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Nitrifying Compartment Studies. Biomass composition

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Introduction

The concept of the MELISSA loop includes a nitrifying bioreactor for the conversion of the NH_4^+ present in the effluent of compartment II before its use in compartment IV. As the main goal of this compartment is such a bioconversion and not the generation of biomass, a packed-bed bioreactor with a mixed population of two strains of *Nitrosomonas* and *Nitrobacter* has been designed. *Nitrosomonas* convert NH_4^+ into NO_2^- in a first step, followed by the conversion of NO_2^- to NO_3^- by *Nitrobacter* cells.

The realisation of the nitrifying compartment experiments requires the obtention of enough cells in order to characterise their composition and prepare the corresponding inoculum. Due to the slow growth of *Nitrosomonas* cells, the results presented in the first version of this technical note were only focused on *Nitrobacter* cells, that have presented a better growth. In the present version the analyses of *Nitrosomonas –Nitrobacter* coculture will presented and discussed. Unfortunately, the slow growth of *Nitrosomonas* makes almost impossible to achieve an enough amount of cells to perform biomass analyses. Also in this second version the results of elemental composition analyses of *Nitrobacter* strain and *Nitrosomonas –Nitrobacter* coculture that have been made in CNRS laboratory at GIF/YVETTE, France will be presented.

Finally, the first version of this TN included the experimental set-up and first tests for a group of three bench-scale bioreactors, with the goal of obtaining complementary results to those given by the pilot scale experiments. With that, the low progress of the experiments due to the low cell growth rate could be partially compensated.

1.- SUSPENSION CULTURES

The main goal of these cultures is to achieve enough amount of biomass, either from every bacteria strain individually, as well as from the coculture. In this way, the inoculation of the nitrifying reactors, as the biomass analyses that will be described during the present technical note will be possible.

Microorganisms and culture medium. In these suspension cultures *Nitrosomonas europaea* and *Nitrobacter winogradskyi* strains (ATCC 19718 and ATCC 25391) have been used. These strains were maintained on agar plates (using the same medium preculture than described below) and incubated at 28 ºC.

The microorganisms were grown in 1 L flasks containing 100 mL of culture medium. The flasks were incubated, at 30º C in separated flasks by means of a shaking-bath system, during several months. The culture medium for *Nitrosomonas europaea* contained per dm^3 demineralized water: 2.51 g $(NH_4)_2SO_4$, 0.68 g KH_2PO_4, 0.71 Na_2HPO_4, 0.0025 g $FeSO_47H_2O$, 0.00012 g $CuSO_45H_2O$, 0.052 $MgSO_47H_2O$, 0.00074 g $CaCl_22H_2O$ (Wijffeels,R); and for *Nitrobacter winogradskyi* contained per dm^3 demineralized water: 0.691 g $NaNO_2$, 0.584 g NaCl, 0.150 g KH_2PO_4, 0.147 g $CaCl_22H_2O$, 0.075 g KCl, 0.049 g $MgSO_47H_2O$, 0.550 g sodium pyruvate, 1.5 g yeast extract, 1.5 g peptone (Laanbroek, H.J. and Gerards, S., 1992).

The pH of the *Nitrosomonas europaea* culture was maintained at 7.4 with enough buffering capacity provided by phosphate, and for *Nitrobacter winogradskyi* the pH was kept at 8.2 also with phosphate buffer.

Due to the low growth velocity of *Nitrosomonas* cells, the biomass obtained, up to now, still not enough to start-up neither a batch culture of reasonable volume (2L) nor a continuous culture in such conditions that would not produce wash-out phenomena or another unwanted effects on the culture system.

Although the *Nitrobacter* cells have also a slow growth, using a mixotrophic medium it has been possible to obtain enough biomass in several months and to conduct a

continuous culture with a moderated dilution rate, biomass concentration and conversion. The dilution rate was fixed taking into account the limited literature available about these bacteria in continuous cultures under different dilution rates (Laanbroek and Gerards, 1993). The dilution rate applied to the system in continuous mode was fixed at 0.005 h^{-1}.

