmission electron micrograph of *Rm. Vannielii*.
Cells are ovoid in shape and attached by filaments which is a characteristic of phototrophic bacteria in the genus of *Rhodomicrobium*. (Bar = 2µm)

Growth profile

Growth of *Rm. vannielii* was measured as optical density at wavelength of 660 nm showed the sigmoid curve (Figure 2). Growth was favored by incubation in anaerobic-light condition, where it entered the exponential phase after 24 hours of incubation and stationary phase after 48 hours of incubation. *Rm. vannielii* was able to undergo photoautotrophic growth in anaerobic-light condition (Brune, 1989). Growth in aerobic-dark condition was not favorable and the cells declined rapidly after 24 hours of incubation, although the initial growth rate was higher compared to anaerobic-light culture. This showed that cells were also capable of growing chemoheterotrophically in the dark.

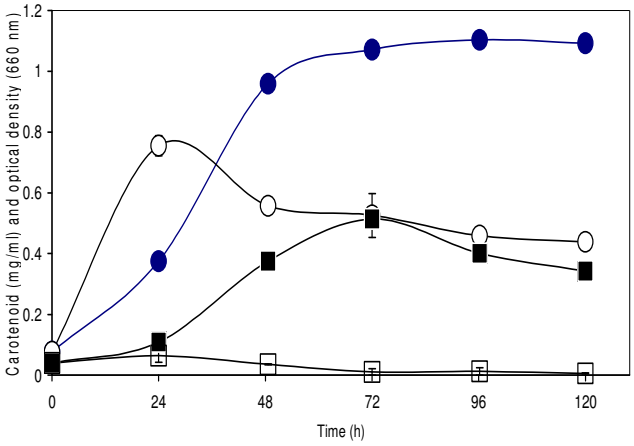

Figure 2: Growth and carotenoid production by *Rm. vannielii* in anaerobic-light and aerobic-dark conditon. The bacterial growth higher in anaerobic-light compared to aerobic dark.
● OD anaerobic-light, ○OD aerobic-dark, ■ carotenoid anaerobic-light, □ carotenoid aerobic-dark

Optimum carotenoid production was observed between 24-72 hours of incubation in anaerobic-light culture, which coincided with the time when cells entered exponential growth phase. This showed a possible link between the increase of carotenoid production with increase in cell biomass. Comparatively, carotenoid production in aerobic-dark culture was lower by 98%. This observation was similar to previous findings that synthesis of carotenoid is repressed in the dark and in the presence of oxygen (Lascelles 1968).

Effect of different light intensities on cell growth and pigment production

The optimum light intensity for carotenoid production was 2000 lux when compared to the other light intensities tested (Figure 3). At 4000 lux, carotenoid was not detected and possibly because the cells were killed by heat generated in the medium. At a low light intensity of 1000 lux, cells remained in a non-reproductive motile stage; which is another characteristics of *Rm. vannielii* (Dworkin 1992), thus reducing cell multiplication and a low in total carotenoid content. At the optimum light intensity, cells enter the budding phase, with increase in the number of daughter cells and subsequently, the total carotenoid content.

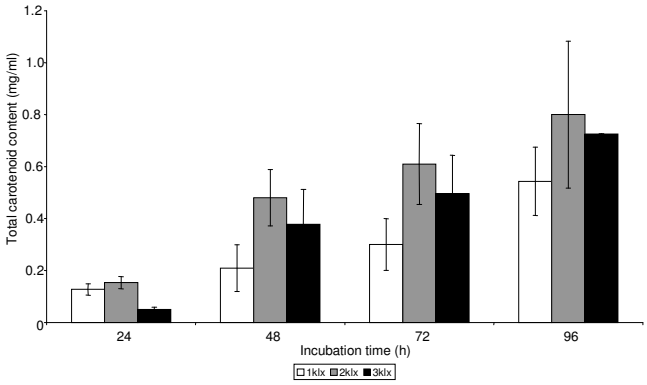

Figure 3: Carotenoid production by *Rm. vannielii* grown at different light intensities.

Effect of different pH medium on cell growth and pigment production

Neutral pH favored the growth of *Rm. vannielii*, since the pH of water samples were neutral (Figure 4). Acidic or alkaline pH will denature the intracellular enzymes or alter the cell structure, thus killing the cells and decreasing the carotenoid production. Almost all carotenoids undergo disintegration, dehydration or isomerization in acidic condition, especially in the presence of light (Britton and Young, 1993).

