CHAPTER 1

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GENERAL INTRODUCTION

PHOTOTROPHIC BACTERIA

Phototrophic bacteria are procaryotes characterized by their ability to grow in the absence of air when exposed to light. Three major groups of these bacteria are known: (a) the purple and brown non-sulphur bacteria (*Athiorhodaceae*); (b) the purple sulphur bacteria (*Thiorhodaceae*); and (c) the green sulphur bacteria (*Chlorobacteriaceae*).

The photosynthetic mode of life of the bacteria occurs without oxygen evolution under anaerobic conditions only. Bacterial photosynthesis depends on the presence of external electron donors. Their photosynthetic pigments are bacteriochlorophyll *a*, *b*, *c*, *d* or *e* and different carotenoids which function in the transformation of light into chemical energy and give the bacterial cultures a distinct colouration. The principal electron carriers are mainly ferrodoxins, group-specific quinones and cytochromes. All components of the photosynthetic apparatus (Fig. 1) are incorporated into the cytoplasmic membranes. Anoxygenic phototrophic bacteria absorbs light at longer wavelengths yielding less energy for photosynthesis. They need electron donors more reduced than water, like reduced sulphur, organic compounds and molecular hydrogen (Pfennig, 1977; Sasikala *et al.*, 1993). Quite recently, even growth with reduced iron as electron donor has been demonstrated with some phototrophic bacteria (Widdel *et al.*, 1993).

The purple non-sulphur bacteria (PNB)use only one photosystem and the photosynthetic apparatus consists of three components that are closely integrated with one another in both structural and functional aspects:

a) an antenna of light - harvesting pigments

b) a limited number of photochemical reaction centres that always contain chlorophyll in a special state, and

c) an electron transfer chain (Kondratieva *et al.*, 1981) (Fig. 1).

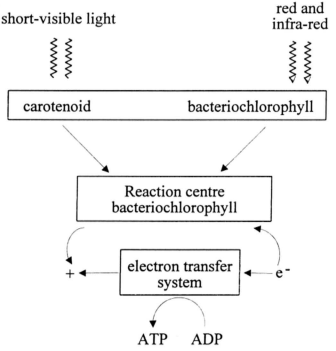

Fig. 1. A schematic diagram to show the functions of the three components of a photosynthetic apparatus in PNB (Kondratieva *et al.*, 1981)

Phototrophic bacteria obtain their energy from light by photophosphorylation. They assimilate carbon dioxide to obtain the necessary cell carbon and to dispose of the excess hydrogen and electrons from the substrates which they oxidize. The usable range of carbon sources is wide and includes carbohydrates, organic acids, alcohols, amino acids and aromatic compounds. The requirement for organic growth factors is widespread, where often more than two vitamins are needed for growth, most commonly biotin, thiamine, *p*-aminobenzoic acid and nicotinic acid (Imhoff, 1995). Complex organic nitrogen sources, for example yeast extract, are known to give increased growth rates (Getha, 1995). This explains the heterotrophic tendencies of these bacteria. However, because of not being able to break down organic macromolecules such as starch, cellulose, lignin and proteins in natural habitats, they are dependent on the preceding activity of chemo-organotrophic bacteria capable of such degradation (Pfennig, 1967).

In nature, anoxygenic phototrophic bacteria occur in fresh waters, brackish waters, marine habitats, wastewaters, hot spring waters, hot sulphur spring waters, moist soils, paddy fields, sewage waters and in extreme conditions of the Antarctic (Sasikala *et al.*, 1993).

PURPLE NON-SULPHUR BACTERIA

Classification and Growth

The purple non-sulphur bacteria (PNB) are the best studied and the most diverse group of the phototrophic bacteria (Imhoff and Trüper, 1989). This diversity is reflected in varying morphology, internal membrane structure, carotenoid composition, utilization of carbon sources and electron donors, among others. However, the variations of deoxyribonucleic acid (DNA) base ratios within each species are surprisingly small and the molecular percentage guanine and cytosine for the whole family lies between 61-70. The cells are rod-shaped to ovoid and multiply by budding. They are gram negative. The cells grow in microaerophilic conditions but may grow under strict anaerobic conditions if sufficient light is present. The classification of purple non-sulphur bacteria is presented in Table 1.

