CHAPTER 3

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IMMOBILIZATION OF *Rhodopseudomonas palustris* STRAIN B1 FOR THE POLLUTION REDUCTION OF SAGO EFFLUENT

INTRODUCTION

Hydrogen could be the fuel of the future and thus, photobiological production of hydrogen by photosynthetic bacteria is being extensively studied (Kim *et al.*, 1982a,b; Bolliger *et al.*, 1985; Vincenzini *et al.*, 1986). Furthermore, the process seems to be economical when enough cheap waste substrates high in carbohydrate content and low in nitrogen are available. Wastewater from sago processing factory may be a suitable substrate for microbial conversion into a fuel as it has a high C to N ratio. The wastewater obtained after starch extraction contained about 5.4 g glucose/L, 0.11 g/L total nitrogen and 0.67 g/L crude protein. The total solids and suspended solids were about 7.0 g/L and 2.8 g/L respectively (Getha, 1995). *Rhodopseudomonas palustris* strain B1 has been observed to photoassimilate starch and produce biomass 4-fold greater when grown in organic carbon sources (Ch. 2, pp74).

It has been reported that phototrophic bacteria contribute to the purification of polluted water in nature because they have the metabolic ability to photometabolize many organic substances (Kobayashi and Haque, 1971; Balloni *et al.*, 1980; Kobayashi, 1982). Based on the findings from these studies, the use of phototrophic bacteria might be a preferable choice for the treatment of sago factory processing wastewater (Getha, 1995).

The feasibility of using immobilized cells of *R. palustris* strain B1 for the treatment of agro-industrial effluents was also studied (Vincenzini *et al.*, 1981,1982b; Sasikala *et al.*, 1992). Immobilization of whole cells is more stable to the intracellular enzymes and obviates the often laborious and expensive steps involved in cell and product separation at the end of the treatment process.

Thus, the aims of this investigation were

(1) to immobilize the isolate strain B1 in a suitable matrice.

(2) to assess the use of the immobilized cells for effective pollution reduction of sago starch processing wastewater.

MATERIALS AND METHODS

Pre-culture preparation

Rhodopseudomonas palustris strain B1 was grown in screw-cap bottles completely filled with GM medium at $30^{o} \pm 2\ ^{o}C$ at 5 klux under continuous anaerobic-light conditions. After 72 h of incubation, the optical density of the bacterial suspension was recorded.

Cell mass determination

The cell mass was determined by measuring the absorption of the bacterial suspension at 660 nm. The bacterial dry weight was calculated with the following empirical relation determined in our laboratory (Hirayama *et al.*, 1986).

Absorbance at 660 nm of 1.2 = 2 mg/mL of dry weight cells of *R. palustris* strain B1 (Appendix A3)

Immobilization of cells

Carrageenan, agar and calcium alginate were tested as the materials for immobilizing the cells.

(i) Calcium-alginate method

A 20 mL of a 72 h culture in GM medium containing about 80 mg cells on dry weight basis was thoroughly mixed with 20 mL of 4% calcium-alginate solution (w/v in distilled water). The mixture was dropped into sterile distilled water containing 50 mM calcium chloride. The resulting gel beads were washed once with distilled water and used as immobilized cells (von Felten *et al.*, 1985).

(ii) Carrageenan Method

About 20 mL of a 72 h bacterial suspension in GM medium containing about 80 mg cells on a dry weight basis was thoroughly mixed with 20 mL of 2.1% carrageenan. The mixture was dropped using a syringe into 0.3 M KCl solution at 4 ^{o}C. The resulting beads were washed once with sterile distilled water and used as immobilized cells (Hirayama *et al.*, 1986).

(iii) Agar Method

About 20 mL of a 72 h culture in GM medium at exponential growth phase containing about 80 mg dry weight of cells were harvested by centrifuging at 5000 rpm for 30 minutes. The supernatant was discarded. The cells were resuspended in 5 mL of sterile phosphate buffer (pH 7.0) (Appendix B3) and thoroughly mixed with 20 mL sterile agar solution in distilled water maintained at 43 ^{o}C to give a final concentration of 1.0%. This concentration has been reported to have a good equilibrium between a good mechanical strength and a high cell retention capacity (Vincenzini *et al.*, 1982a). The solution was pumped dropwise through a syringe into a beaker containing sterile ice-cold distilled water covered with 2 to 3 mm layer of sterile paraffin oil. The mixture solidified into spheres of 2-4 mm diameter. Polymerization occurred in the apolar layer of the paraffin layer. The beads were washed thrice with sterile distilled water (von Felten *et al.*, 1985).

