CHAPTER 2

CHAPTER 2

CHARACTERIZATION AND OPTIMIZATION OF GROWTH OF *Rhodopseudomonas palustris* STRAIN B1

INTRODUCTION

Traditionally, differentiation of species, genera and even families of phototrophic bacteria was based on a number of morphological and chemical properties, such as cell form and size, flagellation, pigment composition, DNA base ratio, and also physiological properties such as carbon and nitrogen substrate utilization and ability to respire aerobically and anaerobically in the light or dark (Pfennig and Trüper, 1974; Trüper and Pfennig, 1981; Imhoff and Trüper, 1989).

Previous studies have shown that most representatives of purple non-sulphur bacteria can grow under anaerobic conditions with sufficient light present. Growth may be further improved by optimizing other parameters such as temperature, pH, inoculum size, growth factors and nutrients.

In this study, the determination of the nutritional requirements and growth parameters of *R. palustris* strain B1 was dependent on its ability to grow rapidly under optimized conditions. The aims of this investigation were to:

(i) characterize *R. palustris* strain B1 based on morphology, photopigments and the growth profile under anaerobic-light and aerobic-dark conditions.

(ii) determine the nutritional requirements of *R. palustris* strain B1.

(iii) determine the effects of physical parameters on the growth of *R. palustris* strain B1

(iv) study the growth profile of strain B1 under optimized conditions.

MATERIALS AND METHODS

Bacteria

The purple non-sulphur phototrophic bacteria *Rhodopseudomonas palustris* strain B1 was obtained from the IPT culture collection. Master stock cultures were maintained as stab cultures on glutamate-malate (GM) medium (Appendix B1) solidified with 1.5% agar and sealed with sterile paraffin oil. After growth at 30 °C and with continous illumination with tungsten bulbs, the cultures were stored at 4 °C. The cells may also be stored for indefinate periods after lyophilization.

Inoculum Preparation

Both the isolates *R. palustris* ATCC 17001 and *R. palustris* strain B1 were grown separately in screw-cap bottles completely filled with GM medium at $30^{o} \pm 2$ °C under anaerobic-light conditions. Continuous illumination was provided

by a row of 60 W tungsten bulbs. After 72 h incubation, the cells were harvested under sterile conditions by centrifugation at 5000 rpm for 30 min. Cells were resuspended in sterile GM medium to obtain a cell density corresponding to OD_{660nm} of 0.3. An inoculum size of 10% (v/v) was used for subsequent tests unless otherwise stated. The culture used in growth studies was transferred every 3 to 4 days by using a 10% inoculum into fresh GM medium.

Characterization of *Rhodopseudomonas palustris* Strain B1

Rhodopseudomonas palustris strain B1 was characterized based on growth, morphological characteristics and the photopigments present. A comparison of this strain was made with the type culture *R. palustris* ATCC 17001.

Morphological studies

Rhodopseudomonas palustris strain B1 and ATCC 17001 were cultured separately in GM medium in screw-capped bottles under anaerobic-light conditions. Cultures were also grown on plates of GM medium solidified using 1.5% agar. The determination of colour of the cell suspensions and microscopic examinations such as cell shape by gram staining techniques (Appendix A1) and motility were observed in exponential growth phase cultures of 72 h.

Electron microscopy

Cultures were grown under anaerobic-light conditions in GM medium for 72 h. Specimens were prepared by placing 2 to 3 drops onto nucleopore membranes and were processed as given in Appendix A3. The specimens were examined with a Phillips Scanning Electron Microscope 515.

Growth studies

The cultures were grown in GM medium under anaerobic-light conditions at $30^{O} \pm 2$ °C in 100 mL Pyrex bottles. The cultures were illuminated at 3 klux with a row of 60 W tungsten bulbs (Plate 1). The heat produced by the bulbs was dissipated using a fan.

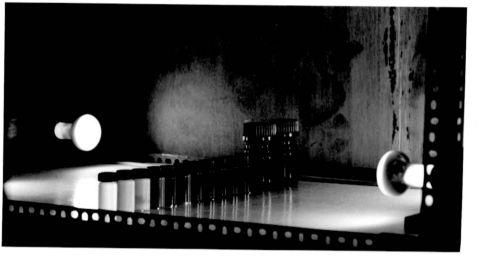

Plate 1 : Purple non-sulphur bacteria grown in GM medium under anaerobic-light conditions at 30° ± 2 °C and 3 klux.

In aerobic growth experiments, 90 mL of GM medium in 500 mL Erlenmeyer flasks were inoculated with 10 mL of inoculum and agitated in a rotary shaker (New Brunswick Scientific Co-G25) at 150 strokes per min. The temperature was controlled at $30^{0} \pm 2°C$. The aerobically growing cultures were kept in the dark by wrapping the conical flasks with aluminium foil.

The culture suspensions, in triplicates, were monitored for absorbance at 660 nm and cell dry weight of the biomass (Appendix A2). The absorption determinations were carried out using Shimadzu UV-160A spectrophotometer. Readings were recorded daily for a period of 7 days.

Absorption spectra of intact cells

In saturated sucrose

Both the *R. palustris* strain B1 and ATCC 17001 were grown separately under anaerobic-light conditions in screw-capped bottles filled with GM medium at $30° \pm 2$ °C. Cells were harvested in exponential growth phase and the pellet resuspended in 60% saturated sucrose which reduced light scattering (Dow, 1982). The absorbance spectrum was determined using a Shimadzu UV-160A spectrophotometer. The procedure followed in this study is described in Fig. 2.

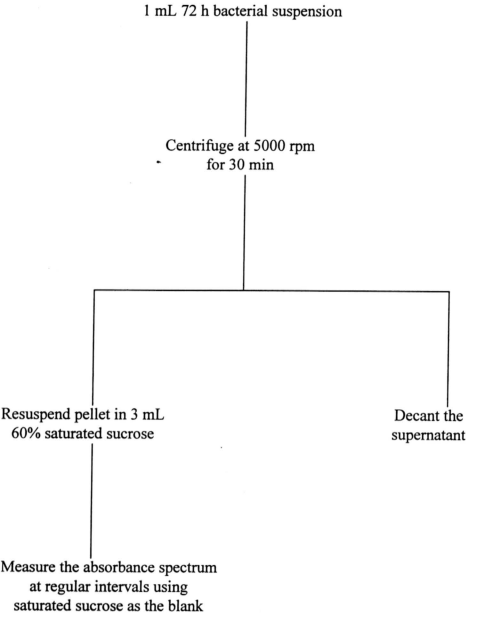

Fig. 2 : Absorption spectrum of intact cells

In 25% bovine serum albumin (BSA)

Cultures grown under anaerobic-light conditions for 72 h were harvested and the cells resuspended in 25% bovine serum albumin. The absorbance of the suspension was determined as described in Fig. 3 (Sojka *et al.*, 1970). Shimadzu UV 160A spectrophotometer was used in these studies.