It is to be noted that a number of changes have recently been made concerning the taxonomy of purple non-sulphur bacteria. *Rhodopseudomonas blastica* has been recognized as a species of the genus *Rhodobacter* (Kawasaki *et al.*, 1993). *Rhodobacter marinus* has been described as a new species (Burgess *et al.*, 1994). And thus, all the marine species of *Rhodobacter*, *Rb. adriaticus*, *Rb. euryhalinus* and *Rb. sulfidophilus* have been transferred to the new genus *Rhodovulvum* with *Rhodovulvum sulfidophilum* as type species (Hiraishi and Ueda,

Table 1 : Classification of purple non-sulphur phototrophic bacteria (Sasikala *et al.*, 1993; Imhoff, 1995)

Genus and Species	
Genus I	*Rhodospirilium*
Species	*R. rubrum, R. photometricum, R. fulvum, R. molischianum, R. salexigens, R. salinarum, R. centenum*, R. mediosalinum and R. sodomense*
Genus 2	*Rhodophila*
Species	*R. globiformis*
Genus 3	*Rhodobacter*
Species	*R. capsulatus, R. sphaeroides, R. veldkampii, R. blasticus*
Genus 4	*Rhodopseudomonas*
Species	*R. palustris, R. viridis, R. sulfoviridis, R. acidophila, R. rutila, R. julia, R. cryptolactis*
Genus 5	*Rhodomicrobium*
Species	*R. vannielii*
Genus 6	*Rhodocyclus*
Species	*R. purpureus, R. tenuis*
Genus 7	*Rhodoferax*
Species	*R. fermentans*
Genus 8	*Rubrivivax*
Species	*R. gelatinosus*
Genus 9	*Rhodovulvum*
Species	*R. sulfidophilum, R. marinus, R,. adriaticus, R. euryhalinus*
Genus 10	*Rhodoplanes*
Species	*R. roseus, R. elegans*

*It has been proposed to transfer this species to a new genus as *Rhodociste centenaria* (Kawasaki *et al.*, 1992).

1994a). *Rhodopseudomonas rosea* has been transferred to the new genus *Rhodoplanes* as *Rhodoplanes roseus* and was designated as the type species of this new genus (Hiraishi and Ueda, 1994b). In addition, a new species of this genus, *Rhodoplanes elegans*, has been described (Hiraishi and Ueda, 1994b).

Mass accumulation of PNB is rarely found in nature. This is because natural habitats contain sulfates which inhibit the growth of the bacteria. However, under anaerobic conditions, sulfide is formed from sulfate by sulfate-reducing bacteria. The sulfide encourages the growth conditions for red and green sulfur bacteria. The latter organisms eventually reduce the sulfide concentrations to such low levels that non-sulphur purple bacteria can coexist with them (Pfennig, 1967).

The preferred growth mode of all species is photoheterotrophic under anaerobic conditions in the light with various organic substances. Many species are also able to grow photoautotrophically with hydrogen and carbon dioxide (Hallenbeck *et al.*, 1990), chemoautotrophically with hydrogen, oxygen and carbon dioxide (Madigan and Gest, 1979); chemoheterotrophically (Sasikala *et al.*, 1990), mixotrophically (Kampf and Pfennig, 1980) and by fermentative metabolism (Madigan and Gest, 1979). These special physiological properties are used for enrichment of these bacteria during isolation from nature.

Environmental conditions such as pH (Biebl and Pfennig, 1981) light intensity, specific substrates (Madigan and Gest, 1988) and salinity (Biebl and Pfennig, 1981) have been used for enrichment, too. Successful enrichment also

depends on a number of other factors, among which the composition of the medium, the inoculum size and age are of importance (Gest *et al.*, 1985).