Continuous culture conditions for Nitrobacter. The culture conditions were: temperature 28 ºC, pH = 8.2 controlled by means of CO_2 gas addition. The level of dissolved oxygen was kept at least at 80%, controlled using a cascade control based on air addition and variable stirring rate. The stirring rate was ranging from 300 to 500 rpm in order to avoid excessive (foaming) or not enough mixing in the system (mass transport problems). After proper inoculation of the bioreactor (BIOSTAT B from Braun Biotech International, 2L of working volume), initially with only 1 L of culture medium, the time evolution of the biomass and conversion was followed. When conversion reached significant values fed-batch operation was started until 2 L of total volume. Then the continuous start-up was carried out. The reactor was covered with foil paper in order to avoid any deleterious effect by light.

The continuous culture operating conditions and its medium composition were the same as in batch and fed-batch operations.

From this culture it was obtained a enough amount of biomass to enable the determination of the main cellular components. Thus, 1 L of volume with a concentration of 0.94 g dry biomass/L was obtained to determine the following parameters or cellular components:

- protein content,
- carbohydrates,
- lipids,
- DNA and RNA.
- elemental composition

As stated in TN 25.330, the estimation of the amount of inoculum necessary to start the pilot bioreactor shows that the amount of biomass obtained in 1 L of volume will be high enough to achieve a successful start-up.

Culture conditions for the coculture Nitrosomonas-Nitrobacter production. The culture conditions were: temperature 28 ºC, pH= 8.2 controlled by means of sodium carbonate solution (40 g/L) addition. The level of dissolved oxygen was kept at least at 80%, controlled using a cascade control based on air addition and variable stirring rate. The stirring rate was ranging from 300 to 500 rpm in order to avoid excessive (foaming) or not enough mixing in the system (mass transport problems). After proper inoculation of the bioreactor (BIOSTAT B from Braun Biotech International, 2L of working volume), initially with only 1 L of culture medium, the time evolution of the biomass and conversion was followed. When conversion reached significant values fed-batch operation was started until 2 L of total volume. Then, a set of batch operations was repeated several times, obtaining the enough amount of culture broth to perform the biomass analyses described in the next point (point 2). During all the operation the reactor was covered with foil paper in order to avoid any deleterious effect by light.

During this set of batch operations the composition of the fresh medium used was kept constant, except the ammonium concentration, which was increased to 0.6 gN-NH_4^+/L to increase the cell concentration in the liquid phase increased.

Once 6 L volume of culture broth was obtained, an amount of 120 mg of dry biomass were achieved. This quantity allow to determine the following parameters or cellular components:

- protein content,
- carbohydrates,
- lipids,
- DNA and RNA.
- elemental composition

2.- BIOMASS ANALYSES

2.1.- Previous treatment.

The obtained biomass in the continuous culture was centrifuged and re-suspended several times with phosphate-buffered solution (0.4M NaCl, 50 mM phosphate buffer at the pH of the growth medium, 50 mM $MgSO_4$) to minimise the salts content. After that, the biomass was freeze-dried. A portion of this freeze-dried biomass was separated to conduct elemental composition analyses.

2.2.- Determination of total protein content from Nitrobacter*.*

The method extracts all the cell proteins after dissolving the suspension of cells in NaOH, heated to 100 ºC and treated with a Na_2CO_3-$CuSO_4$-tartrate reagent (Herber, Phillips and Strange, 1971). The measurement of the protein concentration was made measuring absorbance at 750 nm using an spectrophotometer (UVIKON 941, KONTRON INSTRUMENTS). The detailed procedure is given in Appendix 1.

With a set of standard protein solutions is possible to prepare a standard curve by plotting optical density reading, against micrograms of protein in the cell samples (**Figure 2.1**).

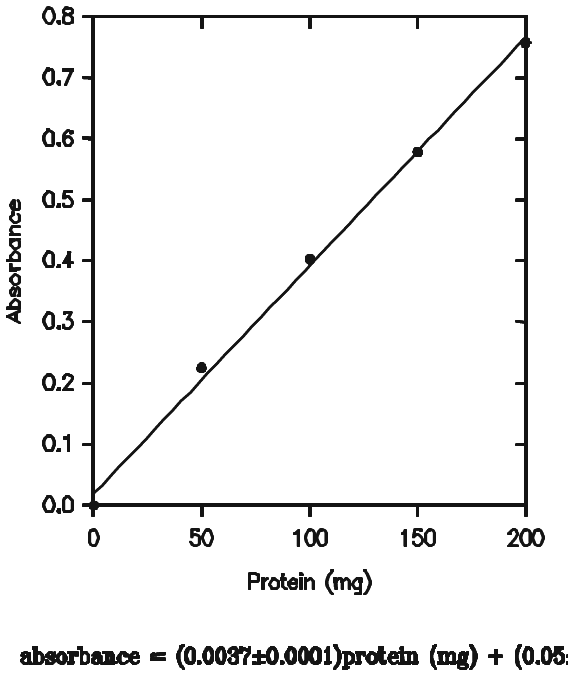


Figure 2.1.- Standard curve for determination of protein content.