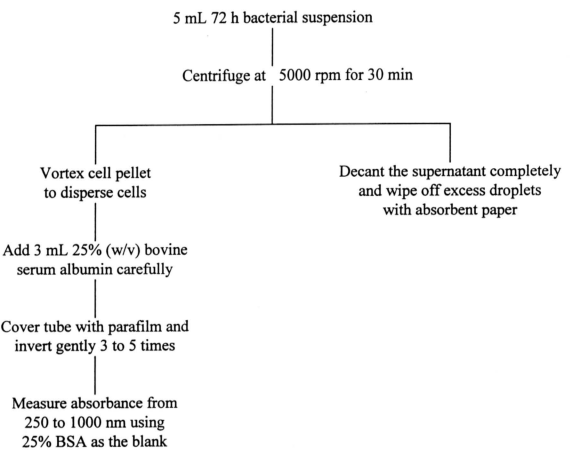

Fig. 3 : Determination of *in vivo* spectrum of photosynthetic pigments

(Sojka *et al.*, 1970)

Optimization of growth of *Rhodopseudomonas palustris* Strain B1

Optimization of growth of *R. palustris* strain B1 was based on its nutritional requirements and the effects of physical parameters.

(a) Nutritional requirements

The nutiritional requirements of the isolates strain B1 and ATCC 17001 were studied on small-scale cultures grown photosynthetically in completely filled screw-capped bottles (20 mL) at 30° ± 2 °C and 3 klux light intensity.

(i) Utilization of complex organic compounds

The utilization of complex organic compounds were tested by growing the cultures in GM medium (Appendix B1) at pH 6.8 to 7.0 under anaerobic-light conditions. The complex organic compounds were added at concentrations of 3.0 g/L in place of DL-malic acid.

(ii) Utilization of simple organic compounds

Tests for the utilization of simple organic substrates (sodium salts were used in case of organic acids), however, were performed by adding the substrates from sterile stock solutions (final concentration 0.3% (w/v)) to the autoclaved GM medium (pH 6.8 to 7.2) in which the DL-malic acid was omitted.

All tests were carried out in triplicates. A 10% inoculum was added (Ch 2, pp28) and the bottles were incubated at 30° ± 2 °C at a light intensity of 3 klux.

Initially, bottles showing good growth (reddish brown) were marked visually after 5 days of incubation. The growth of the cultures were then measured turbidometrically using a Shimadzu Spectrophotometer UV160-A.

(iii) Effect of starch

The ability to utilize starch by *R. palustris* strain B1 was investigated by replacing DL-malic acid with 3.0 g/L of various types of starch available locally before autoclaving. Tests for utilization of raw soluble starch, however, were performed by sterilising the raw starch at 120 °C for two hours before adding the autoclaved GM medium without the DL-malic acid. From the above test, potato starch was better utilized by the strain B1. Hence, the effect of starch concentration on growth of strain B1 was investigated with potato starch as the sole carbon source at concentrations of 0.3%, 0.5%, 1.0%, 2.0%, 3.0%, 4.0% and 5.0% (w/v) respectively. All tests were performed in triplicates at $30^{O} \pm 2^{O}$C and 3 klux. Cell growth was mesured turbidometrically after 72h.

(iv) Growth factor requirements

To determine vitamin requirements, thiamine, nicotinic acid, yeast extract and biotin were tested by omitting the vitamins one at a time from the GM medium.

Growth was observed visually after incubation for 72h at 30° ± 2 °C and at a light intensity of 3 klux.

The requirement of growth factor, was tested by using GM medium at pH 6.8 to 7.2 supplemented with concentrations of 0 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL of *p*-aminobenzoic acid (PABA). Cell growth was measured turbidometrically at 660 nm using a Shimadzu Spectrophotometer UV160-A after 72h of growth at 30° ± 2 °C and 3 klux light intensity . All tests were performed in triplicates.

(b) Effects of physical parameters on cell growth

Culture bottles containing fresh, sterile GM medium were inoculated with a 10% inoculum prepared as given in Ch. 2, pp28 (unless otherwise stated) and were incubated under anaerobic-light conditions. The response of *R. palustris* strain B1 and ATCC 17001 to :-

(i) temperature

25 °C, 30 °C, 35 °C, 37 °C, 40 °C, 45 °C, 50 °C and 55 °C

(ii) pH

4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5

(iii) light intensity

1 klux, 2 klux, 3 klux, 4 klux, 5 klux and 6 klux

(iv) salinity

0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% of NaCl,

(v) inoculum age

12 h, 24 h, 36 h, 48 h, 60 h and 72 h, and

(vi) inculum size

5%, 10%, 15% and 20%

were noted turbidometrically at 660 nm after 72 of incubation.

Growth Profile of Strain B1 and ATCC 17001 Under Optimized Conditions

From the above two experiments a modified version of the GM medium was prepared using the optimum carbon source, vitamin and growth factor requirements. The medium was then autoclaved at 121 °C for 15 min at 15 psi and cooled to room temperature. Tests were performed for the growth profile under the optimized physical parameters of temperature, pH, salinity and light intensity. The inoculum of optimum age and size were inoculated into the culture bottles and incubated under anaerobic-light conditions.

Triplicate bottles were analysed after 72 h for culture pH, cell dry weight and optical density at 660 nm.

Statistical Analysis

Analysis of variance (ANOVA) was done to test the significance of the carbon source, starch concentration, *p*-aminobenzoic acid concentration, temperature, pH, light intensity, salinity, inoculum age and inoculum size on the growth of *R. palustris* strain B1 and *R. palustris* ATCC 17001. ANOVA was carried out for cell biomass data obtained on the third day of growth where maximum cell mass production was recorded in nearly all the treatments studied.

RESULTS AND DISCUSSIONS

General morphological and cultural characteristics

Rhodopseudomonas palustris strain B1 and ATCC 17001 were found to have almost identical morphological characteristics. The cultures grown in GM medium under anaerobic-light conditions were reddish-brown in colour after 48 h incubation at 30° ± 2 °C (Plate 2). They formed small red colonies generally of 2 to 3 mm in diameter (Plate 3A and 3B) on agar plates cultured in anaerobic-light conditions; the pigmentation depended to some extent on the cultural conditions. Cultures grown aerobically in the dark were found to be very pale grey-pink.

The pure culture isolates of *R. palustris* strain B1 and ATCC 17001 were found to be gram negative motile rods with terminal swellings. The cell population showed a range of sizes and not all cells were motile. The cells were found to have a tendency to form rosette-like clusters. Examination of wet mounts under phase contrast at a magnification of 1000 x showed dumbbell shaped organisms (Plate 4A and 4B). Similar observations were also made by Qadri and Hoare (1968).