Most of the purple non-sulphur bacteria have an obligate requirement for one or more water soluble vitamins for phototrophic growth (Weaver *et al.*, 1975; Pfennig, 1978). They can grow at a pH range of 6.0 to 9.0, depending mainly on the substrate used. However, few acidophilic organisms (*Rhodopseudomonas acidophila* and *Rhodophila globiformis*) are known to tolerate an acidic pH of 4.0 (Pfennig, 1969, 1974). The optimal temperature for growth usually ranges between 25 °C and 35 °C (Sasikala *et al.*, 1993). Most of them do not grow at temperatures above 47°C but a few thermotolerant strains have been reported (Watanabe *et al.*, 1981; Favinger *et al.*, 1989; Resnick and Madigan, 1989; Stadtwald-Demchick *et al.*, 1990 a,b). The thermotolerant strains are *Rhodopseudomonas cryptolactis* and *Rhodospirillum centenum.*

Higher light intensities are required for optimal growth of the PNB (Hillmer and Gest, 1977a,b). Both quality and quantity of light are important for the development of PNB as discussed in detail by Imhoff (1992).

Carbon assimilation

Purple photosynthetic bacteria exhibit great diversity in the metabolism of simple carbon compounds (Madigan and Gest, 1979). Carbon assimilation by PNB depends on environmental metabolic variables like the substrates used for growth

and on growth conditions. The PNB are primarily heterotrophic. Autotrophic assimilation of carbon dioxide by PNB in either light or dark conditions requires hydrogen or hydrogen sulfide as electron donor. Autotrophic carbon dioxide fixation by the PNB is carried out mainly by the reductive pentose phosphate cycle (Fuller *et al.*, 1961).

Apart from autotrophic carbon dioxide fixation, photoheterotrophic carbon dioxide fixation has also been shown in the PNB. In *Rhodospirillum rubrum* grown photoheterotrophically on L-malate, one of the major pathways of photometabolism of carbon dioxide was found to be through glycolic acid (Anderson and Fuller, 1967). Similarly in *Rhodobacter capsulatus*, both photoproduction of hydrogen and autotrophic growth are reported to be affected by carbon dioxide (Willison *et al.*, 1984). Large amounts of carbon dioxide are produced from organic acids during photoassimilation and this carbon dioxide forms a reservoir of potential carbon that would increase cell yields (Rolls and Lindstrom, 1967a).

Assimilation of one-carbon compounds such as methanol, formic acid, methane and carbon dioxide for growth is exceedingly slow (Qadri and Hoare, 1968). The ability to photoassimilate formate, the presence of formate hydrogen lyase, and the presence of hydrogenase all appear to depend on the growth of organism on a medium containing formate (Qadri and Hoare, 1968). *Rhodopseudomonas palustris* isolated using formate as sole carbon source could also utilize organic compounds such as alcohols, fatty acids and hydroxyacids with biotin

and *p*-aminobenzoic acid (PABA) as essential growth factors, thus appearing to have properties similar to heterotrophs (Qadri and Hoare, 1968).

Photosynthetic bacterial growth in carbon monoxide has been accidentally discovered in enrichment cultures of carbon monoxide oxidising bacteria (Hirsch, 1968). Carbon monoxide dehydrogenase is assumed to be the enzyme responsible for carbon monoxide oxidation in PNB (Bonam *et al*, 1984). *Rhodospirillum rubrum* ATCC 11170 was found to have the ability to oxidize carbon monoxide to carbon dioxide and hydrogen. Apart from *Rubrivivax gelatinosus* (Uffen, 1976) and *Rhodospirillum rubrum* (Bonam *et al.*, 1989), there are no reports about the assimilation of carbon monoxide by related enzymes in the other species of PNB.

Methanol is utilized as carbon source by a few strains of PNB. *Rhodopseudomonas acidophila* could tolerate methanol concentrations of 15-250 mM (Quayle and Pfennig, 1975). Growth on methanol was dependent on the simultaneous presence of bicarbonate (Siefert and Pfennig, 1979; Fujii *et al.*, 1982) which functions as an electron acceptor. Enrichment in methanol-bicarbonate medium also resulted in the isolation of *Rubrivivax gelatinosus* (Quayle and Pfennig, 1975). In all cases, the pH optimum for growth on methanol was considerably higher than for other carbon sources.

