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Photoproduction of hydrogen by photosynthetic bacteria from sewage and waste water

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Abstract. Numerous prokaryotes, belonging to physiologically and taxonomically different groups, are able to produce hydrogen. Some photosynthetic bacteria have the property of light-dependent production of hydrogen from organic substrates. We isolated several photosynthetic purple and green bacteria from enrichment cultures made from the water of a waste-water pond of a cool-drink refilling station. After testing them for their ability to use various organic compounds as carbon source, and sulphide, thiosulphate and organic compounds as electron donor, we selected the fastest-growing isolate, a *Rhodopseudomonas*, for a study of its ability to produce molecular hydrogen in presence of light. Immobilized cells of this isolate produced significant amounts of hydrogen from both sewage and waste water.

Keywords. Photosynthetic bacteria; immobilized cells; hydrogen production; *Rhodo-pseudomonas·*

1. Introduction

The phototrophic purple and green bacteria are found in nearly all aquatic environments. Purple and green sulphur bacteria can be easily recognized when they form water blooms (Van Niel 1971). Purple nonsulphur bacteria, on the other hand, rarely appear in visible concentrations; their presence in nature, therefore, can only be evaluated from results obtained by enrichment techniques (Kaiser 1966) or by membrane filtration (Biebl and Drews 1969; Swoagar and Linderstrom 1971). Purple nonsulphur bacteria can photoassimilate a wide variety of organic compounds, and their presence in a water body is particularly dependent upon the extent to which it is polluted with organic matter. Their growth contributes to purification of heavily polluted water exposed to sunlight, as, for example, in sewage lagoons (Holm and Vennes 1970; Cooper *et al* 1975). In Japan, phototrophic bacteria are used in the main purification stage of organic waste-water treatment (Kobayashi *et al* 1971; Kobayashi 1975).

Under suitable conditions, the photosynthetic purple nonsulphur bacteria of the family Rhodospirillaceae can photometabolize organic substrates, forming carbon dioxide and hydrogen according to the equation

$$C_x H_y O_z + (2x - z)H_2O \rightarrow xCO_2 + (y/2 + 2x - z)H_2.$$

The light-dependent production of hydrogen by photosynthetic purple bacteria was

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first observed with cultures of *Rhodospirillum rubrum* (Gest and Kamen 1949). Since the evolution of hydrogen by these photosynthetic bacteria is catalysed by nitrogenase, it is inhibited by ammonium salts and competitively by molecular nitrogen. As hydrogen could well be the fuel of the future, photobiological production of hydrogen by photosynthetic bacteria has been discussed in the light of solar energy conversion through biological systems (Mitsui *et al* 1980; Weaver *et al* 1980; Kumazawa and Mitsui 1982). Since naturally adapted organisms may prove to be better suited for photoproduction of hydrogen from dilute carbon source (Weaver *et al* 1980), we examined production of hydrogen by photosynthetic bacteria isolated from the waste water of a cool-drink refilling station.

2. Methods

The source of microorganisms was a pond 100 metres from the bottle-washing unit of a "Thums Up" and "Limca" soft-drink refilling station at Moula-Ali, Hyderabad. Samples were collected in the summer of 1990 from the water at a depth of about 30 cm and also from the surface of the sediment present. The pH (8·5) and temperature (32°C) of the water sample were recorded during sampling. The water sample was also analysed for dissolved oxygen (2·9 mg/1) and carbohydrate (6·0 mg/1) in the laboratory by standard procedures (APHA). Two sets of water samples (5 ml and 10 ml) were incubated in 30 ml of modified Pfennig medium (van Neil 1971) in presence of light (2000 lux) at 3 0±2°C under anaerobic conditions. The nitrogen source used was ammonium chloride. Single colonies were isolated by agar shake method after 10^{-2} to 10^{-14} dilution (Biebl and Pfennig 1981). After one month of incubation in the light, single colonies were isolated from the agar. These were further purified to the axenic state on agar plates under anaerobic conditions. After several weeks of growth in the light, the cultures were examined by light and phase-contrast microscopy. For characterization of cell pigments, absorption spectra from 350 to 900 nm of the whole cells in sucrose were obtained in a Beckman DU40 UV-Visible spectrophotometer according to Truper and Pfennig (1981). The isolates were screened for growth at several pH (from 4·5 to 8·0) and on various carbon sources (8 mM concentration each). We selected the fastest-growing isolate, SM1NSOU, a *Rhodopseudomonas*, for hydrogen production. SM1NSOU was grown in 1 litre culture vessels in the modified Pfennig medium under nitrogen-limiting conditions (no NH_4C1) to induce nitrogenase. The cells were centrifuged at 20,000 *g* for 10 min, washed twice with distilled water, and immobilized in 2% sodium alginate. A known quantity of the immobilized cells (4 g dry wt of cells) was transferred to a bioreactor. The reactor was a 3·5-litre-capacity column. The total volume of alginate containing the immobilized cells was 2 litres, and 1·44 litres of liquid (substrate diluted with medium) was used, leaving 60ml of gas-phase volume.

