MELISSA

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Technical note 11

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Study of pure cultures of *Thiocapsa roseopersicina*.

The study about *T. roseopersicina* gives some information about the bacterium's ability to colonize the second compartment (=phototrophic) of MELISSA. The experiments are looking for the growth possibilities of *T. roseopersicina* in function of the MELISSA needs. This means degradation of volatile and non volatile fatty acids, low molecular compounds, amino acids and urine, more specifically the consumption of carbon dioxide, hydrogen and hydrogen sulfide.
The tests are done on synthetic unprocessed media where different concentrations and different kind of energy, carbon and nitrogen sources are experimented. Growth conditions as light, dark, anaerobiosis and aerobiosis are compared when the bacterium is grown on identical media. Next to these physiological tests, the biomass composition of *T. roseopersicina* is analyzed. The reason for this analysis is the possible nutritional value of the produced biomass of *T. roseopersicina*.

RESULTS

1. Physiology of *T. roseopersicina*.

In a first stage, we wanted to check the ability of *T. roseopersicina* to grow efficiently at the expense of the main expected products of the MELISSA first compartment. This liquefying compartment was designed to liquefy various wastes which could be expected in a space ship or in a CELSS system (faeces, cellulose,...). The substrates which focused our attention were : sulfide, hydrogen, carbon dioxide, acetate, lactate, ethanol, butyrate, urea, gelatine and cellulose. For each compound in question a synthetic medium was composed and the consumption of these substrates was followed by taking into account the various growth strategies of *T. roseopersicina* (all of them in anaerobiosis or microaerophily). The different strategies were : photolithoautotrophy (light, sulfide, carbon dioxide), photomixotrophy (light, sulfide, carbon dioxide, acetate or other carbon source), chemomixotrophy (sulfide, carbon dioxide, acetate or other carbon source). There was preliminary evidence for a possible growth under chemolithoautotrophic conditions (sulfide, carbon dioxide). This was in any way quite slow and deserved further confirmation.

1.1. The role of oxygen : aerobic (20 % O_2)/microaerophilic (0-5 % O_2)/anaerobic (0-0.5 % O_2) conditions and light.

T. roseopersicina however an anoxygenic phototroph, was able to grow efficiently in presence of small amounts of oxygen. This is important for the design of space bioreactors or in case of any leak. When we were investigating the growth possibilities of *T. roseopersicina* for the same medium composition, there was more biomass produced in the light than in the dark (**table 1a**). Moreover the best biomass yield was achieved under microaerophilic to anaerobic conditions (**table 1b**).

Table 1a : Comparison of the optical density (=O.D.) of *T.r.* 6311 in the light and the dark under mixotrophic conditions (O.1 % $Na_2S.9H_2O$, 0.15 % HCO_3^-, 0.05 % Ac^- - incubation time : 1 week).

Strain	O.D.reference	O.D.light	O.D.dark
6311	0.03	0.80	0.15

Table 1b : The optical density (=O.D) of *T.r.* 6311 in the light under aerobic, microaerophilic and anaerobic conditions (0.07 % $Na_2S.9H_20$, 0.15 % HCO_3^-, 0.05 % Ac^- - incubation time : 1 week).

Strain	O.D.aerobic	O.D.microaer.	O.D.anaerobic
6311	0.10	0.66	0.66

1.2. The carbon source

A photolithoautotrophic growth condition, which contained enough carbon dioxide (=more than the concentration in the air) or 0.15 % bicarbonate, caused a slight increase of the biomass production after 5 to 6 days against a condition with no sulfide and no addition of carbon dioxide or bicarbonate (**table 2**). We observed no increase of the biomass production when we added higher concentrations of bicarbonate.

