CBB DEVELOPPEMENT

PRODUCTION OF FREEZE-DREID BIOMASS OF *RHODOSPIRILLUM RUBRUM* :

FURNITURE AND ANALYTICAL RESULTS

T.N. 29.2

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INTRODUCTION

This project is a contribution to the MELISSA (Micro-Ecological Life Support Alternative), conceived for a future biological life support system for manned space missions (Lasseur, 1994). The aim of MELISSA is to recover edible biomass from waste, carbon dioxide, minerals using light as energy source. The objective of the present work was the pilot production of lyophilized biomass of *Rhodospirillum rubrum*. This phototrophic bacteria has to be grown under anaerobic conditions in the Segers and Verstraete medium (1983). Previous experiments on the ability of *Rhodospirillum rubrum* to degrade organic compounds have been done with cultures in 2 litres photobioreactors (Viprey, 1994). Such scale is not big enough to provide the needed biomass for the toxicological and nutritional studies, which have been planned to perform its ability for human consumption.

The study of the effect of extreme environments on the physiology and the biochemistry of photosynthetic microorganisms is the main research thematic of the Marine Biotechnology Laboratory (CNAM-INTECHMER) . To cultivate these microorganisms, the laboratory is equiped with different photobioreactors, essentially used for aerobic cell growth. So the challenge of the present work was to adapt the available equipment to produce one Kilogram of freeze-dried biomass of a photohetrotrophic bacteria.

I -MELISSA project and the production of *Rhodospirillum rubrum*

Mathematical models and experimental works have already supported the concept of the Micro-Ecological Life Support Alternative project (MELISSA). According to this model, photosynthetic bacteria will be used to degrade the compounds, such as organic acids, produced during the thermophilic anaerobic fermentations (Lasseur, 1995). Different works have shown that the bacteria *Rhodospirillum rubrum* is the most appropiate species for this photoheterotrophic transformation (Albiol, 1994). Studies have stated the relations between the biochemical composition of the biomass and culture conditions. It was shown that nitrogen limitation induces accumulation of PHB and glycogen at the expense of protein (Viprey, 1994).

II - Production of starter cultures

a - Maintenance of the strain.

The strain used for this pilot production was *Rhodospirillum rubrum*, ATCC 25903, obtained as freeze dried cells from the American Type Culture Conditions in a test tube (batch 91-09). Freeze dried cells were revived using R8AH medium, as recommended by ATCC (Appendix 1). *R. rubrum* was revived and subcultured using R8AH medium. Bacteria were incubated at 30°C under anaerobic conditions (cultures were argon flushed) and under 100µM quanta $.m^{-2}.s^{-1}$.

b - Adaptation of laboratory reactors for photoheterotrophic cultures.

Inoculums were prepared in two laboratory bioreactors adapted for photoheterotrophic cultures. The characteristics of these reactors are depictited in the Table 1. The illumination was provided through the pyrex wall of the reactors by cylindrical fluorescent tubes (OSRAM L32 W/25C). Light intensity was adjusted to 300 µM quanta $.m^{-2}.s^{-1}$ by the number of tubes installed along the outer side of the reactor wall.

Characteristics	SETRIC	BIOLAFITTE
Working volume	2 litres	2 litres
Agitation	Rushton turbine	Propeller
Regulation of temperature	Inner heat exchangers	Outer thermostated jacket
Reactor geometry	Cylindrical	Cylindrical with rounded bottom
pH regulation	LEVEL II	JENCO 6301

Table 1: Main characteristics of the two reactors used to provide inoculums for biomass production.

III - Biophotoreactors for pilot production of *R. rubrum.*

To fulfill the objective of the project, we have chosen a configuration of reactors at the pilot laboratory scale (20 litres working volume) and easy to work.

a - Adaptation of pilot reactors for photoheterotrophic cultures.

