MELISSA

TN 3

TN 3: PROPOSALS FOR EXPERIMENTATION

1. The thermophilic compartment: proposal of testing.

2. The thermophilic compartment: results of the analysis of a MELISSA substrate (rat faeces)

3. The Spirulina compartment: experimental approaches.

TN 3: PROPOSALS FOR EXPERIMENTATION

1. The thermophilic compartment: proposal of testing.

In this first phase, emphasis will be laid on the study of the thermophilic liquefaction of waste, on the potential toxicity related to this process and on the optimization of the Spirulina compartment.

1. Thermophilic liquefaction of waste.

Rat faeces are proposed as a typical substrate for the liquefaction compartment. They can be obtained on a regular basis from a laboratory animal farm (SCK/CEN), where rats get a diet with a controlled and stable composition. Rats are known to be omnivorous whereas most other laboratory animals are almost exclusively herbivorous (rabbits, mice, monkeys, ...). The diet of rats is more similar to that of the crew. The faeces can be collected without litter and sterilized in different ways (wet and dry sterilization, γ-irradiation).

During this initial phase, wet sterilized rat faeces will be used, without considering the possibility of an adapted feed composition (e.g. a Spirulina containing diet), the age or the sex of the animals.

Two thermophilic clostridia (Clostridium thermocellum, Clostridium thermosaccharolyticum) are proposed for the liquefaction of the rat faeces in batch experiments under axenic conditions.

Three different substrate treatments are proposed :

- sterilized rat faeces in suspension (10 g DW/l)
- sterilized and sonicated rat faeces in suspension (10 g DW/l)
- sterilized rat faeces in suspension (10 g DW/l) and cellulose (1 g/l).

The liquefaction of these substrates is studied in combination with each of the strains seperately and combined. Also a blank is added, consisting of the rat faeces suspension without inoculum.
The tests will be performed in 50 ml anaerobic flasks, connected to a gas collector, which are placed in an incubator at 55 - 60 °C during a period of 15 days.

Some of the parameters which will be monitored, are :

- the gas production and composition (H2, CO2, CH4); methane is an unwanted product, but can be used as an indicator for non-axenic conditions
- CODt, CODs, CODgas phase
- kjeldahl-N, NH4+-N

The outcome of these tests will give an idea about the conversion efficiency, the conversion rate and the suitability of the strains proposed.

2. The potential toxicity.

The liquefaction compartment is by far the most critical as far as potential toxicity is concerned. Therefore, toxicity assays will be conducted, especially with regard to the growth of Spirulina and the consumers. For the latter, laboratory animals could be used as a model system.
The toxicity can be assayed in various ways.

Firstly, the toxicity of the colonizing strains themselves (Clostridium thermocellum, Cl. thermosaccharolyticum, and possible additional strains being Cl. thermoaceticum, Cl. thermosaccharolyticum var. sartarivorum) can be studied on sterilized extracts, on living cells (but not growing) and on spores.

Secondly, the potential toxicity of the growth medium should also be verified. This can be done on sterilized and non-sterilized extracts of cultures in standard media or in MELISSA conditions (waste).

Within the framework of MELISSA 89, we suggest to limit the toxicity tests to the level of the cells, meaning that only the potential toxicity of living cells, sterilized cells and spores on Spirulina cultures will be investigated.

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2. The thermophilic compartment: results of the analysis of a MELISSA substrate (rat faeces)

1. D.W. (Dry Weight)

Method: The substrate has been dried at 105°C untill constant weight. In practice, we see that constant weight is reached when dried during one night.

Result: Average of 3 measurements: 72.6 ± 0.7%

..ny other results will always be expressed in D.W.. Therefore, everytime we use a new sending of substrate, the D.W. must be measured again.

2. C.O.D. (Chemical Oxygen Demand)

Method: The organic matter is oxydized by an excess of dichromate. Argentsulfate serves as catalyser. The amount of dichromate that is used is a measure for the amount of initial oxydizable organic matter. The interferention of chloride is suppressed by mercurysulfate and the interferention of nitrate by sulfamic acid.

