Contract N° 958058

# MELISSA

# A MICROORGANISM BASED MODEL FOR CELSS DEVELOPMENT

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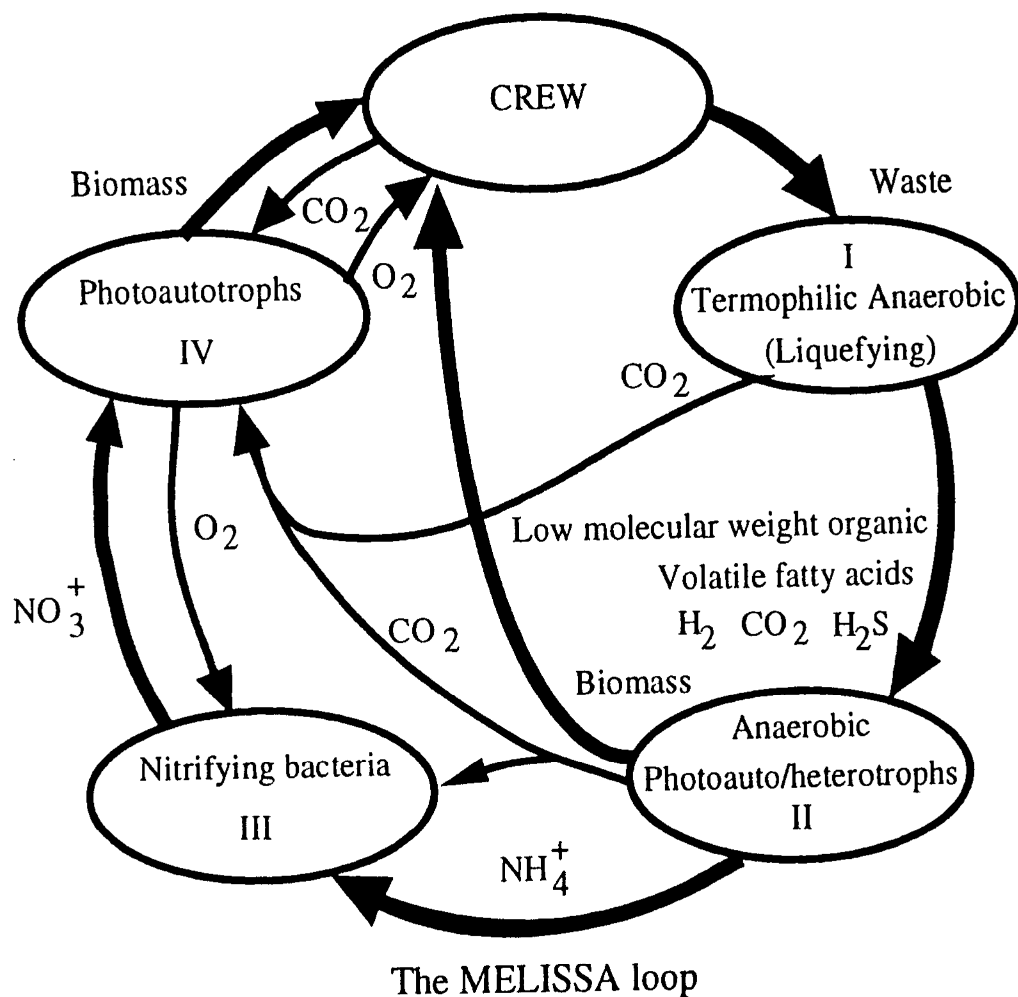
# I. INTRODUCTION

The objective of this project was the study of the second compartment of the MELISSA concept. In this phototrophic compartment different bacteria will be used together. These bacteria are *Thiocapsa roseopersicina*, *Rhodobacter capsulatus* and *Rhodospririllum rubrum*. The second compartment must further degrade the compounds formed in the anaerobic liquefying compartment I. These compounds are volatile and non-volatile fatty acids, carbon dioxide, peptides, aminoacids, ammonium ions, hydrogen and hydrogensulfide. In order to fulfil this task a combination was necessary between photo-autotrophic and photo-heterotrophic bacteria. Therefore the compatibility between *Thiocapsa* and *Rhodobacte*r or *Rhodospirillum* is an essential factor for the design of the second compartment.

The compatibility of the growth media was studied followed by the determination of possible toxic effects of compound produced by one of the strains. In order to distinguish *Thiocapsa* from the *Rhodobacter* or *Rhodospirillum* strains a specific growth medium was tested. In order to be able to make mass balances the chemical composition of the strains was analysed. Finally it was decided to construct a lab scale reactor in which the *Thiocapsa* was separated from the *Rhodobacter* by a dialysis membrane in order to prevent the mixing of the bacteria and to allow free transport of the compounds in the solution. Finally this reactor was transformed in a continuous reactor system.




The MELISSA loop

## II. THEORY

The need for the incorporation of *T. roseopersicina*, which is a sulfide oxidizer, is caused by hydrogen sulfide itself produced by the fermentative thermophilic Clostridia of the liquefying compartment (Mortimer, 1981). This hydrogen sulfide has a toxic effect on the microorganisms of the other compartments (chemical reactions and pH changes) and on humans (10 ppm = threshold value). As a matter of fact there is a real need for a sulfur cycle.
*T. roseopersicina* is a purple non sulfur bacterium (= Chromatiaceae) which belongs to the anoxygenic phototrophic bacteria. This means that *T. roseopersicina* is a bacterium growing under anaerobic conditions with light as energy source and without production of oxygen. The electrons which are activated by the light to produce energy are available from inorganic compounds (minerals). *T. roseopersicina* uses sulfides as inorganic compound. The sulfides are oxidized to sulfur, which is stored in the bacterium as granules and then further oxidized to sulfates.
H. Van Gemerden made the observation that as long as there are some sulfides left in the medium, the hydrocarbons will be stored and only used when the sulfides will be disappeared (Van Gemerden, 1968). At that moment the sulfides will oxidize to sulfur, 42% and to sulfate, 58%. The quantity of synthetised cell material is dependent of the amount of reducing power used.

The environment where *T. roseopersicina* can grow is not rigorously defined. Some experiments showed that the bacterium is able to grow in light and dark anaerobic conditions and in light and dark aerobic conditions. In these conditions, the two possible systems for energy gain are photosynthesis and respiration, which are in competition for their electrons, because both systems use the same redox couples. The situations with the best growth are in the light under anaerobic conditions and in the dark under aerobic conditions with regular addition of thiosulfate for example.

In the nature the bacterium is found in fresh and salt stationary water. In fresh water, a concentration of about 5 ppm hydrogen sulfide is enough for the growth of the bacterium. In salt water the sulfate concentration is much higher than in fresh water (28 mM against 150 μM). The presence of high sulfate concentration results in a higher amount of sulfate reducing bacteria. These bacteria produce a lot of hydrogen sulfide (± 500 μM = 16 ppm $S^{2-}$). This hydrogen sulfide results in the growth of phototrophic sulfur bacteria, such as *T. roseopersicina*. At the upper layer an algae population will grow. This structural consortium of algae, sulfide oxidizing and sulfate reducing bacteria is called a sulfureticum or microbial mats and are mostly present in estuaries.