One fermentation unit was composed of a "bonbonne Biotech" made of polycarbonate (PC), which support sterilization at 121°C. **Agitation** was provided inside the reactor with a magnetized bar (159 x 27 mm) rotated at 150 rpm by an industrial magnetic stirrer (Maxi MR1 Digital IKA and Thermolyne 25 500 Maxi-Stirrer). **Lateral outputs** were managed for a) aseptic inoculation, b) pH electrode - METTLER and NaOH inlet for pH regulation and c) gas inlet and outlet for Argon sparging through 0,22µm sterile filter inside the culture. The **working volume** of the reactor is 20 litres. The reactor was filled with the culture medium and sterilized at 121°C during 20 minutes. pH was regulated with 1N NaOH via a Biocontroller APPLIKON ADI 1030. Illumination was obtained using fluorescent tubes (TLE 32W and TF30 MAZDAFLUOR; total power 9 000W). A photon flux density of 300 µmoles quanta.m-2.s-1 was provided by adjusting the number and the proximity of the fluorescent tubes. Appendix 2 shows the schema of the assembly of the reactor. Temperature was maintained at 30 ± 1°C by running the bioreactions in a thermostated room (Hall Biotechnology).

Up to January 1996, the production was realized with four units of fermentation. This capacity was doubled at this time to provide 160 litres of cultures by week. The production was run in these conditions by successive 4 days batch cultures.

Result: **1520 litres of cultures to produce 5,182 KILOS of wet bacterial biomass.**

b - Control of the culture parameters

The bacterial biomass was produced in the Segers and Verstraete medium (Appendix 3). Lactic acid was used as carbon source with pH adjusted to 6.9 +/- 0.1 UpH with 3 M NaOH as described by Segers and Verstraete (1983). After sterilization at 121°C during 20 minutes, the pH of the medium was at about 5.6 UpH. The pH was regulated before inoculation of the culture medium. Anaerobiose was obtained by flushing the medium with Argon during 10 minutes.

The pH of the medium was controlled before sterilization with a SCHOTT CG 825 pH meter.

Temperature of the bioreactor room was recorded by ORPHY GTS unit connected to SONDOR software within personnal computer (DELL 316SX).

Photon Flux Densities (PFD) in the bioractors were measured with a spherical LI-193 4π sensor (LI CORR) for Photosynthetic Active Radiations (PAR 400-700 nm). The sensor connected to a LI 1000 data loger (LI CORR) was placed in the middle of reactor and the PFD were measured when full of culture medium but free of bacterial cells.

IV - Harvesting, storage and freeze-drying of the bacterial biomass.

The biomass was harvested after five days of culture, corresponding to the stationnary phase of culture. The collected biomass has been stored at -40°C before final freeze- drying.

a - Centrifugation of the bacterial culture.

Bacterial cells were harvested by continuous centrifugation at 20 000 rpm with a CEPA Z41 model equiped with a refrigerated circulating system. The cylinder of the centrifuge (2 litres capacity) was washed by ultrasonic waves and rinsed with sterile water before utilization.

Result : **dry weight of the bacterial biomass: 20.8 ± 0.38 % (n = 10).**

b - Storage of the biomass

Biomass was stored in polyethylene bags at - 40°C in a FORMA SCIENTIFIC freezer.

c - Freeze-drying.

Freeze-drying of the biomass was realized by a specialized company LYOPHAL. The operation was ordered (our order N° 96-49) according to the specifications indicated in the devis N° 603177 (Appendix 4). The expedition to Salon-de-Provence was conducted in order to maintain the temperature of the biomass below O°C; the freezed biomass was packed in the presence of freez packs (24 hours at - 40°C before expedition) inside an insulated container. The transport lasted less than 14 hours. The biomass has been stocked at - 40°C the moment it arrived in the LYOPHAL building.

Result: 1.174 KILOS of freeze-dried bacteria from 5.182 KILOS of wet biomass

V - Biochemical analysis of *Rhodospirillum rubrum* produced in pilot photoreactors.

Biochemical composition of the final biomass was characterized by different methods.

a - Protein

Protein was extracted by alkaline hydrolysis (NaOH 1N) at 100°C during 5 minutes. It was shown that the classical pretreatment of the biomass by 10% trichloracetic acid did not improve the efficiency of the extraction. Protein analysis was performed according to the method of Lowry *et al.* (1951) modified as described by Stoscheck (1990). Bovine Serum Albumine was used as standard.