Properties of the Immobilized Cells

The resulting beads from the three entrapment methods were visually observed for growth, viability, rupture resistance and storage.

Electron microscopic studies

Direct investigation of the agar-immobilized bead was done by microscopic observation using the scanning electron microscope (SEM). The immobilized cells were processed as described in Appendix A3 and observed using a Philips SEM 515. A bead was fractured and the internal surface was observed by SEM.

Immobilized Phototrophic Cells for the Treatment of Sago Effluent

Immobilized *R. palustris* strain B1 cells in agar, with a cell load of 3.2 mg on dry weight basis/mL was used to treat sago effluent. The effect of effluent concentration, cell loading, mixing and reusability on the pollution reduction of the wastewater was observed.

Substrate

Sago starch processing wastewater (henceforth referred to as sago effluent) was collected from a factory in Batu Pahat, Johore Darul Takzim,

Malaysia. The samples were stored in plastic containers at -20 °C. They were thawed at 4 °C and brought to 28° ± 2 °C prior to analysis.

Settled sago effluent was prepared by allowing the sago effluent to settle for an hour to partially remove the suspended solids. The supernatant was analysed for Chemical Oxygen Demand (COD) (APHA, AWWA, WPCF, 1989).

Reduction in the Chemical Oxygen Demand of Unsettled and Settled Sago Effluent

The unsettled and settled sago effluent was adjusted to pH 7.0 and dispensed separately into 100 mL Schott bottles prior to autoclaving at 121 °C for 15 min. The reduction in COD (Appendix A4) of the unsettled and settled sago effluent was studied using a 10% inoculum of free (Ch. 2, pp28) and agar-immobilized whole cells respectively. The bottles were incubated at 30° ± 2 °C at 4 klux under anaerobic-light conditions. A fan was used to dissipate the heat generated from the tungsten lamps placed on both sides of the culture bottles.

Triplicate bottles for each treatment were analyzed for COD reduction at 24 h intervals over five days.

Effect of sago effluent concentration on COD reduction

From the above experiment, the COD of the unsettled sago effluent was found to be reduced significantly and hence selected for further tests. The concentration of unsettled sago effluent prepared were 25%, 50%, 75% and 100% (v/v in distilled water). Each concentration was adjusted to pH 7.0 and dispensed into 100 mL Schott bottles and autoclaved at 121 °C for 15 min. A 10% inoculum of the agar-entrapped cells of *R. palustris* strain B1 was used. For each concentration three bottles were set up. Incubation was carried out at 30° ± 2 °C and continuous illumination at 4 klux was provided. The COD was assayed after 72 h of incubation.

Effect of mixing on COD reduction

The unsettled sago effluent (100%) was adjusted to pH 7.0 and dispensed into three 100 mL Schott bottles prior to autoclaving. A 10% inoculum of agar-immobilized cells was used. The bottles were kept at 30° ± 2 °C under anaerobic-light conditions. Illumination was provided by four tungsten bulbs with a light intensity of 4 klux on the surface on both sides of the bottles. Continuous mixing during cultivation was provided at 150 rpm by a rotary shaker. The COD was analysed at 24, 48 and 72 h intervals after inoculation.

Effect of inoculum on COD reduction

The unsettled sago effluent after adjusting the pH to 7.0 was dispersed into 100 mL Schott bottles and autoclaved at 121 °C for 15 min at 15 psi. Three batches of three bottles each were prepared and each batch was inoculated with 10%, 15% and 20% of the agar-entrapped cells respectively. The bottles were incubated at 30° ± 2 °C under anaerobic-light conditions at 4 klux. The COD was assayed after 72 h of incubation.

Reusability of beads

A 10% inoculum of agar-immobilized *R. palustris* strain B1 was introduced into the autoclaved sago effluent. The COD reduction of the sago effluent was assayed after 72 h. The inoculum was then repeatedly used four times in different batches of sago effluent for 72 h each. The bottles were incubated at 30° ± 2 °C and at 4 klux light intensity. In between each treatment, the immobilized cells were washed once with sterilized distilled water. After each cycle, the effluent was tested for COD reduction. Tests were performed in triplicates.

