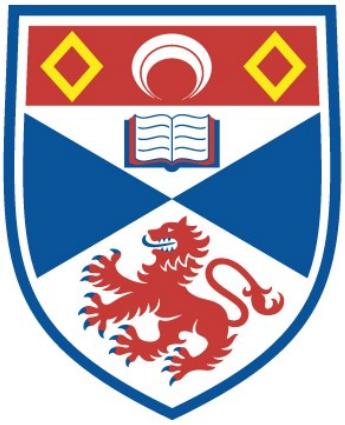
AMMONIA ASSIMILATION IN 'SACCHAROMYCES CEREVISIAE' UNDER CHEMOSTATIC GROWTH

Maria Virginia Campos Lacerda

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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AMMONIA ASSIMILATION IN *SACCHAROMYCES CEREVISIAE* UNDER CHEMOSTATIC GROWTH .

A thesis presented by

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to the University of St. Andrews

in application for the degree of

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DECLARATION

I hereby declare that this thesis is based on work carried out by me, that the thesis is of my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, University of St. Andrews, under the direction of Dr. W. M. Ledingham.

CERTIFICATE

I hereby certify that Maria Virginia Campos Lacerda has spent nine terms engaged in research work under my direction and that she has fulfilled the conditions of Ordinance General Nº 12 of Resolution of the University Court 1967, Nº 1, and that she is qualified to submit the Accompanying thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I graduated with the degree of Bachelor in Biosciences in 1977 from the Federal University of Pernambuco, Recife, Brazil.

I graduated with the degree of Master in Biochemistry in 1981 from the Biochemistry Department of the Federal University of Pernambuco, Recife, Brazil.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in December 1987.

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1. INTRODUCTION

1.1 SCIENCE AND TECHNOLOGY IN BRAZIL

Although Brazil had been discovered by the Portuguese in 1500 , it is not until the 19th century that one can talk about science and technology in that country. Because of the Napoleon war , the Portuguese royal family moved to Brazil in 1808 , taking with them artists , intellectuals and scientists . King Joao VI opened Brazilian ports to the world trade and lifted restrictions on local manufacturing . Also many institutions of science and technology were developed . As a consequence , Brazil was elevated from the category of "Colony" to the "Kingdom United to Portugal".

During the 20th century , many faculties were created , specially in the Southeast area (Rio de Janeiro / Sao Paulo) , because of their economic and political power (1) .

Between the last century end and the beginning of the present century thousands of immigrants like Italian , Japanese , German and Portuguese went to Brazil mainly to the area of coffee plantation in Southeast , helping to develop the local industry . But all the technology was foreign . At that time , Brazil

had only three Universities , two in the Southeast and one in the South (2).

From 1948 , some institutions were created with the purpose of science stimulation . Such institutions like "The National Council for Science and Technology Development" (CnPq) or "The Council to Improve the Training of University Teachers" (CAPES) could provide grants to individuals for research and for fellowship (1) .

In 1956 , the Brazilian President Kubitscheck launched his administration aiming at "fifty years of development in five" . He favoured private investment and opened Brazil to foreign penetration by transnational corporations , through offers of tax incentives and easy profit remission . However, in part due to the massive state spending on the construction of Brasilia and the road system , and in part to the neglect of agriculture , inflation had risen by 1961 to almost 50% per year (3). A succession crisis followed , with the emergence of new forms of popular and worker organization such as "Peasant's League" in the Northeast . In response , the generals took over the power and a New Regime was installed (3).

The "boom" known as the Brazilian "economic miracle" started in 1968 . The government subsidised the purchase of farm machinery and the agricultural sector became a leading consumer of

industrial products and export earner. But the importation of technology made national technology an unimportant matter.

The Brazilian economy , more than any other epitomized the oil age . The country's entire industrial and transport infrastructure had been developed on the assumption of cheap petroleum . With the tripling of oil prices in 1974 , the increased bill for fuel imports , coupled with the effects of recession in all the major Western economies , put paid to this rapid expansion (3).

In 1975 , Brazil developed the "National Alcohol Program" - PROALCOOL , with the aim of substitution of gasoline and petrochemicals by alcohol. The use of ethanol as a fuel in internal combustion engines is technically feasible : although ethanol has a caloric content that is lower than petrol , when it is burned in a properly designed engine it delivers more power than petrol. Furthermore , the amount of pollutants emitted is lower (4). And also, being a tropical country, Brazil has a variety of microorganisms that could be studied aiming to an improvement in ethanol production and other purposes.

In recent years novel development in the ability to select and manipulate microorganisms and their genetic material have led to an unprecedented interest in their industrial use.

By definition , microbiology is the study of microorganisms. But it is important to recognize that microorganisms are very heterogeneous . And also , compared with the vast number of known species , the number of organisms that have been used commercially is extremely small. The participation of microbial physiologists , biochemists and geneticists is required to further exploit the potential of both known and newly discovered organisms.

The ability of microorganisms to modulate their cell structure , chemistry and function is remarkable . Microbial physiology is concerned with the study of the interaction between an organism and its environment and more particularly with attempts to unravel the relationship between metabolic capability and changes in the environment in which the organism exists.

After the realization of "1st Seminar on Energy from Biomass in the Northeast " in 1978 , the basis for the establishment of a biomass program was delineated in Brazil (5).

Although struggling with the highest foreign debt the world has ever known , Brazil government has invested money in the universities with the purpose of developing a "Brazilian technology" , exploring the natural resources and making the best use of them. It is within this context that a biomass program presents itself as one of the best strategies for development

The Biomass Conversion Program (PROALCOOL) and special grants for Biotechnology researches have provided an opportunity for scientist and technologists to acquire skills in the subject , with the aim of developing a technology appropriated to the Brazilian reality.

In the present research we intend to study the kinetics of genetically engineered yeasts under chemostatic culture conditions . The skills acquired with such a study could be applied to other microorganisms and other problems in Brazil .

These studies will involve the use of a microprocessor-controlled bioreactor , build in the Biochemistry Department of the University of St.Andrews by Lima Filho (6). This controller was designed using Z - 80 chip technology. Z-80 technology chips are freely available at reasonable cost in Brazil.

The microorganism chosen for this project was the baker's yeast *Saccharomyces cerevisiae* . Most of the nitrogen required for its growth in large scale is usually supplied as added ammonia . Consequently , the pathways of ammonia assimilation are of great importance in the production of yeast biomass. Although the enzymes of the pathways and some of the factors affecting their regulation have been determined , there are still considerable gaps in our knowledge, e.g. which pathway is

physiologically more important under different growth conditions. Our proposed research programme aims to study the effect of a GOGAT$^-$ mutation on the growth characteristics of *Saccharomyces cerevisiae* in order to improve our understanding of the physiology of ammonia assimilation.

1.2 A SUMMARY OF THE NITROGEN METABOLISM IN *S.cerevisiae*

S.cerevisiae is able to use, as nitrogen source, a variety of amino acids , uracil and purine derivatives , urea and ammonia . However , only a limited number of these compounds and their metabolic fates have been studied .

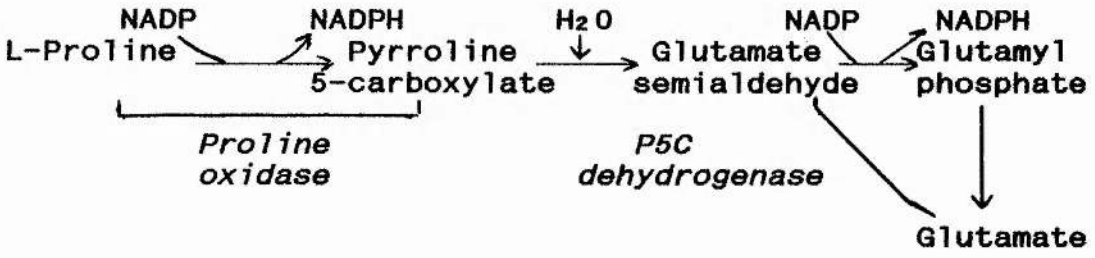
The pathways of nitrogen catabolism may be conveniently divided on the basis of their end products . Some systems , like those degrading allantoin , urea or asparagine , generate ammonia as the final product. Then , a route is required to convert ammonia to glutamate , since the primary products of ammonia assimilation by *S.cerevisiae* are L-glutamate and L-glutamine. Other systems , like proline and arginine metabolism , generate glutamate directly . These facts emphasize the central

position of ammonia and glutamate at the interface between nitrogen catabolic and anabolic reactions .

1.2.1 Glutamate as end product

1.2.1.1 Proline degradation

Proline is degraded in reactions which are just the reverse of its synthesis although catalysed by different enzymes . The first reaction is the oxidation of proline , catalysed by the Proline oxidase , followed by hydration . The Pyrroline-5-carboxylate dehydrogenase catalyses the two final steps (7).