Organic acids are assimilated by PNB through the citric acid cycle (Ormerod *et al.*, 1961) during anaerobic-light conditions. Although PNB prefer organic acids for their growth, they can also metabolize aromatic carbon compounds

but require longer periods. However, substrates that can support only slow growth still serve as significant carbon sources in natural environment, particularly where there are relatively few organisms which compete for their use (Gibson, 1995). The affinity of whole cells for aromatic substrates is very high and virtually complete assimilation of carbon from aromatic substrates into photosynthetic bacterial biomass has been noted repeatedly (Wright and Madigan, 1991). Degradation of diverse aromatics are mediated by separate metabolic pathways during aerobic and anaerobic conditions (Harwood and Gibson, 1988). Anaerobic degradation appears to follow one or both of two main routes: (1) the ring is either aromatized to yield benzoate as an intermediate or (2) it is metabolized by a β-oxidation-like sequence, involving coenzyme A thioester derivates, before ring opening and step-wise conversion to common metabolic intermediates (Kuver *et al.*, 1995). The enzymes involved in the photometabolism of aromatic acids are inducible and appear to lack substrate specificity (Dutton and Evans, 1969). *Rhodopseudomonas palustris* is found to be far more versatile in terms of aromatic degradation than previously demonstrated (Biebl and Pfennig, 1981; Harwood and Gibson, 1988; Madigan and Gest, 1988; Khanna *et al.*, 1992). Typically, anaerobic degradation of benzoate is mediated by a benzoate-coenzyme A ligase (Hutber and Ribbons, 1983; Geissler *et al.*, 1988; Kamal and Wyndham, 1990). ω-phenylalkane carboxylates and *trans*-cinnamate also formed excellent substrates for growth of *Rhodopseudomonas palustris* (Elder *et al.*,1992). A recent report included phenol

among substrates that support photosynthetic growth of a strain of *Rhodopseudomonas palustris* isolated from the effluent of a petrochemical waste water treatment plant (Khanna *et al.*, 1992).

The presence of thiosulfate also significantly affected the carbon assimilation of *Rhodopseudomonas palustris* (Rolls and Lindstrom, 1967b). The cell yields were increased by the addition of thiosulfate. Hansen and van Gemerden (1972) reported that sulfate was the only product of the conversion of sulfide without intermediate accumulation of elemental sulfur.

Experiments of Willison *et al.* (1984) on photoproduction of hydrogen by *Rhodobacter capsulatus* indicated that there was an important relationship between photoproduction of hydrogen by nitrogenase and the pathways of carbon assimilation in the cell. An effective carbon assimilation system is very important for achieving high hydrogen production values.

APPLICATION OF PHOTOTROPHIC BACTERIA IN BIOTECHNOLOGY

Large quantities of agro-industrial wastes are discharged into the environment throughout the world. For example, in Malaysia, 30-50 cm^3 of wastewater is produced for every tonne of sago flour processed, and about 6000 tonnes of sago flour is processed a year (Shim, 1992). The palm oil and rubber industries discharge about 36.5 and 15 million tonnes of waste effluent a year respectively into the Malaysian waterways (Anton *et al.*, 1994; Geetha *et al.*, 1994). Treatment or reuse of agroindustrial wastes therefore, holds much appeal and economic importance. Biogas and single cell protein (SCP) production together with Chemical Oxygen Demand (COD) reduction may well become a desired process for wastewater treatment. This will enhance the overall feasibility and economic viability in terms of practical application.

Purification of wastewater and production of single cell protein (SCP)

Much attention has been paid to the utilization of PNB to produce SCP from agroindustrial wastes (Shipman *et al.*, 1975; Kobaysahi and Kurata, 1978; Sasaki *et al.*, 1981). The biomass of the PNB contains protein of about 60-70 percent on a dry weight basis as compared to 50-60 percent of protein of algae and yeast on dry weight basis (Noparatnaraporn *et al.*, 1987) (Table 2). In addition, these bacteria contain essential amino acids comparable to those of soy bean and egg proteins (Sasaki *et al.*, 1981) or higher than other SCP (Nopartnaraporn *et al.*, 1987).