Result: **49.2 ± 5.7 % of the dry weight (n = 4)**

b - Glycogen

Glycogen was extracted using Palmsternia modified method (Palmsternia, 1956 *in* Albiol, 1994) and analyzed by the phenol method. The results are given as glucose equivalents per unit af sample dry weight.

Result: **10.5 ± 0.2 % of the dry weight (n = 4)**

c - Poly-Hydroxy-Butyrate (PHB)

PHB was extracted and analyzed using the Law and Slepecky method (1960) modified according Albiol (1994). For extraction sample was incubated 1 hour with sodium hypochlorate before purification by successive treatment with acetone, ethanol and chloroform. Colorimetric reaction was obtained by

treating the chloroform extract by concentrated sulfuric acid in a boiling bath during 10 minutes. Calibration curves were obtained by using poly-ß-hydroxybutyric acid (SIGMA P-8150).

Result: **16.6 ± 3.9 % of the dry weight (n = 10)**

d - Desoxyribonucleic acid (DNA)

DNA was extracted by ultrasonic waves (VIBRO-CELL 600 W, 3 minutes at 15 °C) in a solution containing 1 M NaCl, 10 mM Na_2EDTA and 0.7 % Sodium Dodecyl Sulfate in distilled water. Cell extracts were treated by chloroform before precipitation of DNA by absolute ethanol. After centrifugation at 20 000 g during 10 minutes pellets were washed twice with 70% ethanol. The DNA content was finally determined in bacterial extracts according to the colorimetric method of Burton (1956). Samples and standards (FLUKA 31160) were treated with perchloric acid 2N at 70°C during 20 minutes before the addition of the Burton reagent (1.5% diphenylamine and 1.5% concentrated sulfuric acid in glacial acetic acid (v:v)) followed by the action of acetaldehyde (0.16% in distilled water (w:v)). The absorbance was read at 600 nm after incubation of the samples at 25°C during 24 hours.

Result: **0.47 ± 0.06 % of the dry weight (n=5)**

VI - Discussion - Conclusion

The choice of polycarbonate reactors has been driven by considerations which imply security and usefullness. By using such materials we have no serious problem, except some troubles due to bad running of autoclave.

The mean biomass of *Rhodospirillum rubrum* obtained after 4 days of culture in pilot reactors was about 0.8 $g.l^{-1}$. About 10% of this biomass was lost before harvesting (not recuperated within the bacterial paste). As shown by the results obtained by Viprey (1994) in laboratory photoreactors, the productivity of the pilot reactors could be improved, i.e. by varying the intensity of the agitation. Further studies are needed to optimize such culture process. Different technical problems have been resolved to allow the production of 1 kilogram of freeze-dried biomass.

The biochemical composition of the biomass was determined by different colorimetric methods. Table 2 summarizes the results obtained. It appears that this biomass contains high amounts of proteins, about 49% DW. Its contents in PHB and glycogen is significantly higher than those reported in other analytical reports (Viprey, 1994; Albiol, 1994). The bacterial biomass contain about 0.5% DNA. This value is lower than that reported by Aiking and Sojka (1979) for *Rhodopseudomonas capsulata*. Total nucleic acid(DNA + RNA) should also be measured for comparison with data obtained from continuous cultures. Indeed, the biochemical data could be characteristic of

bacterial cells harvested from cultures at the end of the logarithmic phase of growth. In batch culture, growth conditions (from ligth to nutrient availability) change continuously. So differences in DNA content expressed as percentage of the dry weight could reflect the the change in percentage of the other components.

Compound	% DW	S.D.	n
Protein	49.2	5.7	4
Glycogen	10.5	0.2	4
PHB	16.6	3.9	10
DNA	0.47	006	5

In conclusion, one kilogram of freeze-dried biomass was obtained within pilot photoreactors. This bacterial biomass was mainly characterized by high protein content.

