MELISSA

ESTEC/CONTRACT 8125/88/NL/FG

Technical note 6

MELISSA CCN1 TN 6

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TECHNICAL NOTE 6.1

GROWTH OF CLOSTRIDIUM THERMOCELLUM ON DIFFERENT CARBON SOURCES.

Objective

To test the growth of the anaerobic thermophile *Clostridium thermocellum* on different carbon sources.

Methods

Cultures (50 ml) of *Clostridium thermocellum* (ATCC 27405) were grown anaerobically at 60°C on medium GS-2 and medium MJ as described by Johnson et al.(1981). Medium GS-2 is a rich non-defined medium while medium MJ is chemically defined. Different carbon substrates were tested using medium GS-2 omitting cellobiose.

Results

Substrate spectrum of *Clostridium thermocellum* is listed in Table 1.

Suggestion

Due to severe problems with the thermophilic Clostridium strains of the ATCC collection (oxygen sensitivity is too high), the development of the first compartment is retarded. We aim to screen for a proteolytic thermophilic Clostridium in the DRANCO-plant in Ghent.

Table 1 : Growth of Clostridium thermocellum on different media.

	Medium	Growth
	GS-2 cellobiose	+
	cellulose	+
	glucose	+/-
	xylose	-
	starch	-
	MJ cellobiose	-

TECHNICAL NOTE 6.2

1. UTILIZATION OF VARIOUS CARBON AND NITROGEN SOURCES BY THE PHOTOTROPHS OF COMPARTMENT TWO

Introduction

Rhodobacter capsulatus and *Rhodospirillum rubrum* are both capable of utilizing lactic acid, butyric acid and acetic acid as sole carbon source (Pfennig & Trüper, 1989) *R. rubrum* possesses an inducible alcohol dehydrogenase and can therefore metabolize ethanol (Pfennig & Trüper, 1989). Ethanol is an important transformation product expected from the liquefaction compartment. In principle the fatty acids and ethanol expected from the first compartment can be metabolized in the phototroph compartment.
The nitrogen compounds expected in the supernatant from the first compartment are ammonia, amino acids, ureum and amines. *R. rubrum* can utilize ammonia, dinitrogen or purines as sole nitrogen source (Pfennig & Trüper, 1989). *R. capsulatus* is capable of using a wide variety of nitrogen sources - ammonia, dinitrogen and several amino acids. Certain strains can also utilize nitrate, purines and pyrimidines. Certain amino acids from the first compartment may not be assimilated in the second.

Objective

To compare growth of *R. capsulatus* and *Rh. rubrum* on various carbon and nitrogen sources in a synthetic medium.

Materials and methods

Cultures, culture media and conditions. *Rhodobacter capsulatus*

ST407 and *Rhodospirillum rubrum* ATCC 11170 were maintained on the basal medium of Segers & Verstraete (1983) supplemented with 30mM lactic acid and 0.5g/l $(NH_4)_2SO_4$. The cultures were incubated in 20ml screw-topped test tubes at 25°C and at a light intensity of about 60 $\mu E/m^2.s$. Tubes are inoculated with 0.5ml of a 1-3 week-old culture.

Carbon sources. 30mM lactic acid, Na-acetate, Na-butyrate and ethanol were added to the media as sole carbon sources. Yeast extract (0.001%) was omitted in later experiments to obtain a totally defined medium. In the case of acetic acid and ethanol, 5mM lactic acid was added to the medium to stimulate growth.

Nitrogen sources. Nitrogen sources were tested with various carbon sources *i.e.* 15mM Ca-lactate, 30mM Na-acetate and 30mM Na-butyrate. The nitrogen sources $(NH_4)_2SO_4$, ureum, betaine and glutamate were added to the media at a concentration of 105mg N/l. No yeast extract was added to the media in these tests.

Growth curves. To determine the increase in biomass and the reduction of COD and nitrogen with time a reactor was set up. The reactors tubes contained 550-600ml medium with 15mM Ca-lactate and 0.5g/l $(NH_4)_2SO_4$ as carbon and nitrogen sources respectively. No yeast extract was added to the medium and the buffer capacity of the basal medium was doubled. Initial reactor experiments on *R. capsulatus* were done with 30mM lactic acid and biomass was measured as volatile suspended solids (VSS).

Analyses. Analyses were performed about 3 days after growth could be observed. Samples (20ml) were centrifuged at 12000rpm for 15min. The COD of the supernatant was determined by the dichromate method as an indirect measurement of carbon source utilization. The total nitrogen content of the supernatant was determined either by Kjeldahl-N or NH_4-N depending on the nitrogen source added to the medium. The protein content of the pellet was measured by the Bradford method (1976) as an indication of biomass

production.

Results and discussion

Results for carbon source utilization are illustrated graphically in Figs 1-5. Those for nitrogen utilization in Figs 4-9. The figures illustrate the % reduction of COD and % assimilation of nitrogen and also the biomass protein produced over the time course of the experiment.

Protein production is illustrated graphically in 2 ways ie. protein production per day (Figs 3a, 5a & 8) and total production after incubation (Figs 3b, 5b & 9). This was done because *Rh. rubrum* cultures were incubated longer and per day production could thus be compared. The lag phase of *Rh. rubrum* is longer ie. growth is only observed after 4-5 days incubation whereas *R. capsulatus* cultures are red after 2-3 days. Total protein production gives an idea of the harvesting potential of these organisms. Between 60-70% of the dry weight of *R. capsulatus* is crude protein (Driessens *et al*, 1987). Vrati (1984) reported 69% crude protein for *R. capsulatus* and the crude protein content of a fermenter containing photosynthetic bacteria was 66,4±12% (Toerien *et al*, 1980). If the crude protein content is assumed to be 65% than the highest yield obtained for *R. capsulatus* on Ca-lactate and ureum (Fig 9a) is about 0.605 g/l in 6.5 days. That for *Rh. rubrum* is 0.49 g/l on Ca-lactate and ammonia in 8.5 days (Fig 9a). Yields for photosynthetic bacteria in batch culture experiments reported in the literature range between 1 to 2g dry weight/l (Driessens *et al*, 1987). Driessens et al (1987) achieved a maximum yield of 10.41g biomass/l.day in a continuous flow-through reactor on a synthetic medium.

Ethanol was the only carbon source, which was not utilized by either organism (Fig. 3), the others were all utilized to produce biomass (Figs 3 & 5). Ethanol was not detrimental to the growth of *R. capsulatus* as there was a 20% reduction of COD when lactic

acid was added to stimulate growth (Fig. 1). It is not known to what extent the 2 carbon sources influence the COD *.i.e.* how much of the COD is attributable to the ethanol and how much to the lactic acid. *Rh. rubrum* reduced the COD of the ethanol-lactic acid medium by about 40% (Fig. 2). As only 5mM lactic acid was added to the medium (cf. 30mM ethanol) some of the COD reduction must be due to ethanol utilization. It therefore appears that the ethanol is utilized by *Rh. rubrum* if growth is stimulated by another carbon source. Growth on butyrate was poor (cf Figs 3 & 5), but the cultures were sampled a day earlier than the previous tests and no yeast extract was added to the medium. Yeast extract seems to enhance the growth of *Rh. rubrum*, but *R. capsulatus* produced more protein in the absence of yeast extract (Fig 3a).

Growth on acetate was poor compared to that on lactic acid (Fig. 3b). Weaver *et al* (1975) reported moderate to good growth on acetate for various strains of *R. capsulatus*, but growth was usually only observed after 5 days incubation. The isocitrate lyase of wild strains of *R. capsulatus* is barely detectable, but spontaneous mutants have been reported , which grow vigorously on acetate (Pfennig & Trüper, 1989). Growth of *R. capsulatus* on acetate was only observed after 4-5 days incubation.

Ammonia, ureum and glutamate were used by both organisms as sole nitrogen source (Figs 4, 6 & 7). Utilization seems to depend to some degree on the carbon source as *R. capsulatus* does not assimilate ureum in an acetate medium (Fig 7a). *Rh. rubrum* did not grow on glutamate and Ca-lactate (Fig. 6b). Previously however it had been cultured on the media, but growth was only observed after a week of incubation.

Betaine could only be assimilated by *R. capsulatus* growing on Ca-lactate (Fig 6a). Betaine, an amine, was tested because it is thought that amines will be present in the supernatant of the first compartment.

It is important that enough ammonia passes on to the denitrifying compartment. The phototrophic organisms are capable of using both organic and inorganic nitrogen sources. Experiments should be conducted to determine which nitrogen source is used preferentially to find out whether enough ammonia will remain for

the next compartment.
The initial reactor experiments (Fig 10) show the growth curve for *R. capsulatus* when grown on lactic acid and ammonia. The lag phase is about 2 days, logarithmic growth between day 2 and 5 and the stationary phase from day 6. A similar graph was obtained for the second experiments (Fig. 11). The lag phase is not properly indicated because samples were not taken before day 4. The maximum protein production for *R. capsulatus* was ∓0.5g/l ie. 0.77g/l dry weight (Fig 11a).
The lag phase for *Rh. rubrum* (Fig 12) appears to be about 4-5 days.

At present experimentation is continuing on nitrogen sources ie. amino acids and organic vs. inorganic sources. Photoautotrophic growth with CO_2 and H_2 will also be tested. An attempt is being made to link the first and second compartment by growing the phototrophs on the supernatant obtained from experiments with Clostridia. Further studies to be carried out by this lab will be elaborated on in Tn 6.2.