The reason of using *T. roseopersicina* is its ability to oxidize reduced sulfur compounds to sulfate, through and internal formation of S-granules together with light as energy source. The bacteria is also able to assimilate different organic compounds and can grow in different conditions (versatility).

# III. TN16.1 : COMPATIBILITY OF *THIOCAPSA* WITH *RHODOSPIRILLACEAE*

## III.1. DIFFERENT USED STRAINS

The following strains were used: *Rhodobacter capsulatus* ATCC 2372 (R1), *Rhodobacter capsulatus* ST 407 (R2), *Rhodospirillum rubrum* ATCC 1117 (R3), *Thiocapsa roseopersicina* (9314, 6311) from W. Pfennig and H. G. Trüper of the University of Konstanz and *Thiocapsa roseopersicina* (M1) from H. Van Gemerden of the University of Groningen.
In future *Rhodospirillum rubrum* ATCC25903 will be used. The ATCC1117 did first arrive in the laboratory.
Transfer of heavy metal resistance genes was done into *Rhodobacter sphaeroides* NCIB8253. The *R. sphaeroides* was used as a test strain because this strain was quite sensitive to heavy metals.

## III.2. GROWTH MEDIA

Before testing the suitability of the bacteria to the second compartment, cultivation in standard medium is necessary. Therefore we are using the medium of Pfennig, described in 'The Prokaryotes' (1981) with some adaptations.

Description of the Standard nutritional conditions

The medium consists of :

| | | |
|---|---|---|
| **Solution 1 :** | 0.22% salts : | 0.34 g/l $KH_2PO_4$<br>0.34 g/l $NH_4Cl$<br>0.34 g/l Kcl<br>0.5 g/l $MgSO_4.7H_2O$<br>0.25 g/l $CaCl_2.2H_2O$ |
| **Solution 2 :** | $5.10^{-4}$% trace-elements : | 1 ml/l trace solution (pH = 6)<br>/→ 3 g/l EDTA-di Na<br>1.1 g/l $FeSO_4.7H_2O$<br>190 mg/l $CoCl_2.6H_2O$<br>50 mg /l $MnCl_2.2H_2O$<br>42 mg/l $ZnCl_2$<br>24 mg/l $NiCl_2.6H_2O$<br>18 mg/l $Na_2MoO_4.2H_2O$<br>300 mg/l $H_3BO_3$<br>2 mg/l $CuCl_2.2H_2O$ |
| **Solution 3 :** | $2.10^{-6}$% vitamin B12 : | 1 ml/l of a 0.002% solution of vitamin B12 |
| **Solution 4 :** | 0.15% nabicarbonate : | 20 ml/l of a 7.5% solution of $NaHCO_3$ |
| **Solution 5 :** | 0.1% $Na_2S.9H_2O$ : | → 4 ml/l of a 10% solution $Na_2S.9H_2O$ **(opl. 5a)**<br>→ 20 ml/l of a 3% solution $Na_2S.9H_2O$ (pH = 8) **(opl. 5b)** |
| **Solution 6 :** | 0.05% Mg and $NH_4$-acetate : | 10 ml/l of a 5% solution $Mg/NH_4$ acetate<br>→ 25 g/l $(CH_3COO)_2Mg$<br>→ 25 g/l $CH_3COONH_4$ |

To prepare the medium solutions 1 and 2 are brought together and autoclaved. The other 4 solutions are each individually sterilized. The vitamin B12 solution and the Na-bicarbonate solution are sterilized through a membrane filter. This prevents the destruction of the vitamin B12 at the high temperatures. reached during autoclavation and the loss of Na-bicarbonate caused by gas formation ($CO_2$). The right quantities of solutions 3 and 4 are mixed with the autoclaved solutions 1 and 2, after cooling down.
The solutions 5a and 5b are prepared in bottles with rubberized plugs and then autoclaved. Also solution 6 is autoclaved. The appropriate quantities of the solutions 5 and 6 are added to the previous solutions. The whole is put at the ideal growth pH 7.3 (7 - 7.5).
The solutions 5b and 6 are supplementary solutions which could be added regularly during the growth of cultures.
The reason to sterilize all the different solutions individually is to avoid too much precipitations. When the solution is ready, we pour it in sterilized bottles or into testtubes closed with a plug.

When necessary to work with agar plates, 1.5 to 2% agar is added to the solution, composed to solutions 1 and 2, before autoclavation. The other solutions are added after sterilization, the whole is mixed and the plates are poured.

Some remarks have to be done about the chemical situation of the different compounds in the medium. In the medium at pH 7.3, the different added compounds are under more or less dissociated form. The supplied $Na_2S.9H_2O$ ($S^{2-}$) will appear as $HS^-$ and $H_2S$, but in a concentration which is half of the $HS^-$ concentration. The bicarbonate is mostly under the $HCO_3^-$ form. A low quantity of $H_2CO_3$ ($H_2CO_3 \rightarrow H_2O \rightarrow CO_2$) will also be formed. For 1 $H_2CO_3$, there are 8 $HCO_3^-$ in solution. Acetate is present as $CH_3COO^-$ and ammoniumchloride as $NH_4^+$.
The ideal growth temperature for these microorganisms is about 30°C. This temperature determines which compounds will be soluble. The concentration of $H_2S$ which is maximal soluble at 30°C is 3 g/l, for $HS^-$ it is 6 g/l, for $CO_2$ 1.3 g/l and for $HCO_3^-$ 99 g/l. The solubility degree of $CH_3COO^-$ and $NH_4^+$ in the medium is of less importance because their $K_S$ values are very high. $H_2$ has a very low solubility degree, at 30°C 1.5 mg/l.

When the bacteria are grown under phototrophic conditions, 'Sylvania Gro Lux' lightenings are chosen as light source. These lightenings have an emission area between 400-500 and 600-700 nm, which corresponds with the absorption area of the bacteriochlorophyll a and the carotenoids (spirilloxanthin series).