The measurements for the biomass samples and the average value were:

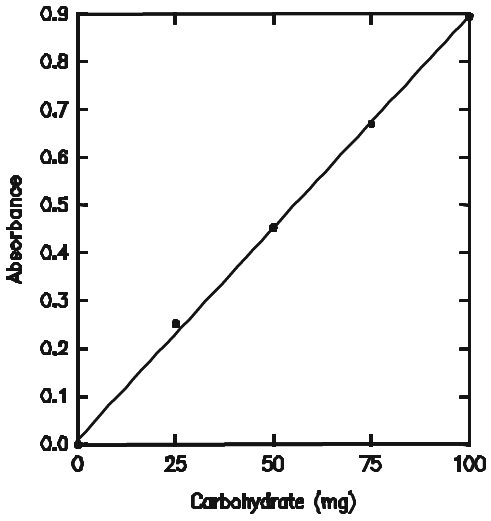
Samples	Absorbance
1	0.590
2	0.593
3	0.591
4	0.588
average	0.591
standard deviation	0.002

The average value obtained was **% Protein = 61.8 g protein / 100 g sample**

2.3.- Determination of total carbohydrate from Nitrobacter *cells.*

The method used was the denominated phenol method and measures the absorbance at 488 nm after adding to the suspension of cells phenol and sulphuric acid (Herber, Phillips and Strange, 1971). The detailed procedure is given in Appendix 2.

The standard solutions are prepared with glucose and the curve of the **Figure 2.2** was obtained as a calibration curve for total carbohydrate.



Absorbance = (0.0088±0.0002) carbohydrate (mg) + (0.01±0.01)

Figure 2.2.- Standard curve for determination of total carbohydrate.

The measurements for the biomass samples and the average value were:

Sample	Absorbance
1	0.112
2	0.117
3	0.115
4	0.135
average	0.120
standard deviation	0.010

The average value obtained was **% Carbohydrate = 12.2 g carbohydrate / 100 g sample**

2.4.- Extraction and determination of lipids of Nitrobacter cells.

Extraction of lipids: after extraction of low molecular weight components with $HClO_4$, the method extracts the lipids with a mixture of chloroform-methanol (1:1).

Determination of lipids: the method is based on the decreasing of absorbance at 350 nm as the dichromate is reduced by increasing amounts of lipids (Hellebust and Craigie, 1975). The detailed procedure is given in Appendix 3.

With the lipid standard solutions (dissolving palmitic acid in chloroform) is possible to draw the standard curve (**Figure 2.3**).

The average value obtained was **% Lipids = 13.0 g lipids / 100 g sample**

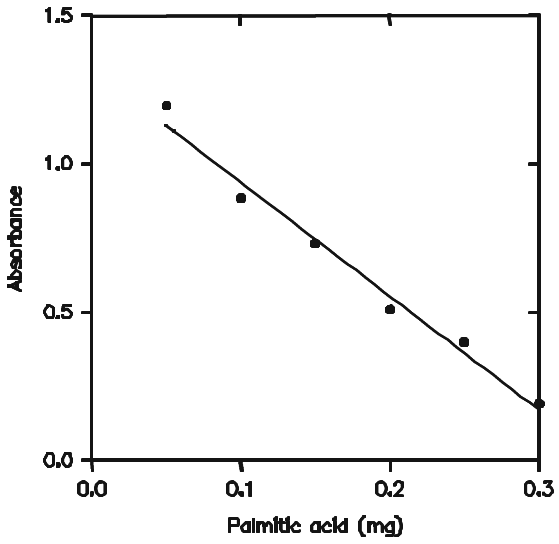


Figure 2.3.- Standard curve for determination of lipids.

2.5.- Determination of DNA of Nitrobacter cells.

The method measures the absorbance of the cell suspension at 600 nm (free of lipids) after the addition of $HClO_4$ and diphenilamine reagent (Hellebust and Craigie, 1975). The detailed procedure is given in Appendix 4.