It has been shown that the production of Proline oxidase and P5C dehydrogenase is inducible by proline . Also , addition of arginine and ornithine to the culture result in partial induction of Proline

appear to be repressible by growth in the presence of readily used nitrogen sources . However it seems that the transport system is depressed in cells growing in minimal ammonia medium (9).

1.2.1.2 Arginine degradation

The products of arginine degradation by Arginase have been identified as ornithine and urea (10). Arginase has a pH optimum of 8.5 - 9.0 and a Km of 5 - 7 mM for arginine . It is a trimeric enzyme of 39 kD (11) . The ornithine formed is converted to glutamate semialdehyde by the Ornithine transaminase . It has been shown that proline is an essential intermediate in the arginine degradation. The glutamate semialdehyde formed is converted to proline via P5C reductase in a biosynthetic reaction in the cytosol (12) . *S.cerevisiae* does not contain urease activity to degrade urea to ammonia , however, an alternative mode of urea degradation in *Chlorella* , *Candida utilis* (13) and *S.cerevisiae* has been reported (14) . This degradative reaction is catalysed by two enzymes : Carboxylase , which requires Mg^{++} and K^{++} , and Allophanate hydrolase .

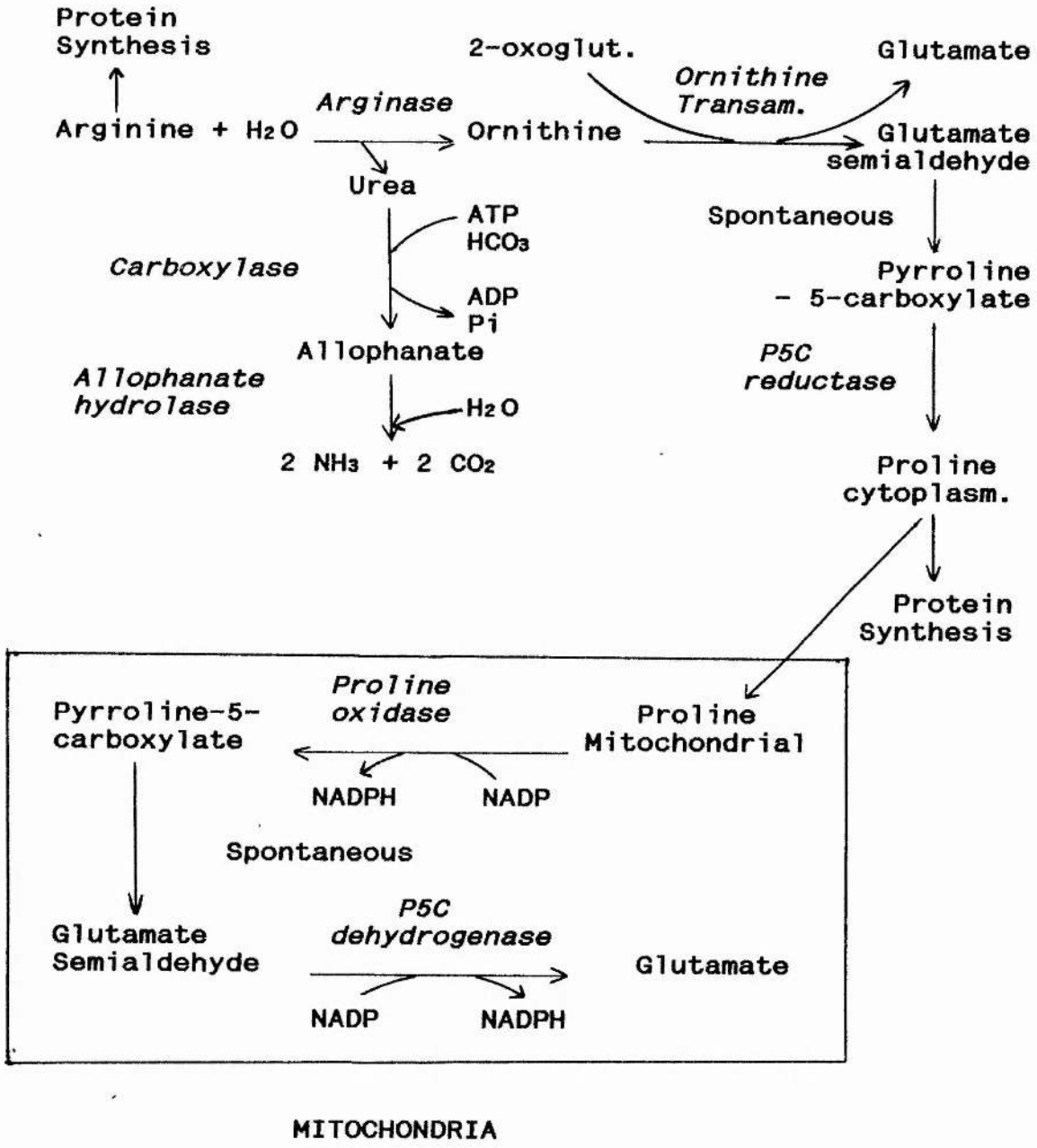
1st reaction

$$\text{Urea} + \text{ATP} + \text{HCO}_3 \xrightarrow[\text{Mg}^{++}\ ,\ \text{K}^{++}]{\textit{Carboxylase}} \text{Allophanate} + \text{ADP} + \text{Pi}$$

2nd reaction

$$\text{Allophanate} \xrightarrow{\textit{Allophanate hydrolase}} 2\ \text{CO}_2 + 2\ \text{NH}_3$$

Arginase and Ornithine transaminase have been reported to be cytosolic enzymes (15) . On the other hand , Proline oxidase is postulated to be active only in the presence of a functional electron transport system . Also P5C dehydrogenase is a mitochondrial enzyme (8) . It appears that proline is synthesized from glutamate or ornithine in the cytosol and is degraded exclusively in the mitochondria .



Enzymatic reactions associated with arginine degradation in *S.cerevisiae.*

1.2.2 Ammonia as end product

1.2.2.1 Allantoin degradation

Allantoin is a product of guanine and adenine catabolism. In many organisms , included *S.cerevisiae* , it can serve as a sole nitrogen source (16) .

Allantoin degradation system involves different enzymatic steps . First , the hydantoin ring of the molecule is opened in a hydrolytic reaction catalysed by Allantoinase . Then the reaction product , allantoate , is degraded in one molecule of urea and one of ureidoglycollate by the Allantoicase (17). The hydrolytic cleavage of ureidoglycollate to glyoxylate and another urea molecule is then catalysed by Ureidoglycollate hydrolase . The degradation of urea has previously been discussed (see 1.2.1.2)

1.2.2.2 Asparagine degradation

In some strains of *S.cerevisiae* the degradation of asparagine involves one enzyme :

Asparaginase I . In other strains , it involves Asparaginases I and II (18).

Asparaginase I is found in the cytosol , and has been reported to possess a K_m for asparagine of approximately 0.25 mM and a pH optimum of 8.5 . Mutants which lack the gene for Asparaginase I showed normal levels of L-glutaminase activity , suggesting that degradation of glutamine is independent of asparagine catabolism .

A second form of enzyme , called Asparaginase II has been found in some strains of *S.cerevisiae* . It has been shown to be an extracellular glycoprotein , and possess a K_m of approximately 0.2 mM for asparagine (19) .

1.2.3 Ammonia and glutamate interconversion

The interface between biosynthesis and degradation of nitrogen compounds is represented by the interconversion of ammonia and glutamate . Both metabolites participate , along with glutamine , as the major nitrogen donors in biosynthetic reactions . Furthermore , as glutamate can be synthesized by the amination of the intermediate of the tricarboxylic acid

2-oxoglutarate , the enzymes which catalyse the interconversion of ammonia and glutamate are also at the carbon and nitrogen interface . These reactions are catalysed by two Glutamate dehydrogenases (GDH) either NADPH or NAD dependent .

High levels of NAD-GDH (E.C.1.2.4.1.2) have been reported when cells were grown on glutamate and low levels when ammonia was provided as the nitrogen source (20). This prompted the conclusion that NAD-GDH is a catabolic enzyme .

$$\text{L-glutamate} + \text{NAD} \xrightarrow{GDH} \text{2-oxoglut.} + \text{NADH} + NH_4^+$$

Roon and Even have shown that NADPH-GDH (E.C.1.4.1.4) has maximal activity on ammonia , allantoin or urea (compounds which degrade directly to ammonia) as the nitrogen source . It was concluded that NADPH-GDH is a metabolic enzyme .

$$NH_4^+ + \text{NADPH} + \text{2-oxoglut.} \xrightarrow{GDH} \text{L-glutamate} + \text{NADP}$$

Mutants with altered levels of NAD-GDH and NADPH-GDH activities have been isolated (21), (22) . Mutants which lack NADPH-GDH activity grow very slowly

in minimal ammonia medium . Grenson et al. (22) suggested that the alternative pathway for ammonia assimilation derived from the operation of NAD-linked GDH. However, the isolation of mutants that overproduce NAD-GDH does not mean that other possibilities are not plausible .