A 10 times concentrated spore solution is composed of :

| | |
|---|---|
| $NiSO_4.6H_2O$ | 0.5 g/l |
| $MnCl_2.4H_2O$ | 0.5 g/l |
| $FeSO_4.7H_2O$ | 0.5 g/l |
| $ZnSO_4.7H_2O$ | 0.1 g/l |
| $CoCl_2.2H_2O$ | 0.05 g/l |
| $CuSO_4.5H_2O$ | 0.005 g/l |
| $H_3BO_3$ | 0.1 g/l |
| $Na_2MoO_4$ | 0.05 g/l |

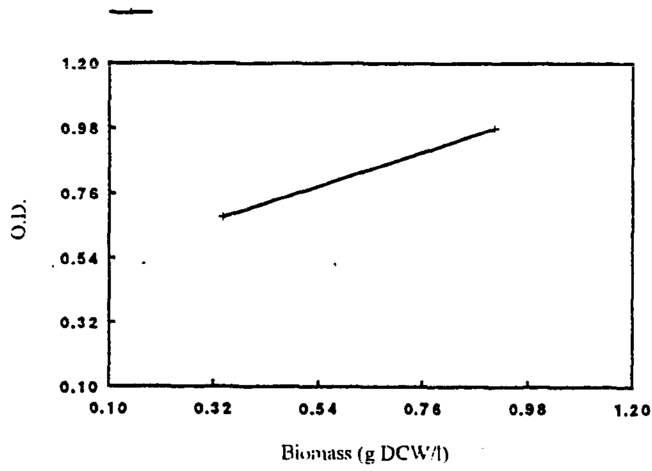
The 10 times concentrated vitamines solution has the following composition :

| | |
|---|---|
| nicotinic acid | 1 g/l |
| biotin | 15 mg/l |
| thiamin | 1 g/l |

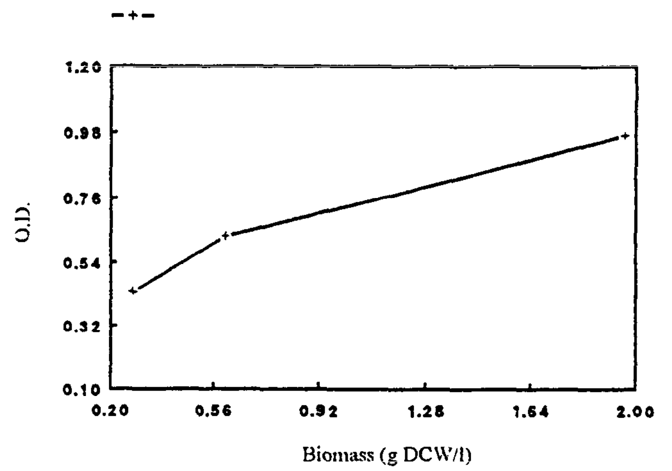
### III.3. GROWTH ON DIFFERENT CARBON SOURCES

The relation between optical density ($OD_{660}$) and biomass weight was calculated by making dilutions of cell suspensions. The OD was measured and the weight of the biomass measured after centrifugation of the solution and two rincings in $10^{-2}$ M $MgSO_4$. The bacteria were grown in erlenmeyers of 1 l at 28°C, 1200 lux and a pH of 6.8.
Figure 2 and 3 present the biomass in function of the OD for *Thiocapsa* and *Rhodobacter* or *Rhodospirillum* respectively.

A.Thiocapsa roseopersicina 9314




B.Thiocapsa roseopersicina M1




C.Thiocapsa roseopersicina 6311

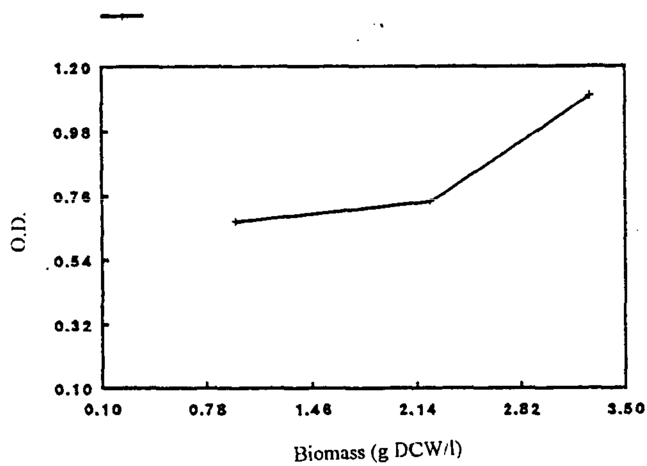
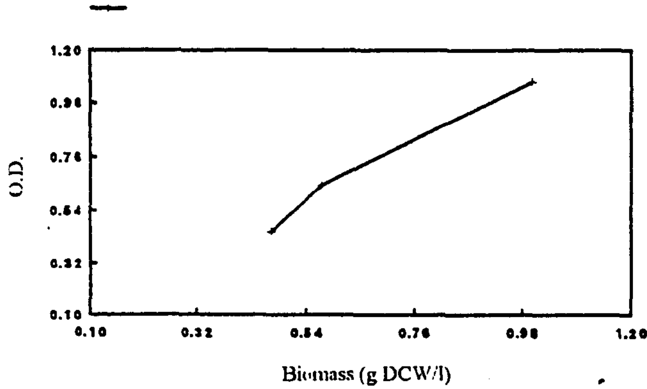
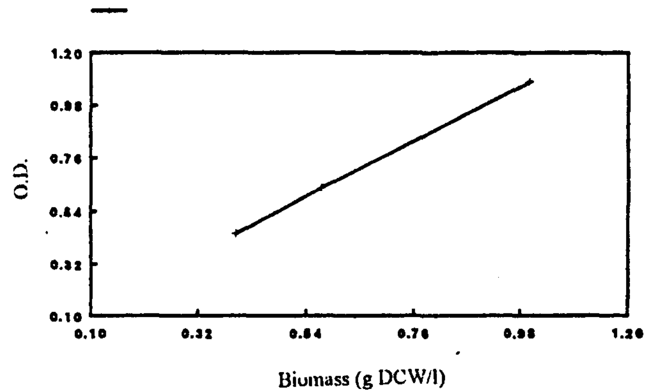



Figure 2. Biomass in function of OD for *Thiocapsa*.

A. Rhodobacter capsulatus
ATCC 23782




B. Rhodobacter capsulatus
ST 407




C. Rhodospirillum rubrum
ATCC 11170

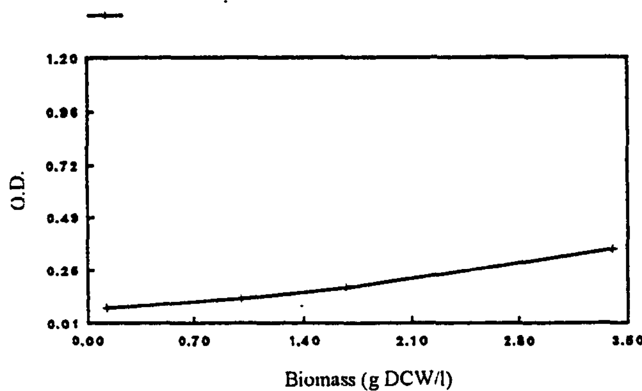



Figure 3. Biomass in function of OD for *Rhodobacter* and *Rhodospirillum*.