Effect of different yeast-extract concentration on cell growth and pigment production

Yeast-extract is an important source of nitrogen and thiamine for the synthesis of amino acids and nucleic acids in bacterial cells. Addition of yeast extract to *Rm.*

vannielii culture did not result in increase carotenoid production after 24 hours incubation, while addition of 0.1% yeast extract produced highest production of carotenoid after 48 hours of incubation (Figure 5). This result showed that in the initial growth period, the cells did

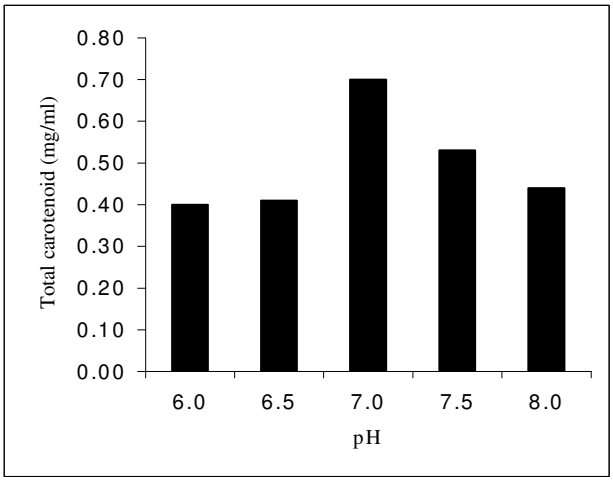

Figure 4: The effect of different pH on the carotenoid production by *Rm. vannielii*

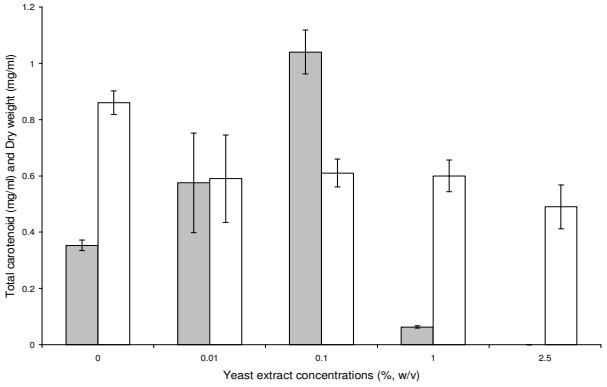

Figure 5: Total carotenoid content and dry weight of *Rm. vannielii* after 48 hours incubation in GMM with different concentration of yeast extract.
(■), total carotenoid and (□), dry weight.

not use yeast extract to support the growth and production of carotenoid. The source of nitrogen from ammonium salt and glutamate in the GMM is enough to support the growth of the cells. However, when this nitrogen source was finished, yeast extract was used to support the growth. No carotenoid was produced at 2.5% of yeast extract after 48 hours of incubation. Castenholz (1973) showed that the addition of 2 g/l of yeast extract increased the bacteriochlorophyll c in *Chloroflexis* by 235%. Prasertsan *et al.,* (1997) also showed addition of 3.0 g/l of yeast extract has an effect on growth, pigment synthesis and COD removal from cultivation of *Rhodocyclus gelatinosus* R7 in tuna condensate medium.

The dry weight of cells were varies with different concentrations of yeast extract. The dry weight was doubled in 0.1% of yeast extract after 48 hours of incubation, which corresponds directly with increase in carotenoid production. Although the dry weight increased three times in culture without yeast extract after 48 hours of growth, the decrease in carotenoid production by 78% showed that most of the cells had entered the stationary phase or were dead. This result showed that yeast extract may be important as inducer in the synthesis of carotenoid at low concentration (Castenholz, 1973).

Effect of different carbon sources on cell growth and pigment production

The carbon sources that enhanced the cell growth (OD at 660 nm > 0.5) were acetate, lactate, pyruvate, citrate and succinate were selected for further tests (Table 1). Moderate growth occurred with D-xylose and no growth was observed in malate and negligible growth in benzoate and tartrate. Further test showed the cells growth and carotenoid production was highest when succinate or acetate used as substrate after 48 hours of incubation (Figure 6). Different concentration of succinate has an effect in the amount of carotenoid production at different incubation period. Addition of succinate at 2.0% (w/v) was found to give a maximum carotenoid production after 24 hours, while addition of 0.5% of succinate also increased carotenoid production, but at a slower rate until 96 hours (Figure 7). Succinate is important since it is involved in the ALA-synthase pathway present in purple nonsulfur bacteria, where 5-aminolevulinic acid (ALA) is produced as the precursor molecule in bacteriochlorophyll biosynthesis (Senge and Smith, 1995).