Under chemolithoautotrophy for same medium composition as for photolithoautotrophy, there was a very little growth. When 0.03 % sulfide was added daily, a much higher growth density was observed under chemolithoautotrophy. Table 2 shows the results for *T.r.* 9314 after an extra addition of 0.12 % sulfide. In this situation the growth condition became more anaerobic, with about 0-2 % of O_2, due to the reductive character of $Na_2S.9H_2O$. (The evidence of growth still needs to be examined).

Table 2 : The optical density (=O.D.) values of *T.r.* 9314 in photo- and chemolithoautotrophy under microaerophilic conditions (0.1 % sulfide, 0.15 % HCO_3^- - incubation time : 1 week).

Condition	O.D.reference	O.D.	O.D.0.1 % + 0.12 % sulfide
photolithoauto-	0.04	0.25	
chemolithoauto-	0.02	0.08	0.63

When an organic compound was added together with or without carbon dioxide/bicarbonate, the optical density of the culture increased strongly (**table 1 <--> table 2**) in the same way as its growth velocity (μ=0.01/h for autotrophy against μ=0.04/h for mixotrophy, *T.r.* 6311).
The four expected fermentation products in the gaseous and liquid phase of compartment 1 of MELISSA are acetate, lactate, butyrate and ethanol. The results shown in **tabel 3** indicate that acetate, lactate and ethanol were very quickly and completely removed from the medium. The bicarbonate, always present in the effluent, didn't show any influence on the removal of the organic compounds. The lactate reduction seemed to be slower when the strain was grown in an acetate/lactate medium, but we established same optical density values, as for lactate or acetate medium.

Table 3 : Consumption of carbon sources after a certain incubation time by *T. roseopersicina* 6311 in the light and under microaerophilic condition.

Carbon source	concentration (%;w/v)	incubation time	consumption(%;w/v)
acetate/HCO_3^-	0.05/0.15	1 day	95
lactate/HCO_3^-	0.05/0.15	1 day	95
acetate/lactate/ HCO_3^-	0.025/0.025/0.15	1 day	91
lactate	1	3 weeks	84
lactate	1.5	3 weeks	87
lactate	0.1 --> 0.5	3 weeks	90-100
lactate/HCO_3^-	1.5	3 weeks	85
lactate/HCO_3^-	0.1 --> 1	3 weeks	90-100
acetate/HCO_3^-	0.1 --> 0.5	3 weeks	90-100
acetate/HCO_3^-	1 --> 1.5	3 weeks	60-70
ethanol/HCO_3^-	0.01 --> 1.5	4 weeks	90-100

After 1 day incubation, 95 % of the organic compounds (0.05 %) were reduced without evolution of biomass. *T. roseopersicina* probably first assimilated the organic compounds and accumulated them as storage materials, such as fat (poly β hydroxybutyric acid), glycogen and polyphosphate granules. These materials may be viewed as granules by adapted colorations and with a microscope (X1000).
In the case of butyrate only a slight growth was observed from 0.001 % to 0.05 % (O.D. values for *T.r.* 1711 from 0.10 to 0.25), with concentrations higher than 0.5 % no growth was observed.

Next to the fermentation products the effluent may contain complex organic compounds (gelatin, cellulose), which could not be fully degraded in the first compartment. *T. roseopersicina* was able to hydrolyze gelatin, but couldn't use it as carbon source (tested in different concentrations). *T. roseopersicina* possessed the β-glucosidase. Each strain degraded glucose to acetate and lactate and these acids are assimilated by the bacteria. Concentrations of glucose higher than 0.5 % (w/v) killed the bacteria after 9 to 10 days growth. This was due to the accumulation of the acids in the medium (**table 4**), which decreased the pH to 4.

Table 4 : The quantity of lactate and acetate formation after degradation of glucose by *T. roseopersicina*. in the light under microaerophilic conditions (incubation time : 1 week).

concentration of glucose (%;w/v)	lactate (%;w/v)	acetate (%;w/v)
1.5	0.06	3.98
1	0.06	0.91
0.5	0.06	0.59
0.1	0.0001	0
0.05	0	0
0.01	0	0

Other compounds as mannose, mannitol, arabinose, N-acetylglucosamine, maltose and gluconaat were assimilated by the different strains.