Electron microscopic examination of the cultures showed that the cells were rod to ovoid shaped. *Rhodopseudomonas palustris* ATCC 17001 was 2.0 to 2.5 mm long and 0.5 to 0.7 mm wide (Plate 5A). *Rhodopseudomonas palustris* strain B1 was slightly shorter, being 1.5 to 2.0 mm long and 0.3 to 0.6 mm wide, with ridges running the whole length of the cell (Plate 5B). Both the cultures showed polar cell growth and multiplied by budding. Short, straight chains of up to 4 cells were sometimes present, although the rosette-like cell clusters were dominant. This has been discussed at length by Westmacott and Primrose (1976).

Plate 2 : *Rhodopseudomonas palustris* (a) ATCC 17001 and (b) strain B1 grown photosynthetically in GM medium

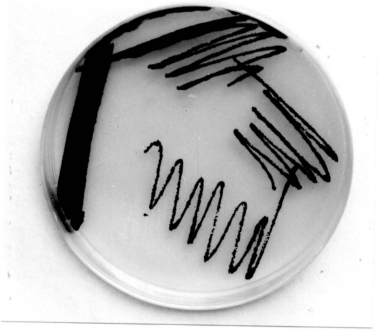

Plate 3A : Pure culture of *R. palustris* strain B1 on GM medium agar plate

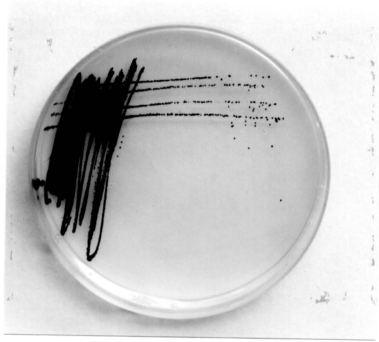

Plate 3B : Pure culture of *R. palustris* ATCC 17001 on GM medium agar plate

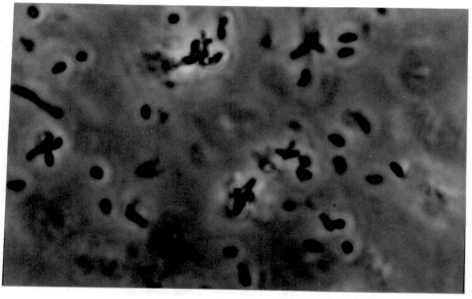

Plate 4A : Phase contrast photomicrograph of crystal violet stained *R. palustris* strain B1 (x1000)

Plate 4B : Phase contrast photomicrograph of crystal violet stained *R. palustris* ATCC 17001 (x1000)

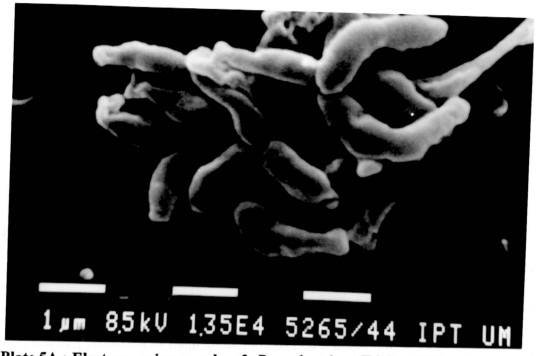

Plate 5A : Electron micrograph of *R. palustris* ATCC 17001 showing the characteristic dumbbell-shaped cells forming rosettes and polar budding

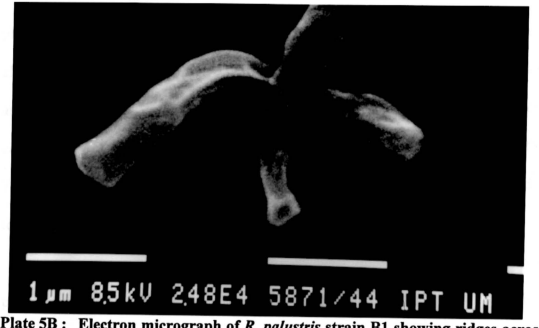

Plate 5B : Electron micrograph of *R. palustris* strain B1 showing ridges across the cell length

Growth Studies

The growth profiles of *R. palustris* strain B1 in GM medium under anaerobic-light and aerobic-dark conditions are shown in Figures 4A and B and also in Appendix C1. The growth profiles of *R. palustris* ATCC 17001 under anaerobic-light and aerobic-dark conditions are listed out in Appendix C2 and also Figures 5A and B.

The GM medium turned reddish-brown during the growth of *R. palustris* strain B1 and ATCC 17001 under anaerobic-light condition. Intense pigmentation was observed after 48 h of incubation and reached a maximum at 96 h. The biomass of strain B1 increased four fold after 96 h of incubation. The organisms grown under anaerobic-light conditions obtain their energy from light through photophosphorylation, therefore, the organic molecules are utilized as carbon and electron sources. Thus, the metabolism of anoxygenic photoheterotrophic bacteria is assimilatory and the organic carbon source is utilized to build biomass (Balloni *et al.*, 1987). At the later stage of the growth, the cells tend to clump together. After 7 days of anaerobic-light growth, the culture turned brown. This was probably due to the decrease in nutrient content and the subsequent death of the cells.

Cultures grown under aerobic conditions in the dark were very pale grey-pink in colour. This indicated that pigment synthesis was suppressed if sufficient aeration was provided (Carr, 1969). The growth studies of the cultures indicated that these bacteria do grow in the dark, thus confirming reports that they are

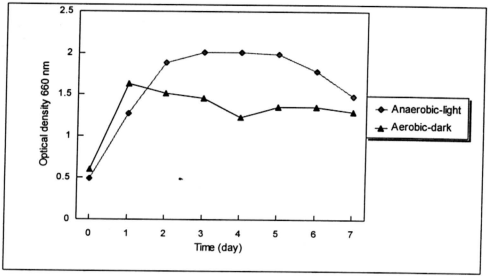

Fig. 4A : The growth profile of *R. palustris* strain B1 grown in GM medium under anaerobic-light and aerobic-dark conditions

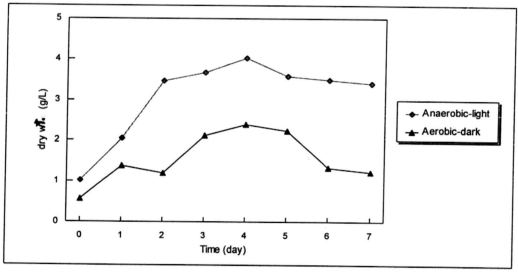

Fig. 4B : The cell dry weight over time of *R . palustris* strain B1 grown in GM medium under anaerobic-light and aerobic-dark conditions