In order to distinguish the strains several carbon sources were tested and the results are presented in table1. From the growth on the carbon sources could be concluded that *Thiocapsa* does grow on gluconate and citrate where *Rhodobacter* and *Rhodospririllum* do not grow. This means that gluconate and citrate can be used on platings to distinguish between Thiocapsa and Rhodobacter or Rhodospirillum. None of the strains did grow on threonine. These results were confirmed by growing the strains on solid medium containing either Pfennig or S & V medium with the different carbon sources. On the other hand *Rhodobacter* and *Rhodospririllum* did grow on Pfennig medium. However *Thiocapsa* could

not grow on pure S & V medium. So the use of Pfennig medium with lactate allows the total counting on the phototrophic cells. The use of Pfennig medium with gluconate or citrate allowed the selective counting of *Thiocapsa.*

| RHODOSPIRILLUM and RHODOBACTER | | | | | | |
|---|---|---|---|---|---|---|
| C-source | M1 | 6311 | 9314 | R1 | R2 | R3 |
| azelate | + | + | + | - | - | ± |
| citrate | + | + | + | - | ± | - |
| gluconate | + | + | + | - | - | + |
| lactate | ± | + | + | + | + | ? |
| glucose | - | + | + | ± | + | + |
| valeriate | + | ± | + | ± | ± | + |
| proline | + | + | + | + | + | + |
| threonine | ± | ± | ± | ± | ± | ? |
| mannitol | + | + | + | ± | ± | ? |
| histidine | - | - | - | - | - | + |
| propanol | + | + | + | + | + | + |
| butanol | + | + | + | ± | ± | ± |
| ethanol | + | + | + | ± | ± | ? |
| methionine | ? | ? | ? | - | ? | + |

M1 = *Thiocapsa roseopersicina* M1 ; 6311 *Thiocapsa roseopersicina* 6311 ; 9314 = *Thiocapsa roseopersicina* 9314 ; + : good growth ; ± : slow growth ; - no growth ; R1 = *Rhodobacter capsulatus* ATCC 3-82 ; R2 = *Rhodobacter capsulatus* ST 407 ; R3 = *Rhodospirillum rubrum* ATCC 11170 ; ? = not tested.

Table 1. Carbon sources used by *Thiocapsa, Rhodospirillum* and *Rhodobacter*.
Table 1 is always based on growth curves.

Figures 4 and 5 give some examples of these growth curves.

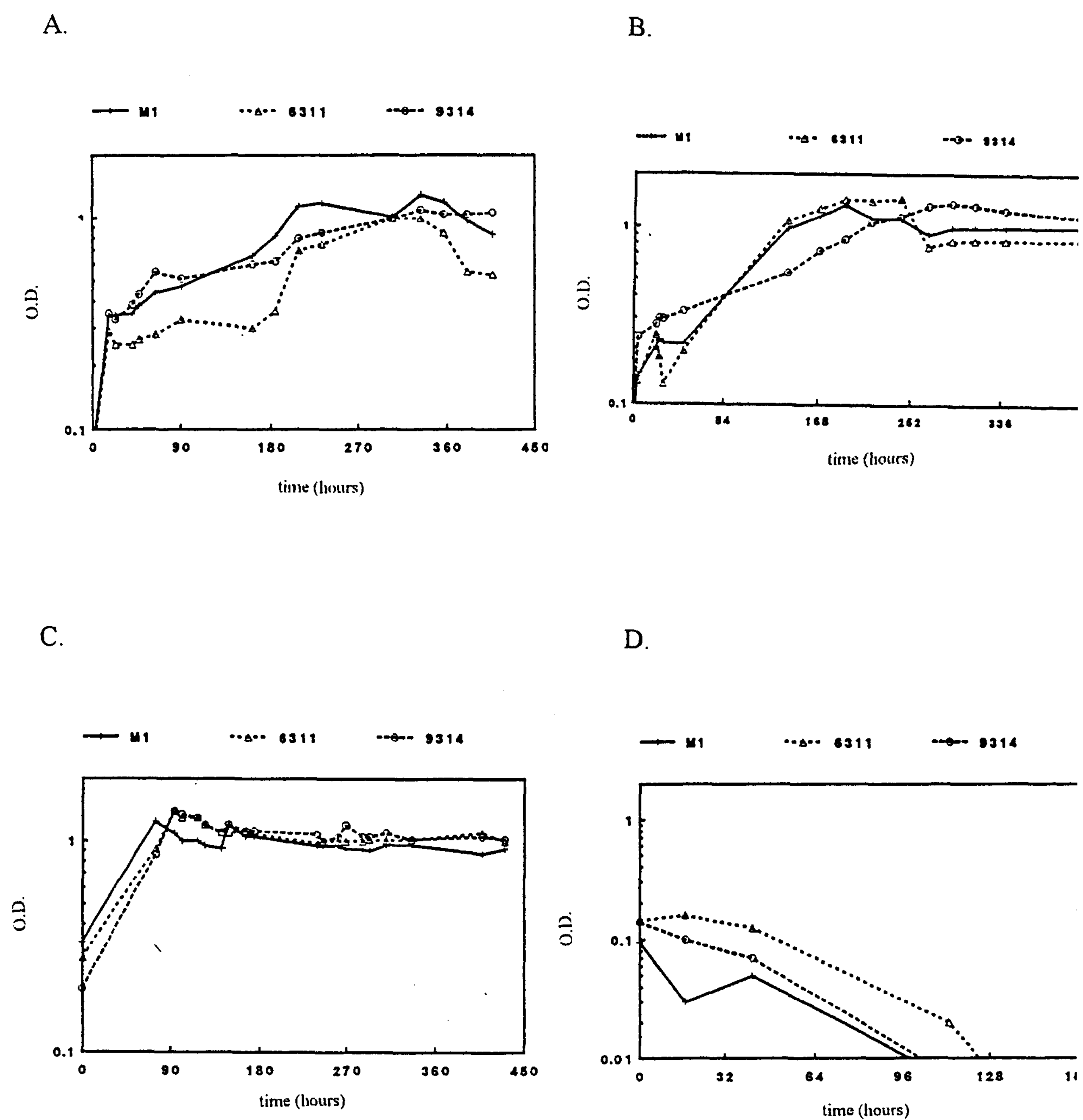



Figure 4. Growth curves for *Thiocapsa* on azelate (A), citrate (B), gluconate (C) and histidine (D).