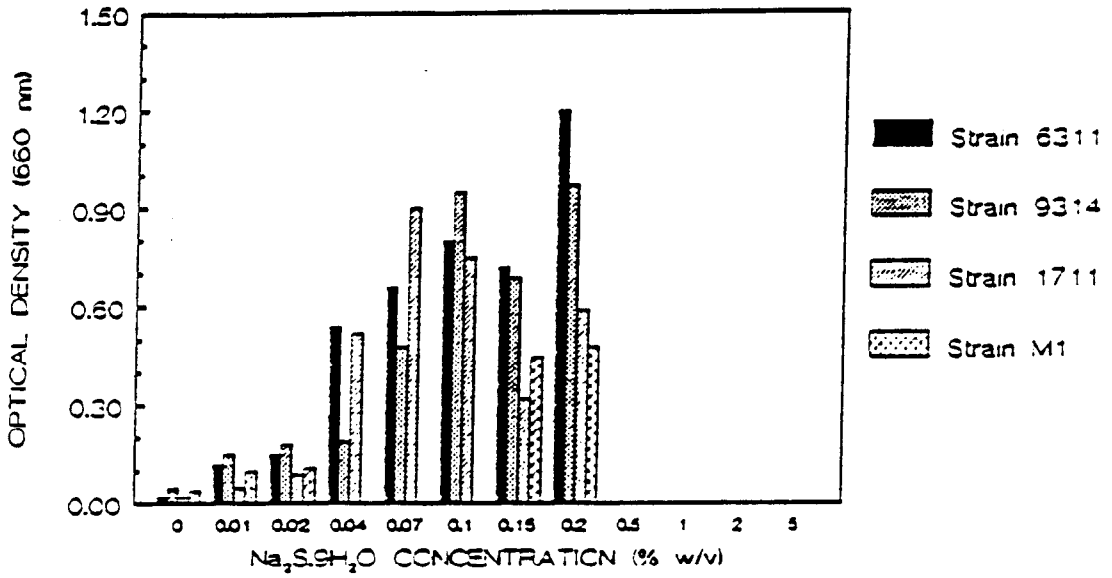
Generally the ideal concentration of the fermentation products, succinate and pyruvate (two compounds closely related to the Krebs cycle) for optimal growth was situated between 0.1 and 0.5 %. The growth started at a concentration of 0.05 % organic compound and remained active until 1 to 1.5 % was added.

In chemomixotrophic conditions the organic compounds increased also the biomass production, but for the same medium composition, the final yield was lower than in photomixotrophic conditions. A regular addition of sulfides, as energy source, enhanced the yield.

1.3. The energy source.

- First we observed the situations where light was used as energy source and sulfide was used as electron donor. At higher sulfide concentrations, until 0.2 %, *T. roseopersicina* showed increasing growth densities. At 0.5 % sulfide no growth was observed anymore (**fig. 1**).

Figure 1 : The growth of *T. roseopersicina* with different concentrations of $Na_2S.9H_2O$ in the light under microaerophilic conditions (0.15 % HCO_3^-, 0.05 % acetate).



When we started at low sulfide concentration and increased the sulfide concentration during the growth of the culture to a certain final value we observed higher optical densities (**table 5**) compared to a situation where we added immediately the high sulfide concentration. If the addition of sulfide took place in the beginning of the exponential growth phase, even higher biomass production and growth velocity were obtained. The carbon source is also consumed faster.

This made us believe that *T. roseopersicina* is suitable for a continuous system, such as a plug stream reactor.

Table 5 : The effect of the added sulfide concentration at the beginning and the final total added concentration on the growth of *T.r.* 6311 (0.15 % HCO_3^-, 0.05 % CH_3COO^-).