Result: Average of 2 measurements: 748 mg C.O.D./g D.W.

We can say that 74.8% of the D.W. are organic matters. This means also that the other 25.2% of the D.W. are ashes.

3. B.O.D. (Biological Oxygen Demand)

Method: To determine the BOD the amount of dissolved oxygen is measured at the beginning and after an incubation at 20 °C during 5 days (BOD5). The inoculate consists of variated bacteria. A nitrificationinhibitor is added so that the no oxygen is used for nitrification. While using the Sapromatapparatus the oxygen disponibility is kept at a constant level. The latter is done by electrolytic generating of oxygen. The CO2 that is liberated is adsorbed.

Result: 243 mg O2 / g D.S.

The biodegradable organic matter (bCOD=BOD∞) is said to be 1.5 times the BOD5. So 36% of the dry matter exists of biodegradable organic matter.

The biodegradable fraction is found by the equation:

$$Bf = \frac{bCOD}{COD} = 50\%$$

This method is using a mixed biota. How much of the organic fraction can be used by the Clostridia is not known yet. Clostridia can not be inoculated here because they grow strictly anaerobic. Anaerobic and aerobic metabolisms are biochemical very different but it seems that for most of the compounds the biological degradation is analogous. This is not the case for aromatic compounds.

4. Nitrogen

4a. Kjeldahl-N

Method: The nitrogen of the sample is reduced in strong acid environment and $(NH_4)_2SO_4$ is formed. NH_3 is then destillated in strong alkaline environment and titrated with H_2SO_4.

This method determines the total amount of both organic and ammonical nitrogen.

Result: Average of 2 determinations: 77.8 mg N/g D.W.

4b. Ammoniacal nitrogen

Method: NH_3 is destillated in slight alkalic environment and titrated with H_2SO_4.

Result: Average of 2 determinations: 21.9 mg NH_4-N/g D.W.

From the difference between de Kjeldahl-N and the ammoniacal N the organic N is known: 55.9 mg org-N/g D.W.

Rude estimation of the protein: 6.25 X 56 mg = 350 mg/g D.W. This value almost equals the value of the total biodegradable organic matter, i.e. not all the N-compounds are biodegradable.

5. Volatile fatty acids

Method: The fatty acids are extrahated with diethylether and injected in a gas chromatograph. The concentrations are known by using a internal standard.

Result: Averages of 2 determinations:
Acetic acid: 7.0 mg/g D.W.
Propionic acid: 0.5 mg/g D.W.
Isobutyric + butyric acid: 0.7 mg/g D.W.
Isovaleric + valeric acid: 0.2 mg/g D.W.

6. pH

The pH is measured with a glasselectrode.

Result: pH=5.5

The Clostridia can grow at this pH. Therefore it is unnecessary to buffer the substrate.

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3. The Spirulina compartment: experimental approaches.

Concerning the *Spirulina* photosynthetic compartment, the experimental approaches consisted in the choice of a suitable strain and in the determination of culture conditions and growth parameters in view of the mathematical modelisation of the compartment.

I - AXENISATION OF *SPIRULINA* STRAIN

None of the suitable *Spirulina* strains were devoided of contamination. We succeeded, in collaboration with the Institut Pasteur, in axening a strain of *Spirulina platensis* n° 8005. Axenization of other strains (*Spirulina platensis* and *S. maxima*) received from Göttingen (Germany) is in progress.

II - CULTURE CONDITIONS

Axenic or non axenic strains of *S. platensis* were grown in batch cultures in the medium described by AIBA (1) modified as follows :

$NaHCO_3$: 10.8 g/l
Na_2CO_3 : 7.6 g/l

in order to directly obtain a pH of 9.5. The temperature was usually regulated at 35°C, and cultures were performed either in Roux flasks or in a bioreactor.

