MELISSA

ESTEC/CONTRACT 8125/88/NL/FG

Technical note 8

MELISSA CCN1 TN 8

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T.N. 8-1

TOXICITY TESTS OF *RHODOBACTER* EFFLUENTS ON *SPIRULINA*

A *Rhodobacter* culture were divided into two samples:
- sample 1 was filtered through 0.45 µm Millipore filters
- sample 2 was sterilized by heat for 30 min at 140°C.

Starting from an initial culture of *Spirulina*, toxicity tests were performed as follow:
- the control consisted in one volume of initial culture of *Spirulina*, diluted with the same volume of fresh *Spirulina* culture medium (Zarrouk),
- Two erlenmeyers containing the initial culture of *Spirulina* were diluted in the same proportion with sample 1, that corresponds to the filtered *Rhodobacter* culture,
- two others erlenmeyers containing the initial culture of *Spirulina* were diluted in the same proportion with sample 2 that corresponds to the heated *Rhodobacter* culture.

Such different cultures of *Spirulina* were allowed to grow for 13 days at 35°C, under a light intensity of 5 w/m^2 and in the presence of 2% CO2. Growth was turbidimetrically followed by measuring OD750nm on samples of each culture. Numerical results (OD750), corrected from the turbidity of the used effluents of *Rhodobacter* cultures, and the corresponding amount of biomass (in g/l)are presented in the following table.

Time (h)	Control		Filtered 1		Filtered 2		Heated 1		Heated 2	
	OD	g/l	OD	g/l	OD	g/l	OD	g/l	OD	g/l
0	.02	.027	.021	.027	.023	.029	0	0	0	0
48	.048	.061	.045	.057	.043	.055	0	0	0	0
148	.36	.461	.24	.31	.25	.32	0	0	0	0
218	.586	.75	.464	.60	.488	.63	0	0	0	0
310	.85	1.09	.74	.95	.73	.93	0	0	0	0

By comparison with the control, growth of *Spirulina* appeared to be only slightly (~ 15%) reduced by the filtered culture medium of the used *Rhodobacter* culture. Moreover, no effects on the morphology, the chloropyll or the phycocyanin content of the algae were observable. By contrast, the heat sterilized culture appeared to totally inhibit growth of *Spirulina*, a result that is consistent with previous observation that organic matter impairs growth of *Spirulina*.

In conclusion, when filtered, effluents of *Rhodoobacter* cultures are not toxic to *Spirulina* and do not exert significant effect on their physiology. By contrast, heat sterilized effluents of compartment 2 totally inhibit growth of *Spirulina*.

TN 8.2

Theoretical considerations.

INTRODUCTION.

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. This hydrogen sulfide has a toxic effect on the micro-organisms 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.

THEORETICAL STUDY OF *THIOCAPSA ROSEOPERSICINA*.

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. 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 uses 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 an 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).

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Pure cultures of T. roseopersicina, cultivation on synthetic media.

To study the bacterium *T. roseopersicina*, we used pure cultures. We received strains isolated by the laboratory of prof. Dr. N. Pfennig and Dr. H.G. Trüper of the University of Konstanz (strains 6311, 9314, 1711, 1813 and 6713) and by the laboratory of Prof. Dr. H. van Gemerden of the State University of Groningen (strain M1).

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
0.34 g/l NH_4Cl
0.34 g/l KCl
0.5 g/l $MgSO_4.7H_2O$
0.25 g/l $CaCl_2.2H_2O$

Solution 2 : 5.10^{-4} % trace-elements : 1 ml/l trace-solution (pH=6)
/--> 3 g/l EDTA-di Na
1.1 g/l $FeSO_4.7H_2O$
190 mg/l $CoCl_2.6H_2O$
50 mg/l $MnCl_2.2H_2O$
42 mg/l $ZnCl_2$
24 mg/l $NiCl_2.6H_2O$
18 mg/l $Na_2MoO_4.2H_2O$
300 mg/l H_3BO_3
2 mg/l $CuCl_2.2H_2O$

Solution 3 : 2.10^{-6} % vitamin B_{12} : 1 ml/l of a 0.002 % solution of vitamin B_{12}

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**)
-> 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_4acetate
--> 25 g/l $(CH_3COO)_2Mg$
--> 25 g/l CH_3COONH_4.