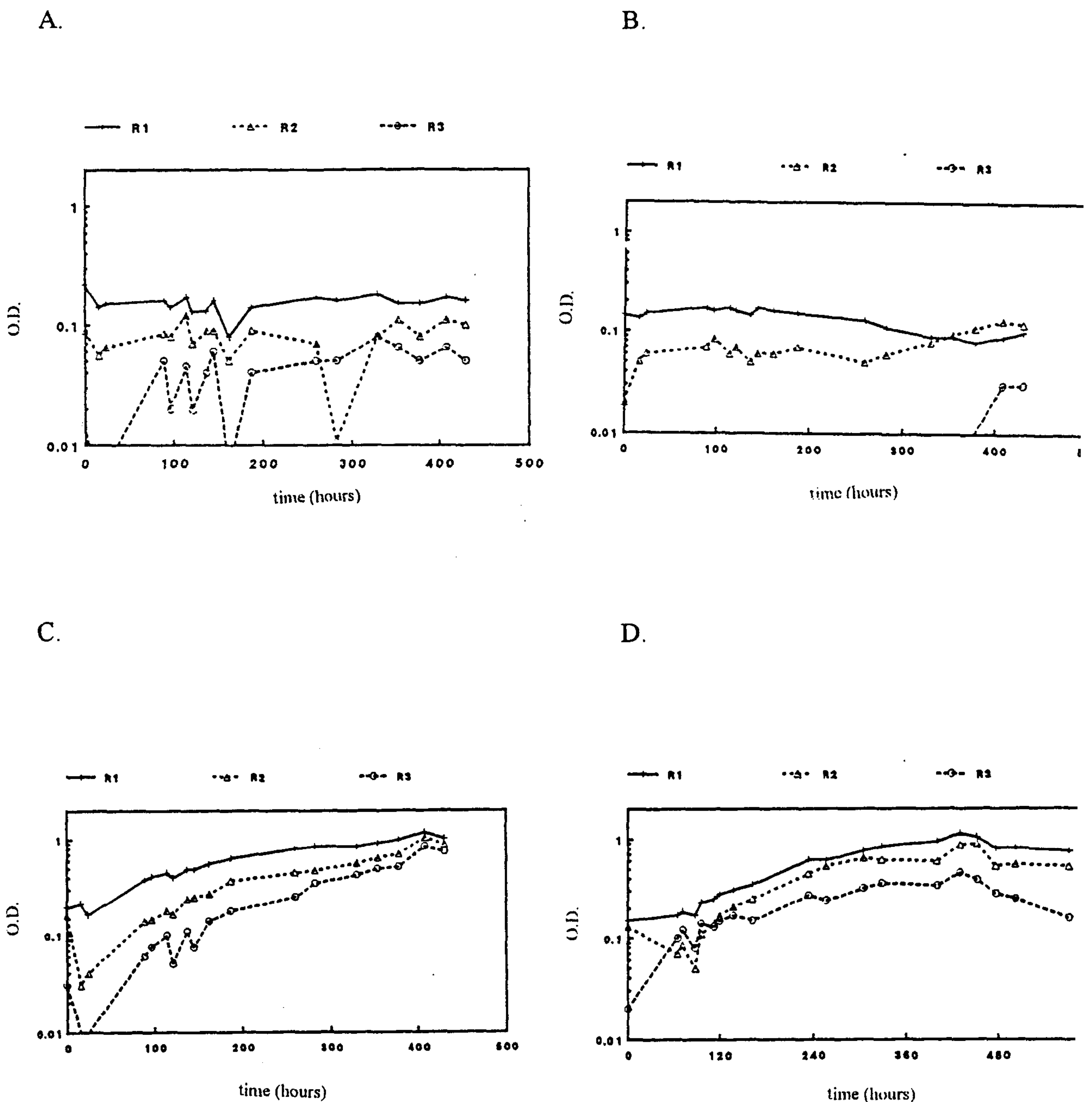



Figure 5. Growth curves for *Rhodobacter* and *Rhodospirillum* on azelate (A), citrate (B), valeriate (C) and propanol (D).

In order to find an identification technique to differentiate *Thiocapsa* and the *Rhodospirillaceae* it was suggested to compare their growth on petridishes with the carbon sources lactate, threonine, gluconate and citrate. The results are presented in table 2. It could be observed that both strains are able to grow on lactate and both do not grow on theonine. However only *Thiocapsa* was able to grow on gluconate and citrate and the *Thodospirillaceae* did not.

| THIOCAPSA | | | | |
|---|---|---|---|---|
| C-source | M1 | 6311 | 9314 | Besluit |
| lactate | + | + | + | + |
| threonine | - | - | - | - |
| gluconate | + | + | + | + |
| citrate | + | + | + | + |

| RHODOBACTER & RHODOSPIRILLUM | | | | |
|---|---|---|---|---|
| C-source | R1 | R2 | R3 | Besluit |
| lactate | + | + | ± | + |
| threonine | - | - | - | - |
| gluconate | - | - | - | - |
| citrate | - | - | - | - |

Table 2. Growth of *Thiocapsa, Rhodobacter* and *Rhodospirillum* on petridishes with different carbon sources.

## III.4. Compatibility of the Two Growth Media

The compatibility of the two growth media was controlled by making dilution series of one medium with the other. The media were composed as follows:

| % Pfennig | 100 | 95 | 90 | 80 | 60 | 40 | 20 | 10 | 5 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| % S & V | 0 | 5 | 10 | 20 | 40 | 60 | 80 | 90 | 95 | 100 |

Table 3 presents the results of the reciprocal growth. As already indicated *Rhodobacter* and *Rhodospririllum* can grow in Pfennig as well as in S & V medium. *Thiocapsa* needs at least 40 % of Pfennig medium.

| | THIOCAPSA | | | RHODOBACTER EN RHODOSPIRILLUM | | |
|---|---|---|---|---|---|---|
| % Pfennig medium | M1 | 6311 | 9314 | R1 | R2 | R3 |
| 100% | ± | ± | ± | ± | ± | ± |
| 95% | ± | ± | ± | ± | ± | ± |
| 90% | ± | ± | ± | ± | ± | ± |
| 80% | ± | + | + | ± | ± | ± |
| 60% | ± | + | + | ± | ± | ? |
| 40% | + | + | + | ± | + | + |
| 20% | - | ± | + | + | + | + |
| 10% | - | - | - | + | + | + |
| 5% | - | - | - | + | + | + |
| 0% | - | - | - | ± | + | + |

Table 3. Growth of *Thiocapsa, Rhodobacter* and *Rhodospririllum* in Pfennig medium diluted with S & V medium.

### III.5. PRODUCTION OF TOXIC COMPOUNDS

In order to find out if some toxic compounds could be formed by one of the strains that were toxic for the other bacteria the following test procedure was established. The strains were grown in their proper media. After growth the cells were removed by centrifugation. Carbon source was added and in the case of *Thiocapsa* also sulfides were added. Table 3 presents the results. It shows that *Rhodobacter* can grow perfectly on the Pfennig supernatant because the sulfides were already removed by the *Thiocapsa* growth. Some tests will be necessary to define the toxic sulfide concentration for Rhodobacter. *Thiocapsa* could only grow on new Pfennig medium or on S & V medium after addition of sulfides. In both cases no toxic compounds seem to be formed. Supernatants were recovered from cultures of 10 to 20 days old.

| Strains | 100% Pfennig | 90% Pfennig | 50% Pfennig | 10% Pfennig | 0% Pfennig |
|---|---|---|---|---|---|
| Rhodobacter | + | ± | + | + | + |
| Thiocapsa | - | - | - | + | + |

Table 4. Growth of *Thiocapsa* and *Rhodobacter* on their reciprocal supernatants.