Roux flasks offer the advantage of simple culture conditions although not very well controled : pH was maintained roughly constant by bubbling 0.5 % CO2 in air ; the temperature was that of the thermostated culture room (35°C) and light was provided by daylight fluorescent tubes (usually 10 $w.m^{-2}$). Such cultures are suitable for routine purpose and for determination of light effects on growth since the flat Roux flasks present mainly a one dimensional geometry.

More controled cultures were performed in a Applikon bioreactor (7 liters, 5 liters work volume). Aggregation of the algae was avoided by a minimum 300 rpm agitation. Light was provided by two 500 W halogen lamps (Philips type R.7.S) placed at appropriate distance of the bioreactor in order to obtain the desired light intensity. Temperature was maintained constant by regulating a hot and cold circuit. pH was maintained at 9.5 by acid-base addition, which allows fine regulation and which eventually allows to estimate the amount of CO_2 fixed by the algae by titrating the carbonate - bicarbonate remaining in the medium.

III - DETERMINATION OF GROWTH PARAMETERS

For a fixed pH and temperature and under constant and equal light intensity at the level of each microorganisms present in the culture (theoretical condition which can be approached in very diluted cultures), growth of *Spirulina* is exponential and is defined by a specific growth rate μmax for these conditions. μmax therefore depends on pH, temperature and light intensity.

3.1- Estimation of growth rate

Growth rate was estimated from biomass production. The relation between biomass concentration in the culture and the absorbance at 750 nm which only result from light scattering by the microalgae has been established in order to allow measurements of biomass concentration by the simple spectrophotometric method.

3.2- pH effects

Values given by Zarrouk (2), and which are reported on figure 1, show that pH does not exert important effect between 8.5 and 10. We therefore grew *Spirulina* at pH 9.5 since this value appears to be optimal for photosynthesis.

3.3- Temperature effects

The effect of temperature on μmax has been calculated from values provided by Zarrouk (2) and from our own measurements. Results are plotted on figure 2. It appears from this curve that the optimal temperature is 36°C.

In addition, and from a theoretical point of view, this curve can be described with the following formula :

$$\mu_{max} = \frac{A.C_E . \exp\left(-\Delta h_E^{*} / RT\right)}{1 + \exp\left(\Delta S_d^{\circ}/R\right) \exp\left(-\Delta h_d^{\circ} / RT\right)}$$

where

A = constant in $m^3 . kg^{-1} . h^{-1}$

CE : concentration of a theoretical catalytic factor in $kg . m^{-3}$.

It then becomes possible, from the experimental curve (Fig. 1) to calculate several parameters which characterize the energetics of the studied organism (3). For *Spirulina Platensis*:

Δh^{*}_E or activation enthalpy = 100 kg/mole

$\Delta S°d$ or standard denaturation entropy = 0.57 kg/mole

$\Delta h°d$ or standard desactivation enthalpy = 175 kg/mole

$A.CE = 3.9 . 10^{15}\ h^{-1}$

3.4- Light effects

Light intensity

When optimal pH (9.5) and temperature (36°C) conditions are satisfied, the specific growth rate μmax increases with light intensity to a Mmax growth rate which is obtained at a plateau corresponding to light saturation. However, increasing light intensity also exerts an inhibitory effect on μmax thus transforming the plateau in a decreasing curve. μmax is therefore obtained for an optimal light intensity of 30 k lux for *Spirulina* (see Fig. 3), as determined from data of Zarrouk (1) and from our own measurement. In this case, μmax ~ 0.7 h^{-1}, corresponding to a doubling time of 10h.

The evolution of μmax as a function of light intensity can be expressed by the following formula

$$\mu max = Mmax \frac{F}{K_F + F + k F^2}$$

where

F is the luminous flux in $w.m^{-2}$.
K_F Monod constant, which expresses the effects of light limitation on μ_{max}
kF^2 expresses a light inhibition on μ_{max}

Remark : The exponential specific growth rate μ_{max} as well as the maximum growth rate Mmax are mostly theoretical constants, as a result of self shadowing by cells in the culture which does not allow to maintain a fixed light intensity at the level of the microorganisms in the culture. In fact, as the opacity of the culture increases during growth, the apparent growth rate decreases so that the exponential biomass production rapidly becomes linear.