Statistical Analysis

Analysis of variance (ANOVA) was done to test the significance of the effluent concentration, the effect of inoculum size and mixing on the COD removal of the unsettled sago effluent after 72 h of treatment.

RESULTS AND DISCUSSIONS

Immobilization of *Rhodopseudomonas palustris* strain B1

In calcium-alginate

Immobilization with Ca-alginate produced beads that were soft and fragile. The immobilization of *R. palustris* strain B1 in Ca-alginate was very simple to carry out. The gel matrice was relatively inert and the cells could be entrapped within this gel under mild conditions without affecting the viability of the cells. Immobilization in alginate gel was safe, fast, mild, simple, cheap and versatile as described by Bucke (1987). But as no solid beads could be formed, the cells tended to get washed out.

In carrageenan

In this study, the beads formed were not distinct and collapsed after a few days. According to Tosa *et al.* (1979), a preparation of greater stability can be obtained by further treatment with hardening agents such as glutaraldehyde and hexamethylenediamine, but in doing so, the cost of the immobilization process increases. Of the three known types of carrageenans (lambda, kappa and iota), only the kappa (κ) form is considered to be suitable as a support for immobilization of cells. The immobilized cells can be formed by cooling with a gel-inducing agent under very mild conditions without destroying the desired activity. The pore size of this gel matrice is small enough to prevent high molecular-weight compounds, such as enzymes from leaking out, although the lower molecular-weight substrates and products easily pass through (Tosa *et al.*, 1979). Full metabolic activity and viability of the cells are retained after entrapment in carrageenan. This gel type exhibits an overall stabilizing effect on microbial cells and a column packed with them can be used for continuous enzyme reaction for long periods (Tosa *et al.*, 1979).

In agar

Immobilization with agar gave rigid gels and the beads formed were of a uniform size. When the immobilized cells of *R. palustris* strain B1 were kept

illuminated with medium, the agar spheres turned variably but homogeneously red from the growth of the cells within a few days (Plate 7). This was because the cells grew up to the surface of the beads in order to be in direct contact with the substrate (Rehm and Omar, 1993).

So far, cells of phototrophic bacteria have only been immobilized successfully in agar (Bennett and Weetall, 1976; Ikemoto and Mitsui, 1984). Agar and agarose (a purified preparation of agar) are polysaccharides isolated from a marine red algae. It is readily soluble in boiling water and solidifies at 37 °C. Agar is also resistant to microbial attack. Large scale operations have been almost entirely restricted to agar (Mitsui *et al.*, 1980; Hirayama *et al.*, 1986; Vincenzini *et al.*, 1986; Mignot *et al.*, 1987). Agar was also the preferred immobilizing agent with respect to hydrogen production rates as well as stability (von Felten *et al.*, 1985).

In this study, it was found that agar was a suitable material for immobilization of *R. palustris* strain B1. Advantages of this method are that the immobilization can be performed under very mild conditions and can be achieved without the use of chemicals that may change the structure of enzyme proteins of microbial cells. One further advantage of using agar is that various shapes of immobilized cells can be formed, such as cube, bead and slab to suit the reactor used

for commercial applications (Matsunaga and Mitsui, 1982; Vincenzini *et al.*, 1982a; von Felten *et al.*, 1985; Planchard *et al.*, 1989).

SEM observation

Direct evidence of cell immobilization was difficult to determine. The sites of adherence between the cells and the matrice existed as a result of complex physical and chemical interactions which often did not survive the damage caused by the procedures of sample preparation for SEM observation.

Direct visual evidence of cell viability was observed as an increase in cell population (Plates 8A,B). The surface of the bead became sponge-like after incubation and the growth of the cells caused the gel surface to rupture, giving a pore size of approximately 4 μm in diameter. However, no cell leakage was observed, because the medium did not turn turbid even after extended periods in the light with sufficient nutrients. The size of the immobilized cells on the gel surface was generally smaller than those of free cells. The microcolonies formed were dense and had no particular formation (e.g. rosette). Due to the dense microcolonies, the cells were probably faced with diffusional limitations of gases and substrates, resulting in smaller cells (Gosmann and Rehm, 1988).

In the fractured bead, dead cells were observed in the interior parts. The microcolonies were very dense and the cells were long and thin and seemed

emancipated. In certain areas, especially in the centre of the carrier granule cell lysis had occurred (Plate 8C).

The immobilized agar bead was found to be rigid since shearing forces arising from stirring and from collision among the particles and the reactor wall did not cause much damage to the gel. However, carrier rupture due to gas formation was not investigated.