Glutamine is synthesized from ammonia and glutamate in a reaction catalysed by ATP-dependent Glutamine synthetase (E.C.6.3.1.2) . It has been shown that this is the sole route for glutamine biosynthesis in *S.cerevisiae* . *Aerobacter aerogenes* grown in ammonia-limited chemostat culture has intracellular ammonia concentration less than 0.5 mM , which is below the K_m for ammonia (3-4 mM) of the GDH of the *A.aerogenes* (23). An alternative pathway for glutamate biosynthesis with a low Km for ammonia should exist . This alternative pathway could be achieved by coupling the Glutamine synthetase with a Glutamate synthase - GOGAT , which catalyses the transfer of the amide group of glutamine to 2-oxoglutarate , a reaction which is NADPH-dependent for bacteria and NAD-dependent for yeast .

1st reaction :

$$\text{L-glutamate} + \text{ATP} + NH_4^+ \xrightarrow[\text{synthetase}]{\textit{Glutamine}} \text{L-glutamine} + \text{ADP} + \text{Pi}$$

2nd reaction :

$$\text{L-glutamine} + \text{2-oxoglut.} + \text{NAD(P)H} \xrightarrow{\textit{GOGAT}} 2\ \text{L-glutamate} + \text{NAD(P)}$$

Cells grown in minimal ammonia medium posses the greatest amount of GOGAT activity , but even then there was tenfold less GOGAT activity than NADPH-GDH (24) . It has been suggested that the GS-GOGAT pathway serves as an auxiliary means of producing amino acids from the amide nitrogen of glutamine without passing through the ammonia pool , or a scavenging pathway to function under conditions of ammonia limitation .

1.2.4 Biochemistry of ammonia assimilation enzymes

1.2.4.1 NADPH-Glutamate dehydrogenase (NADPH-GDH)

The *S.cerevisiae* NADPH-GDH has been reported to be a homohexamer composed of subunits of Mw 54000 (25) . The K_m was found to be 1 mM for NADPH (22) , but there are considerable variations in the K_m value for ammonia , from values of 2 mM up to 11 mM (22), (26).

Studies have been carried out with the activity of *N.crassa* NADPH-GDH and the effects of ammonia concentration (27) . The plot *activity* x *ammonia concentration* was found to be biphasic with a transition point at about 2 mM. The researchers examined the published values for the K_m ammonia and concluded that the differing values were probably due to this biphasic interaction with ammonia. Similar biphasic interactions could exist for the *S.cerevisiae* enzyme , and that could explain the widely differing K_m values .

There is a complex relationship between pH , NADPH and 2-oxoglutarate . The enzyme has maximum activity at high pH (above 7.2) . NADPH alone favours the inactive form of the enzyme , but in the presence of 2-oxoglutarate an active quaternary complex is formed . At pH values below 7.0 , the inactive form is stabilised by protons (28) .

It has been proposed that 2-oxoglutarate functions as an activator , and NADPH modifies this activator effect .

1.2.4.2 NAD-Glutamate dehydrogenase (NAD-GDH)

The *S.cerevisiae* NAD-GDH has been proposed to be a tetramer of identical subunits , although the unclear disagreement in the suggested size (29) . The enzyme is inactive in the phosphorylated form . The dephosphorylation restores its activity .

The dephosphorylated-GDH has a K_m value for NAD at 0.86 mM and 20 mM for glutamate . The K_m for glutamate is much higher , 128 mM , for the phosphorylated enzyme (30),(31) .

The two GDHs found in *S.cerevisiae* have been located in the cytosol . There is evidence that both enzymes have two cofator binding domains (32) .

1.2.4.3 Glutamine synthetase (GS)

Unlike the NADPH-GDH , there are considerable differences in the structural and functional properties of GS from bacteria and fungi .

The bacterial enzyme is a dodecamer of identical subunits (33) . The enzyme activity is regulated in response to covalent modifications and feedback inhibition . The unadenylated form is active , and activity decreases with increasing adenylation . The adenylation degree is modulated by Mg^{++}, Mn^{++}, ATP , glutamine and 2-oxoglutarate levels , plus other metabolites . There is also feedback inhibition by end products of glutamine metabolism (34) . The K_m value for ammonia is much lower than that of NADPH-GDH , below 1 mM in *A.aerogenes* (24) .

GS from *S.cerevisiae* has been demonstrated to be more similar to the bacterial than to the *C.utilis* (35),(36) or *N.crassa* enzymes (37). It is a multimeric enzyme of 10-12 subunits (38) . The addition of glutamine to glutamate grown cells cause a sharp loss of activity due to cessation of subunit synthesis . Removal of glutamine results in reactivation of GS from *S.cerevisiae* (39),(40) .

1.2.4.4 Glutamine(amide) : 2-oxoglutarate amino transferase oxido-reductase-NAD or Glutamate synthase (GOGAT)

GOGAT activity has been found in many microorganisms (41),(42),(43).

The *S.cerevisiae* GOGAT is a heteromeric protein composed of two different subunits . There is disagreement in the literature about the sizes of these subunits . The K_m value for 2-oxoglutarate varies over 0.04-1.0 mM , and for glutamine the K_m value is 0.25-0.3 mM (43) . The pH optima are at 7.4-7.5 . The fungal and yeast enzyme uses NADH as cofactor , while the bacterial uses only NADPH .

The GS-GOGAT pathway requires more energy (the equivalent of four ATP) than NADPH-GDH (equivalent to three ATP) to fix the same amount of ammonia . The higher energy requirement of the GS-GOGAT pathway is a potential source of inefficiency in the conversion of substrate into biomass .

In a mutant which lacks GOGAT activity it may be that the NADPH-GDH alone could still synthesise sufficient glutamate for the strain to grow as normal . The one ATP saved per ammonia assimilated may be used in the formation of more biomass . A secondary effect of a reduction in NADH reoxidation is that the process becomes less exothermic and requires less cooling to maintain the

culture at the ideal temperature , in large scale culture.

1.3 AIMS

The aims of this project fall into two groups :

GENERAL OBJECTIVES

A broad aim is to acquire skills in fermentation techniques particularly bench scale, batch and continuous (chemostatic) culture, as tools for future work (in a Brazilian environment) in the general area of fermentation technology - particularly in relation to national biotechnology programs. Skills are also necessary in the general area of instrumentation - the ability to construct and maintain simple instrumentation being essential in an environment lacking ready workshop facilities/technical support.

SPECIFIC OBJECTIVES

The specific objectives are to investigate the effect, in physiological terms, of the elimination of GOGAT activity in *S.cerevisiae* .

The pool sizes of ammonia, glutamate and glutamine plus the specific activities of the enzymes involved in ammonia assimilation will be determined under Carbon and Nitrogen limited cultures with a variety of

dilution (growth) rates. An estimation of the contribution of GOGAT towards ammonia assimilation in *S.cerevisiae* is sought.

2. MATERIALS AND METHODS

2.1 Microorganisms

Three *S.cerevisiae* strains were used in this project. They were provided by Dr. A. Racher and Dr. J. R. Kinghorn (University of St. Andrews) .

Strains AR2 and AR5 derived from BC55 [*gogat*$^{+}$ *gdh* 1-6^{-} *leu2*$^{-}$] which carries the plasmid pCYG4. This plasmid was developed by Nagasu and Hall (44). They isolated a gene which showed homology to the *Neurospora crassa* gene and conferred NADPH-GDH activity in yeast. This gene was cloned into the *Escherichia coli*-yeast shuttle vector CV13 (YEp13) BamHI site in *Saccharomyces cerevisiae* (*gdh*$^{-}$ *leu2*$^{-}$ strain BC55) directing substantial overproduction of NADPH-GDH .

Strain AR2 has a different deletion in the gene for NADPH-GDH activity [*gdh* 1-1^{-}] and it bears approximately 30 copies of the plasmid pCYG4.

Strain AR5 was selected from a BC55 mutant which showed very low GOGAT activity (about 2% of the GOGAT activity of the parental strain). It also carries approximately 30 copies of the plasmid pCYG4 . Any improvement in its growth characteristics would be due to the lack of GOGAT activity.

Strain ξ1278b is an haploid wild type strain.