Wavelength and Photoperiods

Effects of wavelength and of time distribution of light on growth rate and on yields of biomass production are under investigation.

3.5- Nitrates concentration effects

Limitation by nitrates of *Spirulina* growth is under study.

Qualitatively, it appeared that nitrates exhaustion in the medium results in a reversible lost of pigments.

Quantitatively, the analysis of the kinetics of biomass production in nitrate limiting conditions allow to estimate by calculation a very preliminary value of $1.5\ 10^{-2}$ $kg.m^{-3}$ for the Monod constant K_{NO3} which expresses the effect of nitrate limitation on μ_{max}. The validity of this value must be confirmed by parallel measurements of the kinetics of nitrate exhaustion in the culture medium.

3.6- Ionic strength effects

Culture of Spirulina under reduced ionic strength is analyzed in order:

- -to determine its effect on maintenance,
- -to determine the adaptability of Spirulina to different salt concentrations susceptible to be encountered in the MELISSA system.

3.7- Polysaccharides

Spirulina excretes polysaccharides in the medium. Qualitative and quantitative analyses have been undertaken in order to estimate the magnitude of the effects of these polysaccharides on the gas-liquid tansfers and on the yields of biomass production.

3.8- Measured biomass production rates

Cultures were performed in Roux flasks or in a bioreactor under standart conditions:

-modified Aiba medium
-pH = 9.5 and t = 36°C

*inRoux flasks:
-agitation and aeration by 0.5% CO2/air bubbles
*in the bioreactor:
-agitation at 300 rpm and aeration by 30 $l.h^{-1}$ air

	Roux flasks		Bioreactor	
Light intensity w.m-2	4.7	8.3	14	36
Biomass production rate: kg.m-3.h^{-1}	3.10^{-3}	$6.65.10^{-3}$	$2.57.10^{-3}$	$5.6.10^{-3}$

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(2)- ZARROUK, C. (1966) Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de Spirulina maxima (Setch et Gardner) Geitler. Thèse Université de Paris.

(3)- ROELS, J.A. (1983) Energetics and kinetics in biotechnology. Elsevier Biomedical Press.