References

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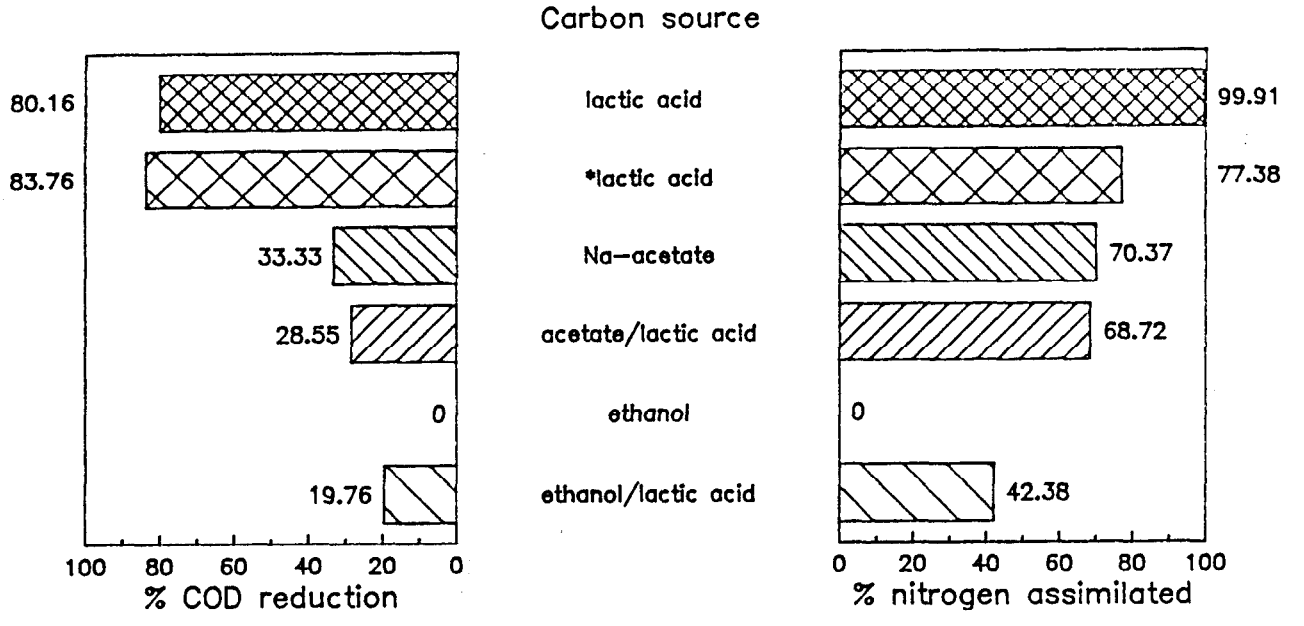
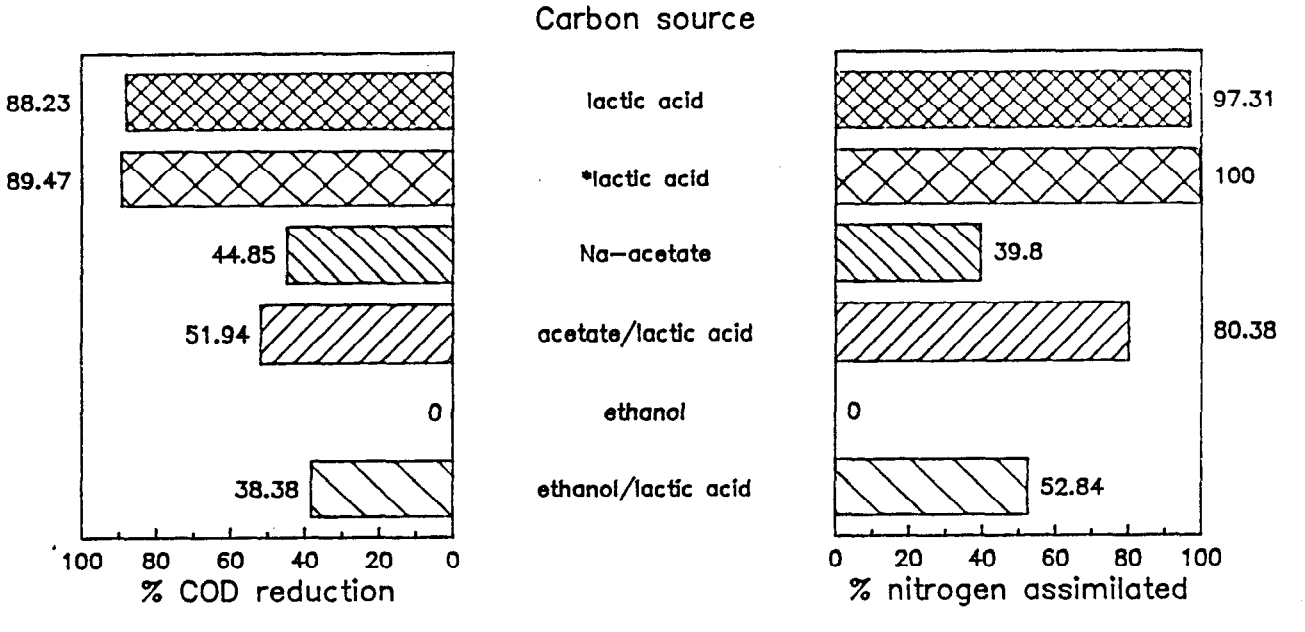
Rhodobacter capsulatus