VII - References

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APPENDIX 1 : R8AH medium ($g.l^{-1}$)

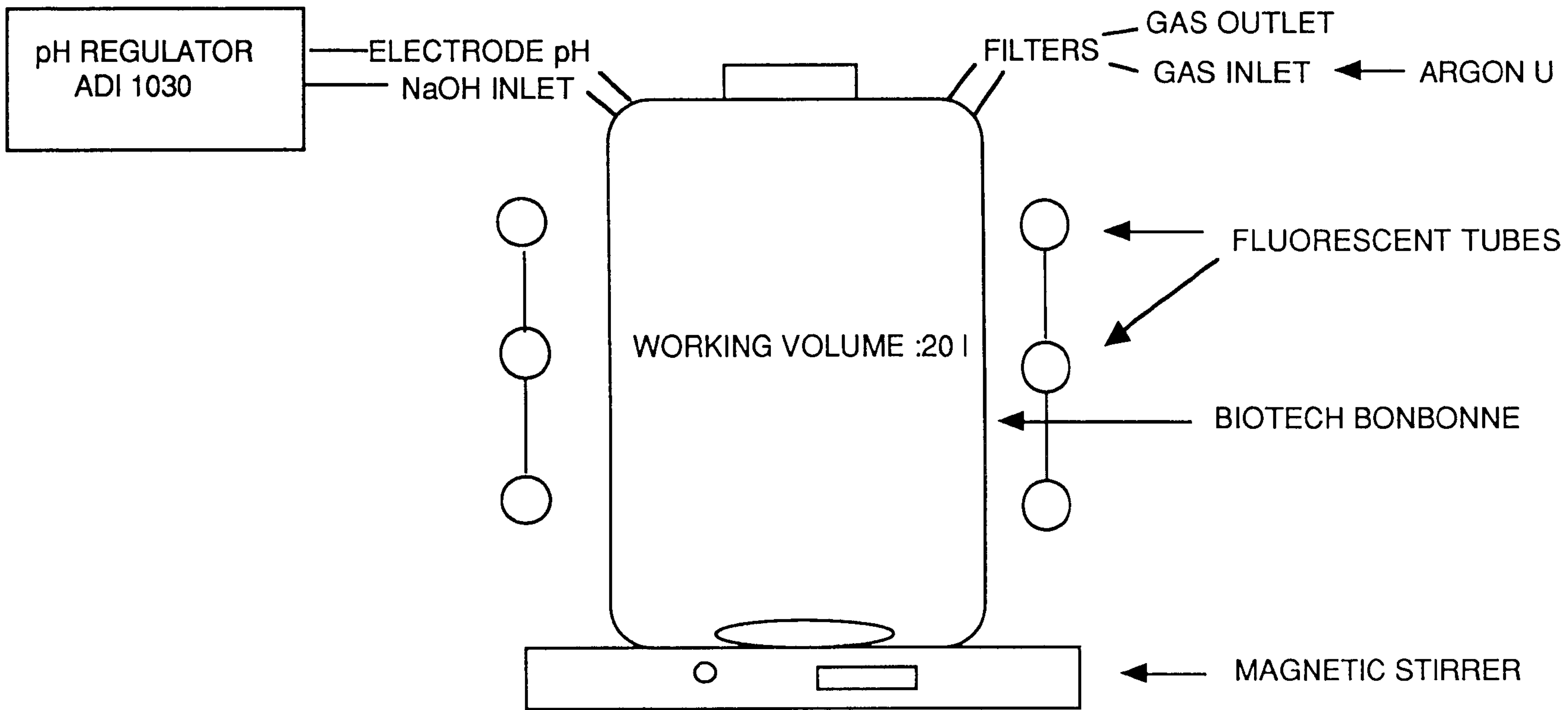
$Mg\ SO_4, 7H_2O$	0,2
$CaCl_2, 2H_2O$	0,07
EDTA	0,02
KH_2PO_4	0,6
K_2HPO_4	0,9
$(NH_4)_2\ SO_4$	1,25
Yeast extract	1
Malic acid	2,5
Fe-citrate	0,01
Agar	20
Trace elements	1 ml
Vitamines	1 ml