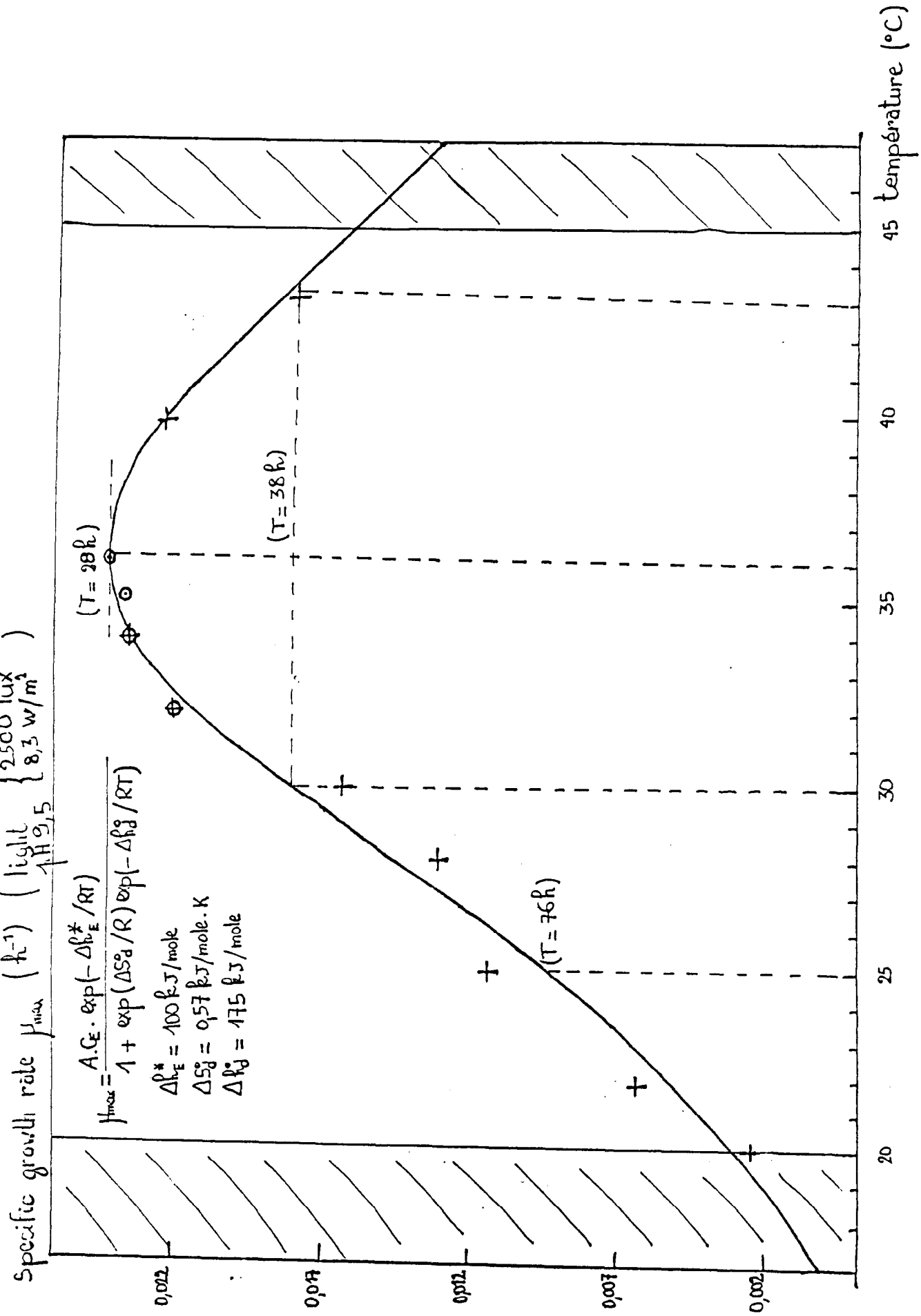
Specific growth rate μ_{max} (h^{-1}) (light 1H 9,5 { 2500 lux / 8,3 w/m²)
$\mu_{max} = \frac{A.C_E \cdot \exp(-\Delta h_E^*/RT)}{1 + \exp(\Delta S_d^\circ/R)\exp(-\Delta h_d^\circ/RT)}$
$\Delta h_E^* = 100$ kJ/mole
$\Delta S_d^\circ = 0{,}57$ kJ/mole.K
$\Delta h_d^\circ = 175$ kJ/mole
(T = 28°)
(T = 38°)
(T = 76°)
0,012
0,009
0,007
0,002
20
25
30
35
40
45 température (°C)