% reduction in 6.5 days
*medium without yeast extract

Figure 1

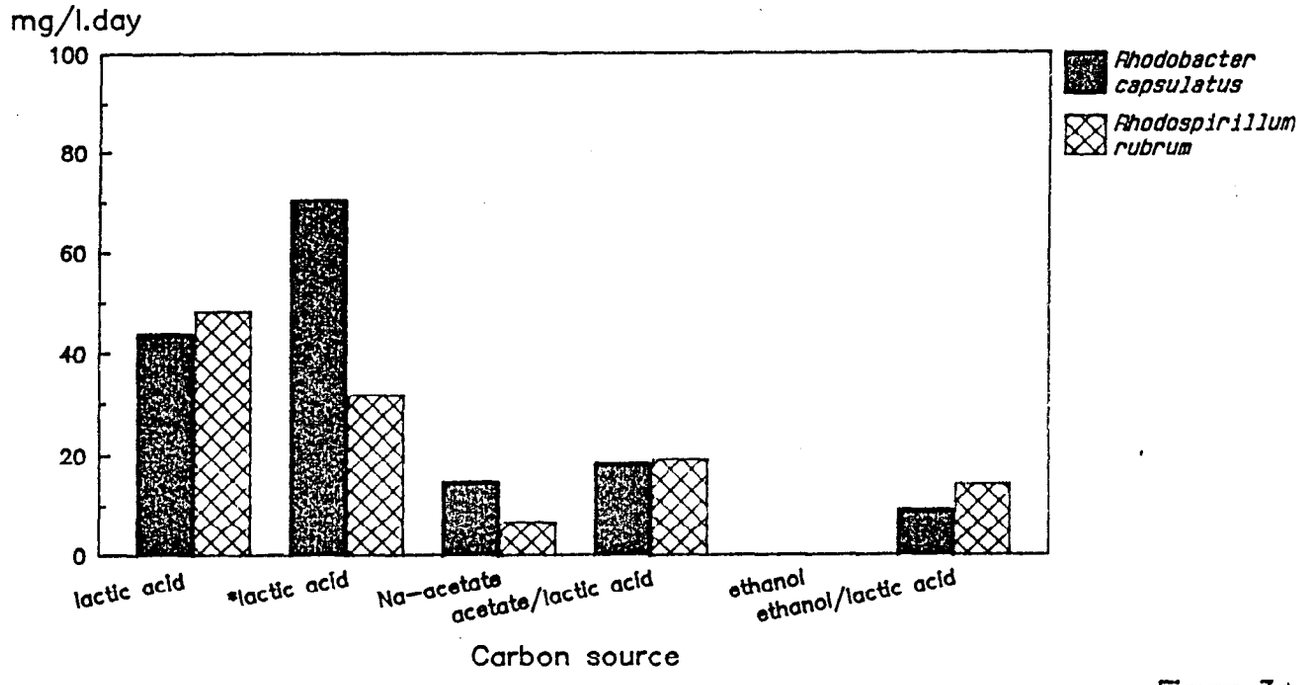
Rhodospirillum rubrum



% removed in 8.5 days
*medium without yeast extract

Figure 2

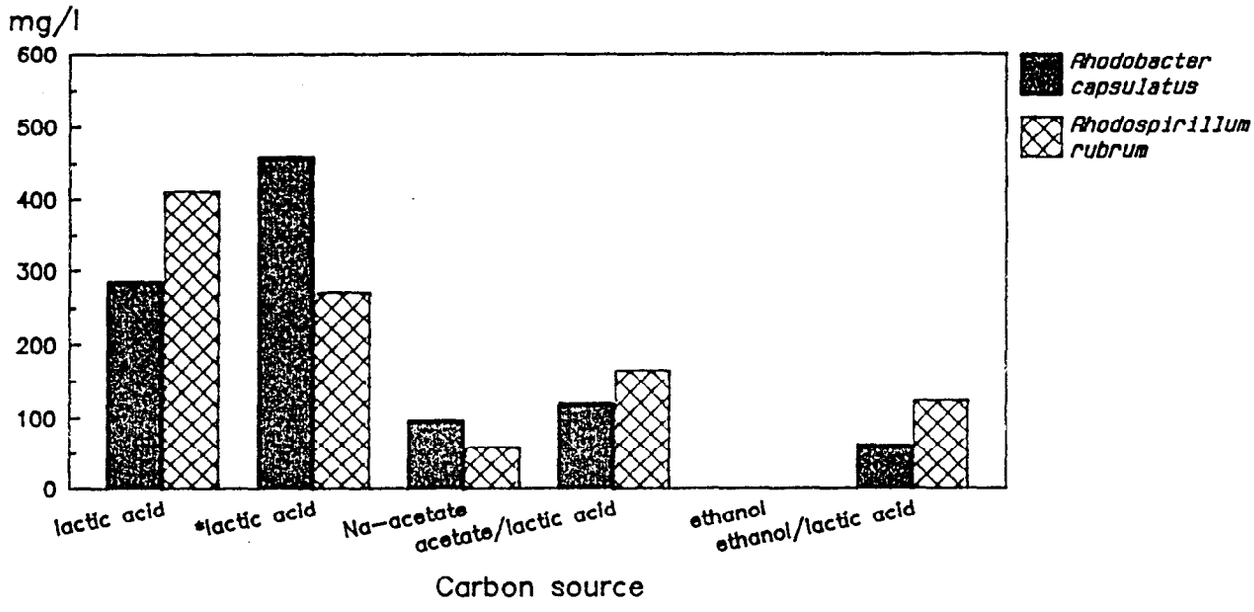
Protein production per day



*medium without yeast extract

Figure 3d

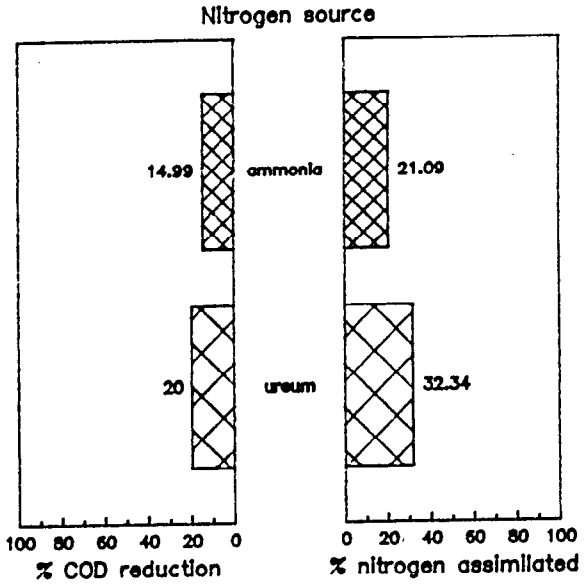
Protein production



Rhodobacter capsulatus 6.5 days
Rhodospirillum rubrum 8.5 days
*medium without yeast extract