### III.6. TRANSFER OF HEAVY METAL RESISTANCE GENES INTO *RHODOBACTER*

The *cnr* operon (Siddiqui et al., 1989) of *A. eutrophus* CH34 cloned in vector pVDZ'2 was transferred to *Rhodobacter sphaeroides* NCI8253. After conjugation (selection done on tetracycline) some transconjugants with an increased Ni resistance could be observed (Table 6). The transconjugants were grown on different Ni and Co concentrations and compared with the wild type. The nickel resistance increased from 0.120 mM in the wild type to 0.625 mM in the new transconjugants. The minimal inhibitory concentration of Co increased from 0.156 mM to 0.312 mM. No increase in Zn resistance could be observed. The Cd, Zn, Co resistance encoded by czc (Mergeay et al., 1985) was transferred via vector pUT mini Tn5. But no transfer could be obtained (Table 5). This way of labelling could be used to distinguish the cells from each other. This way of labelling could be used to distinguish the cells from each other.

## IV. TN16.2 CHEMICAL CHARACTERIZATION OF *THIOCAPSA*

The chemical composition of the *Rhodospirillum* strains was determined at the Service de Microanalyse of the I.C.S.N.-C.N.R.S. (Gif sur Yvette). The results are presented in table 4.

| Bacteria | C | H | N | O | S | P |
|---|---|---|---|---|---|---|
| *Rhodobacter capsulatus* | 40.96 | 6.12 | 7.50 | 20.66 | 1.11 | 3.52 |
| *Rhodobacter capsulatus* | 41.59 | 6.04 | 8.13 | 20.51 | 1.08 | 2.89 |
| *Thiocapsa roseopersicina* | 28.51 | 4.52 | 5.96 | 25.79 | 13.94 | 1.16 |
| *Thiocapsa roseopersicina* | 26.04 | 4.43 | 5.22 | 23.30 | 6.06 | 6.95 |

Table 5. Chemical composition of *Thiocapsa*, *Rhodobacter* and *Rhodospirillum*.

## V. TN16.3 KINETICAL CHARACTERIZATION OF *THIOCAPSA*

The growth of *Thiocapsa* in batch and continuous was studied in TN16.3 as a preparation for the reactor development.
The presented results lead to the idea to split copartment II in two chambers separated from each other by a membrane. This seemed to be necessary because the growth rate of *Thiocapsa* was much slower than the rate of *Rhodobacter*. Therefore a separation was necessary in order to prevent *Thiocapsa* to be overgrown by *Rhodobacter*. The system was first tested in batch and followed by the development of a continuous reactor.

This reactor concept will further be used for the combination with Compartment I and for the kinetical studies.

### V.1. FLAT SHEET REACTOR (FSR)

A Flat Sheet Reactor was used as a first reactor concept. The system is based on a membrane which separates two chambers. In one chamber flows a nutrient to keep the bacteria active and through the other chamber flows an effluent stream to be treated. A biofilm develops on the membrane. So the membrane is used as a separation device and a biofilm support. The membrane is an organo-mineral membrane composed of an organic polysulfone polymer and inorganic $ZrO_2$. The membrane has a cut-off of 40,000 Da, a porosity of 73%, a thickness of 200 µm. The skin side has pore diameters between 0.1 and 0.3 µm and at the open side the pore diameters are between 2 and 4 µm.

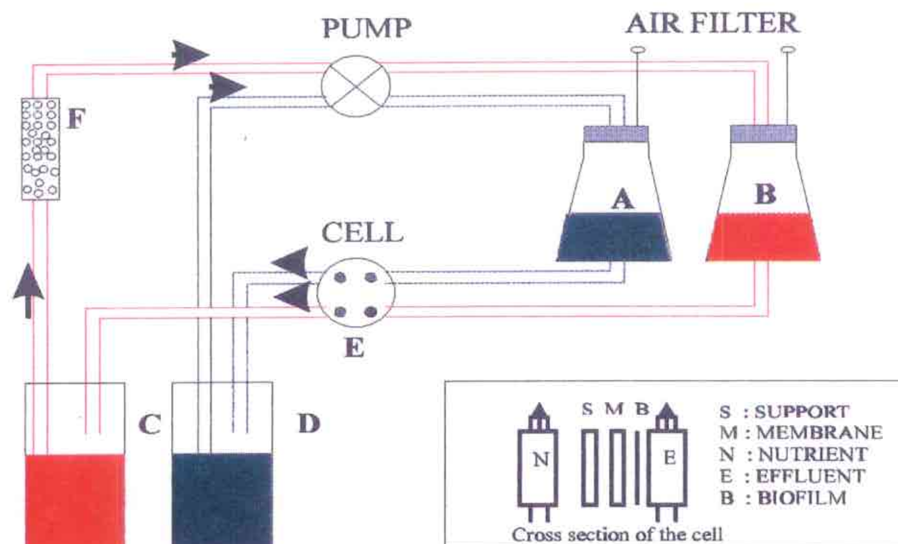



Figure 6. Flow sheet of a Flat Sheet Reactor.

Figure 7 presents a scanning electron microscope photo of the membrane of a FSR with a biofilm of *Alcaligenes eutrophus*. The figure shows the biofilm growth via the formation of microcolonies.