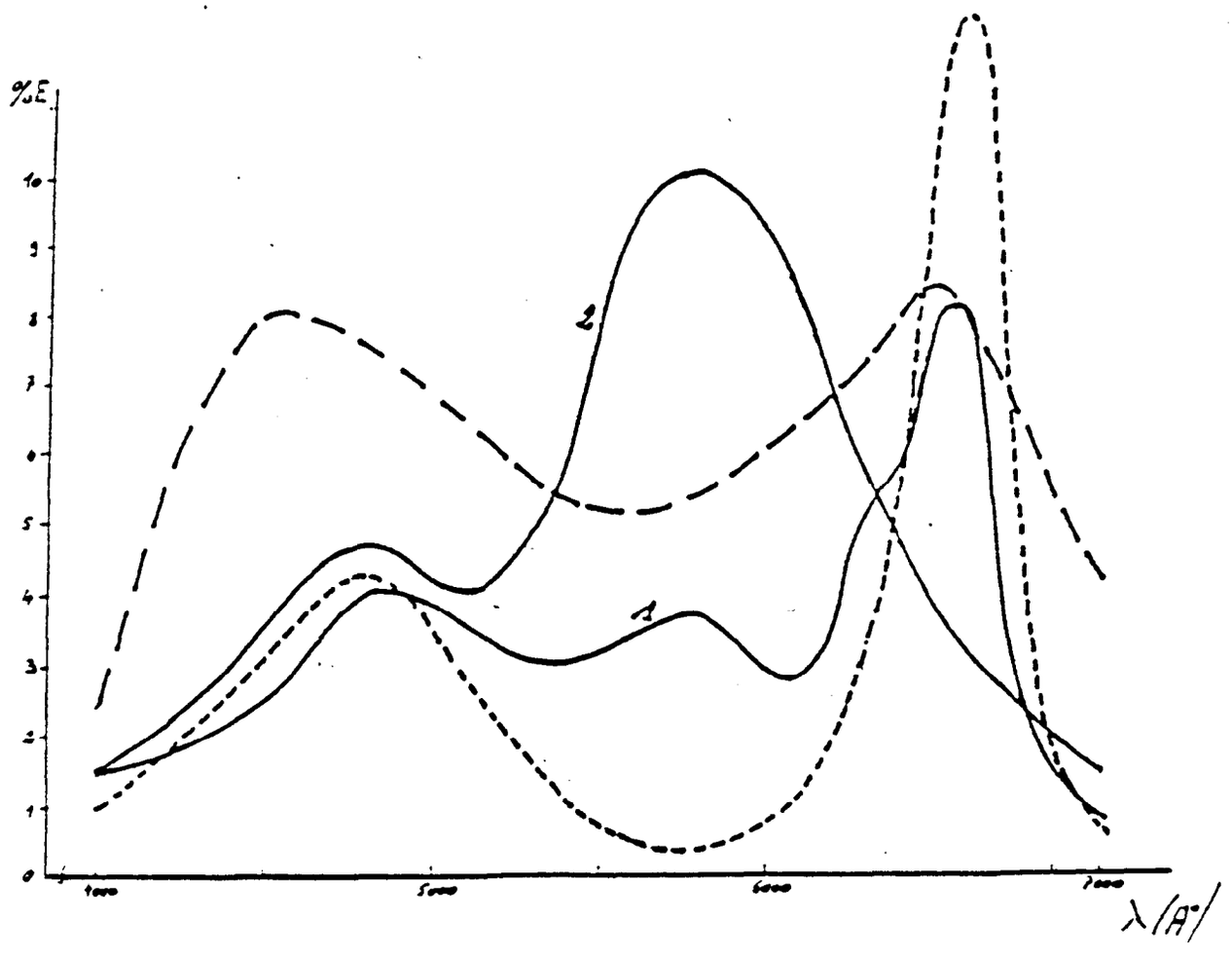
To prepare the medium solutions 1 and 2 are brought together and autoclaved. The other 4 solutions are each individually sterilized. The vitamin B_{12} solution and the Na-bicarbonate solution are sterilized through a membrane filter. This prevents the destruction of the vitamin B_{12} at the high temperatures, 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 to 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 of 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 supplyed $Na_2S.9H_2O$ (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 ($H_2CO_3 \rightarrow H_2O + CO_2$) 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 micro-organisms 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 choosen as light source. These lightenings have an emission area between 400-500 and 600-700 nm (**Figure 1**), which corresponds with the absorption area of the bacteriochlorophyll a and the carotenoids (spirilloxanthin series).
The ideal light intensity for the growth of the bacteria varies between 1000 and 1500 lux. When agar plates are used, these plates are placed in a GASPAK with the light conditions as explained before.