Many morphological changes of immobilized cells, in comparison with free growing cells were observed. Immobilized cells differ in their growth rates and show altered morphological forms of colonies (Rehm and Omar, 1993). The altered growth in microcolonies can be attributed to diffusional limitations of gases, nutrients, light penetration and other parameters. Such colonies can be very dense containing large numbers of cells. Bacterial cells are very often linked together by a net of polysaccharides and the cells normally lengthen after immobilization. The causes of these alterations are not yet known (Rehm and Omar, 1993).

Cells in the outer region had access to better gas and substrate when compared to the interior parts of the beads. This is of great importance for the growth and the metabolism of the microorganism (Rehm and Omar, 1993). The entrapped cells in the centre of the bead depend on the diffusion of gas and substrate through the gel substance and also the microcolonies formed on the surface.

Van Loosdrecht *et al.* (1990) concluded that all differences between immobilized and free cells can be attributed to the surroundings of the cell which has been modified due to the presence of surfaces but not due to the cell itself.

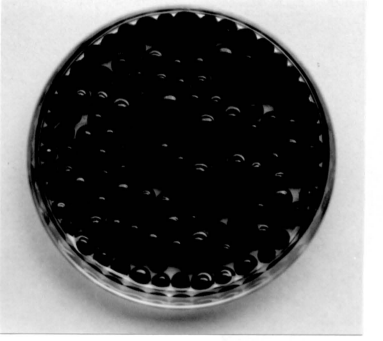

Plate 7 : Immobilized *R. palustris* strain B1 in agar after 96 h at $30^0 \pm 2^oC$ and 4 klux

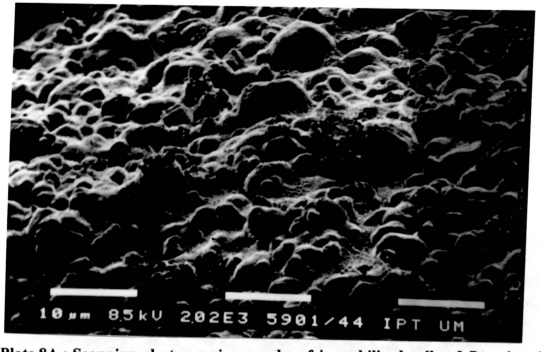

Plate 8A : Scanning electron micrographs of immobilized cells of *R. palustris* strain B1 in agar after 48 h under anaerobic-light conditions at 30° ± 2 °C and 4 klux (whole bead)

Plate 8B : Rupture in the gel film - evidence of budding after 72 h incubation at 30° ± 2 °C and 4 klux (whole bead)

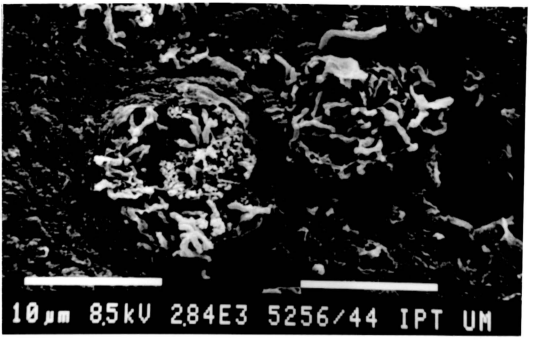

Plate 8C : Cells inside the fractured agar bead after 72 h at 30° ± 2 °C and 4 klux

The chemical oxygen demand (COD) removal of sago effluent

Analysis of the settled and unsettled sago effluent after treatment revealed considerable differences in the COD removal pattern. The results are shown in Fig. 15A and 15B and Appendix C28.

The free cells of *R. palustris* strain B1 were found to be more capable of removing the organic loading in the settled sago effluent than the bound cells. The COD removal from the wastewater was 71% after five days of treatment with the free cells. Similar results were obtained by Getha (1995) using the same organism. The immobilized cells of *R. palustris* strain B1, however, showed slow removal over the first two days and only recorded 55% removal of COD after five days of treatment in the settled sago effluent.