Starting concentration of $Na_2S.9H_2O$ (%;w/v)	total concentration of $Na_2S.9H_2O$ added (%;w/v)	optical density
0.04	0.07	0.90
0.07	0.07	0.66

In autotrophic situation different initial concentrations of sulfide had only little influence on the growth intensity.

Table 6 : Consumption of sulfide by *T. roseopersicina* 6311 in microaerophilic, photomixo- and photoautotrophic conditions.

Condition	compound	concentration (%;w/v)	incubation time	consumption (%;w/v)
photomixo-	sulfide	0.1	2 weeks	85-90
photoauto-	sulfide	0.1	2 weeks	60-70

Table 6 shows the consumption of sulfide in the medium. Sulfide is oxidized to sulfate, which we measured. When we recalculated the measured sulfate concentration to the theoretical concentration of sulfide, which should have disappeared from the medium, we determined that after 2 weeks *T.roseopersicina* 9314 should have assimilated 79 % of sulfide and *T.roseopersicina* M1 91 % (photomixotrophic-microaerophilic condition). These theoretical calculated values fit with the measured consumption of sulfide in the medium, given in **table 6**.
- Secondly, a chemomixotrophic condition was observed where the sulfides were also oxidized to sulfates in microaerophilic to anaerobic conditions. Because in the dark the need for sulfide is higher than in the light for *T. roseopersicina*, it seemed that sulfide ensures the energy formation.

Looking for other energy or electron sources we found that thiosulfate may replace sulfide in the light as well as in the dark (ref. 10). Hydrogen and succinate could be used by *T. roseopersicina* as electron donor in light, not in the dark and this for an incubation time of 3 weeks.

1.4. The nitrogen source.
Urea is a compound which will still be present in the effluents of compartment 1 of MELISSA. Urea is a good nitrogen source, but too high concentrations

such as 0.034 % (w/v) have a negative effect on the growth of *T. roseopersicina*. A concentration of 0.017 % (w/v) was optimal. A mixed nitrogen source, which will be present in the medium had a positive effect on the growth.

1.5. The pH-influence.

The bacteria were resistent to pH fluctuations varying from 6 to 9. At extreme pH's, a reduction of 30 % (w/v) in the optical density values was noted. During the growth the pH of the medium increased and stabilized between 8 and 9. The production of sulfate was not accompanied by a pH decrease.

2. Nutritional Value of *T. roseopersicina*

In the MELISSA-model, the bacterium will get continuously new organic and sulfide compounds. This leads to an increase of the total biomass. If this biomass has any nutritional value, it could be valorized as single cell proteins. Otherwise we will have to use the condition (=chemomixotrophic) which gives less increase in biomass. Some waste water treatment industries try also to valorize their active sludge to a nutritional biomass. Otherwise this active sludge is a new generated waste. In batch process the bacterium produced 1 to 2 g/l dry cell weight. The dry weight contained 35 to 45 % (w/v) proteins, dependent from the strain. The nutritional value of these proteins is function of the amount of essential amino acids, which is given in **table 7**.

Table 7 : The amino acid composition of *T. roseopersicina* whole cell protein and of active sludge (% ; w/w ; g/g proteins).

Essential amino acids	*T.r.* 9314	*T.r.* 6311	*T.r.* 1711	*T.r.* M1	Sludge	FAO
tryptophane	1.97	1.54	1.69	2.14	4.50	
histidine	2.33	1.74	1.13	2.27	1.25	1.9
arginine	5.66	4.29	4.71	5.68	-	
threonine	7.34	4.91	4.92	6.65	6.59	4.0
valine	7.23	5.16	4.65	7.14	4.82	5.0
methionine	3.17	2.37	2.28	2.70	1.73	3.5
isoleucine	5.12	3.63	3.32	4.96	2.71	4.0
leucine	11.02	7.79	6.78	10.74	-	7.0
phenylalanine	7.11	4.65	3.69	6.24	0.94	6.0
lysine	3.97	2.93	2.46	3.95	0.47	5.5