Table 2.1 Three different strains of *Saccharomyces cerevisiae*

Strain	Vector
≤1278b	------
AR2 (a *gogat*$^+$ *gdh*$^-$ *leu2*$^-$)	pCYG4
AR5 (a *gogat*$^-$ *gdh*$^-$ *leu2*$^-$)	pCYG4

2.2 Microbial Growth System

2.2.1 The Microprocessor-controlled Fermenter

A microprocessor-controlled fermenter system was used for all the experiments throughout this work with the three strains. Figure 2.2.1 shows a general diagram of the apparatus for microbial growth. The Processor Control System (PCS) consists of 3 main boards (1,2 and 3); 4 auxiliary boards (4,5,6 and 7); and a connection block (8,9,10 and 11) used to link the PCS with the video terminal, with sensors from the fermenter, with a control box and with other microcomputer.

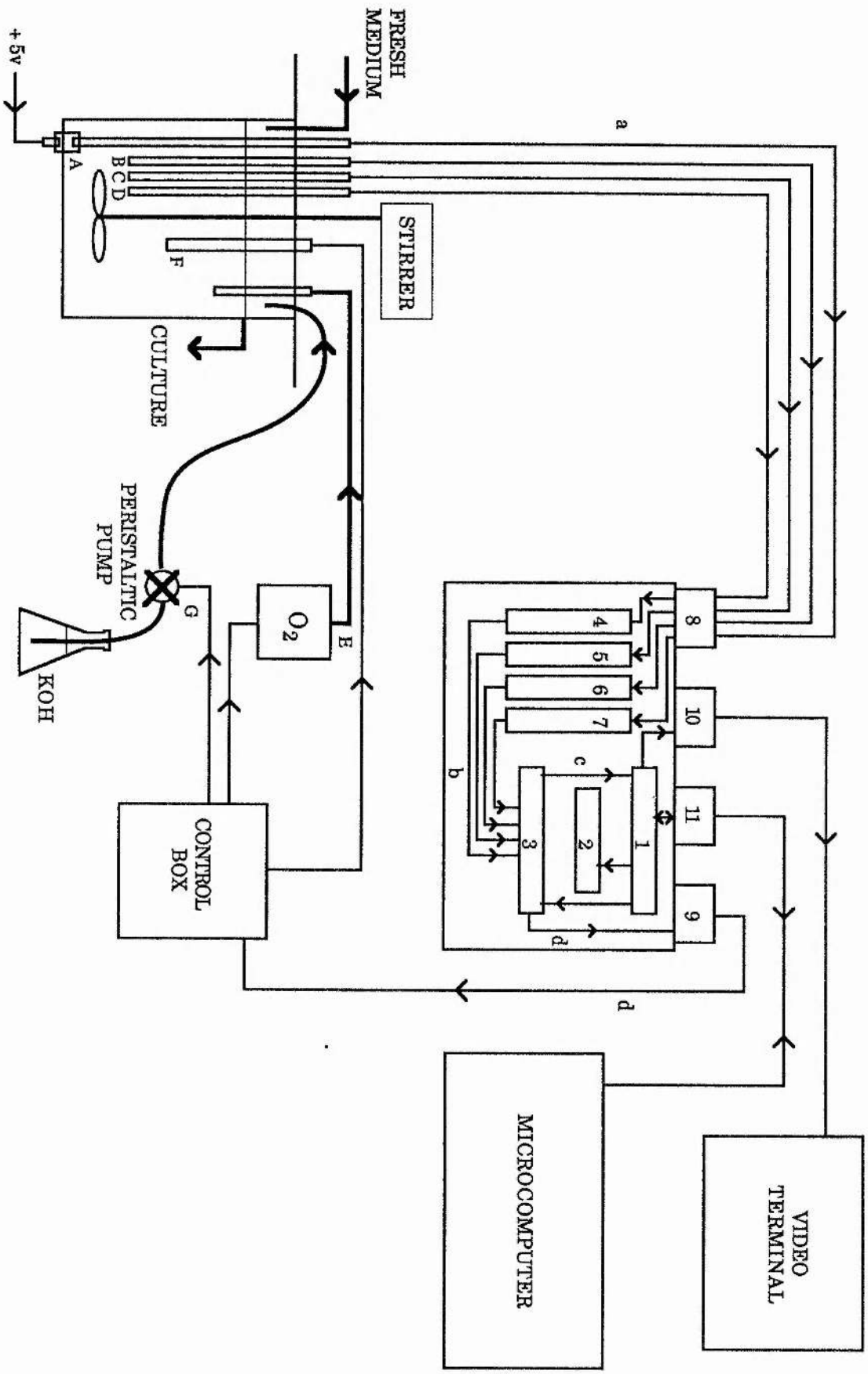


Fig. 2.2.1 - Diagram of the apparatus for microbial growth.

MICROPROCESSOR :

Main Boards

1 - Central Processor Board (CPU-Board)

2 - Memory Board

3 - Analog/Digital Converter (A/D) and (ON/OFF) Switch Board (ADS-Board)

Auxiliary Boards

4 - pH Interface Board

5 - Oxygen Interface Board

6 - Temperature Interface Board

7 - Biomass Interface Board

Connection Block

8 - Connection between Sensors in the fermenter vessel and the Interfaces

9 - Connection between A/D and the Control Box

10 - Connection between the PCS and the video terminal

11 - Connection between the PCS and another microcomputer

SENSORS :

A - Biomass Electrode System

B - pH Electrode

C - Oxygen Electrode

D - Temperature Sensor

CONNECTIONS TO THE CONTROL BOX (CB):

E - Oxygen Pump

F - Heater

G - KOH Pump

SIGNALS

a - Very low voltage or current from electrodes

b - 0 to 2.55 volts d.c.

c - Digital signal to the PCS system

d - Analog signal to the CB

The function of the CPU-board is to process the digital information from the A/D board or from the connecters 10 and 11 (video terminal and another microcomputer), to store the results in the memory board or send them through the connecters 10 or 11 for further data analysis. The conditions of the parameters in the fermenter are also controlled by the PCS which compares data from the transducers with data stored in the memory.

There are two kinds of memory in this system :

1 - Random Access Memory (RAM), in which the content of a memory cell can be erased through a software command or by power cut.

2 - Read Only Memory (ROM), in which the data is permanently fixed in memory cells . A speciall ROM called EPROM (Erasable Programmable Read Only Memory), which allows the data to be erased through ultra violet light exposure is present in the system.

The EPROM memory has been used to store the master software which controls the operation of the microprocessor. For data storage from the fermenter sensors the RAM memory is utilised.

The fermenter is connected to the microcomputer through the Analog/Digital (A/D) converter and ON/OFF Switch board (ADS). This board converts analog data from the fermenter sensors to digital data, before sending

them to the CPU for processing and comparison with set-point data stored in memory. The ON/OFF Switch circuit controls the control box which can switch ON and OFF specific devices to control parameters inside the fermenter vessel (Heater to control the temperature, air pump to control the oxygen level, peristaltic pumps to control the pH).

The auxiliary boards (pH, oxygen, temperature and biomass interface boards) transform the low level from sensors inside the fermenter vessel to suitable levels (0 to 2.55 V. d.c.) for analog-digital conversion.

Four different electrodes are used inside the fermenter :

1 - pH electrode - ACWL 150 steam sterilisable pH electrode (Russel pH Limited, Auchtermuchty, FIFE, Scotland).

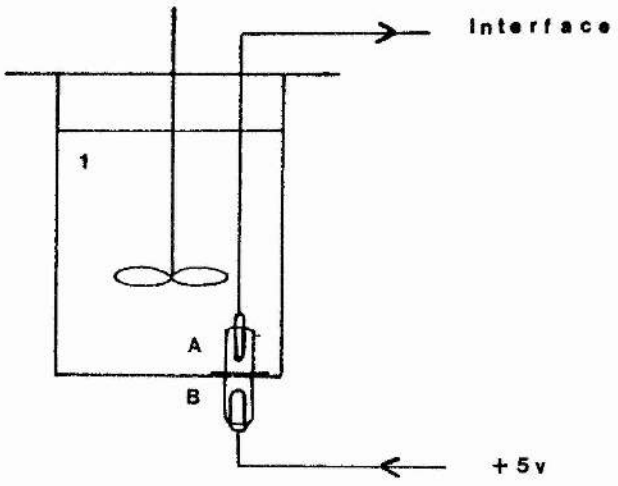
2 - Oxygen electrode - Sterilisable oxygen electrode type G-2 (Uniprobe, Cardiff, England).

3 - Biomass electrode system - The biomass electrode utilized in the microprocessor-controlled fermenter system was developed by Lima Filho (6) . It consists of two components : the emitter and the detector .(See figure 2.2.1a).

The emitter is a light emitting diode (LED) - the H500 (General Electric, USA) . It is a GaA1As diode which has a typically luminous intensity (TLI) of 500 mcd , and a spectral peak at 650 nm .It is fixed outside and to the bottom of the fermenter vessel , and its vertical position is aligned with the detector .