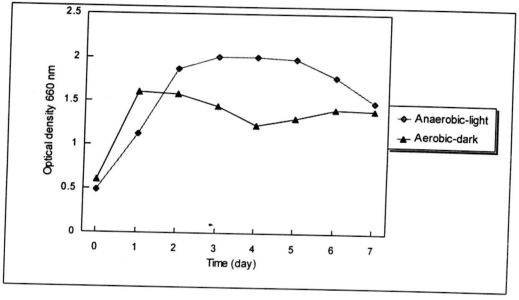

Fig. 5A : The growth profile of *R. palustris* ATCC 17001 grown in GM medium under anaerobic-light and aerobic-dark conditions

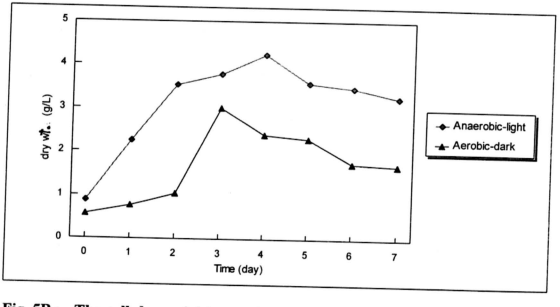

Fig. 5B : The cell dry weight over time of *R. palustris* ATCC 17001 grown in GM medium under anaerobic-light and aerobic-dark conditions

heterotrophic (Ormerod and Sirevag, 1983). The growth rate of these organisms under anaerobic-light conditions was always higher than the rate of growth under aerobic-dark conditions. The biomass produced in aerobic-dark growth was much lower when compared to growth in photosynthetic conditions.

Absorption Spectrum of Intact Cells

In saturated sucrose

The absorption spectrum of cell suspensions exhibited characteristic maxima at 375, 590, 805 and 860 nm (Fig. 6 and Table 4) which indicated the presence of bacteriochlorophyll *a* (Table 5). Similar observations was also made by Akiba *et al.* (1983). Comparison of absorption by strain B1 with the absorption spectrum of ATCC 17001 showed that cell suspensions of strain B1 also absorbed light at 490 nm indicating the presence of carotenoids of the lycopene and rhodopin groups (Table 6).

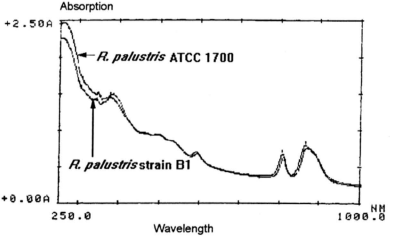

Fig. 6 : Absorption spectrum of cell suspensions of *R. palustris* strain B1 and ATCC 17001 in saturated sucrose

Table 4 : Absorption values corresponding to the wavelengths of cell suspensions of *R. palustris* strain B1 and ATCC 17001 in saturated sucrose

Peak No	Strain B1		ATCC 17001	
	λ(nm)	Abs	λ(nm)	Abs
1	864.0	0.791	865.0	0.768
2	806.0	0.672	806.0	0.643
3	592.0	0.702	592.0	0.684
4	492.0	0.940	-	-
5	379.0	1.453	380.0	1.507

Table 5 : Characteristic absorption maxima of bacteriochlorophylls in living cells (Dow, 1982; Imhoff, 1995)

Bacteriochlorophyll	Absorption maxima (nm)
a	375, 590, 800-810, 830-890
b	400, 605, 835-850, 1015-1035
c	335, 460, 745-760, 812
d	325, 450, 725-745, 805
e	345, 450-460, 715-725
g	370, 419, 575, 670, 780-790

Table 6 : Typical carotenoids and their approximate absorption maxima in living cells (Pfenning and Trüper, 1991)

Major carotenoid components	Absorbance maxima (nm)
Lycopene and rhodopin	463, 490, 524
Spirilloxanthin	486, 515, 552
Spheroidene	450, 482, 514
Rhodopinal	497, 529
Okenone	521

In 25% bovine serum albumin

The absorption spectrum of strain B1 resembled that of *R. palustris* ATCC 17001. The absorption maxima at 379, 499, 591, 806 and 864 nm of ATCC 17001 was nearly identical to absorption maxima at 379, 496, 591, 805 and 863 nm of *R. palustris* strain B1 (Fig. 7 and Table 7). This indicated the presence of carotenoids of the spirilloxanthin group in both the cultures (Akiba *et al.*, 1983; Kompantseva and Gorlenko, 1984) (Table 5). However, *R. palustris* strain B1 also showed peaks at 466 and 528 nm.

Therefore between the two absorption studies carried out, *R. palustris* strain B1 showed extra peaks at 466, 492 and 528 nm which corresponds to the absorption maxima of lycopene and rhodopin which also belong to the normal

spirilloxanthin series (Table 8). Hence, the additional carotenoids possessed by the phototroph probably functions as secondary light harvesting pigments and prevents photo-oxidation within the cells (Dow, 1982).

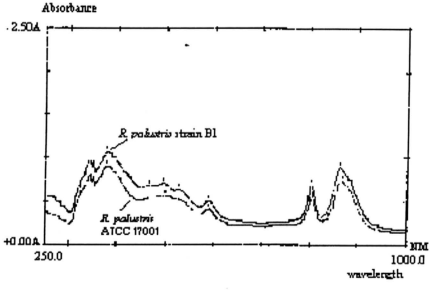

Fig. 7 : Absorption spectrum of *R. palustris* strain B1 and ATCC 17001 cells suspended in 25% BSA

Table 7 : Absorption values corresponding to the wavelengths of cell suspensions of *R. palustris* strain B1 and ATCC 17001 in 25% BSA

Peak No	Strain B1		ATCC 17001	
	λ(nm)	Abs	λ(nm)	Abs
1	863.0	0.894	864.0	0.730
2	805.0	0.702	806.0	0.564
3	591.0	0.523	591.0	0.409
4	528.0	0.654	-	-
5	496.0	0.723	499.0	0.573
6	466.0	0.704	-	-
7	379.0	1.118	379.0	0.901

Table 8 : Major carotenoid groups of anoxygenic phototrophic bacteria (Schmidt, 1978)

Biosynthetic group	Major components
Normal spirilloxanthin series	Lycopene, rhodopin, spirilloxanthin
Rhodopinal series	Lycopene, lycopenal, lycopenol, rhodopin, rhodopinal, rhodopinol
Alternative spirilloxanthin series	Hydroxyneurosporene, sphaeroidene, sphaeroidenone (spirilloxanthin)
Okenone series	Okenone
Isorenieratene series	β-carotene, isorenieratene
Chlorobactene series	γ-carotene, chlorobactene

A Comparison Between *R. palustris* Strain B1 and *R. palustris* ATCC 17001 Based on Morphology, Growth Studies and Photopigments

Rhodopseudomonas palustris strain B1 and the type culture ATCC 17001 had similar morphology and growth profiles. Both the cells were gram negative motile rods with terminal swellings and formed rosette-like clusters. The only difference being that the cells of *R. palustris* strain B1 were slightly smaller in size and showed ridges running through the whole length of the cell. Photosynthetic cultures were red and the bacterium was a facultative photoheterotroph growing either anaerobically in the light or aerobically in the dark. Cultures grown aerobically in the dark were a very pale grey-pink. In general, cultivation under anaerobic-light conditions gave higher specific growth rates (0.7 to 0.8 day^{-1}) and maximal biomass production (3.5 to 4.0 g/L). Growth under aerobic-dark conditions gave specific growth rates of 0.4 to 0.7 day^{-1} and the biomass production was 1.0 to 3.0 g/L. Both the cultures showed absorption maxima for intact cells indicating the presence of bacteriochlorophyll *a* and carotenoids of the spirilloxanthin group. *Rhodopseudomonas palustris* strain B1 also exhibited absorption maxima for lycopene and rhodopin. Some of its characteristics are summarized in Table 9.