With the DNA standard solutions (Calf thymus dissolved in NaOH and with addition of $HClO_4$) is possible to draw the standard curve (**Figure 2.4**).

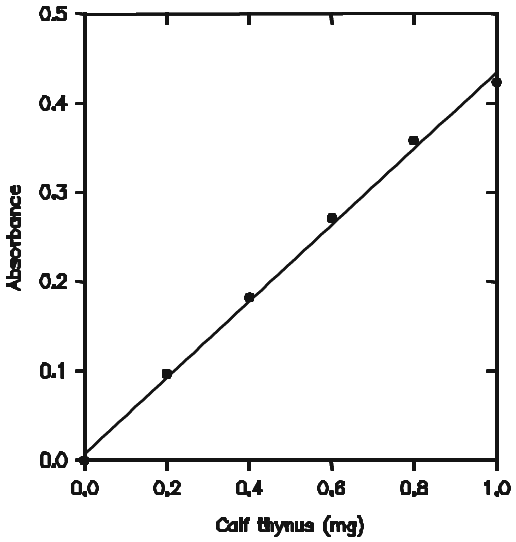


Figure 2.4.- Standard curve for determination of DNA.

The average value obtained was **% DNA = 5.7 g DNA / 100 g sample**

2.6.- Determination of RNA of Nitrobacter cells.

The analysis method measures the absorbance of the suspension of cells (free of lipids) after addition of: KOH (hydrolysis period), $HClO_4$ (until pH 1.0) and orcinol reagent (Hellebust and Craigie, 1975). The detailed procedure is given in Appendix 5.

Plotting the absorbance of RNA standards (dissolving yeast RNA in water) against the RNA concentration the standard curve can be obtained (**Figure 2.5**).

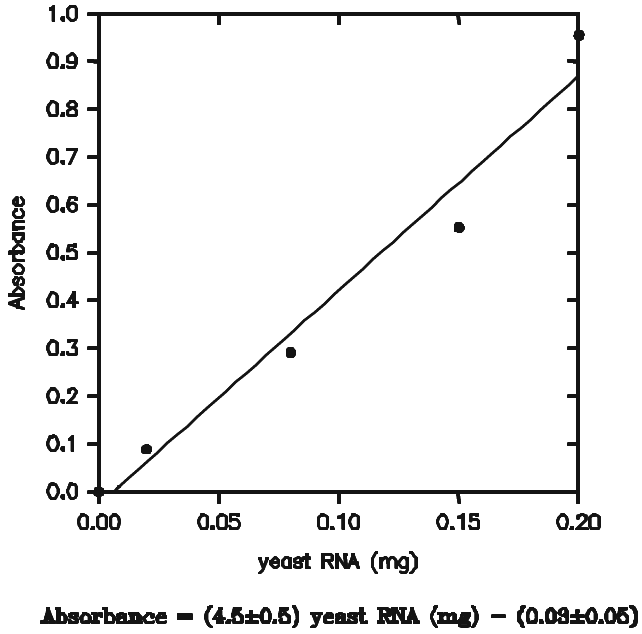


Figure 2.5.- Standard curve for determination of RNA.

The average value obtained was **% RNA = 5.6 g carbohydrate / 100 g sample**

The results of the whole analyses conducted and their precisions are presented in the **Table 1**. The biomass composition is preliminary due to the fact that the *Nitrobacter* cells grew in mixotrophic medium.

Components	Percentage
Protein	(62 ± 4) %
Carbohydrates	(12 ± 2) %
Lipids	(13 ± 1) %
DNA	(5.7 ± 0.1) %
RNA	(5.6 ± 0.7) %

Table 1. Biomass composition for *Nitrobacter* grown in mixotrophic medium.

Comparing these results with the molecular composition of a typical bacterium (Atkinson, B. and Mavituna, F. 1983), it is possible to say that these values are in the expected range.

The protein content of bacteria typically varies from 60 to 65 %, and this percentage does not usually vary dramatically with different growth conditions (Rehm H.-J. and Reed G. 1981).

The amount of DNA per bacteria varies as a function of the growth rate, although the variation of DNA per unit of biomass is low, being always about 5%.

The concentration of some cell component (RNA, lipids and carbohydrates) is strong function of the growth rate and environment. For RNA a typical range is from 5% to 15%.