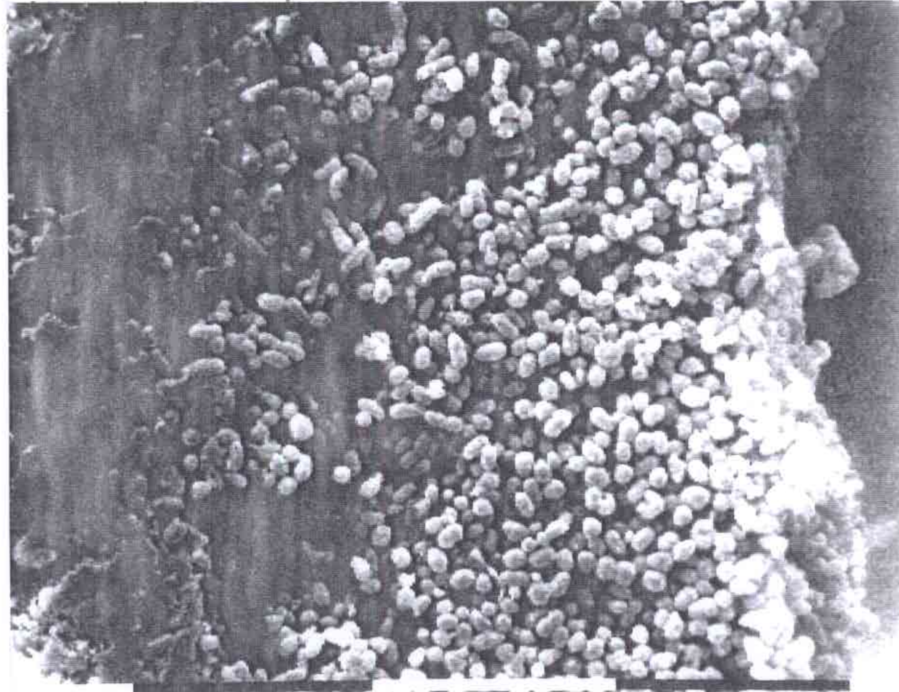
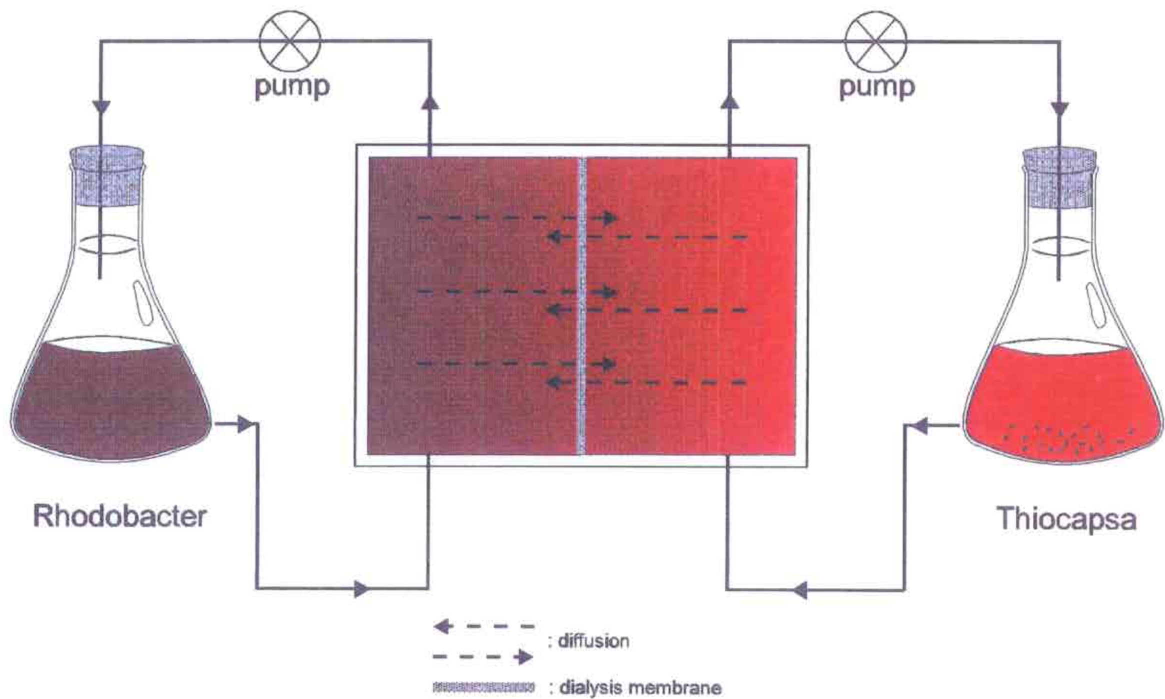


Figure 7. Scanning electron microscope photograph of the growth of an *Alcaligenes eutrophus* biofilm in a Zirfon® membrane.

V.2. BATCH MEMBRANE REACTOR

A batch reactor consisted of two reaction vessels from which liquid was pumped in and out. The liquid was tangentially filtred over a dialysis membrane. The soluble compounds could pass the membrane and the bacteria were stopped by the same membrane. A flow sheet of the reactor is presented in figure 8. The system allowed *Thiocapsa* and *Rhodobacter* to grow without inhibition by each other.




**Flowchart of a Membrane Reactor**

Figure 8. Flow sheet of batch reactor system.

Efforts were made to find a good membrane which allows the diffusion of chemical compounds and no transfer of bacteria. Dialysis membranes and composite membranes (Zirfon®) were tested. It could be concluded that Zirfon® membranes allowed a better diffusion and an easy biofilm formation. A problem arose by the supporting polyester layer. This layer seemed to limit the diffusion although the porosity and pore diameter were very high. In a next project supporting layers with different air permeability characteristics will be tested. Figure 10 shows a photograph of the lab scale batch reactor system. Until now the experiments were done to show that such a combined reactor system could work. Data will be presented in the next report.

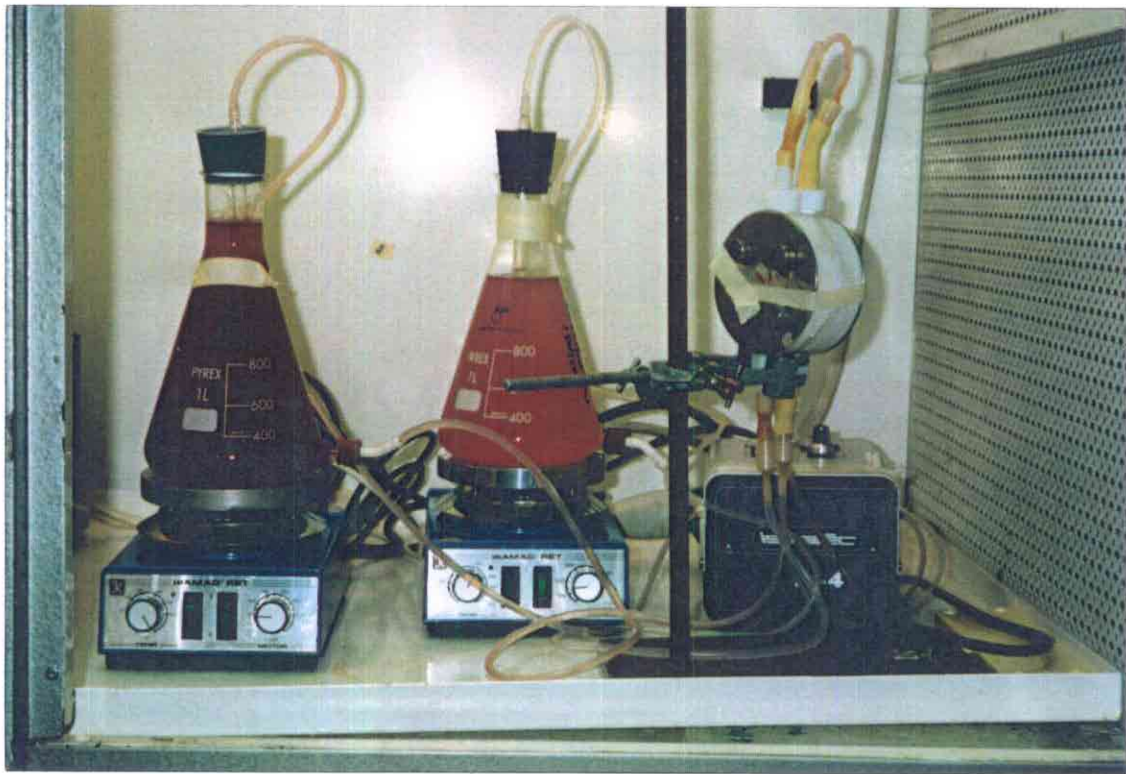



Figure 9. Photo of the lab scale batch reactor system.
The combination of a *Thiocapsa* and *Rhodobacter* culture via a membrane reactor system.