On the other hand, both the free cells and bound cells showed similar COD removal patterns with the unsettled effluent. The free cells showed the highest COD removal of 86% after 3 days while the immobilized cells recorded the highest COD removals of 82% after 4 days. This may be because the immobilized cells probably required more time to get acclimatized to the environment. Therefore, most of the utilization of carbon sources by *R. palustris* strain B1 was during the initial four days of treatment. After the fourth day, the free cells recorded much lower COD removal compared to the immobilized cells. Yang *et al.* (1988) noted that immobilized cells maintained higher solid retention time (SRT) which in turn

offered stable and effective processing of biological wastewater. The decrease in COD removal by free cells on the fifth day of treatment may be due to product inhibition. Inhibition may be derived from the substrate or the carrier material and it may be reduced by using a controlled rate of addition of substrate and rapid removal of products either in a continuous flow system or by dialysis (Philips and Poon, 1988).

The immobilization technique itself may affect and alter development and behaviour of immobilized cells compared to free cells. Diffusion of gas and substrate are the most important parameters which are different for immobilized and free microorganisms. Free microorganisms are able to get carbon dioxide and substrate directly from the surrounding atmosphere or from the liquid medium (Rehm and Omar, 1993). Growth conditions of the cells prior to immobilization, e.g. cell age, light intensity or nutrient composition, were also of primary importance for the activity in the later immobilized state (von Felten *et al.*, 1985). Therefore, the altered conditions mentioned above might have influenced the metabolism of the immobilized *R. palustris* strain B1 resulting in decreased COD removal rates from the settled sago effluent.

The prolonged metabolic activities of immobilized cells has been well documented in many examples of semicontinuous and continuous processes for product formation, for example, ethanol and hydrogen (Vincenzini *et al.*, 1986;

Bisping *et al.*, 1989). Free cells have only a short metabolic activity when used in batch culture. When compared to free cell cultures, immobilized cells exhibited improved production characteristics and stabilities in many systems (Fukui and Tanaka, 1982). Entrapment in polymeric matrices anchors the living cells in a network but allows free diffusion of the substrate and product. Natural polymers like agar or alginate (von Felten et al., 1985) are suitable matrices that avoid loss of activity during the immobilization procedure.

Although growth rate was higher in free cells, enzyme activity was higher in immobilized cells. Shinmyo *et al.* (1982) observed that the growth rate of *Bacillus amyloliquefaciens* was repressed to 1/6 when immobilized in κ-carrageenan beads, but the α-amylase productivity was higher than that of free cells. Sasikala *et al.* (1990) noted that alginate immobilized cells of *Rhodobacter sphaeroides* O.U. 001 gave four-fold increase in hydrogen production, two-fold increase in nitrogenase activity and 1.2 fold increase in hydrogenase activity compared to free cells. Therefore, the prolonged metabolic activity of agar-entrapped *R. palustris* strain B1 may be due to the alteration of enzyme activity. However, the reasons for a longer life and prolonged metabolic activity of immobilized cells are not known (Rehm and Omar, 1993).

The marked difference between the COD removal of settled and unsettled sago effluent by immobilized *R. palustris* strain B1 may be because the nutrients

required by the immobilized cells were removed together with the solids during the settling process. The solids in the effluent mainly contained starch residues (Getha, 1995). As the bottles were kept static, the immobilized beads settled to the bottom of the culture reactor and were not exposed to the nutrient in the sago effluent (Plate 9). Agitation of the mixture or a continuous system may help overcome this problem. In addition, there might have been an initial lag period of acclimatization of the beads to the environment, resulting in decreased removal in COD of the settled sago effluent. This lag period may be shortened by preparing the inoculum in the sago effluent.

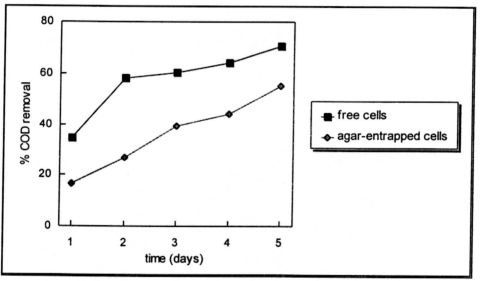

Fig. 15A : COD removal of settled sago effluent by free and agar-entrapped cells of *R. palustris* strain B1

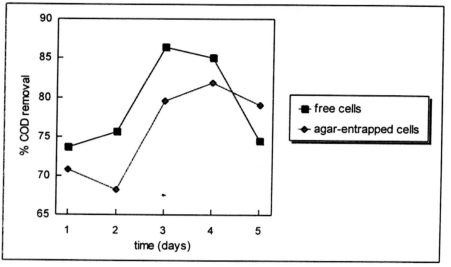

Fig. 15B : COD removal of unsettled sago effluent by free and agar-entrapped cells of *R. palustris* strain B1