Figure 1 : The emission area of different lightenings.
- - - - - : Hoover
--------- : Sylvania Gro Lux
___(1) : ACEC Phytor
___(2) : Sylvania VHO (ACEC H 300 K)



TN 8.3 : ISOLATION OF PROTEOLYTIC, THERMOPHYLIC STRAINS.

1. Introduction.

The input of biological polymers in the first compartment of the Melissa cycle is high; e.g. the crude protein concentration of faeces is estimated to be 20-30% of the total dry weight. Therefore, in order to have an efficient cycling of N and S (as well as C), an excessive degradation of proteins is necessary. Therefore a screening for proteolytic, thermophylic bacteria was set up.

2. Materials and methods.

Enrichment procedure.

Samples from Dranco (Christiaens and Verstraete, 1990) and from fresh rat faeces were used seperately as potential sources of proteolytic bacteria. 10% w/v was inoculated into the following medium (g/l): gelatine, 6; cellobiose, 2.5; yeast extract 0.5; KH_2PO_4, 1.5; K_2HPO_4, 2.9; cysteïne, 0.075. Gelatine and cellobiose were used as respectively major N and C source. Yeast extract was added as a source of vitamins and other cofactors. Cultures were then incubated anaerobically at 60°C for 5 days. In four subsequent steps, a 1% v/v sample was inoculated into the same medium, however with lower concentrations of yeast extract; respectively : 0.5 ; 0.1; 0.05 and 0.025 g/l.

Isolation.

Colonies were isolated on Wilkinson and Chalgren agarose medium (Oxoïd) (g/l) : trypton, 10; gelatin pepton, 10; yeast extract, 5; dextrose, 1; sodium chloride, 5; L-arginine, 1; sodium pyruvate, 1; menadione, 0.0005; haemin, 0.005; agar, 10.
After several purification steps, single colonies were picked up and isolated.

3. Results

The results of the enrichment procedure are shown in Table 1. Gases produced were H_2 and CO_2. For both sources, Dranco and rat faeces, respectively 4 and 9 strains were isolated on Wilkinson and Chalgren medium. Strains were stored at -70°C untill further characterisation (i.e. : proteolysis, amino acid fermentation and amine production).

Table 1 : Gasproduction and pH of different enrichment cultures after 5 days of incubation at 60°C.

Source	Enrichment step	pH	Gas production (ml)
Dranco	1	4.1	70
	2	4.2	26
	3	5.4	35
	4	-	19
Rat faeces	1	5.8	16
	2	4.5	8
	3	4.8	19
	4	-	20

4. Discussion.

Rat faeces produced less gass than the Dranco inoculum. This is proboably due to the high temperature incubation. Indeed, bacteria present in rat faeces are mesophylic micro-organisms. However, the gas production by rat faeces increased in the third and fourth enrichment step. This is probably due to adaptation to thermophylic conditions.
On the other hand, lowering the concentration of yeast extract caused a decrease in gas production of the Dranco inoculum. This may be an indication that the inoculum used the yeast extract as a sole N-source and not the gelatine. A further characterisation of these strains must give a better estimation of their proteolytic capacity.

5. References.

Christiaens H. and W. Verstraete (1990). Anaerobic elements recycling for artificial closed ecosystems. DARA/CNES workshop on artificial ecological systems, Marseille, p. 139-152.

TN 8.4

Evaluation of MELISSA :

A. The loop

The MELISSA includes 5 compartments : the consumer, the liquefying compartment, the phototrophic compartment, the nitrifying compartment and finally the Spirulina.
Spirulines are given as a food source to the consumer, what allows to close the loop. The loop is not conceived as a completely closed ecosystem :

- it is not closed as far as CO_2 and O_2 are concerned
- the consumers will use other nutrients than those provided by the MELISSA loop
- other waste than faeces and urine will be used in MELISSA : mainly cellulosic material (paper, food waste ...)