The detector is the part of the electrode which is set up inside the fermenter vessel .It is an infrared detector (L14F1 - General Electric , USA) , supersensitive NPN planar silicon photo-darligton amplifier .It is autoclavable and has a peak of light detection at 850 nm, but at 650 nm it retains 50% of its sensitivity, enough for the purpose of the system. The detector is connected to an interface in the microprocessor which amplifies the signal from the detector and transmits it to the ADS board , then to the CPU board where the processing of data will be carried out .

Figure 2.2.1a - Diagram of the biomass electrode



1 - Fermenter vessel

A - Detector

B - Emitter

4 - Temperature sensor - It was also developed by Lima Filho (6). The temperature sensor consists of a 590KH temperature transducer (RS Components), which produces an output current proportional to absolute temperature. It operates in the range of -55° C to +150° C, with a nominal current output of 298.2 uA at +25° C and a nominal temperature coefficient of 1 uA/° C.

Originally the sensor should be covered by a single teflon membrane, but we found that a double membrane, although decreasing its response speed would improve its lifetime as it is continuously subjected to sterilisation.

2.2.2 Inoculum Medium

Yeast Nitrogen Base	0.17 g
Glucose	2.00 g
Glutamate	0.15 g
Final volume	100 ml

2.2.3 Batch medium

Yeast Nitrogen Base	0.85 g
Glucose	10.00 g
Ammonium Sulphate	1.32 g
Final volume	500 ml

2.2.4 Chemostatic Culture medium

2.2.4.1 Carbon limiting medium

Yeast Nitrogen Base	34.00 g
Glucose	100.00 g
Ammonium Sulphate	52.80 g
Final volume	20 L

2.2.4.2 Nitrogen limiting medium

Yeast Nitrogen Base	34.00 g
Glucose	400.00 g
Ammonium Sulphate	5.28 g
Final volume	20 L

2.2.5 Maintenance Medium

Yeast Nitrogen Base	0.85 g
Glucose	10.00 g
Ammonium Sulphate	1.32 g
Agar	10.00 g
Final volume	500 ml

2.2.6 Maintenance of the Strains

Subcultures were made from the original strains utilising the maintenance medium (2.2.5) and incubated at 30º C for 72 hours. The purity of the colonies was checked using Gram's stain. Then the new plates were kept at 4º C. Subcultures were made every three weeks.

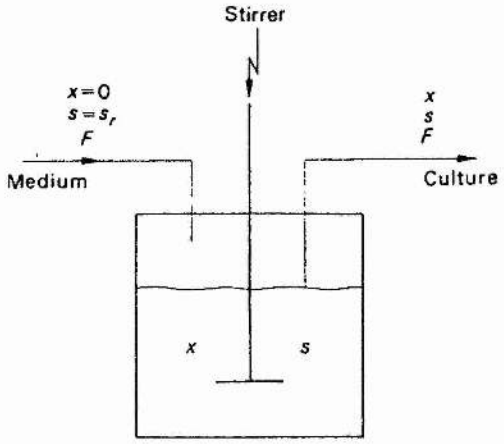
2.2.7 Batch experiments

Cells grew overnight in 100 ml of inoculum medium at 30° C on an orbital shaker. Subsequently 50 ml of the inoculum were added to 500 ml of batch medium in the fermenter vessel. The temperature was 30° C , the oxygen saturation was 30% and the pH maintained at 5 by addition of 2 M KOH.

2.2.8 Chemostatic Culture Experiments

A chemostat culture consists of a mixed suspension of biomass into which fresh medium is continuously introduced at a constant rate and the culture is harvested at the same rate so that the culture volume remains constant. The biomass growth is limited by the concentration of a single substrate, all the other nutrients being in excess (See figure 2.2.8)

Figure 2.2.8 - Diagram of a chemostat



x - biomass concentration

s - growth-limiting substrate concentration

s_r - initial growth-limiting substrate concentration

F - flow rate

Usually, a chemostatic experiment is preceded by growth without addition of fresh medium (batch mode). When the culture achieves the exponential phase, then the flow of medium is connected . The value

of flow rate/volume of working culture (F/V) is known as the dilution rate (D).

If the rate of wash out of the biomass is less than the maximum growth rate ($D < u_m$) the limiting substrate concentration must decrease the specific growth rate until the biomass growth rate equals the wash out rate. Then a steady state may be achieved and the specific growth rate of the biomass will be determined by the dilution rate. However, if the rate of wash out of biomass exceeds the rate of growth ($D > u_m$) then the biomass concentration will decrease and the growth-limiting substrate concentration will tend towards s_r. If the dilution rate is equal to the maximum specific growth rate, the biomass concentration will be zero, and the dilution rate is called the critical dilution rate (D_c). (See figure 2.2.8a).

Figure 2.2.8b shows the rates of biomass output in a chemostat. the steady-state output rate is a function of the dilution rate and it reaches a maximum at a value known as D_m, obtained by the following equation :

$$D_m = u_m \{ 1 - (K_s/s_r + K_s)^{1/2} \}$$

K_s - saturation constant

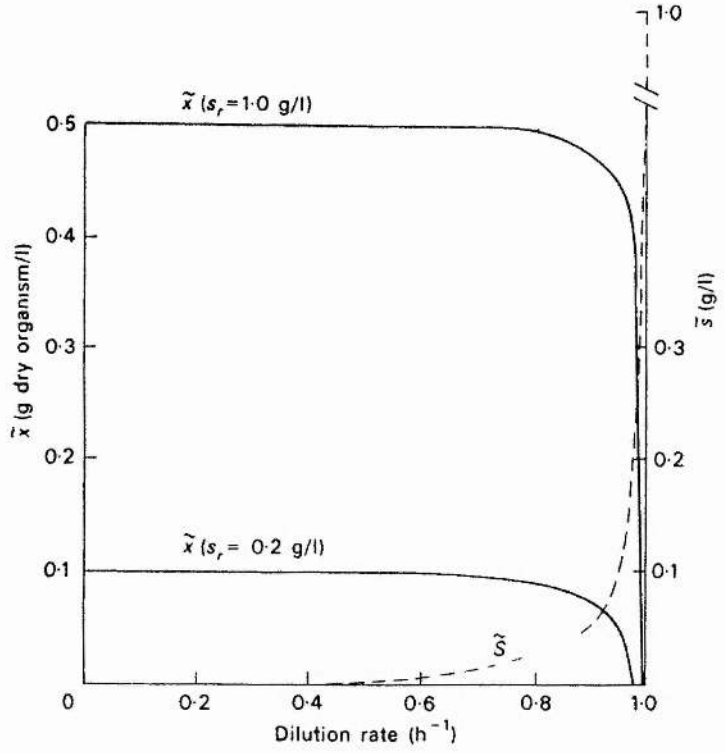


Figure 2.2.8a - (From Pirt S.J. ref.45) Variation of steady-state biomass ($\tilde{x}$) and growth-limiting substrate ($\tilde{s}$) concentrations with dilution rate.

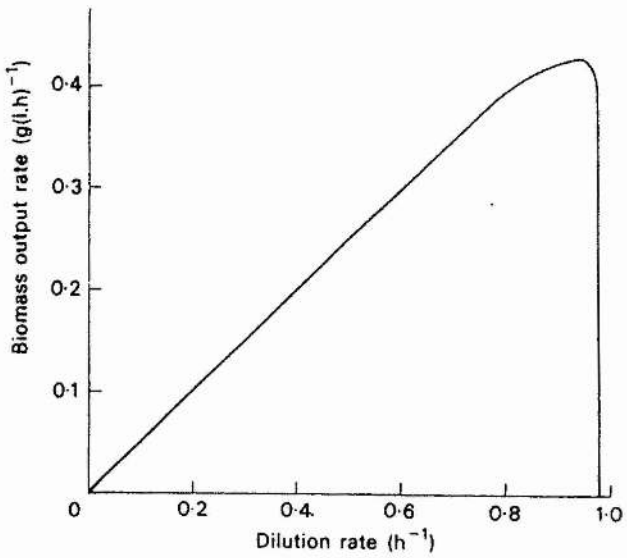


Figure 2.2.8b - (From Pirt S.J. ref.45) Steady-state rates of biomass output in a chemostat.

Under steady-state conditions the mean generation time (td = doubling time) is calculated following the equation below (45) :

$$td = \frac{\ln 2}{u} h$$

where $u = D$

The experiments were carried out under the conditions described in 2.2.6. When the cells achieved late exponential phase in batch culture , the continuous influx of limiting medium was started. The flow rate was determined measuring the time (minutes) to fill a 10 ml volumetric vessel with culture. The dilution rates were calculated dividing the flow rate by the volume of culture inside the fermenter (600 ml). The dilution rates utilized in the experiments were 0.05, 0.10, 0.15 and 0.20/h.