Figure 3b

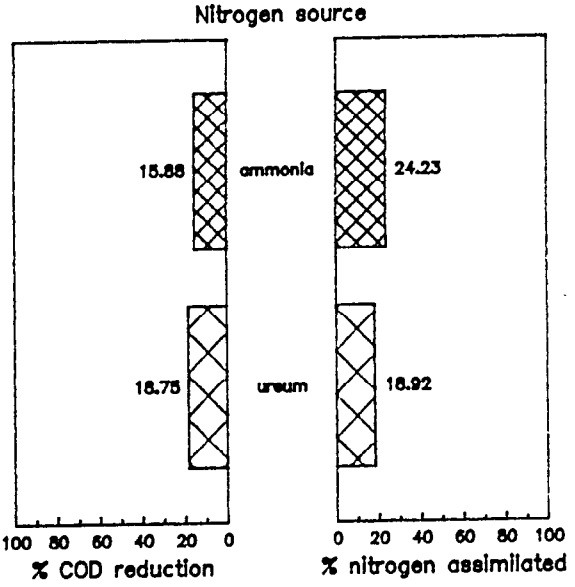
Rhodobacter capsulatus



% reduction in 5.5 days
30mM Na–butyrate
105 mg/l nitrogen

Figure 4a

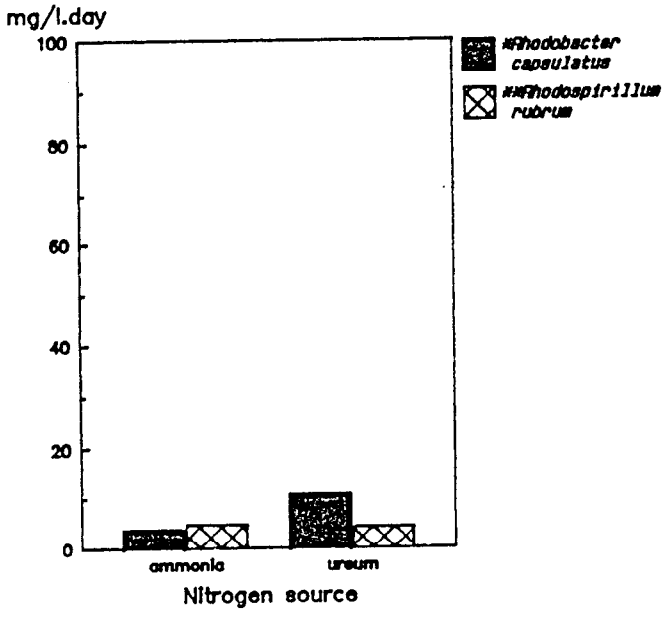
Rhodospirillum rubrum



% reduction in 7.5 days
30mM Na–butyrate
105mg/l nitrogen

Figure 4b

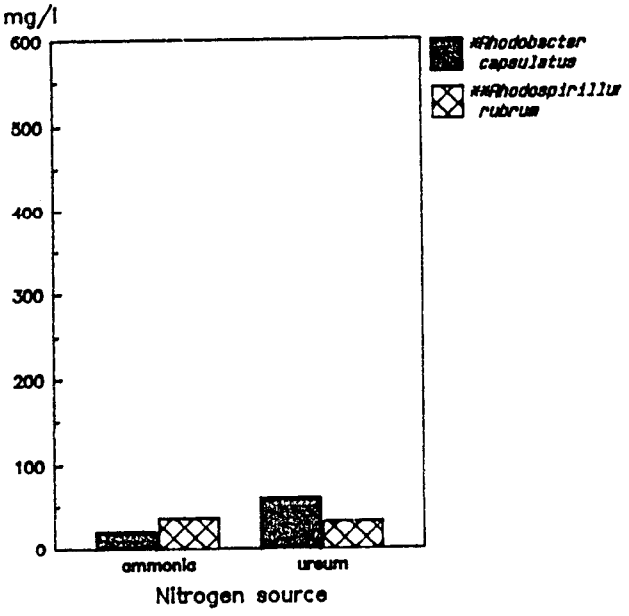
Protein production per day



30mM Na–butyrate
*5.5 days
**7.5 days

Figure 5a

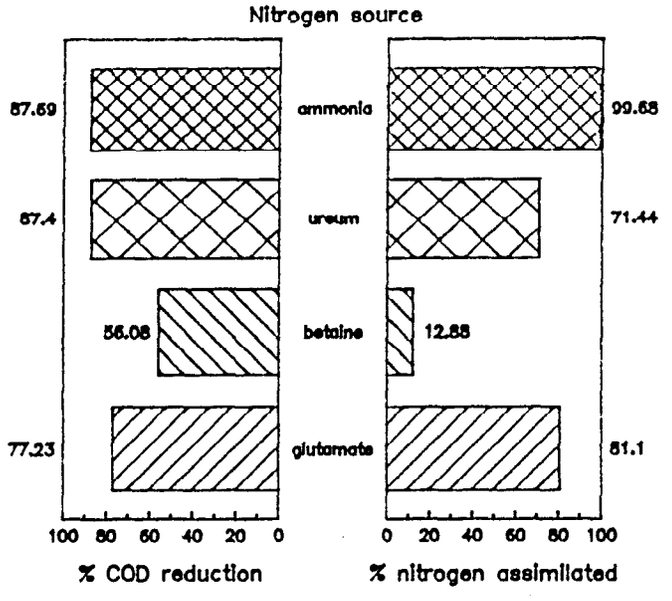
Protein production



30mM Na–butyrate
*5.5 days
**7.5 days

Figure 5b

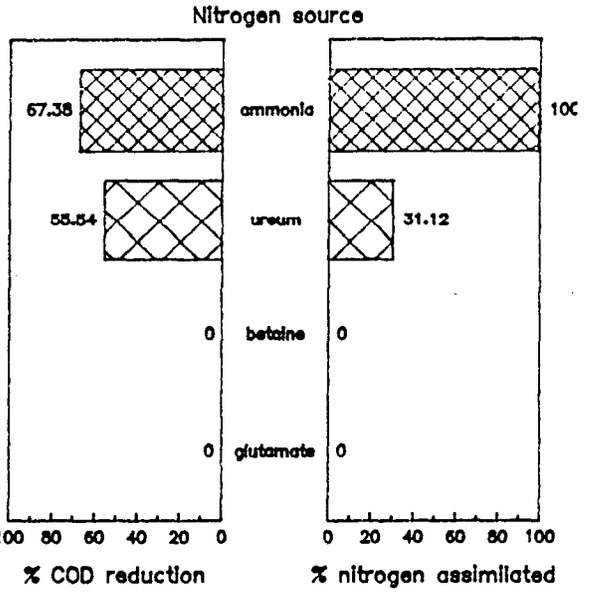
Rhodobacter capsulatus



% reduction in 6.5 days
105mg/l nitrogen
15mM Ca-lactate

Figure 6a

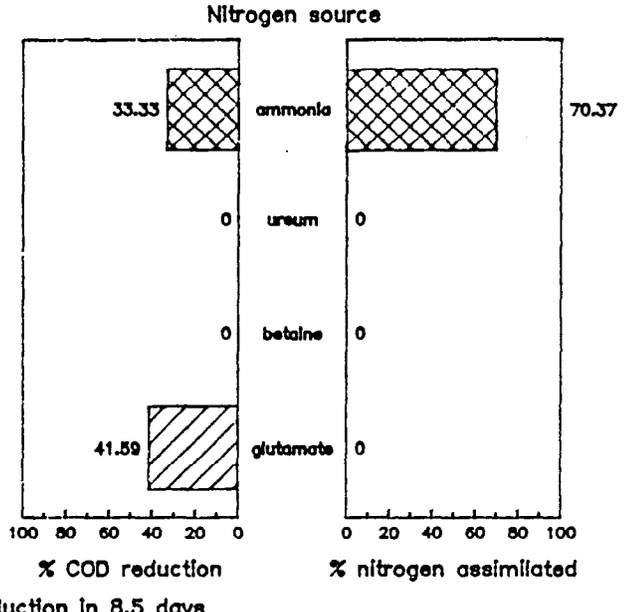
Rhodospirillum rubrum



% reduction in 8.5 days

Figure 6b

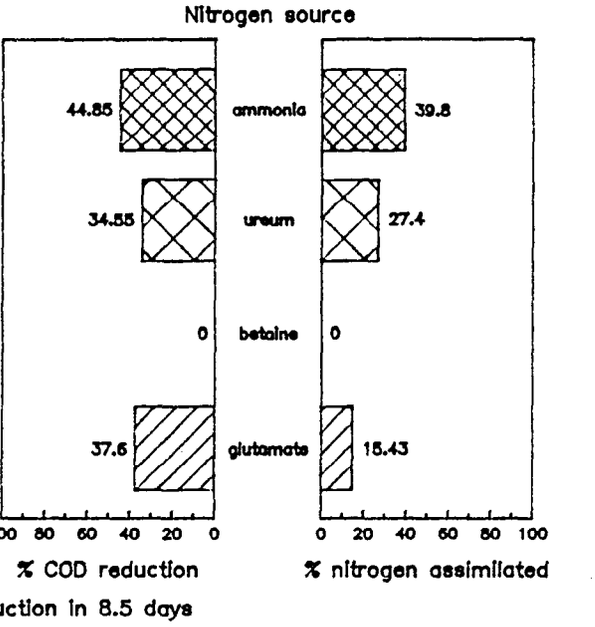
Rhodobacter capsulatus



% reduction in 8.5 days
105mg/l nitrogen
30mM Na-acetate

Figure 7a

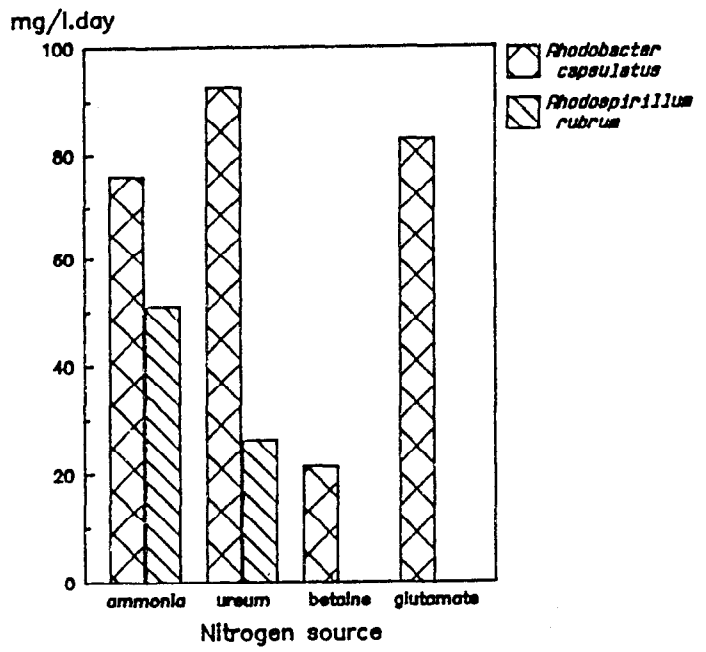
Rhodospirillum rubrum



% reduction in 8.5 days

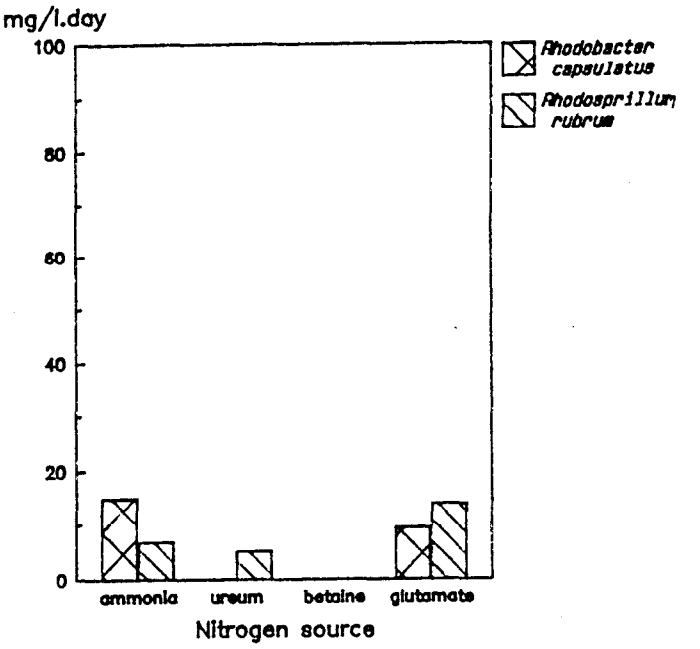
Figure 7b

Protein production per day



15mM Ca–lactate

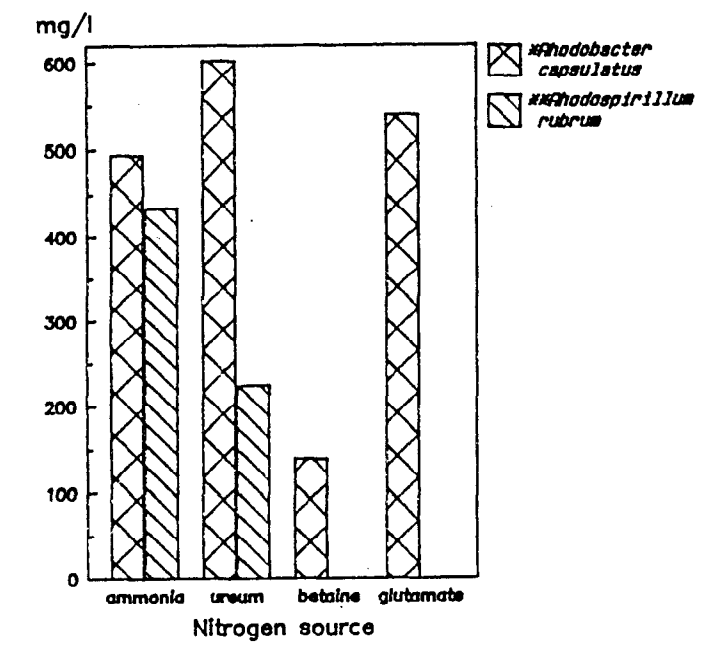
Figure 8a



30mM Na–acetate

Figure 8b

Protein production

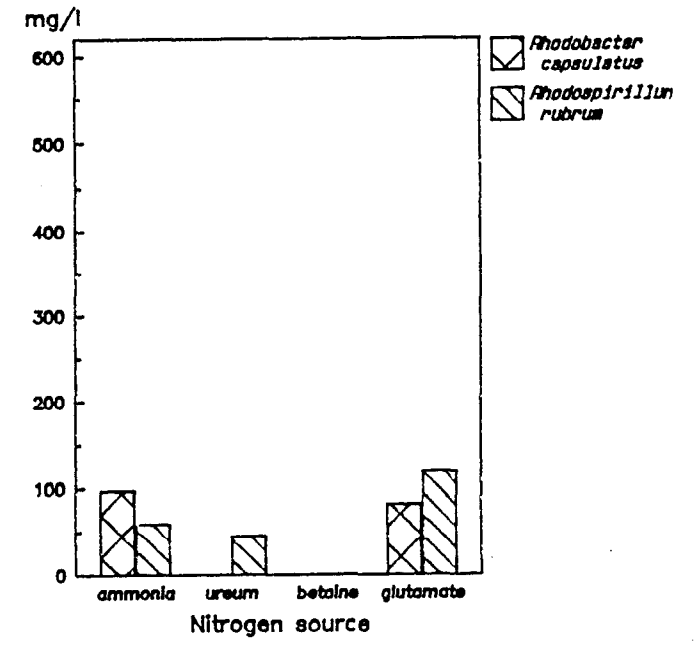


15mM Ca–lactate

* 6.5 days

** 8.5 days

Figure 9a 8.5 days



30mM Na–acetate

Figure 9b

Rhodobacter capsulatus
Reactor 1

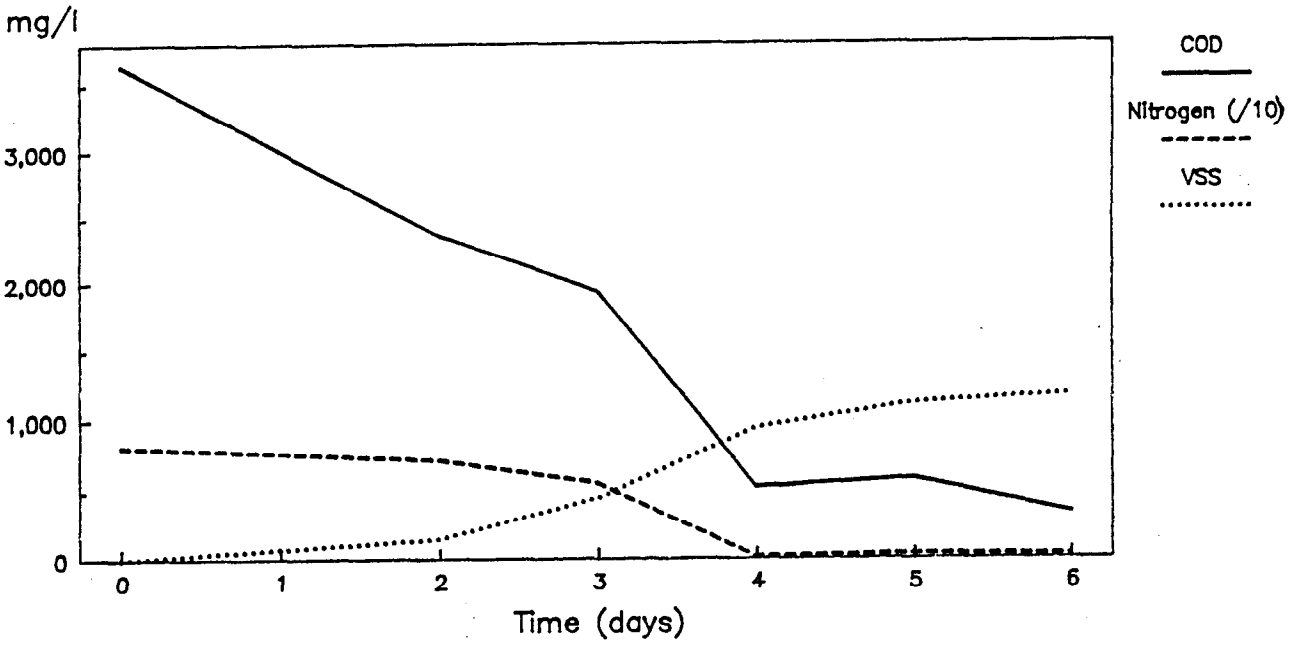


Figure 10a

Rhodobacter capsulatus