Table 1: Utilization of various substrates as electron donor or carbon sources by *Rm. vanniellii*

Carbon source	24 hours	48 hours
Organic acids		
Acetate	+	+++
Benzoate	±	±
Citrate	++	+++
Lactate	++	+++
Malate		
Pyruvate	+++	+++
Succinate	++	+++
Tartrate	±	±
Sugar and alcohol		
D-xylose	+	++
Fructose	+	+
Galactose	+	+
Glucose	+	+
Maltose	+	+
Mannose	±	+
Sucrose	+	+
Mannitol	±	+
Sorbitol	+	+

+++, good growth (OD at 660 nm, > 0.5); ++, moderate growth (OD at 660 nm, 0.2 to 0.5); +, poor growth (OD at 660 nm, 0.1 to 0.2); ±negligible growth (OD at 660 nm< 0.1); -, no growth. All substrates were added at a concentration of 1.0 % (w/v).

In conclusion, *Rm. vannielii* isolated from the hot spring was a photoheterotroph, reproduce by budding, favorable to grow anaerobically in the presence of light at maximum intensity of 2000 lux. This isolate was able to use succinate and acetate efficiently as carbon source.

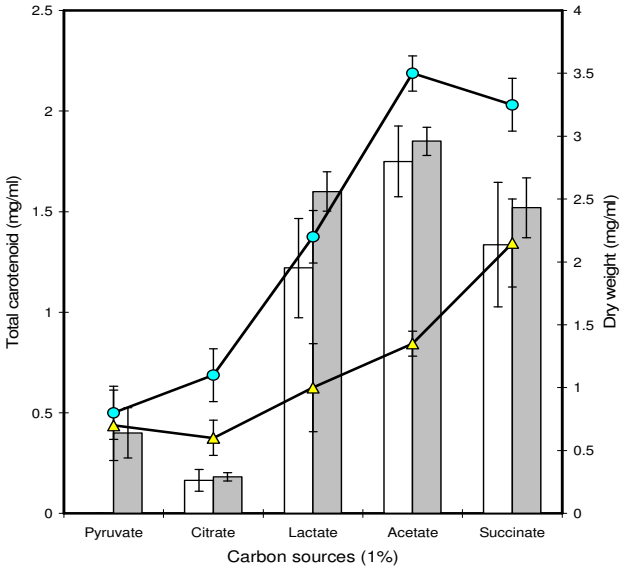

Figure 6: Total carotenoid content and dry weight of *Rm. vannielii* after 24 hours and 48 hours incubation in GMM with different carbon sources.
(□) total carotenoid at 24 h; (■) total carotenoid at 48 h; (-▲-) dry weight at 24 h; (-●-) dry weight at 48 h.

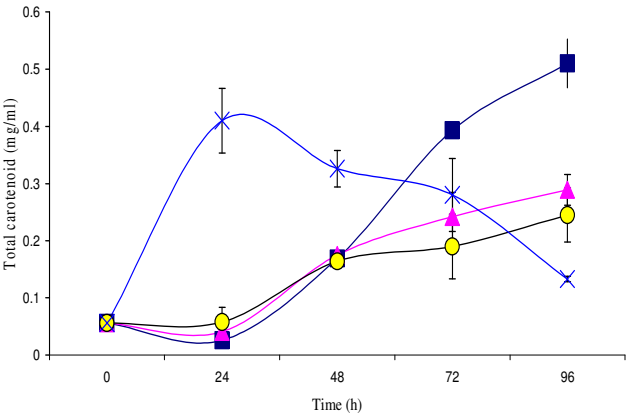

Figure 7: Total carotenoid content of *Rm. vannielii* after 48 hours incubation in GMM with different concentrations of succinate incubated in anaerobic-light at 2000 lux.
Concentration of succinate: ■, (0.05%), ▲, (1%), ●, (1.5%), **x ,** (2%).

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