These bacteria can grow at high growth rates under anaerobic and light conditions. In an activated sludge process, an additional sludge disposal problem is created, whereas PNB treatment of wastes produces a bacterial biomass rich in proteins and many other valuable organic substrates (Balloni *et al.*, 1980). Moreover, because dilution of wastewater is not required, the process can be used in areas of water scarcity and the area required for treatment facilities is decreased (Kobayashi and Kobayashi, 1995).

Table 2 : Composition of phototrophic bacteria, green algae and yeast cells (g/100 g dry weight) (Kobayashi and Kobayashi, 1995)

	phototrophic bacteria	**algae**	**yeast**
Crude protein	60.95	55.52	50.5
Crude fat	9.91	8.07	1.1
Soluble carbohydrates	20.83	21.04	39.3
Crude fibre	2.92	12.09	2.1
Ash	5.39	3.28	7.0

Various agricultural wastes have been used as substrates for SCP production. Many industrial wastewaters are treated economically using phototrophic bacteria too (Sasaki *et al.*, 1981; Noparatnaraporn *et al.*, 1986, 1987; Prasertsan *et al.*, 1993; Getha *et al.*, 1995).

A. Pineapple wastes

These wastes have high organic loading (COD 10 000 mg/L) containing mainly carbohydrates with little protein. *Rhodobacter sphaeroides* P_{47} was used to treat pineapple peel wastes. Noparatnaraporn *et al.* (1986) reported that the culture yielded 26.5 g dry cell/L after 60 h cultivation with a COD removal of 85.3 percent.

B. Soybean wastes

The COD of the wastewater was 20 000 mg/L consisting mainly of carbohydrates and proteins. Growth of *Rubrivivax gelatinosus* on cooked soybean runoff medium showed a COD reduction of 82.9 percent under optimal conditions. The cell biomass obtained had about 62 percent protein and 33 μg vitamin B_{12} per gram dry cells, suggesting the potential use of the cell mass as an SCP source (Sasaki *et al.*, 1981).

C. Starch wastes

Cassava starch

Noparatnaraporn *et al.*, (1983) described *Rubrivivax gelatinosus* VI which utilized cassava starch directly with good results. The cassava liquid wastewater, required supplementation of ammonia and phosphate. The maximum cell mass obtained was 4.7 g dry cells/L and COD removal was 95.6 percent after eleven days culture. Growth of *Rubrivivax gelatinosus* VI on cassava solid waste however, gave 23 μg of vitamin B_{12} per gram of cell and carotenoid content of 0.09 mg/g cell,

making it useful as an SCP source. Cassava solid waste is normally used as a carbohydrate source of animal feed due to its low cost and abundance.

Sago starch

Getha (1995) reported an indigenous strain of *Rhodopseudomonas palustris* that was able to reduce COD of sago starch processing wastewater by 76.8 percent after four days culture. Maximum cell mass obtained was 2.5 g/L and the carotenoid concentration was 1.1 mg/g dry weight.

D. Mandarin orange peel wastes

Rhodobacter sphaeroides S cultivated under both aerobic-dark and microaerobic-light cultures gave COD removal of 85 percent. The growth yield from COD consumed was 0.83 g cell/g COD in microaerobic-light condition, which was 1.6 times higher than that in aerobic-dark conditions. In anaerobic-light culture, remarkably slow growth and COD reduction were observed. Thus, from a practical point of view, a microaerobic-light culture of *Rhodobacter sphaeroides* S may be promising for SCP production (Sasaki *et al.*, 1991).

E. Seafood processing wastes

Prasertsan *et al.* (1993) reported that the growth of *Rubrivivax gelatinosus* R7 in tuna condensate gave a maximum cell mass of 5.6 g/L containing about

50 percent protein. The COD of the wastewater was reduced to 86 percent after five days incubation under optimal conditions. The carotenoid content was 0.98 mg/g dry cell weight and bacteriochlorophyll content was 9.2 mg/g dry cell weight.

F. Cow dung and swine wastes

The PNB was used for the secondary treatment of the effluent after anaerobic digestion. After the anaerobic digestion, the COD was reduced to one-tenth of its original strength to about 3.9 g/L consisting mainly of volatile fatty acids. Single cell protein production from cow dung and swine wastes using phototrophic bacteria was possible, although these wastes have to be treated by anaerobic digestion, followed by the separation of the solid to obtain the liquid medium required.