FIG. 2

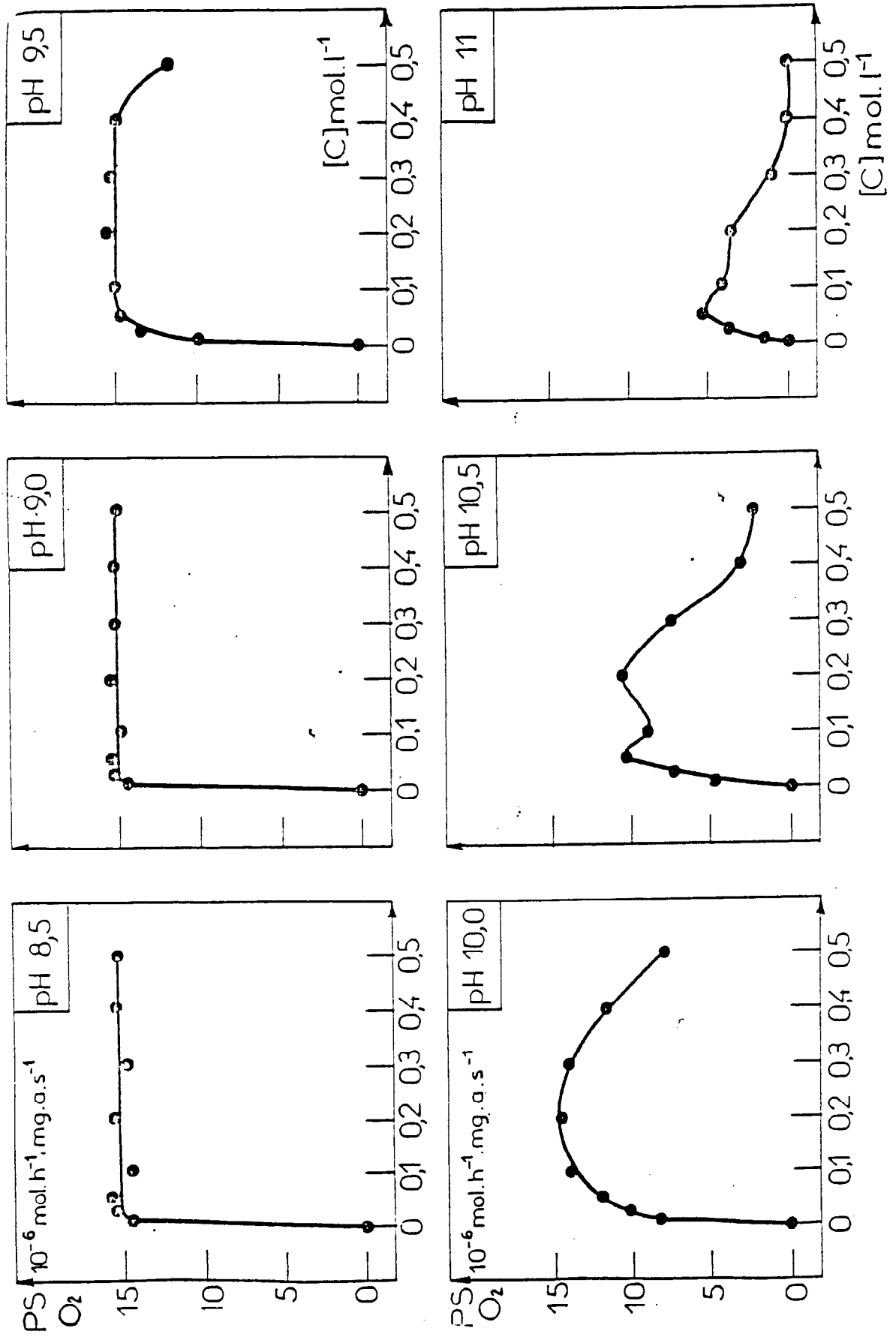

Fig.1 - Influence de la concentration de la source carbonée sur la vitesse de photosynthèse de Spirulina à différentes valeurs du pH.

Eclairement maximal 60 Klux - T = 40°C - Essais de 60 mn

SPECIFIC GROWTH RATE μ_{max} (h^{-1}) VS. LIGHT INTENSITY

($pH = 9,5$ - $T = 35°C$)