Trace elements ($g.l^{-1}$):

$ZnSO_4, 7H_2O$	0.001
$CoCl_2, 2H_2O$	0.02
$CuSO_4, 5H_2O$	0.001
H_3BO_3	0.001
$MnSO_4, H_2O$	0.002
$(NH_4)_6Mo_7O_{27}$	0.002
EDTA	0.05

Vitamines ($g.l^{-1}$):

Nicotinic acid	0.2
Nicotinamide	0.2
Thiamine HCl	0.4
Biotin	0.008



Appendix 2 : Schema of the pilot reactor used to produce *Rhodospirillum rubrum* under photoheterotrophic conditions.

APPENDIX 3 : Segers and Verstraete medium.

	$g.l^{-1}$
Mg SO_4, $7H_2O$	0.2
$CaCl_2,2H_2O$	0.05
$FeSO_4$	0.02
$MnCl_2$, $4H_2O$	0.01
EDTA	0.02
KH_2PO_4	1.2
K_2HPO_4	1.8
$(NH_4)_2SO_4$	0.5
CH_3CH_2COOH	2.7
Trace elements	1 ml
Vitamines	1 ml

Trace elements ($g.l^{-1}$):

$ZnSO_4,7H_2O$	0.1
$CoCl_2,2H_2O$	0.05
$CuSO_4,5H_2O$	0.005
H_3BO_3	0.1
$MnCl_2$, $4H_2O$	0.5
$(NH_4)_6Mo_7O_{27}$	0.002
EDTA	0.05
$NiSO_4,6H_2O$	0.05

Vitamines ($g.l^{-1}$):

Nicotinic acid	1
Thiamine HCl	1
Biotin	0.015

APPENDIX 4: Freeze-drying conditions

SOCIETE INTECHMER
Boulevard Collignon
50110 TOURLAVILLE

Objet : Devis de lyophilisation.

Salon de Provence, le 11 mars 1996

A l'attention de Monsieur GRIZEAU

DEVIS N° 603177

Lyophilisation expérimentale de bactéries.

- Réception et identification des lots,
- Congélation : - 40 °C
- Sublimation : - 30 °C
- Température de désorption : + 30 °C
- Conditionnement final dans des sacs plastique étanche.

Prix départ usine de Salon de Provence, transport en supplément :

- Pour 1 à 6 kilos humides..1 000,00 F H.T la lyophilisation pilote

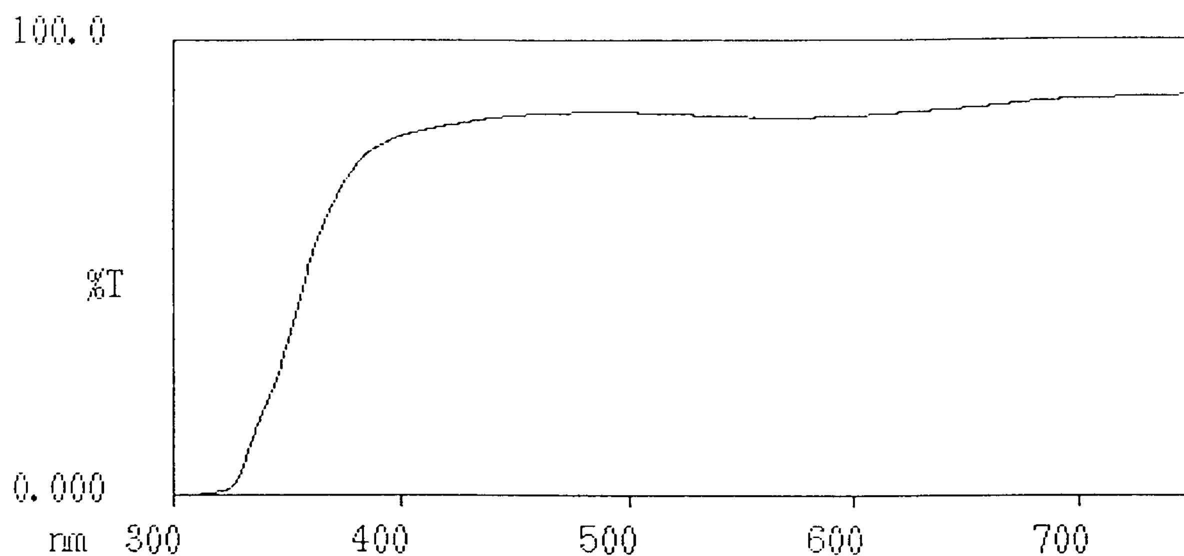
Délai : 1 semaine.