Each experiment with chemostatic culture last from 5 to 10 days , resulting in a different number of generations.

Table 2.2.8 - Parameters for chemostatic culture experiments.

Flow rate (ml/min)	Culture volume (ml)	D (/h)	t_d (h)
30	600	0.05	13.86
60	600	0.10	6.93
90	600	0.15	4.62
120	600	0.20	3.46

2.3 Biomass Measurement

The biomass concentration was measured by two methods :

1 - Relationship of absorbance-dry weight of cells

2 - Continuous recording by the biomass electrode connected to the microprocessor control system.

2.3.1 Absorbance-dry weight relationship

After experiments with C and N limited continuous culture , 600 ml of culture were centrifuged [1500 x g; 10 min] and then washed twice with distiled water before finally being ressuspended to a thick but pipettable solution . From this solution a series of suitable dilutions was made and their absorbances recorded at 610 nm [Unicam SP 600 series 2] spectrophotometer] against a distiled water blank . Three 1 ml samples of this solution were dried for 18 h (oven 200° C) and then weighted accurately until the readings were constant in pre-weighted containers . Since the accurate weight of 1 ml of cells was known the equivalent dry weight of the dilutions could be calculated . Absorbance/dry weight relationship graphs were constructed

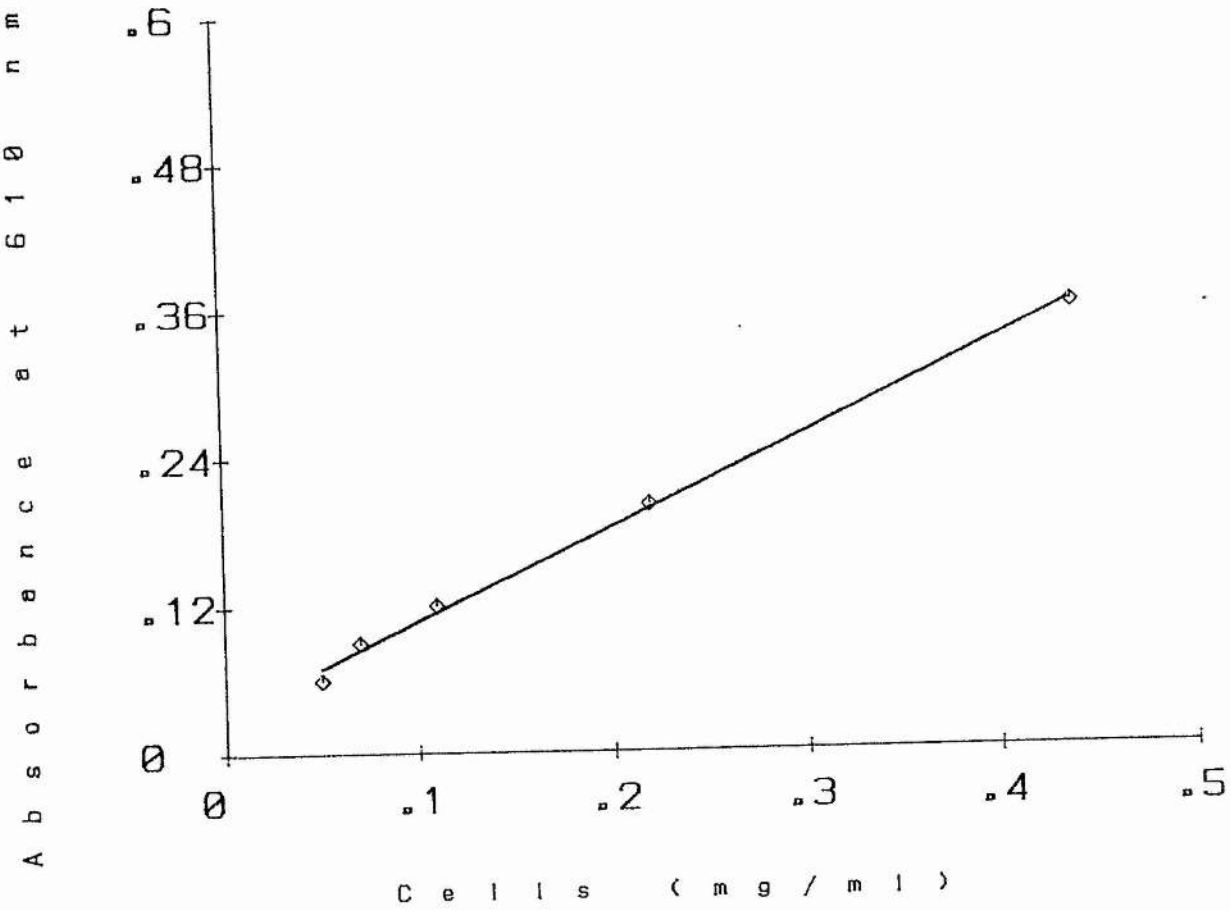


Figure 2.3.1 : Dry weight calibration plot for AR2 strain.

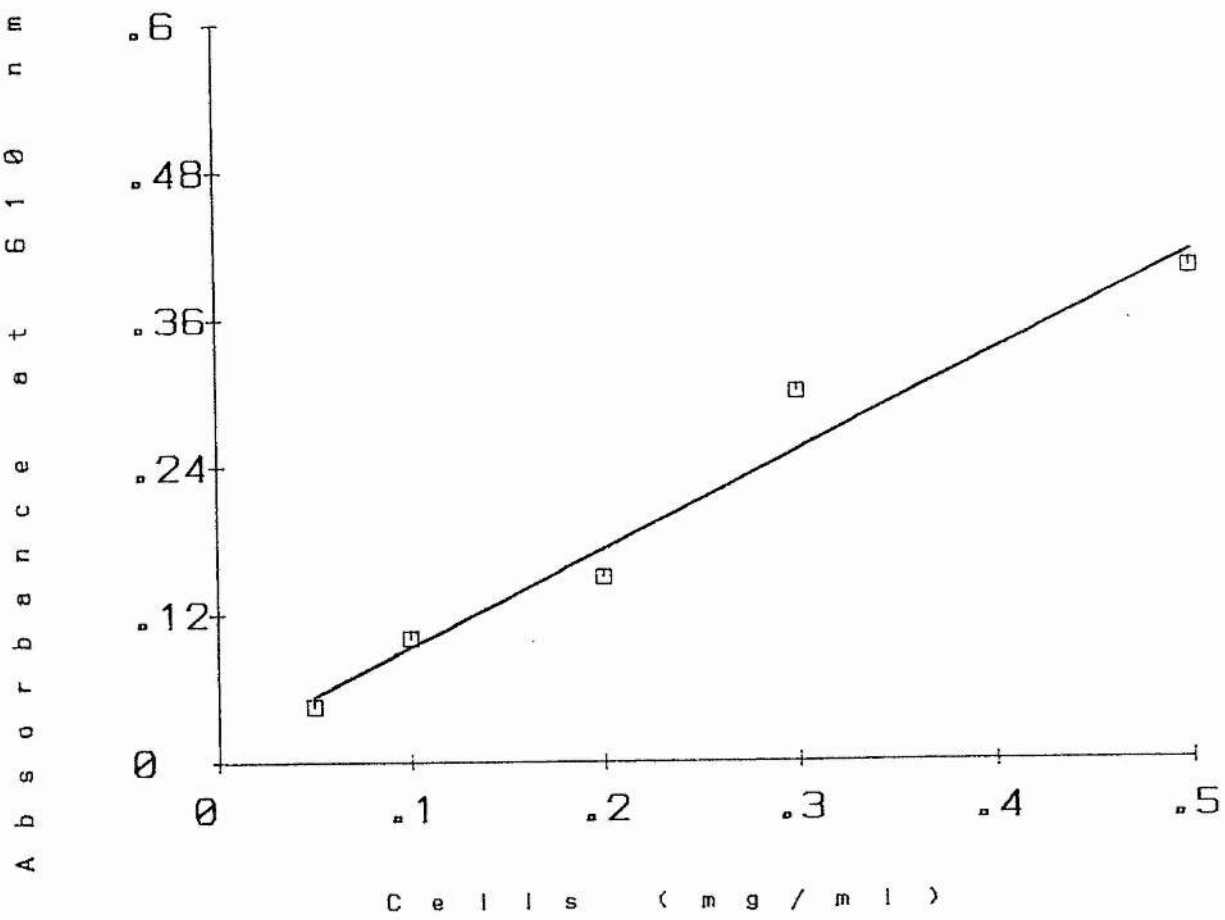


Figure 2.3.1a : Dry weight calibration plot for AR5 strain.

2.4 Detection of Plasmid

The presence of plasmid in the cells was checked using two different approaches :

1 - The determination of NADPH-GDH activity in the cells , which is correlated with NADPH-GDH encoded in the plasmid .