Table 9 : Characteristics of *Rhodopseudomonas palustris* strain B1

Cell shape	Rod to ovoid; With terminal swelling;
	Forms rosette-like clusters;
	Ridges on surface
Mode of division	Budding
Cell size (mm)	
length	1.5 to 2.0
width	0.3 to 0.6
Culture colour	Reddish-brown (anaerobic-light)
	Pale grey-pink (aerobic-dark)
Clumping	Yes
Motility	Yes
Gram stain	Negative
Bacteriochlorophyll	*a*
Carotenoids	Spirilloxanthin, lycopene, rhodopin

Utilization of Carbon Sources and Electron Donors

Growth of *R. palustris* strain B1 and ATCC 17001 on the modified GM medium with single organic sources are summarized in Table 10.

After 5 days of growth under anaerobic-light conditions, strain B1 and ATCC 17001 photoutilized several organic compounds as carbon sources and as electron donors. The carbon source utilized had a significant effect on cell biomass production ($p<0.05$) (Appendix C4 and C5). Of the organic compounds tested,

malic acid, succinate, tartarate, benzoate and pyruvic acid were utilized by both isolates. In addition, *R. palustris* strain B1 utilized starch and its breakdown compound amylopectin. No growth was observed on the simple sugars. It is well documented that the purple non-sulphur bacteria require reduced carbon compounds such as malate and succinate as both electron donors and carbon sources (Pfennig, 1967; Madigan and Gest, 1979). Van Niel (1944) noted as early as in 1944 that the purple non-sulphur photosynthetic bacteria grew anaerobically in the light on diverse simple organic compounds. Many photosynthetic bacteria do not grow particularly well on sugars, probably because they are sensitive to the acidification that can result from sugar metabolism, particularly under oxygen-limiting conditions (Willison, 1993). In general terms, very little information is available on the mechanisms of sugar transport, with the exception of fructose uptake. In some cases, the ability or inability to use fructose is characteristic of the species, particularly, *Rhodospirillum* and *Rhodobacter* (Madigan, 1988).

The spectrum of organic compound utilization has been one of the criteria for classification in the genus *Rhodopseudomonas*. Therefore, the ability of *R. palustris* strain B1 utilizing starch was found to be significant ($p<0.05$).

Table 10 : Utilization of single organic substrates by *R. palustris* strain B1 and ATCC 17001 under anaerobic-light conditions after 96 h at 30° ± 2 °C and 3 klux.

	R. palustris	
Carbon source	**Strain B1**	**ATCC 17001**
Sodium carbonate	-	-
Sodium succinate	+++	++
Sodium acetate	+	-
Sodium thiosulfate	++	++
Sodium sulfite	+++	++
Sodium formate	+++	+
Sodium tartrate	+++	++
Tri-sodium citrate dihydrate	++	+
DL-malic acid	+++	+++
Sodium benzoate	+++	+++
Sodium lactate	+	-
Citric acid	-	-
Soluble starch	+++	-
Dextrin	-	+
Glycerol	-	-
Methanol 3%	++	++
Methanol 1%	+++	++
Methanol 0.3%	+	-
Ethanol 3%	++	++
Ethanol 0.3%	-	-
Pyruvic acid 1%	+	+
Pyruvic acid 0.3%	+++	+++
D(+) xylose	-	-
α-L-rhamnose	-	-

D(+) galactose	-	-
β-D(+)-glucose	-	-
D(+)-glucose	-	-
D(-)-fructose	-	-
β-D(-)fructose	-	-
D-mannitol	-	-
D(+)raffinose	-	-
L(+) arabinose	-	-
D(+) mannose	-	-
Sucrose	-	-
Maltose	-	-

Marked visually after 5 days incubation (also shown in Plate 6).
a = - = no growth (colourless) (as in the control without the inoculum)
b = + = slight growth (pink)
c = ++ = good growth (red)
d = +++ = excellent growth (reddish brown)

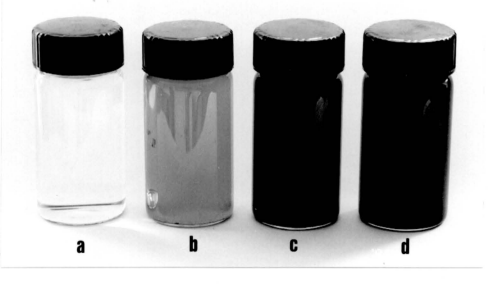

Plate 6 : Growth of *R. palustris* corresponding to Table 10

The experiments with organic compounds which supported good growth of the isolates were repeated under identical conditions and the optical density was recorded after 72 h. The results obtained are summarized in Fig. 8 and Appendix C3.

Rhodopseudomonas palustris strain B1 grew well in media with malic acid, succinate, tartrate, formate, pyruvic acid, starch or amylopectin. The strain grew slightly with benzoate, sulfite or methanol and failed to grow on amylose. Under the same conditions, *R. palustris* ATCC 17001 showed good growth only on malic acid, succinate, benzoate, tartrate, methanol and pyruvic acid. Growth on media containing formate, sulfite or starch was slow.

Amongst the purple non-sulphur bacteria, *R. palustris* has been classically considered as the one strain which may actively metabolize and grow on formate under photosynthetic conditions (Qadri and Hoare, 1968). Methanol, another one carbon substrate may be assimilated by some strains too. However, the growth was CO_2-dependent due to the reduction level of methanol (Tabita, 1995). Bicarbonate (or CO_2) is absolutely required as an electron acceptor for growth of photosynthetic non-sulphur bacteria on reduced organic compounds such as methanol, propionate, glycerol and acetone since they are considerably more reduced than cell material (Tabita, 1995).