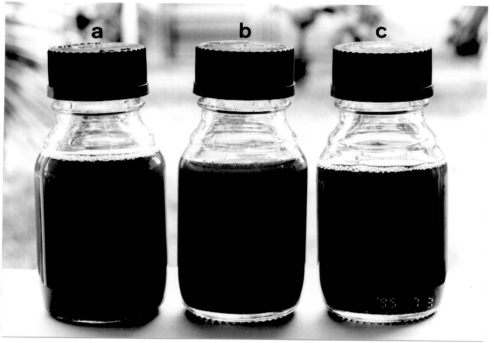

Plate 9: (a) Sago effluent inoculated with (b) free and (c) agar-entrapped cells of *R. palustris* strain B1 after 96 h at 30° ± 2 °C and 4 klux

Effect of sago effluent concentration on COD reduction

The effect of effluent concentration on the COD reduction by immobilized cells of *R. palustris* strain B1 is shown in Table 19.

Table 19 : Effect of sago effluent concentration of COD removal by agar-immobilized *R. palustris* strain B1 after 72h at $30^{o} \pm 2^{o}$C and 4 klux

Sago effluent concentration (% v/v)	Organic loading (g/L)	COD removal* (%)
25	1.5	36.7
50	3.2	46.2
75	4.4	58.0
100	6.9	71.4

*Mean of three values

After 72 h of incubation, the immobilized cells in 25% effluent (v/v) (1.5 g/L) were only able to remove 37% of the organic loading. There was a 10% increase when the organic loading was increased to 3.2 g/L and a further 10% increase when the organic loading was increased to 4.4 g/L. When the undiluted effluent of an organic loading of 6.9 g/L was used, the COD removal was 71.4%. Therefore, it is desirable to use the undiluted sago effluent for COD removal studies as the immobilized cells are capable of utilising the carbon sources in the effluent. At lower effluent concentrations, the poor COD removal could be attributed to the

low levels of soluble carbohydrate in the medium. The effluent concentration used had a significant effect on the COD removal ($p<0.05$) (Appendix C29). A 100% or 75% effluent concentration is necessary so as not to limit the utilization of the sago effluent by the immobilized cells.

Yang *et al.* (1988) reported that higher organic loading resulted in problems of clogging. They suggested the use of lower organic loading rates as it would eliminate possible problems of clogging and also reduce the frequency of rinsing and washing of a carrier. However, from the results so far obtained, it was found that since the solids in the effluent mainly contained the nutrients utilized by the immobilized cells, decrease in organic loading automatically resulted in a decrease in COD removal.

However, Yang and Wang (1990) noted that immobilized cells had a critical loading rate. When the loading rate was increased dramatically, the system could not recover and wash-out was observed. Thus, in this experiment, it was observed that a loading rate of 6.9 g/L was still below the critical loading rate.

Effect of mixing on COD reduction

The effect of mixing on the COD reduction of sago effluent by immobilized *R. palustris* strain B1 is listed in Table 20.

Table 20 : Effect of mixing on the COD removal of sago effluent by agar-immobilized *R. palustris* strain B1 at 30° ± 2 °C and 4 klux

Time (h)	COD removal (%)*
24	39.4
48	49.0
72	51.4

*Mean of three values

There was a 49% of COD removal after 48 h of incubation with the immobilized cells of *R. palustris* strain B1. This was only increased to 51% of removal after a further 24 h treatment. It has also been shown in a previous experiment that the agar-immobilized *R. palustris* strain B1 cells removed 82% of COD from the sago effluent without the aid of agitation (Ch. 3, pp93). Therefore, it can be concluded that mixing did not significantly aid in the removal of the COD from the effluent ($p>0.05$) (Appendix C30).

During treatment, the immobilized cells were agitated and were subjected to forces which tended to rupture them by stirring and from collision among the

particles and with the culture vessel wall. The carrier might have been subjected to considerable wear due to abrasion and rupture from gas formation. This was, however, not confirmed in this study.

The degeneration or rupture of the carrier can adversely affect the ability of the immobilized cells to utilize the nutrients present in the effluent. Since most of the enzymes taking part in the treatment with immobilized cells are intracellular, the damage to the carrier may give rise to added debris in the effluent which might increase the organic loading.