Rhodobacter capsulatus grown on cow dung for about six days gave maximum growth of 0.91 g on dry weight basis (Vrati, 1984). An aerobic cultivation of *Rhodobacter spheroides* S on swine discharges gave a cell mass of 1.8 dry cell/L and COD removal of 80 percent in 20 h.

To be suitable for SCP production, the microorganism should grow at fast rate with high growth yield from diverse carbon substrates and the cell mass should contain balanced nutrients. The growth characteristics of PNB in terms of growth rates, cell mass yields and COD removal from several agroindustrial wastes as reported in literature are summarized in Table 3.

Table 3 : Growth yields and COD removal of PNB from the various wastes

Substrate	Pineapple	Soybean	Mandarin orange peel	Cassava	Sago	Seafood	Swine
Strain	*Rhodobacter sphaeroides* P_{47}	*Rubrivivax gelantinosus*	*Rhodobacter sphaeroides* S	*Rubrivivax gelatinosus* V1	*Rhodopseudomonas palustris* B1	*Rubrivivax gelatinosus* R_7	*Rhodobacter sphaeroides* S
Maximum cell mass obtained (g/L)	26.5	9.3	3.7	4.7	2.5	5.6	1.8
Incubation time (h)	60	22	50	264	96	120	20
% COD removal	85.3	82.9	85.0	95.6	76.8	86.0	80.0
Culture conditions	aerobic-dark	aerobic-dark	microaerobic-light	microaerobic-light	anaerobic-light	anaerobic-light	aerobic-dark

IMMOBILIZATION OF PNB

Cell immobilization can be defined as any technique that limits the free movement of cells. Immobilization of whole cells is not a novel concept but rather a duplication and refinement of phenomena observed in nature (microbial activity in soil, leaching of mineral ores) and in some industrial microbiological processes, where microorganisms or cells are attached to solid surfaces or form films (trickling filters, vinegar production process, tissue culture) (Rosevear, 1984).

The first example of artificially immobilized cells was described by Mosbach and Mosbach (1966). Since then, the field has expanded tremendously and is now a massive research effort. The first catalytic bioprocess based on immobilized microbial cells was production of isosyrup which has gained commercial status (Venkatasubramaniam and Vieth, 1979). However, the real potential of immobilized systems could only be fully understood by a detailed study of multi-step reactions (fermentations and bioconversions) involving entire metabolic pathways.

Whole cell immobilization versus enzyme immobilization

Immobilization of whole cells rather than purified enzymes offers several advantages. It obviates the laborious and expensive steps involved in extracting, isolating and purifying intracellular or extracellular enzymes. Stability of the

specific enzyme is normally improved by retaining its natural environment. Also, cofactor regeneration sites are not disturbed and its requirement can be fulfilled *in situ* using whole cells. A wider scope of reaction is possible including multi-step reactions involving several enzymes.

In several instances, bound cell systems are much more tolerant to perturbations in the environment (e.g. temperature, pH and ionic strength) and are less sensitive to toxic substances (e.g. dissolved oxygen and metal ions) as compared to immobilized enzymes. (Chibata, 1978)

The disadvantages of immobilized whole cells include diffusional and permeability problems that might impair uptake and transport of substrates as well as excretion of products. The other enzymes present in a whole cell might lead to unwanted side reactions. Another difficulty is the maintenance of cell integrity since loss of enzymatic activity is common in non-growing cells. Thus, it might be necessary to supply immobilized cells with nutrients or an energy source to keep the cells viable and active but slowly growing in order to regenerate cofactors needed in complex pathways. (Fukui and Tanaka, 1982)

Immobilized cell fermentations versus conventional fermentations

An immobilized cell fermentation process offers several advantages associated mainly with general productivity and operational flexibility. Batch fermentations can be replaced by continuous column reactions, allowing a drastic

decrease in plant equipment size and costs (Chibata, 1978). There is better process control, reduced operational costs, minimal "down time" and product uniformity. Furthermore, fermenter operation remains possible at high dilution rates without culture wash-out and facilitates recycling or reuse of microorganisms. Continuous removal of toxic metabolites can also contribute to an enhanced productivity (Phillips and Poon, 1988).