Virtually, all purple photosynthetic bacteria are able to grow on pyruvate under photoheterotrophic growth conditions. As an important hub in microbial

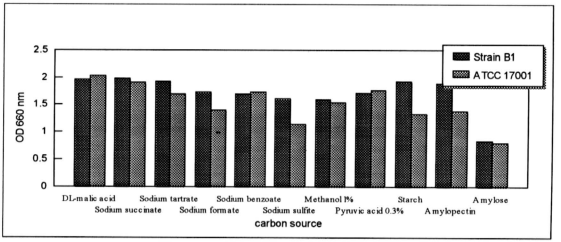

Fig. 8: The effect of various carbon sources on growth of *R. palustris* strain B1 and ATCC 17001 in modified GM medium after 72h at 30°± 2° C and 3 klux

metabolism (Gest, 1981) the reductions leading to and from pyruvate have attracted some interest in purple non-sulphur bacteria, as they readily grow aerobically and anaerobically. Another 3-carbon substrate, glycerol, is also able to support the growth of *R. palustris* and *Rb. sphaeroides* but not the other purple non-sulphur bacteria.

However, it is well known that purple non-sulphur bacteria grow better, with faster doubling times and to higher density when C_4 dicarboxylic acids such as malate or succinate are used as electron donors (Tabita, 1995). Such compounds, along with pyruvate and lactate and other common growth substrates are more

oxidized than the cell material and are able to release CO_2 into the growth medium (Muller, 1933). The transport of C_4-dicarboxylates was first studied by Gibson (1975) who showed an inducible transport system for malate, succinate and fumarate in *Rb. sphaeroides*. The major route for the transport of malate, succinate and fumarate is a high-affinity binding-protein-dependent system (Dct) (Hamblin et al., 1990; Shaw and Kelly, 1991). It is able to bind L-malate, succinate and fumarate with high affinity and D-malate with somewhat lower affinity, but does not bind other organic acids significantly (Armitage *et al.*, 1995). The Dct system is regulated in activity by the intracellular pH value (Shaw and Kelly, 1991).

Growth on Starch

Studies on nutritional requirements revealed that *R. palustris* strain B1 utilized starch. Cultivation of *R. palustris* strain B1 on the various types of starch are summarized in Fig. 9 and Appendix C6.

The type of starch used and its concentration had a significant effect on cell dry weight ($p < 0.05$) (Appendix C7 and C9).

Rhodopseudomonas palustris strain B1 grew well on media with potato, sago, tapioca and soluble starch compared to corn, wheat, glutinous rice or rice starch. The growth was enhanced with the addition of 0.1% yeast extract. Raw (soluble) starch did not support the growth of *R. palustris* strain B1. The optimum concentration of potato starch in the media was at 2.0%. The optical density after

72 h incubation with 2.0% starch was 2.361. The results obtained are summarized in Fig. 10 (Appendix C8).

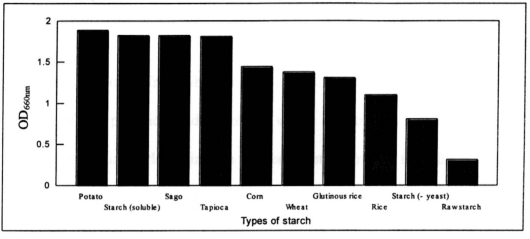

Fig. 9: **The growth of *R. palustris* strain B1 on the various types of starch in modified GM medium after 72h at 30° ± 2° C and 3 klux**

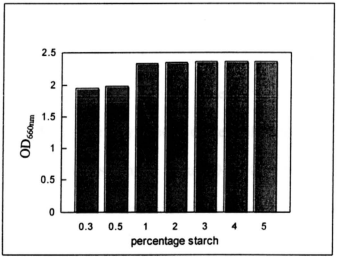

Fig. 10: **The effect of potato starch concentrations on the growth of *R. palustris* strain B1 in modified GM medium after 72h at 30° ± 2° C and 3 klux**

Rhodopseudomonas palustris strain B1 was able to actively metabollize starch, the increase in cell biomass being significant ($p < 0.05$) (Appendix C4). Utilization of starch is arousing increasing interest because of its abundant availability in nature. Starch is a polysaccharide and its monomeric structure is glucose. The chemical structure of starch is essentially the same, regardless of the wide variety of sources from which it can be obtained. Naturally occurring starch is a mixture of two polysaccharides, both of which are polymers of D-glucose. The major component is amylopectin with a branched structure and the minor component amylose is a linear macromolecule.

Rhodospeudomonas palustris strain B1 was able to utilize potato, sago and tapioca starch very well compared to the other types of starches because the amylopectin content in those starches were about 80% (Young, 1984). The amylopectin content in corn and wheat starch was slightly lower than 80%, thus accounting for the decrease in cell mass production. The modern rice starches, however, have a wide range of values among varieties with high-amylose content of up to 40% (Juliano, 1985). The amylopectin content of various starches is shown in Table 11. Most native starches contain about 20-30% by weight amylose and the ratio of amylose to amylopectin is genetically controlled (Young, 1984).

Table 11 : Amylopectin content of starches (Young, 1984; Juliano, 1985)

Starch	Amylopectin (%)
Potato	80.0
Sago	74.2
Tapioca	83.3
Corn	72.0
Wheat	74.0
Rice	60-81.5

Physiological and Biochemical Characteristics

The results of tests for growth factor requirements are summarized in Table 12 and 13.

Studies on the requirement of vitamins revealed that *R. palustris* strain B1 required only biotin for growth at a concentration of 100 μg/ml while *R. palustris* ATCC 17001 required nicotinic acid, thiamine and biotin for growth, but not *p*-aminobenzoic acid or yeast extract. The addition of *p*-aminobenzoic acid to the GM medium does not significantly increase the growth rate of *R. palustris* strain B1 and may even inhibit growth at higher concentration ($p>0.05$) (Appendix C10). However, higher concentrations of *p*-aminobenzoic acid did not affect the growth of *R. palustris* strain 17001 ($p>0.05$)(Appendix C11).

Table 12 : Phytosynthetic growth of *R. palustris* strain B1 and ATCC 17001 on GM medium with specific growth factors omitted after 72 h at 30° ± 2 °C and 3 klux

Growth factor	*R. palustris*	
	Strain B1	ATCC 17001
- nicotinic acid (omitted)	++	-
- thiamine (omitted)	+++	-
- biotin(0 µg/ml)	+	+
+ biotin(50 µg/ml)	+	++
(100 µg/ml)	+++	+++
(150 µg/ml)	++	+
(200 µg/ml)	++	+
- yeast extract (omitted)	+++	+++

Table 13 : Optical density at 660 nm on the growth of *R. palustris* strain B1 and ATCC 17001 in GM medium with *p*-aminobenzoic acid after 72 h at 30° ± 2 °C and 3 klux

p-aminobenzoic acid concentration (µg/ml)	*R. palustris*	
	Strain B1	ATCC 17001
0	1.865	1.846
50	1.915	1.915
100	1.807	1.906
150	1.862	1.873
200	1.848	1.892

Effect of Temperature on Growth

The response of strain B1 and ATCC 17001 to temperature is shown in Fig. 11 and Appendix C12.