Reactor 2

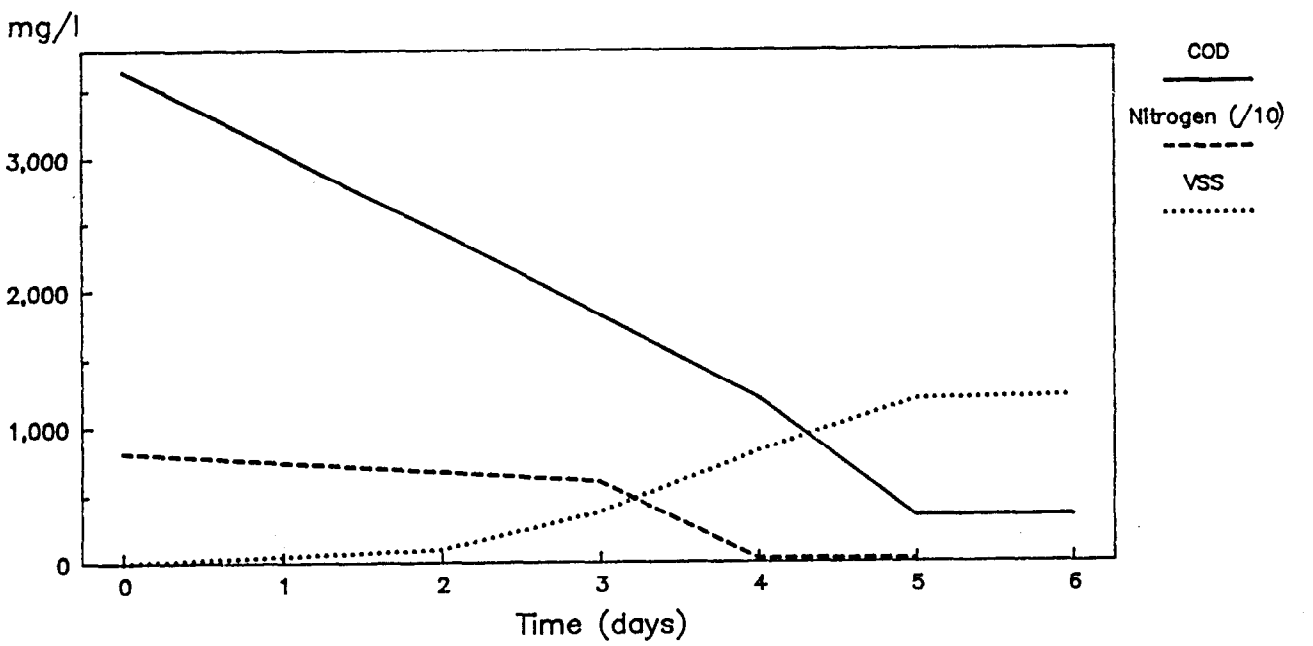


Figure 10b

Rhodobacter capsulatus
Reactor 1

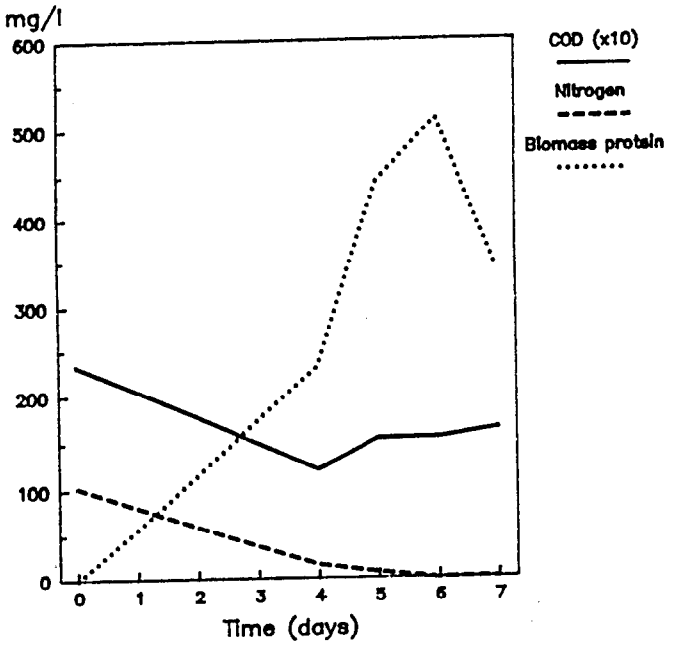


Figure 11a

Rhodobacter capsulatus
Reactor 2

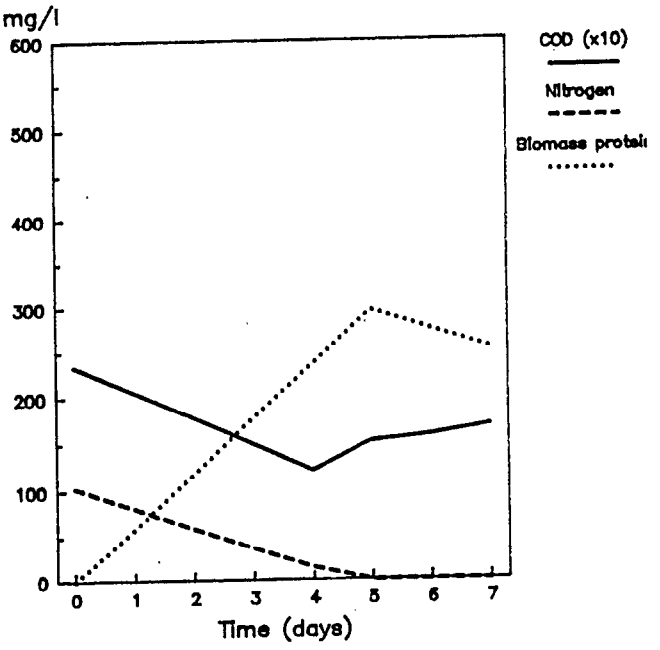


Figure 11b

Rhodospirillum rubrum
Reactor 3

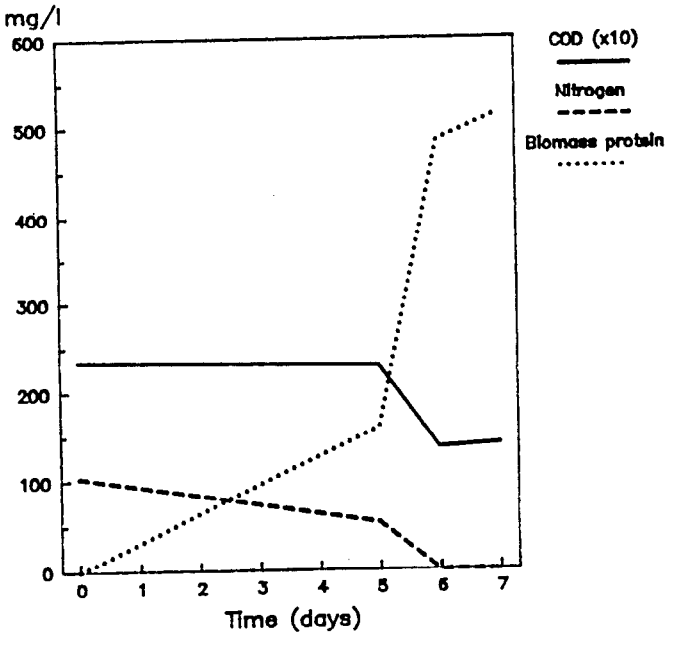


Figure 12a

Rhodospirillum rubrum
Reactor 4

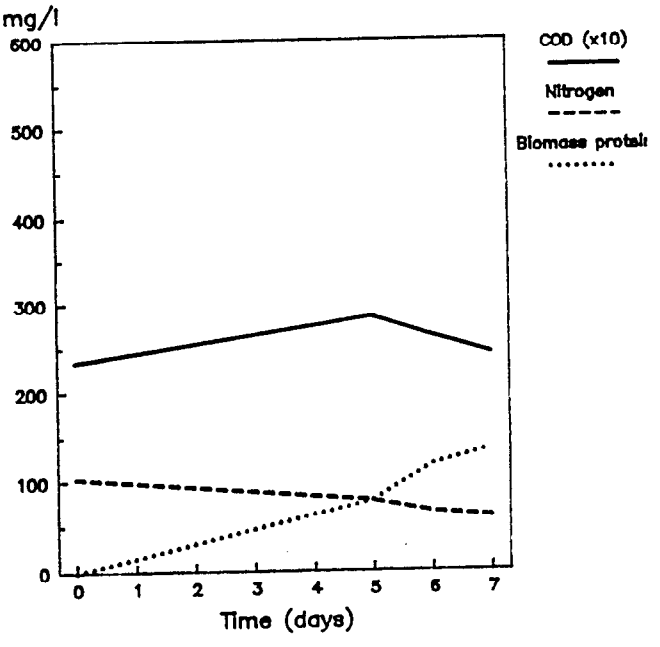


Figure 12b

Combination of carbon and nitrogen sources used by R. capsulatus and Rh. rubrum.

A. CARBON AND NITROGEN SOURCES USED BY RHODOBACTER CAPSULATUS

	Ammonium	urea	betaine	glutamate	dinitrogen
lactic acid	+	+	+	+	ND
acetic acid	+	-	-	+/-	ND
butyric acid	+	+	ND	ND	ND
ethanol	-	ND	ND	ND	ND
CO_2 & H_2	+	ND	ND	ND	-

B. CARBON AND NITROGEN SOURCES USED BY RHODOSPIRILLUM RUBRUM

	Ammonium	urea	betaine	glutamate	dinitrogen
lactic acid	+	+	-	+/-	ND
acetic acid	+	+	-	+	ND
butyric acid	+	+	ND	ND	ND
ethanol	-	ND	ND	ND	ND
ethanol + lactic acid	+	ND	ND	ND	ND
CO_2 & H_2	+	ND	ND	ND	+

ND : not done + : efficient growth ± : poor or less efficient growth - : no growth

2. UTILIZATION OF AN ORGANIC AND AN INORGANIC NITROGEN SOURCE BY THE PHOTOTROPHS

Objective

To determine whether there is a preference for an organic nitrogen source (ureum) or an inorganic nitrogen source (ammonium).

Methods

Medium was prepared as before but different concentrations of ammonium sulphate and ureum were added (Table 1).

Results