Immobilized cells allow the use of increased cell density per unit reactor volume, resulting in higher overall reaction rates and higher product yields. This productivity can be explained by the fact that the microenvironments offered by the carrier are more stabilizing to the organism, which generally shows optimal activity only under narrowly prescribed physical conditions (Phillips and Poon, 1988). Such a system also allows an intermittent regeneration of cells or controlled growth rate within the immobilized cell systems. Living or growing immobilized cell systems are frequently a must to perform multistep or cofactor requiring reactions (Brodelius and van Damme, 1987).

There are also some problems associated with immobilized cell fermentations. Immobilized whole cells have an additional diffusion barrier and thus may limit the possible range of applicable substrates. There are also diffusional limitations where only the peripheral layer of the catalyst is working at an optimal rate. Decreasing particle size and increasing the pore size of the carrier may to some extent overcome this limitation.

Vigorous growth of cells within the matrice may lead to release of cells or even destruction of the matrice which may cause problems in product isolation and clogging of reactor equipment. Techniques have also been developed which allow for immobilization of cells with preserved viability. The initial expense for such a system is usually high and the process usually requires a large reactor. The mechanical properties of the system (microbial cell bound to solid carrier) are more complex than those of free cells and have to be taken into account in order to provide a continuous recycling process and an effective agitation and filtration.

Cell Supports

A large variety of matrices are available for cell immobilization. No ideal general immobilization method is applicable to all types of cells. Therefore, it is necessary to select suitable methods and conditions for immobilization of each type of cell (Chibata *et al.*, 1978). Generally, PNB are immobilized using the entrapment or encapsulation method (Rosevear, 1984). Entrapment is the most extensively investigated immobilization method. The enclosure of a cell within a gel structure is unlikely to change the function of the cell. This is one of the least destructive immobilization method (Rosevear, 1984).

HYDROGEN PRODUCTION FROM WASTE WATERS USING IMMOBILIZED PNB

Immobilized cells systems have been increasingly used in the bioconversion process, including the production of food, beverages, pharmaceuticals, macromolecules such as organic acids and amino acids, other industrial chemicals, fuels such as alcohol, methane and hydrogen and biological wastewater treatment. Immobilized cells systems such as biofilm processes have been widely used for treatment of wastewaters and methane production. These processes are particularly suitable for the treatment of nonconventional or toxic wastewaters which require a longer solid retention time (SRT) (Yang and Wang, 1990).

Generally, immobilization of PNB is for the purpose of hydrogen photoproduction. Immobilization of PNB for the treatment of wastewaters is also for the purpose of producing hydrogen as an important by-product.

As phototrophic bacteria can evolve hydrogen gas via nitrogenase (Gest and Kamen, 1949; Kamen and Gest, 1949), a technology of large scale hydrogen gas development on concentrated organic waste has successfully been established (Kobayashi and Kondo, 1984).

Development of hydrogen as an alternative source of energy is one of the intensely studied areas in the energy scene today (Veziroglu, 1987). Because

molecular hydrogen burns to produce water, it is the ultimate fuel of the future, being both environmentally sound and renewable. Depletion of non renewable fuel supplies and costs associated with environmental protection are expected to eventually make hydrogen economically competitive with traditional fuel supplies (Veziroglu, 1987).

A review of recent data available on immobilization of PNB for hydrogen photoproduction reveals that although there is considerable disagreement regarding enhancement in the rates of hydrogen evolution by immobilized cells compared to free cells, there is a possibility of achieving long term stability of the process in course of time (Sasikala *et al.*, 1993).