- : undetectable

If we compare these results with the one obtained for active sludge of a brewery, we see that the dry weight of sludge contains only approximately 26 % of proteins and has a poor amino acid composition. Only strains 9314 and M1 correspond to the norm of the Food Agriculture Organisation (=FAO). In comparison to the amino acid composition of other single cell organisms (Chlorella) and *Rhodobacter*, we found higher amounts of leucine, phenylalanine and threonine for *T. roseopersicina*. For the other essential

amino acids their amounts were the same. Regarding to meat proteins, the amounts of essential amino acids is higher for *T. roseopersicina*.
These results show that the biomass production of *T. roseopersicina* doesn't seem to be an additional waste production.

DISCUSSION

The results obtained on basis of synthetic media, show that *T. roseopersicina* has a real potential to be used in the MELISSA project. *T. roseopersicina* is a phototrophic bacterium which is also able to grow in the dark. Anaerobiosis provides the best growth conditions, but low concentrations of oxygen (0-5%) don't have any influence on the growth of *T. roseopersicina*. Consumption of sulfide, hydrogen, carbon dioxide, urea, acetate, ethanol and lactate which are expected to be the main fermentation products arising in the first MELISSA compartment, shows the potential of *T. roseopersicina* as a colonizer of the second compartment. Butyrate has probably a toxic effect at concentrations higher than 0.5 % (w/v). But removal of the butyrate can be realized by *Rhodobacter* and/or *Rhodospirillum* which are also candidates for the second compartment with *T. roseopersicina*.
Moreover, the biomass of *T. roseopersicina*, produced during its growth has based on an amino acid analysis, a nutritional value comparable with the FAO norm. Its total protein content is lower than the protein content of photosynthetic bacteria(+/- 40 % against 61 %) but the amounts of amino acids given as percent of protein are for both bacteria the same. Further nutritional research about the fat, fibre and mineral composition is necessary to check the absence of any toxic components. This would give us the possibility of using the biomass of *T. roseopersicina* as single cell proteins.
The current work consists of experiments with processed effluents from the MELISSA liquefying compartment, derived from rat faeces and paper (cellulose) as primary waste source. An other experiment in progress includes the introduction of genetic markers in order to easily check the axenical character of the compartment. The chemolithoautotrophic growth condition is also a further study object.

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1. : paper in conference proceedings
2.+3.+4.+5.+6.+7.+8.+10 : journal papers
9. : book

Compatibility of T. roseopersicina with other phototrophs.

Before testing the compatibility with other phototrophs, we are looking for the growth on processed effluents from compartment 1. From tests done at the laboratory of Prof. W. Verstraete it is already known that *R. capsulatus* and *Rh. rubrum* are able to grow on supernatant from *Clostridium*. The growth density obtained is higher but the efficiency of biomass production is much lower than on synthetic media.
The experiments on processed supernatant will show us the behaviour of the different bacteria. This behaviour is different of the one they would have on their respectively unprocessed media. In unprocessed media the bacteria are not submitted to any stress (= toxic organic compounds, to high sulfide concentrations, ...). It is the adaptation of the bacteria to this stress condition, which will define if they can co-habitate.

UNPROCESSED MEDIA.

The effluents are processed by growing *Clostridium thermocellum* or *Cl. thermosaccharolyticum* or bacteria isolated from household refuse or from rat faeces on a synthetic medium. The composition of this synthetic medium (GS3) is given in **table 1**. The media were incubated during 5 days at 60 °C.

Table 1 : The composition of the synthetic medium GS3 (*l*) (pH=7).