Yet, good indications are provided that the nitrogen cyclus in MELISSA could be completely closed or at least on a satisfactory manner.

B. The consumers

A model system has to be used before taking into account a real situation (crew of a shuttle space). The model system will use rats. The waste to be processed would be made of 1) material external to the loop : paper 2) material produced in the loop : rat faeces.
Up to now rats were fed with ordinary food, later they may be fed with spirulines as an attempt to better simulate a real loop.
Paper cellulose is a potential good substrate of thermophilic Clostridia and is representative of potential real waste to be recycled in MELISSA although strong limitations may soon appear (except may be if plants are included in the CELSS). Therefore, the research has to focus on the recycling of "unavoidable" waste : faeces, urine, CO_2.

C. The liquefying compartment

Thermophilic Clostridia as C. thermocellum and C. thermosaccharolyticum are

plausible candidates for use in MELISSA : they can breakdown cellobiose, cellulose and provide the expected substrates for the next compartment. Yet, they apparently use poorly rat faeces what is a major limitation of the system. Therefore, an additional microorganism has to be found : a thermophilic anaerobe with extensive proteolytic capacities (cfr. CCN3).
Some additional research is nevertheless required to more precisely evaluate the fraction of rat faeces which can be metabolized by thermophilic Clostridia. On the other hand, attention has to be given on microorganisms which are the most efficient in sulfur recycling, mainly through sulphide release.

D. The phototrophic compartment

Two types of substrates have to be processed by the phototrophic bacteria :
- gaseous substrates : CO_2, H_2, H_2S
- dissolved or suspended substrates : fatty acids, alcohols, amino acids ...

Rhodobacter capsulatus and Rhodospirillum rubrum were found to be suitable microorganisms to process most of the expected dissolved substrates. They can provide a biomass with a good food value.
On the other hand, they are also able to grow chemolithotrophically at expenses of CO_2 and H_2. However, this probably has to occur separately from the heterotrophic growth at expense of non gaseaous substrates.
A subdivision of the phototrophic compartment seems thus to be required and should not be too difficult technically to realize.
Another major problem would arise in this hypothesis : the processing of H_2S. The intervention of a third microorganism is postulated : Thiocapsa roseopersicina . As R. capsulates and Rh. rubrum is T. roseopersicina able of both chemolithoautotrophic and heterotrophic growth, and looks thus perfectly suitable to process gases and to recycle completely H_2S. T. roseopersicina can also be mixed with R. capsulatus and Rh. rubrum in the heterotrophic subcompartment, at least to recycle the solubilized sulphides.
As far as T. roseopersicina is concerned, additional research is required on food value, potential toxicity or pathogenicity (no data available). A point which should require attention is the possible modification of the sulfur cycle. T. roseopersicina oxidizes sulphides in sulfur granules which

are stored by the bacteria and which are only used as an energy source when other carbon sources are depleted. Release of sulfate is well observed but the mechanism of sulfate formation is still unclear.
The main point is to avoid useless storage of native sulphur in the biomass.
Another major purpose of the second compartment is the release of NH_4^+ : from the metabolic point of view, the photoautotrophic bacteria are fully suitable. The modelling studies will learn us now to manage this compartment to allow enough NH_4^+ to proceed to the next compartment.

E. The nitrifying compartment

This compartment is actually the main bottleneck in the MELISSA study.
Nevertheless, some growth studies were carried out and show that combination Nitrobacter/Nitrosomonas strains are suitable and may provide enough nitrate for the Spirulina compartment.
Up to now, no other bacteria is expected to colonize this compartment. Information has to be gathered about facultative nitrifiers which would be able of heterotrophic growth in some conditions (publications seem to be not yet available).

F. The Spirulina compartment

This compartment is up to now the best studied from the physiological point of view at least in batch.
Further studies on continuous cultures can be considered as well as management of growth conditions to change the composition of Spirulina (polysaccharides) and to increase the food value. No alternative to Spirulina maxima or S. platenis is foreseen at the moment.
Spirulines grown in various batch or MELISSA conditions or in continuous cultures have now to be given as food source to rats.
Other dietetic studies should be foreseen with R. capsulatus, Rh. rubrum and T. roseopersicina biomass, in separated and in mixed cultures.