It was noted at the end of the experiment that the sago effluent had turned slimy, probably due to the disintegrated agar in the carrier. One way to mitigate this effect is to use a higher concentration of agar when preparing the immobilized cells. The concentration of gel is an important factor to be considered when a balance has to be established between optimum product formation, substrate diffusion and the mechanical strength of the matrix.

Other ways to improve the mechanical strength of the matrice include use of wire mesh particles (Atkinson *et al.*, 1979), reinforcement with polyacrylamide (Kuu and Polack, 1983) or the addition of various mineral solids such as ceramic, silica gel (Kolot, 1981) and glass (Nordin *et al.*, 1967). In general, these carrier particles are superior in mechanical strength, thermal stability and confer a stabilising effect on the immobilized organism. These carriers have also been

demonstrated to be suitable for batch or continuous fluidized bed reactors (Philips and Poon, 1988; Cochet *et al.*, 1990). The cell retention on the support also depends to a great extent on the nature of the solid support, mainly the available surface, porosity and its charge. The stability of the cell retention depends on the pH, ionic strength and even the dynamics of the ion exchange conditions by varying the flow rate of the liquid medium.

The effect of pH on microbial attachment (Marcipar *et al.*, 1978) suggested that a change in pH induced a variation of the carrier retention properties as a consequence of the particle microenvironment modification. Chibata *et al.* (1974) added various metal ions to the organic carriers and greatly increased the capacity of the support for cell immobilization. The data confirms that microbial immobilization depends on pH as well as on ionic strength of the solution.

The main requirement of any immobilization is to prolong and induce a higher activity of the cells in producing the desired substances. In this case, the simple stirred tank reactor does not serve the function. In a batch reactor, agitation does not significantly aid in reducing the organic loading of the sago effluent. Probably, other types of reactors have to be considered.

A more suitable reactor for immobilized whole cells would be the packed bed reactor. The substrate solution is passed through a settled bed of immobilized cells held in a column and the treated effluent is obtained at the outlet. The degree

of conversion depends on the retention time of the fluid in the bed. Contact time is determined by the free volume in the reactor bed divided by the flow rate through the column. Unlike stirred tank reactor, the inoculum in a packed bed is stationary and the effluent moves past the beads. This prevents damage to the beads and increases catalytic activity in the reactor.

Effect of inoculum concentration on COD reduction

The effect of inoculum concentration of the COD reduction of sago effluent by immobilized *R. palustris* strain B1 is shown in Table 21. These results indicate that a 10% inoculum of immobilized cells is sufficient to treat the sago effluent. The analysis of variance showed that the inoculum size had no effect on the COD reduction capability of the immobilized cells ($p>0.05$) (Appendix C31) .

Confining the living cell by immobilization inevitably increases the local concentration of the inoculum and provides higher solid retention time (SRT) compared to free cells. The overall activity per unit volume of reactor is consequently higher than is normally found in free cell systems. This high cell density results in a more rapid conversion of organic matter and places great demands on the supply of nutrients to the active sites (Philips and Poon, 1988).

It can be pointed out that the cell load also plays an important role in the metabolic activity of the immobilized cell. Normally, improved results could be

obtained by increasing the cell load in the gel. Fiβler *et al.* (1995) found that optimal hydrogen production was obtained with 10 mg dry wt of cells/mL gel. However, higher cell loads resulted in decreased hydrogen production. Higher cell densities are known to cause substrate diffusion problems (Vincenzini *et al.*, 1982a; Mignot *et al.*, 1987) and light limitation (Francou and Vignais, 1984) due to a shading effect resulting from the shadowing of beads by one another or by the bacteria inside the beads.

Poor mass transfer is undesirable as the immobilized cells are used inefficiently and the longevity of the system is reduced (Rosevear *et al.*, 1987). A build-up of product also occurs when mass transfer is poor which reduces the activity of the immobilized cell. Mass transfer can be improved by careful choice of the immobilization matrice, by attention to operational conditions and by making the immobilized beads smaller in size.

Contact between immobilized cell and substrate solutions should be maximized in order to increase diffusion of substrates and products. Gel beads with reduced diameter have a better surface to volume ratio, thus exhibiting more efficient biotransformations (Neufeld *et al.*, 1991; Westrin and Axelsson, 1991). The better surface to volume ratio guarantees the substrate easier access and an increased light supply to the immobilized cells.