2.7.- Determination of the molecular composition of a Nitrosomonas - Nitrobacter coculture.

Using the same procedure already described for the analyses of *Nitrobacter* cells, the results detailed in **Table 2** have been obtained for the molecular components.

Components	**Percentage**
Protein	(65 ± 6) %
Carbohydrates	(8 ± 3) %
Lipids	(13 ± 2) %
DNA	(7 ± 1) %
RNA	(5 ± 1) %

Table 2.- Biomass analyses for *Nitrobacter-Nitrosomonas* (coculture).

A major number of tests were impossible to perform due to sample exhaustion. For this reason, the accuracy of the analytical results is lower than in the case of the analysis of the composition of *Nitrobacter* cells.

2.8.- Chemical composition of the microorganisms

In order to determine biomass composition, cells were withdrawn from the bioreactor during continuous culture. The sample was centrifuged and re-suspended several times with phosphate-buffered solution (0.4M NaCl, 50 mM phosphate buffer at the pH of the growth medium, 50 mM $MgSO_4$) to minimise the salts content. After that, the biomass was freeze-dried.

The sample was analysed for composition of C, H, N and S in an elemental analyser 240B (Perkin Elmer). It is considered that the cells are formed mainly of C, H, O, N and S, neglecting the rest of elements (P, K,...). Normally, the coefficients of the other elements in the chemical formulae of the microorganisms are much lower than the N and S coefficient (in molar basis related to C). The coefficient for O is obtained in the following way: Once the quantity in % weight of C, H, N and S is known, the Oxygen is determined by difference (100%- (%C + %N + %H+ %S)). After that, all the percentages are transformed to molar basis related to C.

In order to obtain composition for additional elements, another set of determinations will be performed by the CNRS laboratory at GIF/Yvette, France.

The results obtained in weight percentage and its elemental composition in C molar basis are presented in **Table 3** for the *Nitrobacter* cells.

Element	Weight percentage sample 1/sample 2	Carbon molar basis sample 1/sample 2
C	46.28/46.34	1.000/1.000
H	6.750/6.810	1.750/1.766
O	33.96/33.825	0.550/0.548
N	12.61/12.64	0.234/0.234
S	0.397/0.385	0.003/0.003

Table 3. Elemental composition obtained for *Nitrobacter* cells grown in a mixotrophic medium.

Hence, the average biomass composition is determined as:

$$CH_{1.76\pm0.02}O_{0.549\pm0.002}N_{0.234\pm0.001}S_{0.003\pm0.001}$$

The elemental analyses for the samples obtained from coculture of *Nitrosomonas-Nitrobacter* were determined using the same protocol already described for *Nitrobacter* cells. The results obtained for the CNRS laboratory at GIF/Yvette (France) are presented in **Table 4**.

Element	Weight percentage		Carbon molar basis	
	aliquot 1 sample 1/ sample 2	aliquot 2 sample 1/ sample 2	aliquot 1 sample 1/ sample 2	aliquot 2 sample 1/ sample 2
C	40.98/41.05	41.33/40.79	1.000/1.000	1.000/1.000
H	6.36/6.18	6.15/6.18	1.862/1.807	1.786/1.818
O	24.06/23.91	24.13/24.11	0.503/0.499	0.500/0.507
N	10.15/10.05	10.21/10.19	0.186/0.184	0.185/0.187
S	0.75/0.61	0.91/0.56	0.007/0.006	0.008/0.005
P	3.33/2.86	3.27/--	0.031/0.027	0.031/--

Table 4.- Elemental composition obtained for *Nitrosomonas* - *Nitrobacter* coculture.

Hence, the average biomass composition for the cocoulture is determined as:

$$CH_{1.82\pm0.08}O_{0.502\pm0.007}N_{0.186\pm0.004}S_{0.006\pm0.003}\ P_{0.030\pm0.004}$$

3.- PROPOSAL OF THE TEST PROCEDURES TO BE CARRIED OUT IN THE NEXT PHASE.

The experiments in the pilot plant bioreactor will be completed by the information obtained in a set of smaller size bench column reactors, that will enable to work in parallel at different operational conditions. The objective of the preparation of such a kind of experimental set-up is to obtain more information on the behaviour of the system in the shortest time. This aspect is required because of the slow growth rate of the nitrifying bacteria. It should be stated that, the time necessary to reach the biofilm development will be long, in the order of 3-4 months. After this period, once biofilm would be formed, it is expected to achieve reasonable velocities of ammonia conversion.