KH_2PO_4	1.5 g
K_2HPO_4	2.9 g
urea	2.1 g
$MgCl_2.6H_2O$	1.0 g
$CaCl_2.2H_2O$	150 mg
$FeSO_4.6H_2O$	1.25 mg
cellobiose	5.0 g
yeastextract	6.0 g
cysteine hydrochloride	25 mg
resazurine	2 mg

When the effluents of the synthetic media, which were colonized by the different cultures mentioned before, are inoculated with *T. roseopersicina* no growth is seen. After adaptation of the pH from 6 to 7 and addition of vitamin B_{12}, 0.1 % sulfide to the media and inoculation of the media, there was still no growth. The possible explanation is the production of butyrate (0.09 %) in the effluent processed by *Cl. thermosaccharolyticum* and isovaleriate in the effluent processed by *Cl. thermocellum*. From experiments done previously

we know that butyrate can't be consumed by *T. roseopersicina*. This result was obtained in the condition where next to butyrate there was no other carbon source except carbon dioxide/bicarbonate.

Further investigations on the effluents of compartment 1 are necessary. For instance some tests are in progress with different concentrations of the effluent. An important aspect for these compatibility tests is the fact that the produced gaseous phase is lost after centrifugating the effluent from the bacteria. This means that also the produced hydrogen sulfide, which is necessary for the growth of *T. roseopersicina*, is lost. It is important to construct a standardized method for collecting the effluents of compartment 1.

GROWTH TOGETHER WITH *RHODOBACTER CAPSULATUS* AND *RHODOSPIRILLUM RUBRUM*.

This topic will be first analyzed theoretically. We are investigating the addition of *T. roseopersicina* with the other phototrophic bacteria on the liquid phase or on the gaseous phase of the first compartment. From a previous topic it is already known that at first instance *T. roseopersicina* will be overwhelmed by the other phototrophs when the different cultures are inoculated together on the liquid phase of the first compartment. In this liquid phase the conditions are more heterotrophic, which means a lot of organic compounds. *R. capsulatus* and *Rh. rubrum* can use the organic compounds without any problem as electron donor. This heterotrophic condition is for *T. roseopersicina* not as optimal as for the other phototrophics. The growth velocity of the other phototrophics will be much higher and *T. roseopersicina* can't compete.
It is possible that during the first period, where the organic concentrations are high, only *R. capsulatus* and *Rh. rubrum* will efficiently consume the degradation compounds. But in a second period when the different conpounds (N- and C-sources) are limited, *T. roseopersicina* will activate. *T. roseopersicina* is a bacterium which is very resistant to different conditions and is able to survive in these difficult situations and will come to expression and growth when the conditions are proportionally much worser for the other phototrophics. At that moment *T. roseopersicina* will eliminate the rests of organic compounds and the solubilized hydrogen sulfide.

When *T. roseopersicina* is grown together with the other phototrophs on the gaseous phase of the first compartment, a co-culture seems to be more realistic. The major compounds in the gaseous phase are hydrogen, hydrogen sulfide, carbon dioxide and some low concentrations of low molecular volatile compounds.

The possibility of co-culture will be mainly dependent of the hydrogen sulfide formation in the first compartment.
These are some theoretical considerations which will need some experimental confirmation.

TN 11.3

Theoretical study of *Thiobacillus*.

Thiobacillus is a colorless sulfur bacterium, which can oxidize reduced inorganic sulfur compounds and utilize these compounds as sole energy source (= chemolithotrophs). Such reduced sulfur compounds are hydrogen sulfide, sulfides, polysulfide, elemetal sulfur, thiosulfate, (poly)thionates and sulfite. The two major metabolic products formed are sulfur and sulfate. During the oxidation of the sulfur compounds oxygen or nitrate is used as terminal electron acceptor and there is no formation of intracellular sulfur.