The analysis of variance showed that the cell dry weight was affected significantly by the temperature ($p<0.05$) (Appendix C13 and C14). Both *R. palustris* strain B1 and ATCC 17001 could not tolerate temperatures higher than 45 °C. *Rhodopseudomonas palustris* strain B1 grew within the range of 25 °C to 40 °C with very little difference. While *R. palustris* ATCC 17001 preferred 37 °C for optimal growth and could tolerate ranges from 25 °C to 37 °C.

The majority of photosynthetic bacteria fit into the category of mesophiles, that is organisms which prefer intermediate temperatures (Philips and Mitsui, 1982b). To date, purple non-sulphur bacteria with growth temperature limits above 47 °C have not been isolated in the laboraotry (Resnick and Madigan, 1989; Stadtwald-Demchick *et al.*, 1990a,b). Most earlier described species of the purple non-sulphur bacteria in culture have temperature optimum of 30-35 °C and only two species have optimum as high as 40-42 °C (Imhoff and Trüper, 1989).

Other studies of hot springs species have recovered purple non-sulphur bacteria from temperatures exceeding 50 °C. However, these studies have not demonstrated growth in the 50-60 °C range, since recovery from the water was performed at lower temperatures in the laboratory (Gorlenko *et al.*, 1985, 1987; Yurkov *et al.*, 1992).

The purple non-sulphur bacteria are infamous in being present almost everywhere but extreme thermophiles have yet to be found.

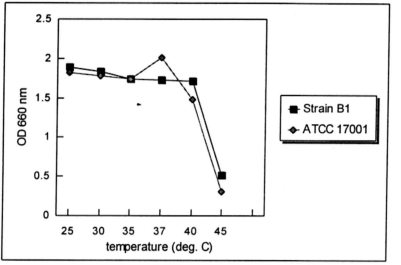

Fig. 11: **The effect of temperature tolerance of *R. palustris* strain B1 and ATCC 17001 in GM medium after 72h at 3 klux**

Effect of pH on Growth

Response of growth of both the strains to varying pH is shown in Fig. 12 and Appendix C15.

The analysis of variance showed that the cell dry weight was affected significantly by the pH ($p<0.05$) (Appendix C16 and C17). The general pH range which supported good growth for both the isolates was between 5.5 to 8.5. Growth at lower pH showed significant decrease in the cell biomass. The pH of the medium after 72 h of growth of both isolates studied was between 7.5 and 8.5.

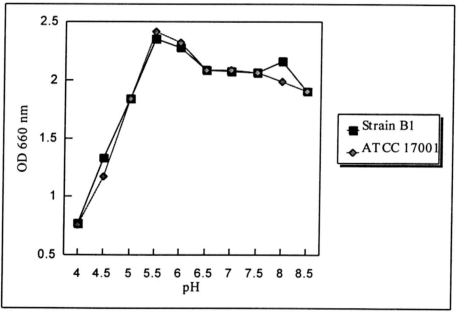

Fig. 12: The effect of initial pH on the growth of *R. palustris* strain B1 and ATCC 17001 in GM medium after 72h at 30° ± 2° C and 3 klux

Effect of Light Intensity on Growth

The effect of light intensity on the growth of *R. palustris* strain B1 and ATCC 17001 is shown in Fig. 13 and Appendix C18.

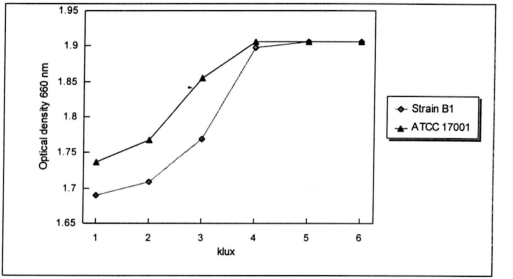

Fig. 13: The effect of light intensity on growth of *R. palustris* strain B1 and ATCC 17001 in GM medium after 72h at 30° ± 2°C

When the effect of light intensity on growth of strain B1 was investigated in the range of 1000 to 6000 lux, it was found that growth was highest at 5000 lux. It was also found that any further increase in light intensity did not increase growth. At low light intensity, the cells grew but at a slower rate, and did not significantly affect the cell dry weight of *R. palustris* strain B1 ($p>0.05$) (Appendix C19). *Rhodopseudomonas palustris* ATCC 17001, however, was significantly affected by

the light intensity ($p<0.05$) (Appendix C20). Highest cell biomass was obtained with light intensities of above 3 klux.

It was observed that the near infra-red wavelengths of light 780 to 920 nm were absorbed by bacteriochlorophyll, in *Rhodopseudomonas* (Pfennig,1967,1977; Imhoff 1995). This was provided with tungsten bulbs and the heat was dissipated using a fan.

The growth of the culture itself, from a faintly turbid solution to a deeply pigmented thick suspension, caused continuous decrease in the light received by each microbial cell due to shading. Thus, to obtain reliable growth, over-illumination should be considered (Getha, 1995).

It has also been reported that the most marked effect of variation of light intensity, other than on growth, is on the concentrations of photosynthetic pigments within the microbial cell. The levels of bacterichlorophyll and carotenoids was found to vary inversely with light intensity (Cohen-Bazire *et al.*, 1957, Lascelles, 1960; Frisow and Drews, 1977). Very high light intensities were found to cause problems with irradiance, exceeding saturation levels for photosynthesis and growth. The excess light intensities was found to be a cause of photoinhibition, especially by photooxidation and ultra violet (UV) light damage (Philips and Mitsui, 1982a). This light effect was explained by the degradation of bacteriochlorophyll and not the repression of bacteriochlorophyll synthesis (Biel, 1986). Therefore, a compromise

between light intensity and good growth and concentration of photopigments is desirable.

Effect of Salinity on Growth

Results of the effect of varying salinity on the growth of *R. palustris* strain B1 and ATCC 17001 are shown in Fig. 14 and Appendix C21.