This set-up is defined in section 4 and a proposal of the experiments to be carried out is presented. The work to be carried out at the bench scale reactors will complete that performed in the pilot plant reactor, as described in TN 25.330.

4.- TEST BENCH COLUMNS.

These small bioreactors have, as main goal, to obtain information about the biofilm growth of the *Nitrosomonas* and *Nitrobacter* co-cultures and to be the first step in the scale-up of the immobilized culture. With these premises, the relationships between bioreactor dimensions at pilot plant and bench scale have been maintained as much as possible **(Figure 4.1)**. Moreover, some lateral ports have been implemented along the external wall of the bioreactor. This will allow to obtain information about internal concentration profiles.

An interesting aspect to investigate in the bench columns, is the separate behaviour of each strain. The incorporation of this knowledge in the control system could allow to use any potential behaviourial difference of the strains, in order to create the conditions

favouring the growth of one particular strain, with respect to the second one. This kind of information will be obtained from the internal concentration profiles for the main compounds in the reaction (NH_4^+, NO_3^- and NO_2^-), and the kinetic behaviour of each strain.

One aspect to be considered is that the information obtained through the concentration profiles of the different compounds should be representative of the intrinsic kinetic behaviour of the immobilized cells and not influenced by limitations of mass transport from the liquid to the solid particles. In principle, the hydrodynamic conditions in the reactor (recirculation ratio, aeration rate), as discussed in TN 25.330, allow to anticipate that there will not be external mass transfer limitations. However, for a given experimental conditions this hypothesis will be tested.

In particular, three bench scale bioreactors, as described above, will be built. The experiments proposed for the next phase using them have been planned to study the following detailed aspects:

- Hydrodynamic flux model in the bioreactors (RTD analysis). For different operating conditions the liquid phase in the bioreactor will present different degrees of mixing that will clearly influence the bioreactor performance. Specially for the recirculation ratio (feed flow/recirculation flow), aeration rate and stirring speed at the reactor inlet. This information can be determined by the stimulus-response technique. The method involve the introduction of a tracer material at the inlet or some other point within the reactor, and the observation of the subsequent response in the effluent stream or some other downstream point within the reactor. The distribution of residence times is obtained from the response and a suitable model for the flow can then be selected by matching the experimental response with that obtained from the mathematical model.

- Gas liquid-oxygen transfer (Kla determination). The dynamic method will be applied, as used in the pilot plant bioreactor, for conditions where there is no reaction. The effect of different operating parameters, like the stirring speed, gas flow rate and recirculation ratio on the volumetric mass transfer coefficient will be

studied. Thus, with the experiments conducted for the RTD analysis and Kla determination the physical characterization of the reactor will be obtained.

- Effect of the different loads of ammonia, on the overall conversion, products distribution and microorganism location along the columns.

- Effect of different level of dissolved oxygen, also on the conversion, products distribution and microorganism proliferation. The changes on the dissolved oxygen will be conducted by changing the oxygen composition of the inlet gas, keeping the gas flow-rate constant, in order to not alter the mixing conditions in the system (TN 25.330).

- Effect of the mixing in the system, with a fixed level of dissolved oxygen and changing recirculation ratio and flow gas-rate (allowing changes on gas composition), on the concentration profiles that would enable different cell distribution along the reactor. As stated in TN 25.330 the main mixing effect will be provided by the aeration at not much low gas flow-rates.

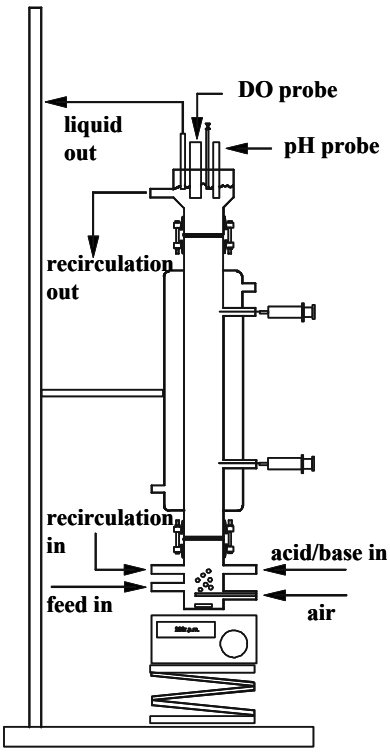


Figure 4.1.- Scheme of the test bench column design.