Results are illustrated graphically as percentage reduction of ammonia nitrogen, organic nitrogen and COD as compared to uninoculated medium. Figure 1 illustrates the percent reduction of the three factors by *Rhodobacter capsulatus* after incubation for four days. Figure 2 illustrates the percent reduction for the three factors by *Rhodospirillum rubrum* after 8 days incubation. Figure 3 illustrates the amount of biomass protein produced by *R. capsulatus* after 4 days and by *Rh. rubrum* after 8 days.

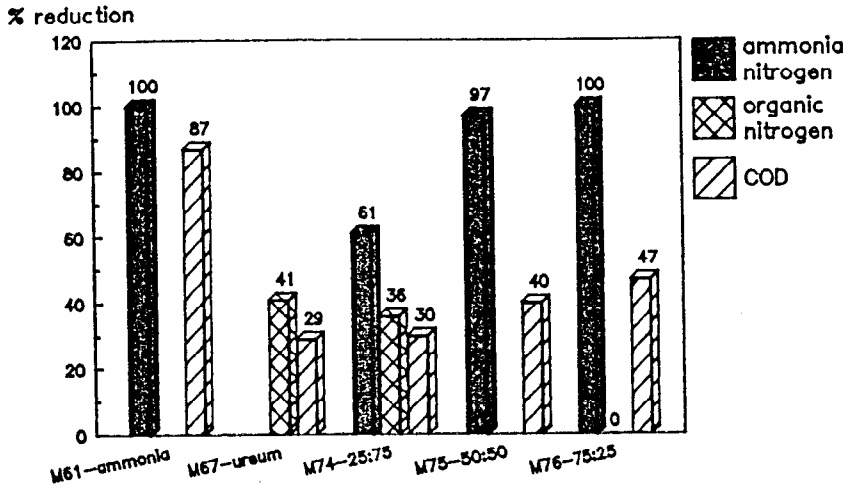
Discussion

The percent reduction of the ammonia nitrogen was always greater than that of the organic nitrogen (Figs 1 & 2). Higher levels of protein were produced when ammonia was the sole nitrogen source and when it comprised 75% of the available nitrogen (Fig 3). COD reduction was greatest when ammonia was the sole nitrogen source (Figs 1 & 2).
Both organisms preferred the inorganic nitrogen source (ammonium) to the organic nitrogen source (ureum).

Table 1. Amount of ammonium sulphate and ureum added to the media

	ammonium sulphate (g/l)	ureum (g/l)
Medium 61	0.500	0.000
Medium 67	0.000	0.272
Medium 74; 25:75	0.125	0.170
Medium 75; 50:50	0.250	0.114
Medium 76; 75:25	0.375	0.057

Rhodobacter capsulatus
4 days incubation



ammonia : ureum

Fig. 1

Rhodospirillum rubrum
8 days incubation

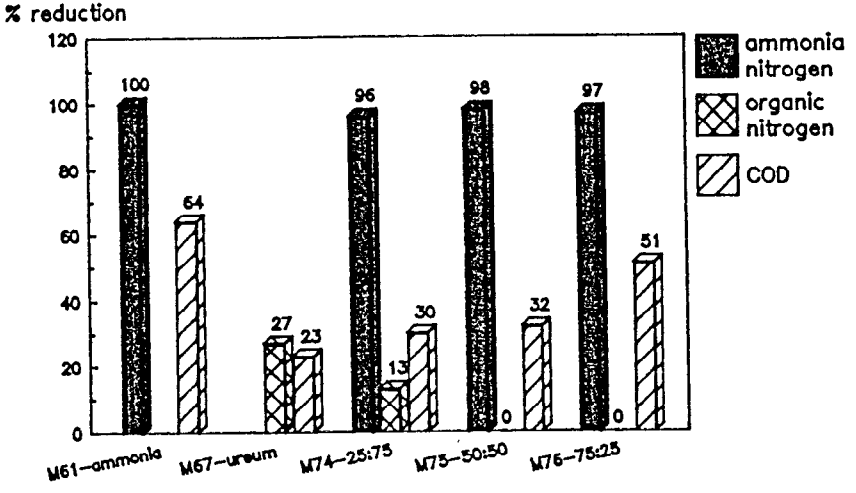
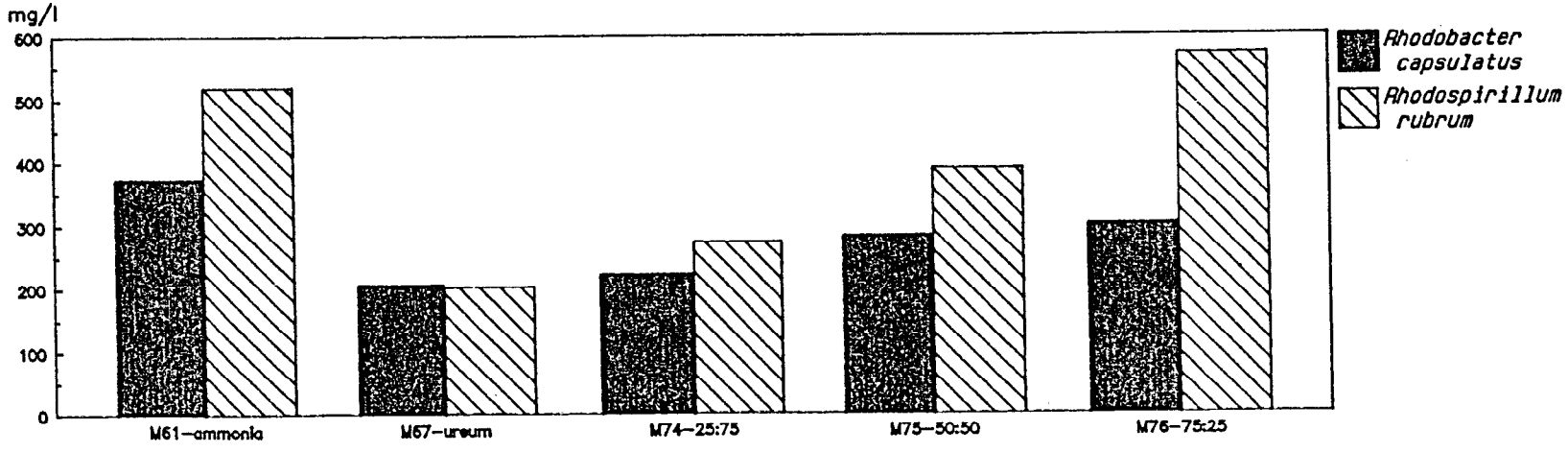