Paiement : traite à 30 jours date de facturation.

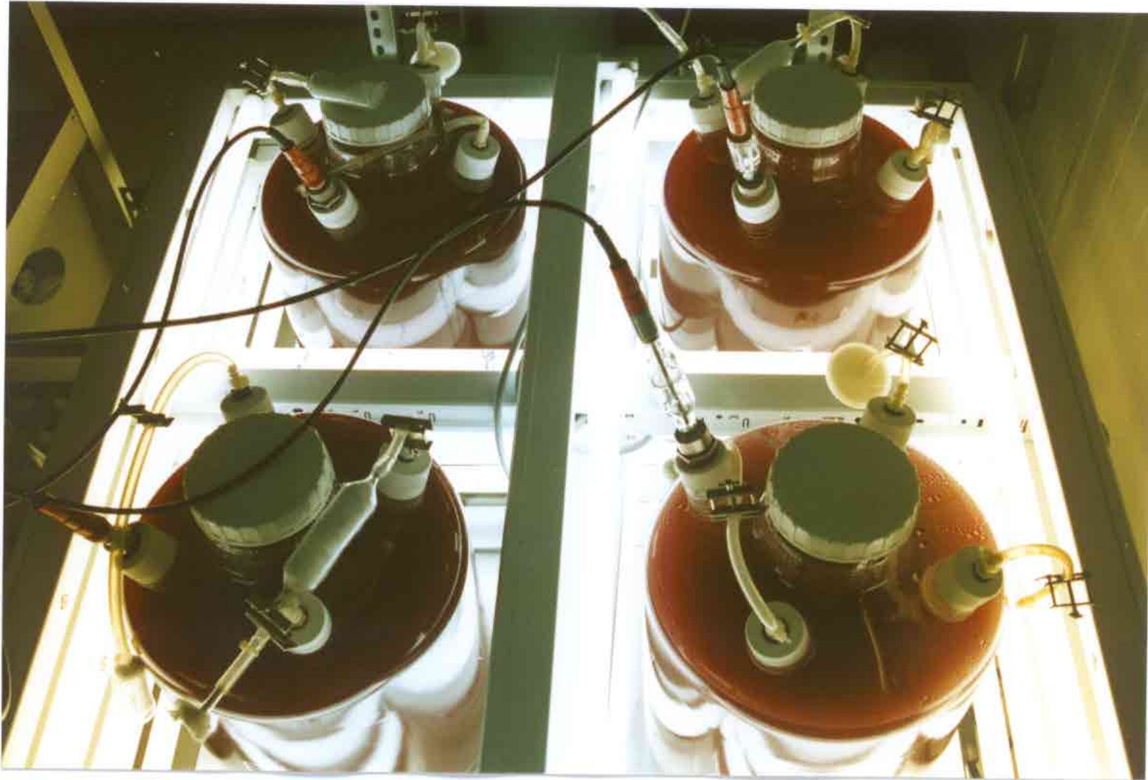
Validation de l'offre : 1 mois à compter de ce jour.

--Toute commande devra être confirmée par écrit - courrier ou fax - et accompagnée d'un RIB--

APPENDIX 5



Transmission of light towards polycarbonate wall of the photoreactors as a function of the wavelength.



Photography of the reactors used for the pilot production of *Rhodospirillum rubrum* biomass.