BENCH COLUMNS COST ESTIMATION

This overall cost estimation is related to the cost of the reactors, their instrumentation and other equipment necessary for the three bench columns to be mounted.

- Measurement and control of pH and dissolved oxygen	2.200.000
- Inlet medium pump	260.000
- Outlet medium pump	260.000
- Acid pump	150.000
- Base pump	150.000
- Pressure reductor	50.000
- Needle valve	100.000
- Rotameter	60.000
- Magnetic stirrer (digital display)	270.000
- Reactors	850.000
TOTAL AMOUNT	4.350.000
IVA 16%	696.000
FINAL COST ESTIMATION (ptas)	**5.046.000**

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APPENDIX 1

DETERMINATION OF TOTAL PROTEIN CONTENT OF MICROORGANISMS.

Reagents. The reagents are as follows:

(i) 5 % Na_2CO_3

(ii) 0.5 % $CuSO_4 5H_2O$ in 1% sodium potassium tartrate.

(iii) To 50 mL of reagent (i) add 2 mL of reagent (ii); prepare immediately before use and do not keep.

(iv) Diluted Folin-Ciocalteu reagent. The total acidity of the concentrated reagent is determined by titrating a sample with 1.0 N NaOH using phenolphthalein as indicator. It is then diluted with distilled water to make the total acidity exactly 1.0 N.

(v) Protein standard: bovine serum albumin, 0.2 mg/mL.

Procedure

Measure 0.5 mL of washed cell suspension (0.1 mg dry weight of cells) into a test tube, add 0.5 mL of 1.0 N NaOH, place in a boiling water bath for 5 min, and cool in cold water. Add 2.5 mL of reagent (iii), alow to stand 10 min, and rapidly add 0.5 mL of reagent (iv) . A blank of reagent containing 0.5 mL of distilled water instead of cell suspension, and a set of standard protein solutions (0.05-0.2 mg protein) are treated in the same way, including the heating stage. After standing 30 min to allow full colour development, measure all optical densities against the blank of reagent at 750 nm.

APPENDIX 2

DETERMINATION OF TOTAL CARBOHYDRATE.

Reagents. The following reagents are used:

(i) 5 % (w/v) solution of phenol in water.

(ii) Concentrated sulphuric acid.

Procedure

Add 1.0 mL of sample containing the equivalent of 0.02-0.1 mg glucose. A blank of reagent containing 1 mL of water, and a set of glucose standards (0.025, 0.05, 0.075 mg glucose, in a volume of 1 mL) are prepared at the same part. To all tubes add 1 mL of 5 % phenol and mix; then from a fast-flowing pipette add 5 mL of concentrated sulphuric acid, directing the stream of acid to the surface of the liquid and shaking the tube simultaneously, to achieve fast and complete mixing. The tubes are allowed to stand 10 min, shaken, and placed in a water bath at 25 to 30ºC for 10 to 20 min before readings are taken. The absorbance of the characteristic yellow colour is measured at 488 nm.

APPENDIX 3

DETERMINATION OF LIPID CONTENT.

Reagents. The following reagents are used:

(i) Palmitic acid.

(ii) Lipid standard solution. 1 mg/ml of palmitic acid in chloroform.

(iii) Dichromate solution. 2.5 g/l of $K_2Cr_2O_7$ in H_2SO_4.

Procedure

Add 0.2 volumes of water to the combined chloroform-methanol extracts of the cells. Shake the solution for 5 min to mix well and centrifuge to separate the phases. Collect the organic (lower) phase, discarding a precipitate that forms at the interphase. Discard the aqueous (upper) phase. Evaporate the chloroform-methanol solution under a stream of N_2 to a final volume of 2 ml. Transfer 0.05, 0.1, 0.15, 0.20, 0.25 and 0.30 ml of the lipid standard solution to marked 5- or -10 ml screw-capped tubes. Transfer 0.1, 0.2 and 0.5 ml of the unknown lipid sample to marked tubes. Evaporate all tubes to dryness under vacuum or a stream of N_2. Add 2 ml of dichromate solution to all tubes and cap with Teflon-lined caps. Place all tubes in a boiling-water bath for 45 min. Shake the tubes two or three times during the heating. Cool the tubes, remove 1.0 ml of each, and dilute to 10.0 ml with water. Read the absorbance of each tube at 350 nm against an H_2O blank. Plot a standard curve with the known lipid samples and determine the unknowns graphically or by Beer's law. Note that the assay is based on the disappearance of absorbance at 350 nm as the dichromate is reduced by increasing amounts of lipids. It is thus convenient to plot the standards as the reciprocal of absorbance against lipid concentration.