Fig. 2

Protein production



R. capsulatus; 4 days incubation
Rh. rubrum; 8 days incubation

Fig. 3

TECHNICAL NOTE 6.3

GROWTH PARAMETERS OF *NITROSOMONAS* AND *NITROBACTER* IN MIXED, BATCH CULTURES

INTRODUCTION

Studies on growth of *Nitrosomonas* and *Nitrobacter* were undertaken in view of mathematical modelling of the nitrifying compartment and of the entire MELISSA artificial ecosystem. The required informations concerned mainly: 1) the composition of the produced biomass, 2) the kinetics of conversion of ammonium to nitrite and nitrate, 3) the effect of limitations by substrates (ammonia, nitrites) and oxygen transfer on these processes, and 4) the yield of such conversions, related to consumed CO2.

Batch cultures appeared the most appropriate conditions for that purpose. However, bibliographic data relate a very low growth rate and biomass production of these nitrifying bacteria, particularly in non-fixed cultures conditions, and culture problems resulting from inhibition of growth by substrates or products (1-4). In consequence, most of available results concern continuous cultures of pure or mixed strains that are not of great utility for our purpose. It therefore appeared that studies of growth parameters of *Nitrosomonas* and *Nitrobacter* in batch, mixed cultures could present some difficulties.

I - MATERIALS AND METHODS

1°) Culture conditions

Nitrosomonas europea (ATCC 25978) and *Nitrobacter winogradsky* (ATCC 25391) were provided by Pr W. VERSTRAETE, (Rijks Universiteit, Gent, Belgium). Both strains were mixed and grown in darkness, at 28°C in the culture medium described by SCHMIDT et al. (5).

Composition of the culture medium

NH_4Cl	0.8	g	
Na_2HPO_4	5	g	
KH_2PO_4	0.5	g	
Sol Fe-EDTA	5	ml	
Sol A	1	ml	
Sol B	10	ml	
Distilled water to	1000	ml	---> pH ~ 8

Solution Fe-EDTA

$FeSO_4;7\ H_2O$	77	mg
EDTA	103	mg
Distilled water to	50	ml.

Solution A			Solution B	
$ZnSO_4$; 7 H_2O	2	mg	$MgSO_4$; 7 H_2O	0.2 %
$CuSO_4$; 5 H_2O	2	mg		
Na_2MoO_4; 2 H_2O	2	mg		
Distilled water to	100	ml		

Precultures were carried out in stirred 700 ml erlenmeyers containing 250 ml culture medium. pH was maintained at 8 by $NaHCO_3$ 0.06 g/l. Experimental cultures were performed in a SETRIC SET 2M reactor, with air bubbling (24 l/h) and stirring at 200 to 400 rpm. pH was maintained constant by controlled addition of sterile NaOH 1M. Modifications of culture conditions are described in the text.

2°) Titration methods

-Ammonium was spectrophotometrically titrated using the Merck kit Spectroquant 14752. Absorbance at 690 nm allowed to titrate ammonium in the culture medium with a sensitivity of 0.02 mg/l.

-Nitrites were spectrophotometrically titrated, according to GRIESS (6), using the Merck kit Spectroquant 14776. Absorbance at 525 nm allowed to titrate nitrites accumulated in the culture medium with sensitivity of 0.03 mg/l.

-Nitrites plus nitrates were spectrophotometrically titrated according to CAWSE (7). Samples of cultures were filtered through 0.45 µm Millipore filters to eliminate cells and to reduce interferences with polysaccharides. The filtrate was diluted with 5 % (v/v) perchloric acid and light absorption by oxidized nitrogen was measured at 210 nm, against fresh culture medium (free of oxidized nitrogen and of organic matter). The obtained values were corrected from absorbance by organic matter by substracting the double of absorbance measured at 275 nm. This method allowed to titrate nitrogen with a sensitivity of 0.002 mg NO_2^--NO_3^-/l.

-Nitrate concentration was obtained by substracting the amount of nitrite from total oxidized nitrogen.

II - RESULTS

1°) Precultures

Both strains were mixed and cultured in the Schmidt medium, in erlenmeyer flasks, in view of inoculation of the 2 l reactor. It appeared that: 1) depending on some still unknown parameter, growth occurred in some flasks and not in others, 2) when successful, the precultures took about 3 weeks before containing enough biomass for inoculation.

Such results could be explained by some inhibitory effect of the high concentration of ammonium in the culture medium. Moreover, in that hypothesis, it would be desirable to verify in those precultures that have grown, that the relative proportion of both strains is still conserved.

In any case, it would perhaps be necessary to grow *Nitrosomonas* and *Nitrobacter* as separate cultures, on mediums containing reduced concentration of ammonium or nitrite, or as mixed cultures in fed batch conditions.

2°) Controlled cultures

Controlled cultures were carried out in the 2 l Setric reactor with non fixed or fixed conditions.

a) Non fixed cultures

One liter of the Schmidt culture medium was inoculated with 20 ml of mixed preculture. Numerical results are presented in Table I.

-Biomass production. Biomass production was spectrophotometrically followed at 560 nm. We did not succeed in correlating OD_{650} to biomass in g/l because not enough biomass was available, even in "dense" cultures.
In these experimental conditions, figure 1 shows that no significant growth occured during a long lag phase of approximately 250 h. Increasing the concentration of the carbon source by addition of 3 mg $NaHCO_3$, or reducing the rate of stirring from 400 to 200 rpm remained without significant effect. A slow accumulation of biomass appeared after 250 h, that coincided with the enrichment of the mixed culture by addition of 7 ml of a dense preculture of *Nitrobacter*.

- Nitrification. Consistent with the duration of the lag phase, the concentration of ammonium in the culture medium did not change during the first 250 h of culture, and therefore neither nitrites nor nitrates accumulate during that time (fig. 2). The subsequent accumulation of nitrites, which is accompanied by a limited accumulation of nitrates could be correlated to the enrichment of the culture in *Nitrobacter*.

Anyhow, these preliminary and ambiguous results express that: 1) the precultures used for inoculation may have been largely enriched in the *Nitrosomonas* partner, 2) growth and nitrification are very slow in non fixed, mixed cultures, and 3) *Nitrobacter* could be largely inhibited by high concentrations of ammonium.

We therefore undertook to grow fixed, mixed cultures under conditions of low concentrations of ammonium.

b) Fixed cultures

Ammonium concentration in the Schmidt culture medium was reduced from 0.28 g/l to 0.028 g/l and small pieces (0.5 cm) of porous polyurethane were added in the reactor for cell fixation. Numerical results are presented in table II.

- Biomass production: Cells fixation impairs any estimation of biomass production.

Fig.1- Growth of Nitrosomonas-Nitrobacter in batch, non-fixed mixed culture.

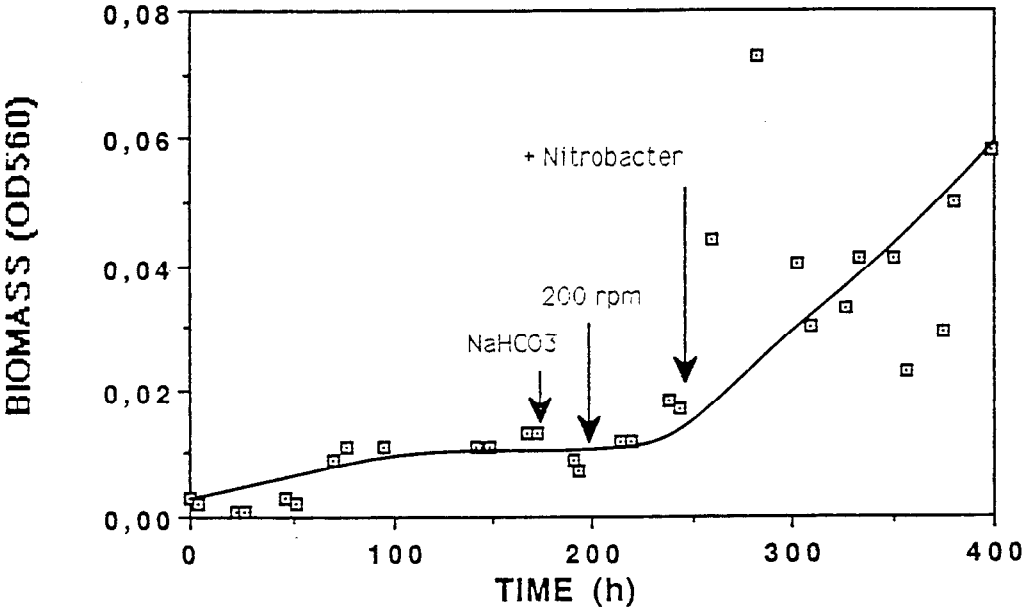


Fig.2- Nitification by mixed, batch culture of Nitrosomonas-Nitrobacter.