Alginate-immobilized cells of *Rhodopseudomonas* sp. BHU 1 (Singh *et al.*, 1990) and *Rhodobacter sphaeroides* O.U.001 (Sasikala *et al.*, 1990) showed a 4-fold increase in hydrogen evolution rate over free cells, whereas a 2- to 10-fold increase was observed in agar-immobilized *Rhodospirillum rubrum* (von Felten *et al.*, 1985). In contrast, carrageenan entrapped cells of *Rhodobacter capsulatus* B10 (Francou and Vignais, 1984) and agar-immobilized *Rhodopseudomonas* sp. Miami PBE 2271 (Matsunaga and Mitsui, 1982) retained only about 67 and 50 percent of the hydrogen evolution rate of free cells, respectively. In all the above cases, the hydrogen evolution by the free cells declined and completely ceased after some time. However, the immobilized cells continued to photoproduce hydrogen at the same rates for a longer time.

Microbial production is likely to be the technology of choice because hydrogen is produced from the renewable resources - water or carbohydrates (Kosaric and Lyng, 1988). Because PNB are able to use either carbon dioxide or a variety of organic substrates as carbon sources, hydrogen production in conjunction with wastewater treatment is a realistic possibility (Zürrer, 1982).

Wastes used as electron donors for photoproduction of hydrogen include sugar refinery waste (Vincenzini *et al.* 1981, 1982b; Bolliger *et al.*, 1985), straw paper mill effluent (Vincenzini *et al.*, 1982b), distillery wastewater (Vatsala and Ramasamy, 1989; Sasikala *et al.*, 1992), lactic acid containing wastes (Zürrer and Bachofen, 1979) and the clarified slurry of biogas plants (Vrati and Verma, 1983).

Agar-entrapped cells of *Rhodopseudomonas palustris* and *Rhodospirillum molischianum* photoproduced hydrogen from sugar-refinery wastes and straw paper mill effluent for over a month at a rate ranging from 50 to 139 μL H_2 /mg cell dry weight /h depending on the organism and on the substrate. The amount of hydrogen evolved per litre of wastewater ranged from 0.78 to 2.6 L with a COD conversion to hydrogen of 28-43 percent (Vincenzini *et al.*, 1982a). Bolliger *et al.* (1985) observed that higher hydrogen production rates were obtained from sugar refinery wastes than from any known synthetic substrates. They also reported that naturally adapted organisms selected from a similar environment proved better for hydrogen production from wastewaters than the laboratory strains.

Rhodospirillum rubrum entrapped in calcium alginate gel was employed for continuous hydrogen production from wastewater containing organic acids such as acetic, propionic and butyric acid (Karube *et al.*, 1984). The highest hydrogen evolution rate was observed for 6 h at a cell content of one percent in calcium alginate under optimal conditions. Vrati and Verma (1983) used effluent slurry from cow dung separated from the solid residue to produce SCP and hydrogen using *Rhodobacater capsulatus*. The only commercially exploited practical system of hydrogen photoevolution from wastewaters is that of Kobayashi and Kondo (1984). In an organic waste with a biological oxygen demand (BOD) higher than 10 000 mg/L *Rhodobacter capsulatus* produced more than 150 L / day / m^3 of hydrogen gas. Klasson *et al.* (1993) reported that *Rhodospirillum rubrum* was capable of converting carbon monoxide and water to carbon dioxide and hydrogen in batch cultures with a hydrogen evolution rate of 87 percent of the theoretical value depending upon light intensity. *Rhodopsudomonas palustris* was capable of producing up to 45 percent hydrogen of the maximal theoretical value from aromatic acids benzoate, *p*-hydroxybenzoate, cinnamate and D- and L-mandelate (Fiβler *et al.*, 1994). Further efforts to utilize agricultural wastes and photoproduce hydrogen were undertaken by Buranakarl *et al.* (1985), Singh *et al.* (1990) and Sasikala *et al.* (1993).

OBJECTIVES OF THIS STUDY

Waste treatment methods coupled with production of value-added products are much more desirable, an attempt is made to apply this knowledge to develop a local strain of *R. palustris* for practical utilization. Possible strategies for the utilization of sago factory wastes by immobilized PNB were initiated. Thus, the aims of this research project were to:-

(1) characterize and optimize the growth of *Rhodopseudomonas palustris* strain B1.

(2) immobilize the *Rhodopseudomonas palustris* cells in a suitable carrier.

(3) assess the use of the immobilized cells for treatment of sago effluent.