Either sewage or waste water was used as substrate in the bioreactor. Sewage was collected from a large drain nearby. It had a pH of 8·2; temperature at the time of collection was 32°C; dissolved oxygen was 0·69 mg/1 and total carbon was 38 mg/1. Prior to use, both sewage and waste water (from which the organisms were isolated) were filtered and autoclaved. Both were added at 50% (v/v) concentration, diluted with nitrogen-free modified Pfennig medium. The bioreactor was flushed with

argon to create anaerobic conditions, and illuminated (2000 lux) with infrared bulbs and fluorescent tubes. Gas production was measured volumetrically by the downward displacement of water and the gas produced was analysed by gas chromatography using nitrogen as the carrier gas.

3. Results and discussion

Both the 5 ml and the 10 ml samples of the original stock gave identical results. We obtained six isolates of sulphur and nonsulphur bacteria. Growing cells of the isolates had virtually the same absorption spectrum, and the microscopic investigation revealed rod-shaped and spherical cells (table 1). We found differences in growth when factors like pH (4·5 to 8·0), and carbon source and electron donor were changed (table 2). All the isolates gave cellular absorption spectra with the characteristic peaks due to bacteriochlorophyll *a* (table 1). One isolate gave the 760 nm peak due to bacteriochlorophyll *c*, which is present in some of the green bacteria. Isolates SM1NSOU and SM2NSOU were incapable of using sulphide or thiosulphate as electron donor (table 2), and are the purple nonsulphur *Rhodopseudomonas*, the other four isolates were all capable of growth in presence of sulphide. This shows that the waste-water pond contained, in additon to nonsulphur bacteria, several sulphur bacteria. Clearly there were sufficient sulphate reducers present to provide these sulphur bacteria with their electron donor sulphide/sulphur.

Table 1. Morphological and pigment characteristics of isolates of photosynthetic bacteria from waste water.

	SM1NSOU	SM2NSOU	SMS1OU	SMS2OU	SMS3OU	SMS4OU
Cell size (μm)						
Length	2–4·5	0·6–3(dia)	2–5	2·4–3·6	2–3(dia)	2·4–3·6
Breadth	1		1·5	1·2		1·2
Motility shape	motile rods slightly curved	motile spheres	motile rods	motile rods	motile spherical cells forming sticky aggregates	motile rods
Colour of cell suspension	purple-red to orange brown	brown orange	blood red	purple violet	pink	green
Cellular absorption peaks (nm)	890 855 520 490 470 460 375 330	890 860 483 450 360 330	890 870 860 610 800 490 487 380	860 804 483 453 449 374 354	864 804 379 374 346 337 332	870 760 680 460 340
Species	*Rhodopseudo-monas* sp	*Rhodopseudo-monas* sp	*Chromatium* sp	*Chromatium* sp	*Amoebo-bacter* sp	*Chlorobium* sp

We selected the fastest-growing isolate SM1NSOU (*Rhodopseudomonas*) for studies of its ability to carry out light-dependent production of hydrogen from

Table2. Biochemical Characters of photosynthetic bacteria isolated from waste water.

Carbon source (8 mM) electron donor	SM1NSOU	SM2NSOU	SMS1OU	SMS2OU	SMS3OU	SMS4OU
Acetate	+	+	+	+	+	+
Benzoate	–	–	–	–	–	–
Citrate	+	+	–	–	–	–
Ethanol	+	+	–	–	–	–
Fructose	–	+	+	–	+	+
Glucose	–	+	+	–	–	–
Malate	+	+	+	+	+	+
Valerate	+	–	–	–	–	–
Mannitol	–	+	–	–	–	–
Thiosulphate (3 mM)	–	–	+	–	+	–
Sulphide (3 mM)	–	–	+	+	+	+
Growth pH	5·5	7·2	6·5	6·5	6·5	6·8

+, Utilized; –, not utilized.

waste water and sewage. With waste water/sewage at a concentration of 50% (v/v), there was a lag period of two days, and then a relatively constant rate of hydrogen production for 3 days. After this period, production of hydrogen levelled off and then stopped, owing perhaps to depletion of substrate or accumulation of inhibitory products. Figure 1 shows the hydrogen production from both sewage and waste water. Hydrogen production from sewage continued until day 7, whereas in the case of waste water hydrogen production stopped after day 5. The highest production was from waste water on day 3. Figure 1 also shows the primary observations, namely total gas produced each day, as well as hydrogen per ml of total gas. The cells remained active after gas production stopped and hydrogen production could be resumed by addition of fresh substrate (data not shown).