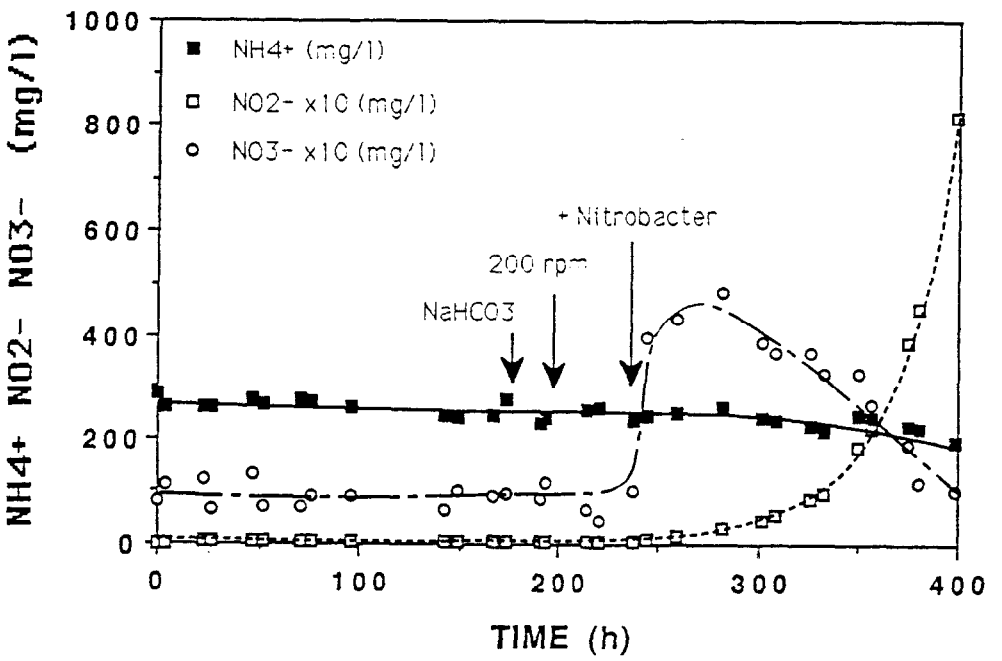


Fig.3- Nitrification by fixed, mixed batch culture of Nitrosomonas-Nitrobacter.

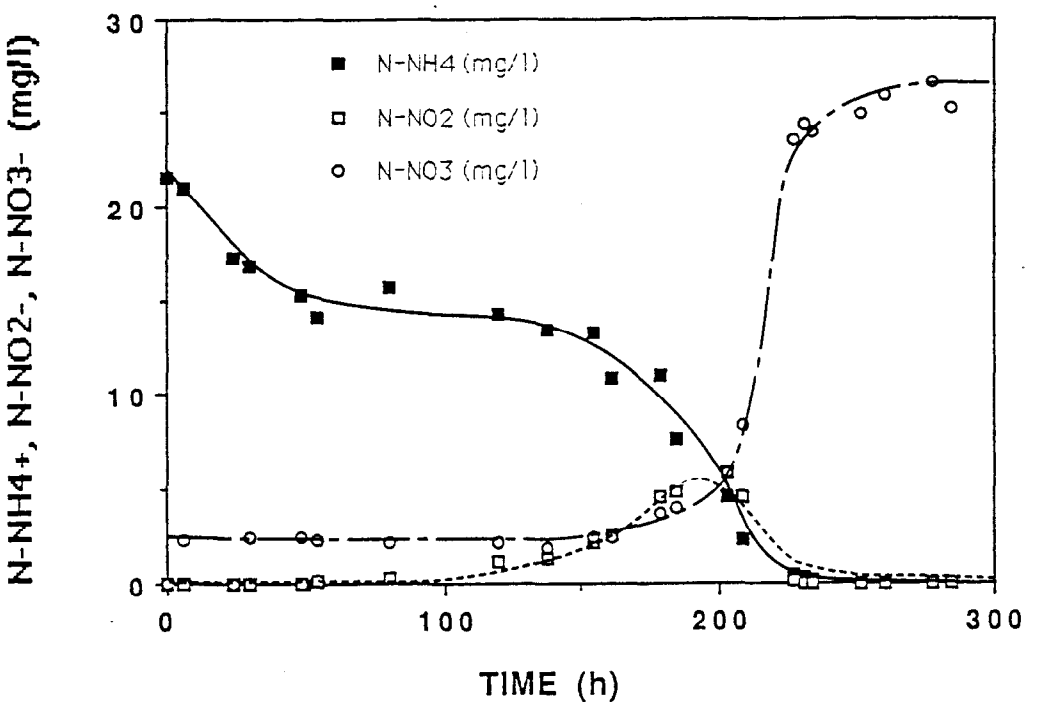


Table I - Biomass accumulation and nitrification during non-fixed culture of Nitrosomonas -Nitrobacter: numerical results.

TIME (h)	BIOMASS (OD560)	NH4+ (mg/l)	N02- (mg/l)	NO3- (mg/l)
0,0	0,003	283,20	0,61	79,20
4,0	0,002	258,60	0,37	110,40
22,0	0,001	258,50	3,06	122,80
27,0	0,001	260,70	3,56	67,10
46,3	0,003	274,10	5,00	133,50
52,3	0,002	263,00	5,85	73,10
70,3	0,009	277,90	6,27	69,20
76,3	0,011	269,10	6,37	93,80
96,0	0,011	257,70	4,92	89,70
142,3	0,011	246,20	5,15	63,90
148,3	0,011	242,20	5,54	103,30
166,3	0,013	244,20	5,75	94,20
172,3	0,013	276,60	6,60	96,50
190,3	0,009	231,47	5,60	87,10
193,3	0,007	241,71	6,27	117,20
214,3	0,012	254,72	5,99	64,20
220,3	0,012	259,33	6,71	45,00
238,3	0,018	232,81	6,95	101,90
244,3	0,017	245,96	8,49	395,80
259,0	0,044	246,60	12,94	431,60
282,0	0,073	258,64	29,50	483,60
302,0	0,040	241,65	48,40	385,60
308,0	0,030	233,56	56,80	365,30
326,0	0,033	225,15	85,30	368,70
332,0	0,041	212,15	98,00	327,00
350,0	0,041	243,96	184,30	325,10
356,0	0,023	239,56	217,00	265,80
374,3	0,029	225,94	387,70	190,00
380,3	0,050	219,55	452,00	116,60
398,3	0,058	195,53	817,30	101,50

Table 2 - Nitrification during fixed culture of Nitrosomonas-Nitrobacter: numerical results.

TIME (h)	N-NH4+ (mg/l)	N-NO2- (mg/l)	N-NO3- (mg/l)
0,000	21,480	0,015	0,000
6,000	21,010	0,000	2,220
23,300	17,220	0,018	0,000
29,300	16,850	0,000	2,360
47,300	15,250	0,061	2,390
53,300	14,200	0,078	2,330
79,000	15,770	0,280	2,170
119,300	14,340	1,110	2,150
137,300	13,490	1,230	1,800
155,300	13,290	2,070	2,400
161,300	10,890	2,580	2,430
179,300	10,970	4,580	3,670
185,300	7,710	4,820	3,960
203,300	4,480	5,760	5,800
209,300	2,330	4,530	8,310
227,300	0,440	0,140	23,560
231,300	0,320	0,012	24,310
234,300	0,150	0,022	23,860
251,300	0,046	0,007	24,970
260,300	0,028	0,006	25,880
278,000	0,025	0,004	26,620
284,300	0,039	0,009	25,180

- Nitrification: conversion of N contained in ammonium to N contained to nitrite or nitrate is illustrated in figure 3. The initial low decrease in ammonium concentration could be not significant, since not accompanied by any accumulation of nitrite or nitrate. Under these new culture conditions, the lag phase is reduced to approximately 100 h, when ammonium begins to be oxidized to nitrite. Subsequently, ammonium is actively oxidized to nitrate, with a transitory and limited accumulation of nitrite.

The sum of total concentration should remain constant at any stage of the culture. The observed deviations probably result from some inaccuracy in experimental titrations, which therefore should be improved.

Anyhow, it appears from this experiment that batch cultures of mixed strains should be performed under conditions allowing cell fixation, and under conditions of low concentrations of ammonium which probably inhibits the *Nitrobacter* partner. The preliminary obtained results allow to gain some insight in the kinetics parameters of oxidation of ammonium to nitrite and nitrate , and on the effects of ammonium limitations, which both are required for mathematical modelling of the compartment.

CONCLUSION

The encountered difficulties in growing mixed cultures of *Nitrosomonas* and *Nitrobacter* confirm that the analysis of this nitrifying compartment requires time and experience in the field of microbiology. Growth parameters required for mathematical modelling of the nitrifying compartment will have to be determined in batch cultures of mixed strains under conditions that allow cell fixation, and under low concentrations of ammonium, that could certainly be achieved by fed batch cultures.

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