2 - The level of Penicillinase activity in the supernatant of the broth culture was measured. This method was based on the Chevallier and Aigle's method (46) which showed that plasmid carrying the Amp^R in a chimeric plasmid derived from the 2u yeast plasmid can produce Penicillinase . Penicillinase activity can be detected by a white halo around the Penicillinase-producing strains, due to reducing action of penicillinoic acid over a deep-blue iodine-starch complex incorporated into the solid medium after growth of strains.

2.5 Specific Growth Rate

The specific growth rate was determined by two different approaches :

2.5.1 Specific growth rate determined through a batch experiment :

The specific growth rate in batch culture was calculated using the following equation :

$$\ln X = \ln X_o + ut$$

Where :

X_o = biomass when t = 0

The plot of *lnX* against *time* in exponential growth will be a straight line which slope is equal to u (45).

2.5.2 Specific growth rate determined through Chemostatic growth

The specific growth rate in continuous culture was determined using the following equation :

$$\ln X = (u_m - D)t + \ln X_o$$

If we make D>Dc in the chemostat wash-out occurs and the slope of *lnX* versus *time* is (u_m - D) (45)

2.6 Determination of Enzymatic Activities

2.6.1 NADPH-Glutamate dehydrogenase (NADPH-GDH) and Glutamate synthase (GOGAT)

2.6.1.1 Reagents

1 - Extraction buffer (0.1 M potassium phosphate pH 7.5 , 5 mM ethylenediaminetetraacetic acid disodium salt (EDTA) , 0.25 % v/v 2-mercaptoethanol , 0.1 mM phenylmethylsulphonylfluoride (PMSF)).

2 - 2-oxoglutarate (0.2 M in phosphate buffer 0.1 M pH 7.5) .

3 - NADPH (tetrasodium salt type I, product no N 1630 by Sigma) (1 mg/ml in phosphate buffer 0.1 M pH 7.5).

4 - NADH (disodium salt grade III, product nº N 8129 by Sigma) (1 mg/ml in phosphate buffer 0.1 M pH 7.5)

5 - Ammonium chloride (0.2 M in phosphate buffer 0.1 M pH 7.5).

6 - L-Glutamine (0.04 M in phosphate buffer 0.1 M pH 7.5)

2.6.1.2 Preparation of extracts

Three 1 ml aliquots of cell culture were centrifuged (Eppendorf : 3 min/13,000 rpm) when the

medium was carbon limited , and twelve 1 ml when the medium was nitrogen limited . The pellets were pooled in one tube and ressuspended in 1 ml of extraction buffer . Glass beads (0.45-0.5 mm) were added up to approximately the same volume as the pellet . Cells were broken with a vortex mixer for 3 min . Supernatants were removed after centrifugation (Eppendorf : 3 min/13,000 rpm) and used as a sample for enzyme assay immediately.

2.6.1.3 NADPH-Glutamate dehydrogenase (NADPH-GDH)

The NADPH-GDH activity was determined by recording the decrease of absorbance at 340 nm in a spectrophotometer fitted with a chart recorder and water-jacketed cell carriage operating at 30° C . The reaction was initiated by the addition of ammonium chloride, and the rate was corrected by subtracting the rate of cofactor oxidation observed in the absence of ammonium chloride (47).

Reaction :

$$NH4+ + NADPH + 2\text{-oxoglut} \xrightarrow{\text{NADPH-GDH}} \text{L-glutamate} + NADP$$

NADP-GDH Assay Conditions

The following reagents were pipetted into cuvettes :

Phosphate buffer	2.5 ml
2-oxoglutarate	0.1 ml
NADPH	0.2 ml
The cuvette was mixed and left for 5 min to equilibrate the temperature.	
Enzyme extract (Background activity)	0.1 ml
Ammonium chloride	0.1 ml

One Unit of GDH activity was defined (using the molar extinction coefficient of 6.22 cm/umole for NADP) as the amount of enzyme which produced 1 umol of NADP in 1 min under the assay conditions . Specific activities were expressed as units per mg protein (U/mg)

2.6.1.4 Glutamate synthase (GOGAT)

GOGAT activity was determined spectrophotometrically as described in 2.6.1.3 , measuring the rate of NADH decrease at 340 nm after addition of L-glutamine , using a molar extinction coefficient of 6.22 cm/umole (24).

Reaction

$$\text{L-glutamine} + \text{NADH} + \text{2-oxoglut.} \xrightarrow{\text{GOGAT}} 2\ \text{L-glutamate} + \text{NAD}$$

GOGAT assay conditions

The following reagents were pipetted into cuvettes :

Phosphate buffer	2.4 ml
2-oxoglutarate	0.1 ml
NADH	0.2 ml
The cuvettes were mixed and left for 5 min to equilibrate the temperature	
Enzyme extract (Background activity)	0.2 ml
L-glutamine	0.1 ml

One Unit of GOGAT activity was defined as the amount of enzyme which produced 1 umol of NAD in 1 min at the assay conditions. Specific activities were expressed as units per mg protein (U/mg).

2.6.1.5 Calculation of Enzyme Activities

The activities of NADPH-GDH and GOGAT were carried out using the formula below :

$$\text{Activity} = \frac{\text{(Vol. of assay mix (ml)) X (Ex./min)}}{\text{6.22 x Vol. of enzyme extract (ml)}} = \text{U/ml}$$

Where Ex is the corrected rate of NADPH or NADH decrease per min.

$$\text{Specific activity} = \frac{\text{Activity (U/ml)}}{\text{Protein concentration (mg/ml)}} = \text{U/mg}$$

Protein were measured using the Bradford method (48).

2.6.2 Determination of Glutamine synthetase (GS) activity

GS activity can be measured by the reversible formation of ɣ-glutamylhydroxamate from glutamine and hydroxylamine :

$$\text{glutamine} + NA_2OH \xrightarrow{GS} \text{ɣ-glutamylhydroxamate} + NH_4^+$$

The ɣ-glutamylhydroxamate formed in the reaction gives a coloured complex with Fe^{+++} salts, which concentration can be determined at 500 nm (Transferase activity) (36).

2.6.2.2 Assay conditions

The following reagents were pipetted into small tubes :

Assay mixture	0.7 ml
Glutamine	0.1 ml
Tris buffer	0.7 ml
The mixture was incubated for 15 min at 37° C.	
Stop mixture	2.0 ml

The assay mixtures were transfered to cuvettes and read at 500 nm against blank prepared with buffer instead of sample. 1 umol of glutamine gives an absorbance difference compared to blank of 0.129 at 500 nm. A calibration plot was constructed using Glutamine synthetase (1 mg protein/ml 20 kU/ml) from Sigma. (Fig. 2.6.2).