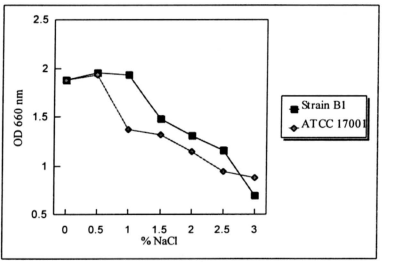

Fig. 14: The effect of salinity on growth of *R. palustris* strain B1 and ATCC 17001 in GM medium after 72h at 30° ± 2° C and 3 klux

The dry weight of biomass for *R. palustris* ATCC 17001 obtained after 3 days of culture with the various concentrations of NaCl did not show any significant differences ($p>0.05$) (Appendix C23). However, the concentration of NaCl

significantly affected the cell dry wieght of *R. palustris* strain B1 ($p<0.05$) (Appendix C22) . *Rhodopseudomonas palustris* strain B1 was isolated from fresh water sources, and therefore it could only tolerate salinities of up to 2.0%. Higher salt concentration showed significant decrease in the growth. It is found that higher levels of sodium chloride in the medium inhibits the growth of *R. palustris* strain B1. This would also be probably true for the type culture if salinity ranges of above 3.0% was tested as the higher salt concentration corresponds to the decrease in cell dry weight though not significantly. At present, only the marine species of *Rhodovulvum*, *Rv. marinus*, *Rv. adriacticus*, *Rv. euryhalinus* and *Rv. sulfidophilus* have been known to occur at salinities ranging from 3.5% to 15% (Burgess et al., 1994; Hiraishi and Ueda, 1994a). Therefore it can be concluded that *R. palustris* strain B1 and ATCC 17001 do not show any requirement for sodium chloride for growth.

Effects of Inoculum Size and Age on Growth Rates

The effect of inoculum size and age on the growth of *R. palustris* strain B1 and ATCC 17001 was studied and the results obtained are shown in Table 14 and 15.

Table 14 : Effect of inoculum size on the specific growth rates of *R. palustris* strain B1 and ATCC 17001 in GM medium at 30° ± 2 °C and 3 klux

% inoculum	Specific growth rate, μ_{max} (gh^{-1})*	
	Strain B1	ATCC 17001
5	0.056	0.050
10	0.052	0.054
15	0.050	0.051
20	0.041	0.045

*mean of three values

Table 15 : Effect of inoculum age on specific growth rates of *R. palustris* strain B1 and ATCC 17001 in GM medium at 30° ± 2 °C and 3 klux

Inoculum age (h)	Specific growth rate, μ_{max} (gh^{-1})*	
	Strain B1	ATCC 17001
12	0.049	0.048
24	0.050	0.048
36	0.049	0.053
48	0.051	0.052
60	0.049	0.053
72	0.052	0.051

*mean of three values

The analysis of variance showed that the inoculum size had significant effect on cell dry weight ($p < 0.05$) (Appendix C24 and C25). Studies on the optimal inoculum size revealed that a 5 to 15% inoculum was more suitable and gave higher specific growth rates for both *R. palustris* strain B1 and ATCC 17001. However, the inoculum age did not significantly affect the specific growth rates of either isolates ($p > 0.05$) (Appendix C26 and C27). In general, an inoculum size of 5 to 15% and an inoculum age of 48 to 72 h was found to be more desirable for effective and optimal cultivation of the isolates under anaerobic-light conditions. Cells in the late log phase of growth are preferred as inoculum as the organism should grow quickly and in high numbers so that the period of incubation required is relatively short.

Comparison of Growth of *R. palustris* Strain B1 under Optimized Conditions

The growth characteristics of *R. palustris* strain B1 in the modified GM-salt medium (1) (Appendix B1(b)) and GM-starch medium (Appendix B1(c)) medium under optimized conditions were compared to the conventional method of growth. The results obtained are presented in Table 16. The conditions for cultivation were at pH 7.0 and a temperature of 25 °C, at a light intensity of 5 klux with a 48 h old 5% inoculum.

Table 16 : Comparison of growth of *R. palustris* strain B1 under optimized conditions after 72h incubation

R. palustris **Strain B1**	**Medium**		
	GM	**GM-salt(1)**	**GM-starch**
OD 660 nm*	1.796	1.753	1.405
Final pH*	7.82	7.97	5.56
Cell dry wt. (g/L)*	2.87	4.00	8.14

*mean of three values

It was found that the maximum biomass growth was obtained when GM-starch was used as the medium. This aspect should be studied in future. Probably, the unutilized starch also contributed to the overall dry weight. The pH decreased from an initial value of 7.0 to 5.6 over 72 h of growth. In other words, pH became more acidic as growth progressed. It could be due to the production of acids in the growth medium. According to Mangels *et al.* (1986), the purple non-sulphur prototrophic bacteria produced acidic compounds during photoheterotrophic growth on carbohydrates or sugars. In contrast, growth on mono- or dicarboxylic acids led to an increase in pH. The increase in biomass in the GM-salt(1) and GM-starch medium might also have been due to the supplementation of *p*-aminobenzoic acid and sodium chloride to the medium.

The optical density of GM-starch grown *R. palustris* strain B1 revealed an interesting observation. The reduction in absorbance of the cell suspension was

due to a decrease in the concentration of pigments. This decrease could probably be due to inhibition by several factors during growth. Pigmentation has been shown to be sensitive to oxygen, heat, light and acids (Jensen and Jensen, 1971). It is thus possible that the strong acidic pH might be one of the inhibiting factors.

Comparison of Growth of *R. palustris* ATCC 17001 under Optimized Conditions

Information on the growth characteristics of *R. palustris* ATCC 17001 in the GM-salt(2) medium (Appendix B1(d)) under optimized conditions is shown in Table 17. Cultivation was carried out at pH 7.0 and at a temperature of 37 °C with a light intensity of 4 klux and a 48 h old 10% inoculum.

Table 17 : Growth of *R. palustris* ATCC 17001 in GM medium and GM-salt (2) medium after 72h incubation

R palustris **ATCC 17001**	**Medium**	
	GM	**GM-salt(2)**
OD 660 nm*	1.855	1.943
Final pH*	8.23	8.28
Cell dry wt. (g/L)*	4.51	5.08

*mean of three values

The marginal increase in the pigmentation and biomass with the GM-salt(2) medium may be due to the supplementation of *p*-aminobenzoic acid and salt to the medium. Generally, a low sodium requirement is found in fresh-water purple non-sulphur photosynthetic bacterium (Imhoff, 1982).

The above results have indicated that 72 h of bacterial cultivation was sufficient to produce a maximal biomass with high pigmentation. Supplementation of 50 μg/ml *p*-aminobenzoic acid and 0.5% sodium chloride increased the biomass of the isolates. In the case of *R. palustris* strain B1, maximum biomass was achieved with starch as the organic carbon source although pigmentation was decreased and the medium became acidic. Illumination was provided by tungsten bulbs and a fan was used to dissipate the heat. The optimal growth conditions are summarized in Table 18.

Table 18 : Optimal growth conditions for *R. palustris* strain B1 and ATCC 17001

Conditions	*Rhodopseudomonas palustris*	
	Strain B1	**ATCC 17001**
Temperature	25 °C	37 °C
pH	5.5	5.5
Light intensity	5 klux	4 klux
Salinity	-	-
Inoculum size	5%	10%
Inoculum age	48 h	48 h
Growth factors required	biotin	nicotinic acid, thiamine, biotin

- not required