### V.3. CONTINUOUS REACTOR

The continuous reactor was principally based on the same system. The *Thiocapsa* compartment was feeded by an input pump. The input feed was the liquid from the liquifying compartment I diluted with Pfennig medium and with additional lactate and acetate in order to measure the degradation of these compounds in the reactor. The *Rhodobacter* compartment was connected with an overflow system to the output. Figure 10. Presents the flow sheet of the continuous reactor. In the output the final lactate and acetate concentrations were measured. The lactate and acetate were completely biodegraded in the system. Further details about lactate and acetate concentrations will be presented in next report. The growth rates of *Rhodobacter* and *Thiocapsa* will also be measured.

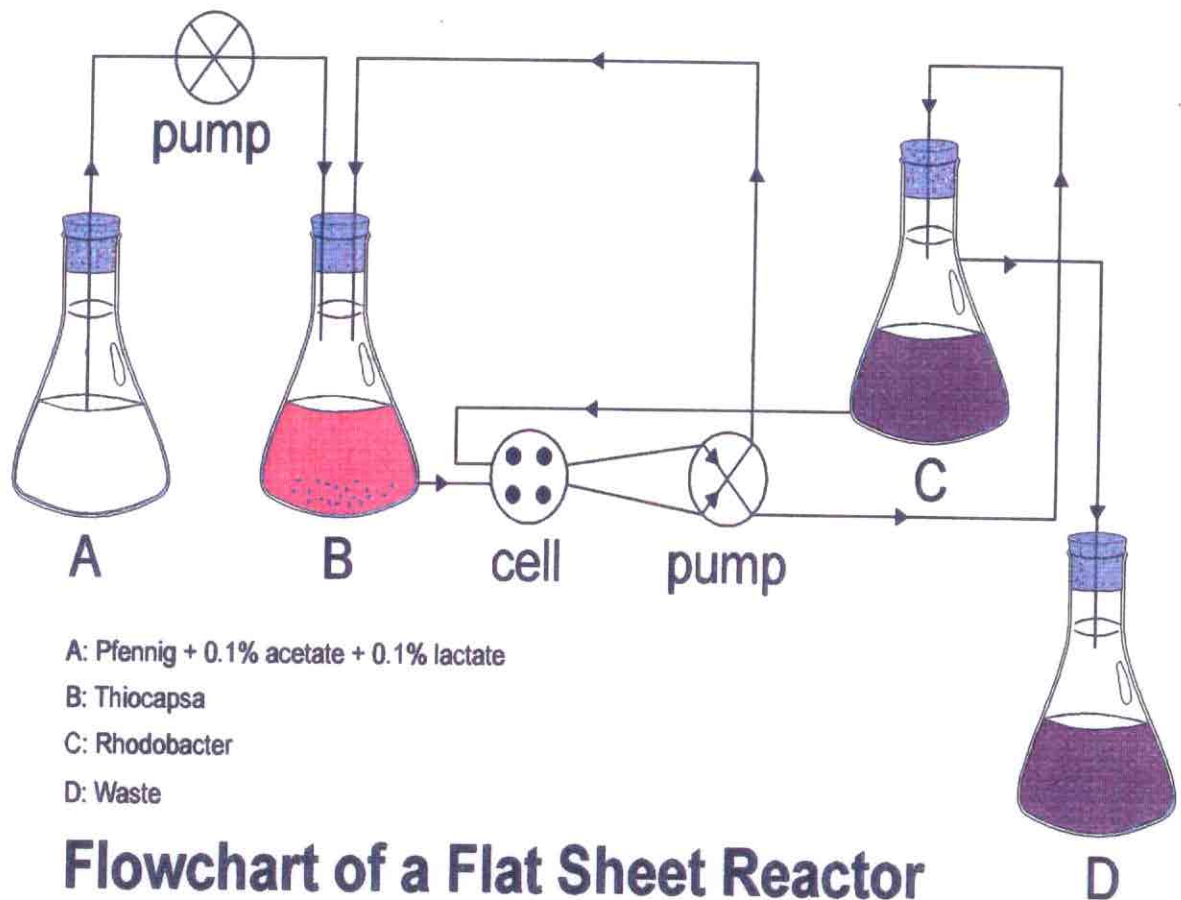



Figure 10. Flow sheet of the continuous membrane reactor.

Lactate and acetate concentrations of 0.1% were added together in the system. At the output concentrations lower than 0.01% were measured. Some first tests were done with solutions of the liquefying compartment and also there a biodegradation could be observed and no inhibition of the cellular *Rhodobacter* or *Thiocapsa* growth was observed.

## VI. Conclusions

The study of the Phototrophic Compartment of MELISSA showed that *Thiocapsa roseopersicina, Rhodobacter capsulatus* and *Rhodospirillum rubrum* are compatible to the Pfennig medium. The three species can also grow in a medium composed of 50% S & V medium and 50% Pfennig medium. *Rhodobacter capsulatus* can grow on the supernatant of Pfennig medium after the addition of lactate. Lactate is a very good carbon source for *Rhodobacter capsulatus* and *Rhodospirillum rubrum*. The growth of *Thiocapsa roseopersicina* is rather slow. The bacteria do not produce too much toxic compounds which can inhibit the growth of the other strains.

These conclusions lead to the development of a rector system in which the photoautotrophic and photoheterotrophic bacteria are separated from each other by a dialysis or Zirfon® membrane. The membrane separates the two bacterial strains and allows a diffusion of nutrients. The bacteria could be distinguished by growth on azelate, citrate or gluconate.

In future the reactor system will further be developed and much attention will be paid to the use of polyester supports which do not limit the diffusion. Further attention will be paid to the degradation of lactate, acetate, butyrate and the fate of sulphur entering the reactor. Also the problem of the increasing biomass will be studied. But as shown in TN11 the amino acid

composition of *T. roseopersicina* whole cell protein corresponds to the Food Agriculture Organisation (FAO) and exceeds largely the FAO values for leucine and theonine.

## VII. References

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