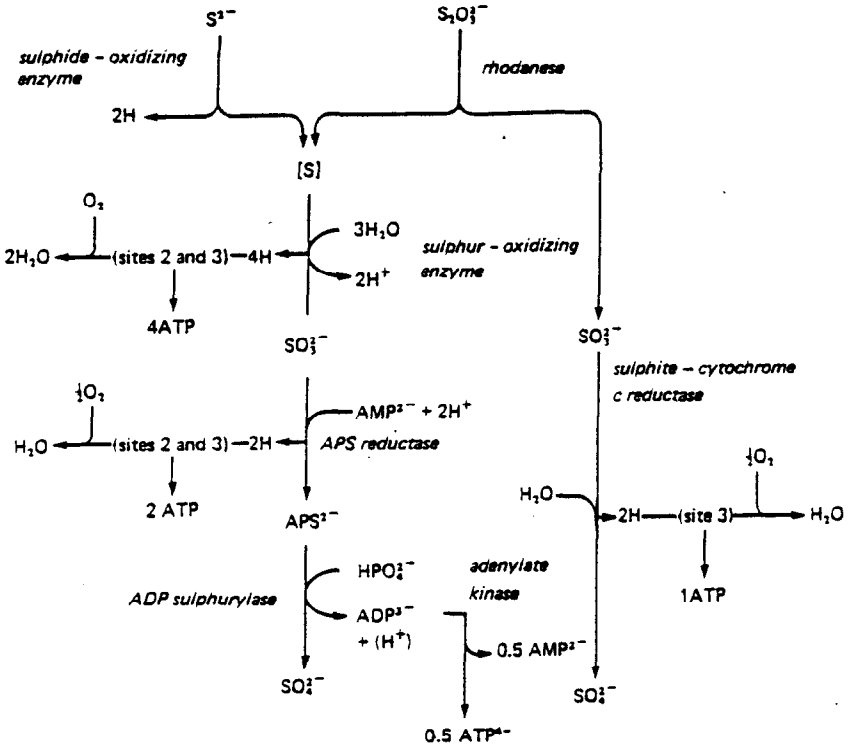
When looking to the *Thiobacillus* genus, there are only four species which are able to grow strictly chemolithoautotrophically with sulfides. Three of these bacteria (*T. thioparus*, *T. niapolitanus* and *T. tepidarus*) are strict aerobic and could only be inoculated in the third compartment. This is the only dark aerobic compartment with only carbon dioxide as carbon source. The problem is that these bacteria are also consuming oxygen, which is a negative aspect.
T. tepidarius is a bacterium, which will need a higher temperature to grow. Its optimal temperature is 43 °C against 28-30 °C for the other one's. This includes the conception of a new compartment if we want to use this bacterium.
One of the four species is facultative anaerobic (*T. denitrificans*) and will use nitrates as electron acceptor. A negative aspect is the fact that nitrates, which are necessary for the *Spirulina* growth, will be reduced to N_2 and a new compartment will be necessary with only an input of CO_2, H_2S, nitrates and some vitamins because of the strict chemolithoautotrophic growth of *T. denitrificans*.
The other species which are facultatively chemolithotrophic or mixotrophic are not able to oxidize sulfides or to grow in anaerobiosis.

Generally one negative aspect is present : the consumption of oxygen or the consumption of nitrates.

Sulfur cycle.

Thiocapsa and *Thiobacillus* use probably the same oxidation system when grown in the dark (**Figure 1**).

Figure 1 : The oxidation of thiosulfate to sulfate (after Kelly, 1978) by various species of *Thiobacillus*.



Next to this oxidative respiration system *Thiocapsa* possesses a photosynthetic system which uses sulfides as electron donors and light. This is an advantage against *Thiobacillus*, where sulfides are the energy source in all circumstances and the quantity of these sulfides is limited. When light is the energy source there are no restrictions about the quantity of disposable energy.

The external storage of sulfur granules and the pH decrease mainly observed in aerobic conditions, has a negative influence on the other strains of the third compartment, which don't happen when *Thiocapsa* is used. *Thiobacillus* also hasn't a survival capacity as large as *Thiocapsa* to hibernate in situations where there are no inorganic compounds.

As conclusion it can be said that *Thiocapsa* will better fulfil the requested goals, without making MELISSA more complexer.

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