APPENDIX 4

DETERMINATION OF DNA

Reagents. The following reagents are used:

(i) DNA standard solution.

(ii) Aqueous acetaldehyde.

(iii) Diphenylamine solution.

Procedure

Add 1 ml of 1 N NaOH to one of the acid-extracted, lipid-free pellets and heat 10 min in a boiling-water bath to dissolve the pellet. Assay aliquots of the sample for protein by the dye-binding assay.

Calf thymus DNA is dissolved in 5 mM NaOH at a concentration of 0.4 mg/ml. A working solution is made every 3 weeks by mixing an aliquot with an equal volume of N $HClO_4$ and heating at 70 ºC for 45 min. Acetaldehyde is cooled and 1 ml is transferred in a cooled pipette into 50 ml of H_2O. This solution is stable at 4 ºC for several months (acetaldehyde is flammable and should be stored in the cold and thoroughly chilled before opening). Dissolve 1.5 g of diphenylamine in 100 ml of glacial acetic acid and add 1.5 ml of conc. H_2SO_4. Stable at 4 ºC for up to 3 months. Just before use add 0.1 ml of aqueous acetaldehyde per 20 ml of reagent.

Add 5 ml of 0.5 N $HClO_4$ to another of the acid-extracted, lipid-free pellets and resuspend the sample by vortexing or inversion. Incubate the sample at 70 ºC for 45 min. Centrifuge and remove the supernatant. Transfer 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the calf thymus DNA stock solution to marked screw-cap test tubes and adjust the volume to 2 ml with 0.5 N $HClO_4$ to serve as a reagent blank. Transfer 0.5, 1.0, and 2.0 ml of the unknown DNA sample to marked screw-crap test tubes and adjust all volumes to 2 ml with 0.5 N $HClO_4$. Add 4 ml of the diphenylamine reagent to all tubes

and mix well. Incubate all tubes at 30 ºC for 16-20 h and read absorbance at 600 nm. Plot a standard curve with the standards and determine un-known DNA concentrations.

APPENDIX 5

DETERMINATION OF RNA

Reagents. The following reagents are used:

(i) RNA standard solution.

(ii) Orcinol reagent.

(iii) Orcinol stock solution.

(iv) Cupric ion solution.

Procedure

Add 1 ml of 1 N NaOH to one of the acid-extracted, lipid-free pellets and heat 10 min in a boiling-water bath to dissolve the pellet. Assay aliquots of the sample for protein by the dye-binding assay.

Dissolve yeast RNA in water to a concentration of 10 µg/ml. Store frozen. Dissolve 0.15 g of $CuCl_2.2H_2O$ in 100 ml of conc. HCl. Stable at room temperature. Dissolve 12.5 g of orcinol to a final volume of 25.o ml in 95% ethanol to obtain the Orcinol stock solution. For the Orcinol reagent mix 2 ml of orcinol stock solution with 100 ml of cupric ion solution. This solution should be freshly prepared for each determination.

Add 2 ml of 0.3 ml N KOH to the remaining acid-extracted, lipid-free pellet and incubate for 18-24 h at 30 ºC. After the hydrolysis period, cool the sample on ice and acidify the solution to pH 1.0 (pH paper) with conc. $HClO_4$. Centrifuge and carefully remove the supernatant. Wash the pellet with 1 ml of cold 0.2 N $HClO_4$. Combine the supernatants. Transfer 0.2, 0.4, 0.8, 1.0, 1.5 and 2.0 ml of the RNA standard solution to marked screw-cap tubes. Transfer 0.1, 0.2, and 0.5 ml of the unknown RNA solution to marked tubes and adjust the volume of each to 2.0 ml with H_2O. Include a blank containing 2.0 ml of H_2O. Add 2 ml of the orcinol reagent to each tube, seal with Teflon-lined caps, and place the tubes in running water and read the absorbance of each tube at 665 nm. Plot the absorbance of the RNA standards against the RNA

concentration to obtain a standard curve. Determine the concentration of he unknowns against the standard curve.