The other component of the gas produced is mainly carbon dioxide. Hence the hydrogen to carbon dioxide ratios can be obtained directly from the figure. For waste water on day 3 and for sewage on day 5, both total gas production and hydrogen production are high; for waste water on day 3, we observed a high proportion of hydrogen in total gas. Although it is premature to attach importance to this observation, it is possible that once stable conditions are established hydrogen production can perhaps be qualitatively correlated with total gas production. The total volume of hydrogen produced, calculated for 1 litre of substrate, is 214 ml for waste water and 290 ml for sewage. We wish to stress that the sewage and waste water and the medium used as diluent were not optimized for any particular parameter, *e.g.* growth or hydrogen production. It is very likely that a better (quantitative) correlation may be seen once the system is properly optimized. Our results show that when crude and undefined substrates without pretreatment, such as waste water and sewage, are used for hydrogen production good results are obtained with organisms selected and enriched from environments that contain such substrates. More positively, a system could be developed to use sewage, sludge or waste waters for growth of such bacterial species. Some photosynthetic bacteria, *e.g*, the Chromatiaceae, are known to take up noxious

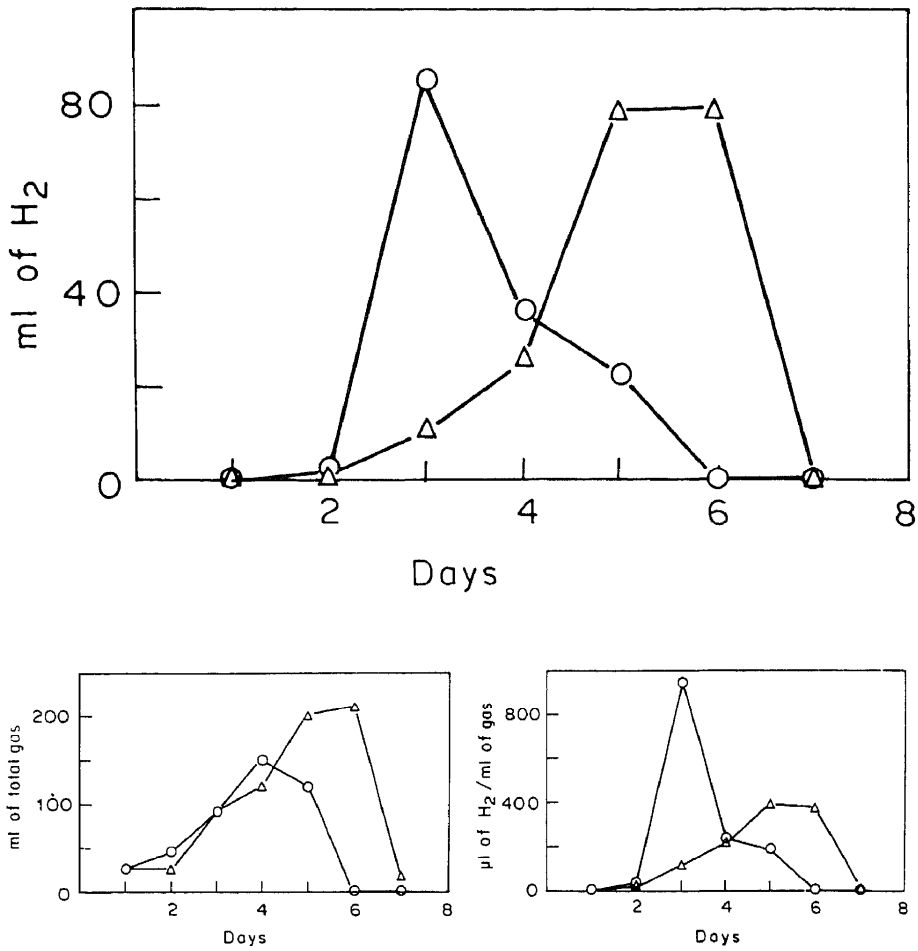

Figure 1. Photoproduction of hydrogen from sewage (Δ) and waste water (O) by immobilized cells of *Rhodopseudomonas* isolate SM1NSOU. The bioreactor contained 4g (dry wt) of immobilized cells and waste water (4·3 mg carbohydrate) or sewage (27 mg carbon).

sulphur compounds and convert them to elemental sulphur (Thiele 1968; Truper 1968, 1980; Imhoff 1980; Imhoff and Truper 1980).

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