Immobilization of *Rhodospirillum rubrum* in agar beads gave higher rates of hydrogen evolution with smaller lag periods when compared to agar blocks, due to the rather long distance for diffusion (von Felten *et al.*, 1985). Beads are also advantageous because they can be prepared more conveniently for use in larger volumes or in fluidized bed reactors (Francou and Vignais, 1984). Fiβler *et al.* (1995) noticed an increased hydrogen production of 62% when the bead size of 3 mm was used. This was, however, increased to 88% when 50 μm microbeads were used.

The most important factors affecting product formation with the immobilized system are the cell density and the age of the cells for immobilization. von Felten *et al.* (1985) recommended the concentration of 1 mg dry wet/mL of original culture suspension which was 70 h old for long-term hydrogen production. The age of the cells could reduce the lag phase until the product formation started. The photoproduction of hydrogen by agar-entrapped cells of *R. palustris* from sugar refinery wastes and straw paper mill effluents were investigated by Vincenzini *et al.* (1982b). On average, 43% of the initial COD from the sugar refinery effluent and 28% of straw paper mill effluent was converted to hydrogen.

Thus, to obtain optimum product formation by the immobilized cells, a good balance between the concentration of gel, diffusion of substrate and product as well as light penetration ought to be studied.

Table 21 : Effect of inoculum concentration on COD removal of sago effluent by agar-immobilized *R. palustris* strain B1 after 72 h at 30° ± 2 °C and 4 klux

Inoculum concentration (%)	% COD removal*
10	61.4
15	69.8
20	69.8

*mean of three values

Reusability of immobilized cells of *Rhodopseudomonas palustris* strain B1

The activity of the immobilized cells used in five different runs of 72h each are shown in Table 22.

Table 22 : The reusability of the agar-immobilized *R. palustris* strain B1 cells in sago effluent at 30° ± 2 °C and 4klux

No. of runs (72h each)	% COD removal*	% activity retained
1st	69.8	100.0
2nd	64.6	92.6
3rd	62.4	89.4
4th	50.5	72.3
5th	36.0	57.6

*mean of three values

The immobilized cells retained 58% of their original activity after 5 repeated uses. The loss of activity in the immobilized cell after prolonged use could be attributed to the limitations of substrate availability, the build-up of toxins and the decrease in mass transfer.

The cell is usually the weakest element in an immobilized preparation and its protection from deactivation is paramount in devising operational procedures. Although the stability of the cell is reported to be enhanced by immobilization, the activity declines gradually when stored or during operation (Chibata, 1978). Factors influencing the decay of cell activity are denaturation, bacterial contamination, inhibition by toxic substrates and incorrect operation. The long-term survival of live cell reactors depends on a number of factors including a steady supply of sufficient nutrients.

Hydrogen production from lactate by agar-immobilized *Rhodospirillum rubrum* was maintained over several months with a loss of activity of 60% after 4 months. In comparison, immobilized *R. palustris* cells exhibited lower stability of hydrogen production from benzoate and had decreased to 10% of the original activity within 35 days (Planchard *et al.*, 1989).

The build-up of toxins can also lead to a loss of cell viability. Immobilized cells have altered resistance against poisons. Ca-alginate immobilized *Pseudomonas putida* exhibited increased resistance against phenol compared to free

cells (Bettmann and Rehm, 1984). Although the reason for this increased resistance of entrapped microorganisms is not yet known, it can be assumed that the behaviour of the microorganisms in the microcolonies found in the beads protect the cells. By forming many colonies at the border of the beads, a diffusional barrier against the toxins can be built up. By entrapment, the formation of microcolonies leads to a connection of membrane gradients of the cells, because of the sharing of a common external milieu. Therefore, the waste secretion from immobilized cells is small compared with that of free cells (Rehm and Omar, 1993).

Planchard *et al.* (1989) noted that it was possible to regenerate and even to increase the initial hydrogen production activity by preincubating the immobilized cells under anaerobic-light conditions in culture medium containing high C to N ratio. They suggested the optimum duration as one day after about every five days of hydrogen production to maintain the hydrogen producing activity over several months.

Rapid changes in substrate composition and flow are also likely to cause osmotic, gas or nutrient stress to the organism. It is also equally important to see that the mass transfer of solutes to immobilized cell is efficient if cell viability is to be retained. Further work is needed to increase the storage stability and the reusability of the immobilized cell for the treatment of biological waste containing starch.