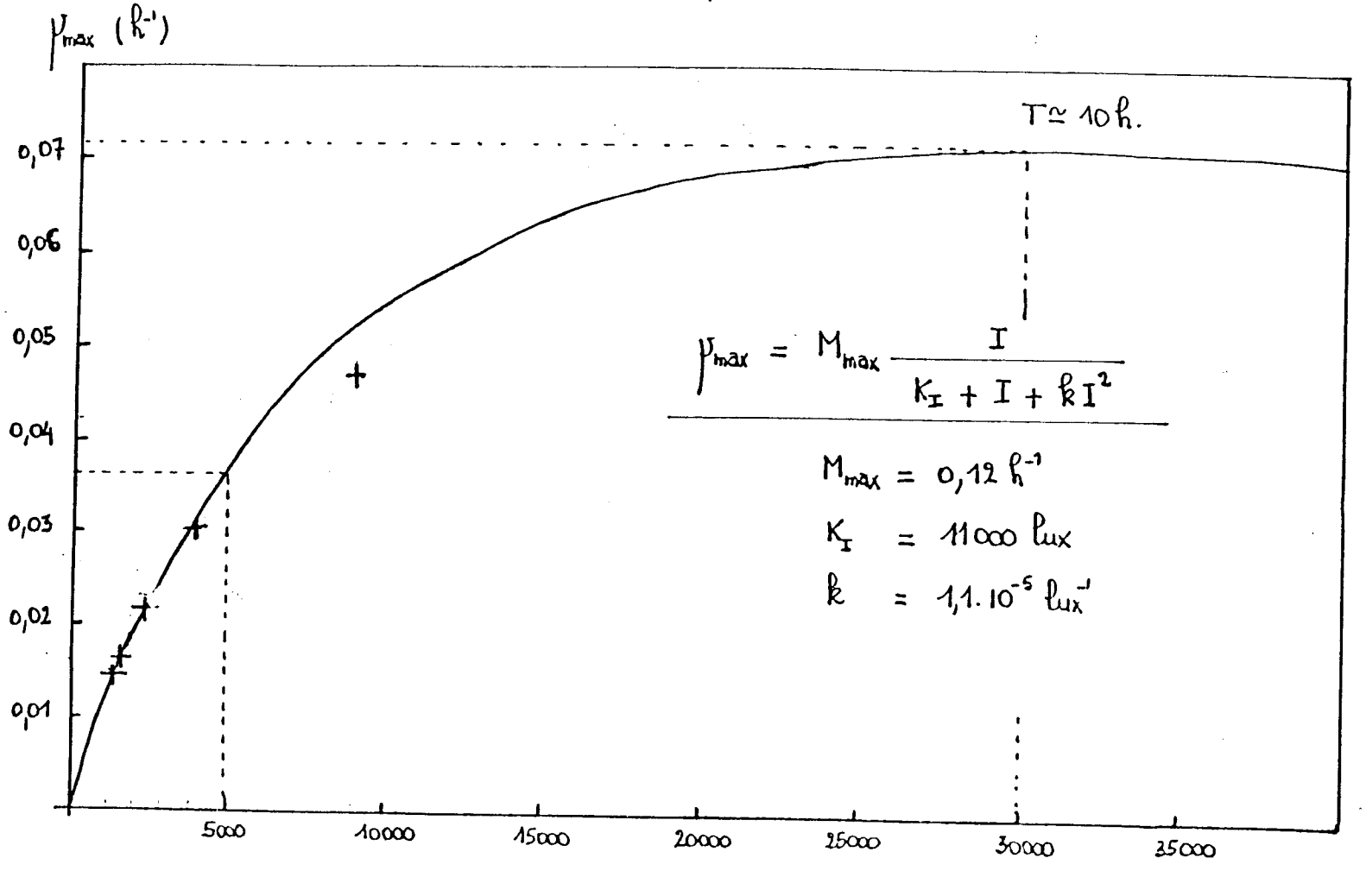

FIG. 3.

4. Attempts of compatibility between Clostridia cultures and Spirulina

Cultures of Clostridium thermosaccharolyticum were performed out at 60°C in rich medium (Brain Heart Infusion, Difco).

Full grown cultures were centrifuged : supernatant was immediately autoclaved. Pellet washed in physiological fluid (0.85% NaCl), resuspended in 1/3 original volume and autoclaved.

Both preparations were mixed Spirulina cultures. Resulting mixtures were incubated at 25°C under light, but growth of Spirulina was immediately inhibited. No H_2S smell was detectable and the pH remained compatible with Spirulina growth. At first view, it seems that the Brain Heart Infusion is much too rich and would impair the growth of any autotrophic microorganism as Spirulina.

The same would be said of the high cell densities mixed with the Spirulina inoculum.

Experiments are now repeated with dilutions of supernatant and autoclaved cell suspensions and with sterile medium.

New cultures of Clostridium thermosaccharolyticum should be prepared in synthetic conditions (sugar - salts) much more adequate for correct toxicity tests.