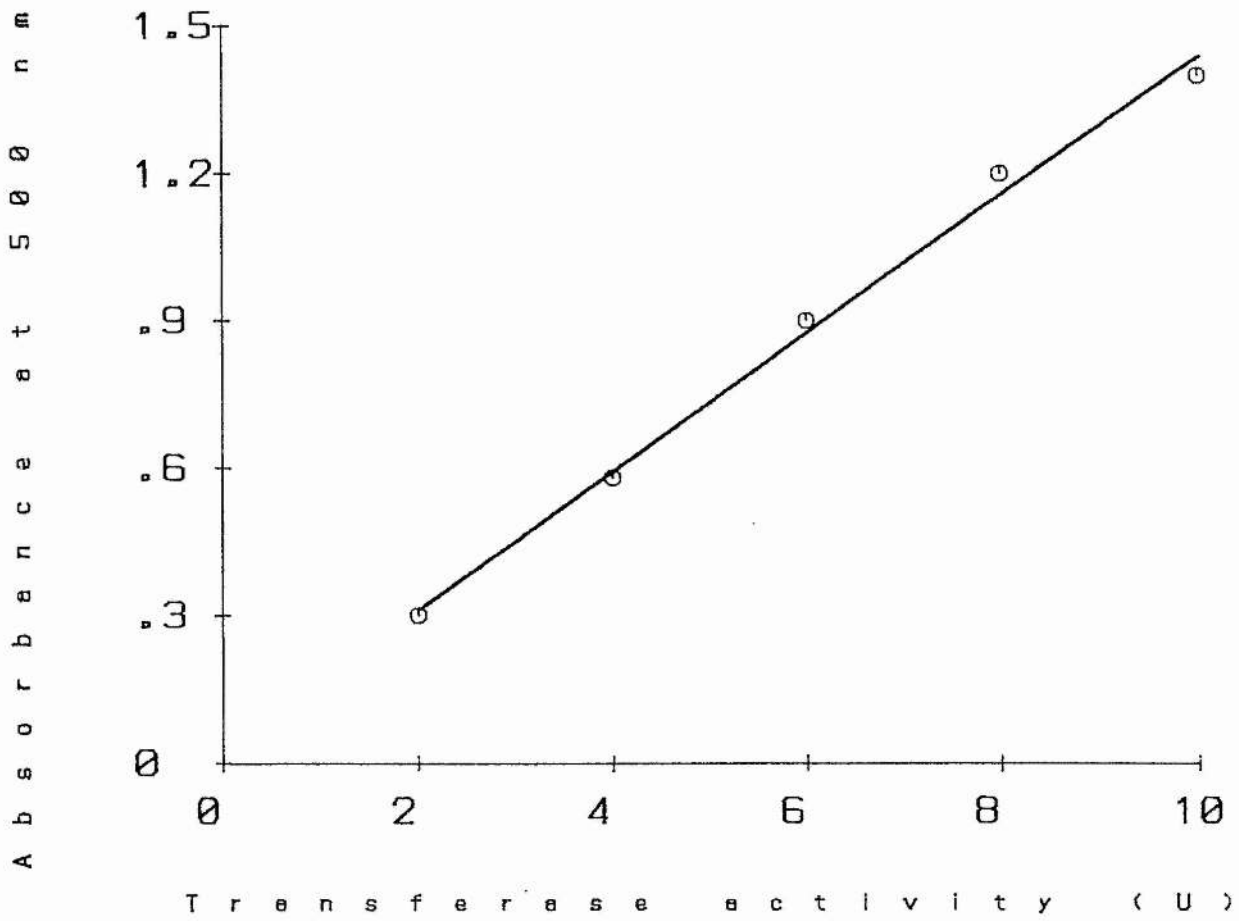


Figure 2.6.2 : γ-Glutamyl-transfer activity calibration plot.

2.6.3 Determination of extracellular Penicillinase activity

Penicillinase activities were determined in a simple and rapid fixed-time assay in which the product of penicillin hydrolysis (penicillinoic acid) reduces iodine to iodide - a reaction which is detected colorimetrically (50),(51).

2.6.3.1 Reagents

1 - Stock iodine solution : 0.32 M iodine and 1.2 M potassium iodide (20.3 g of resublimed iodine and 100 g of potassium iodide in 500 ml of distilled water)

2 - Iodine reagent : 5 ml of stock iodine solution in 95 ml of acetate buffer pH 4.0 (Acetate buffer : 80 g of anhydrous sodium acetate adjusted to pH 4.0 with acetic acid in 2 litres of distilled water)

3 - 20,000 units of Penicillin-G (Benzylpenicillin potassium salt from Sigma) in 0.5 ml of distilled water.

2.6.3.2 Preparation of the culture supernatants

Culture supernatants were obtained by centrifugation of 5 ml of culture broth (Eppendorf 3 min/13,000 rpm). The pH was adjusted to 7.0 by using 0.1 M phosphate buffer.

2.6.3.3 Assay conditions

The following reagents were pipetted into tubes :

Culture supernatant	2.5 ml
Penicillin G sol.	0.5 ml
The assay mixtures were incubated for 30 min	
Iodine reagent	5 ml
After a quick mix the assay mixtures were transfered to cuvettes and read at 499 nm	

Three tubes were prepared simultaneously as controls :

a : Phosphate buffer instead of sample.

b : Phosphate buffer instead of penicillin G.

c : Phosphate buffer only.

Absorbance of the control a = Aa

Absorbance of the control b = Ab

Absorbance of the control c = Ac

Absorbance of the sample = As

The final absorbance (FA) was calculated following the equation below :

$$FA = Aa - (Ac - Ab) - As$$

A calibration plot was constructed measuring the activity of a Penicillinase (B-lactamase I; EC 3.5.2.6, *Bacillus cereus* type I,product nº P 0389 from Sigma) in different concentrations following the procedures described above . (Fig. 2.6.3).

One unit of activity was defined as the amount of enzyme which hydrolyzes 1 umol of Penicillin G per min at pH 7.0 at 25º C.

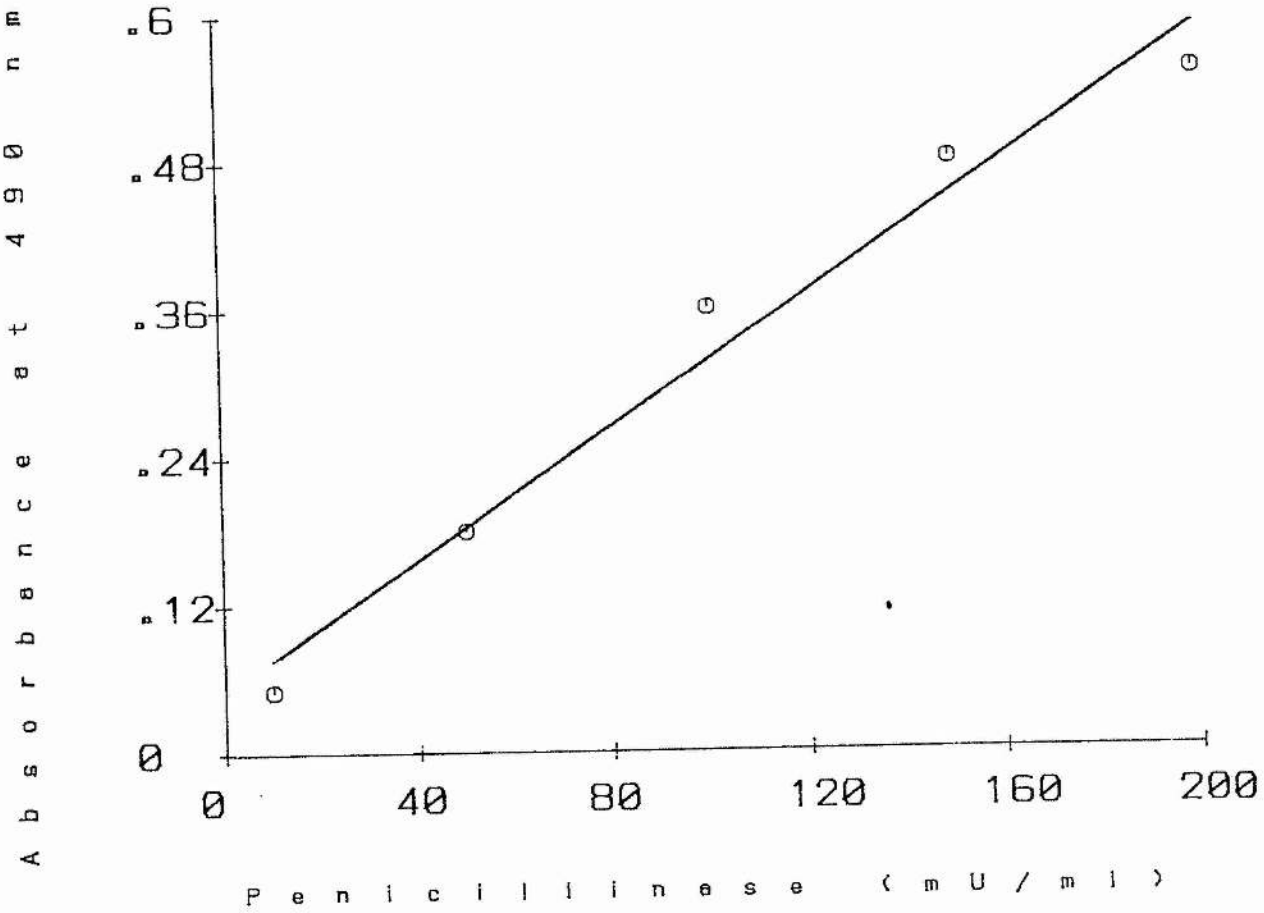


Figure 2.6.3 : Penicillinase activity calibration plot.

2.7 Protein Concentration Assay

Protein concentration was measured using the Bradford reaction (48). This method is a rapid and sensitive one for the measurement of microgram quantities of protein utilising the principles of protein-dye binding.

2.7.1 Reagent

100 mg Coomassie Brillant blue G dissolved in 50 ml 95% ethanol and mixed for 20 min. To this solution 100 ml 85% (w/v) phosphoric acid was added. The solution is diluted to 200 ml, then filtered twice. The final volume is adjusted to 1 litre.

2.7.2 Assay conditions

The following reagents were pipetted into tubes :

Protein sample	0.1 ml
Bradford Reagent	5.0 ml

After 5 min incubation at room temperature the absorbances were read at 595 nm against a blank prepared with buffer instead of sample.

The protein concentration was determined against a calibration plot pre-constructed with known albumen concentration samples.

The reagent was refiltered and a new calibration plot constructed every 2 weeks. Figure 2.7 shows one of the calibration plots used during the research.

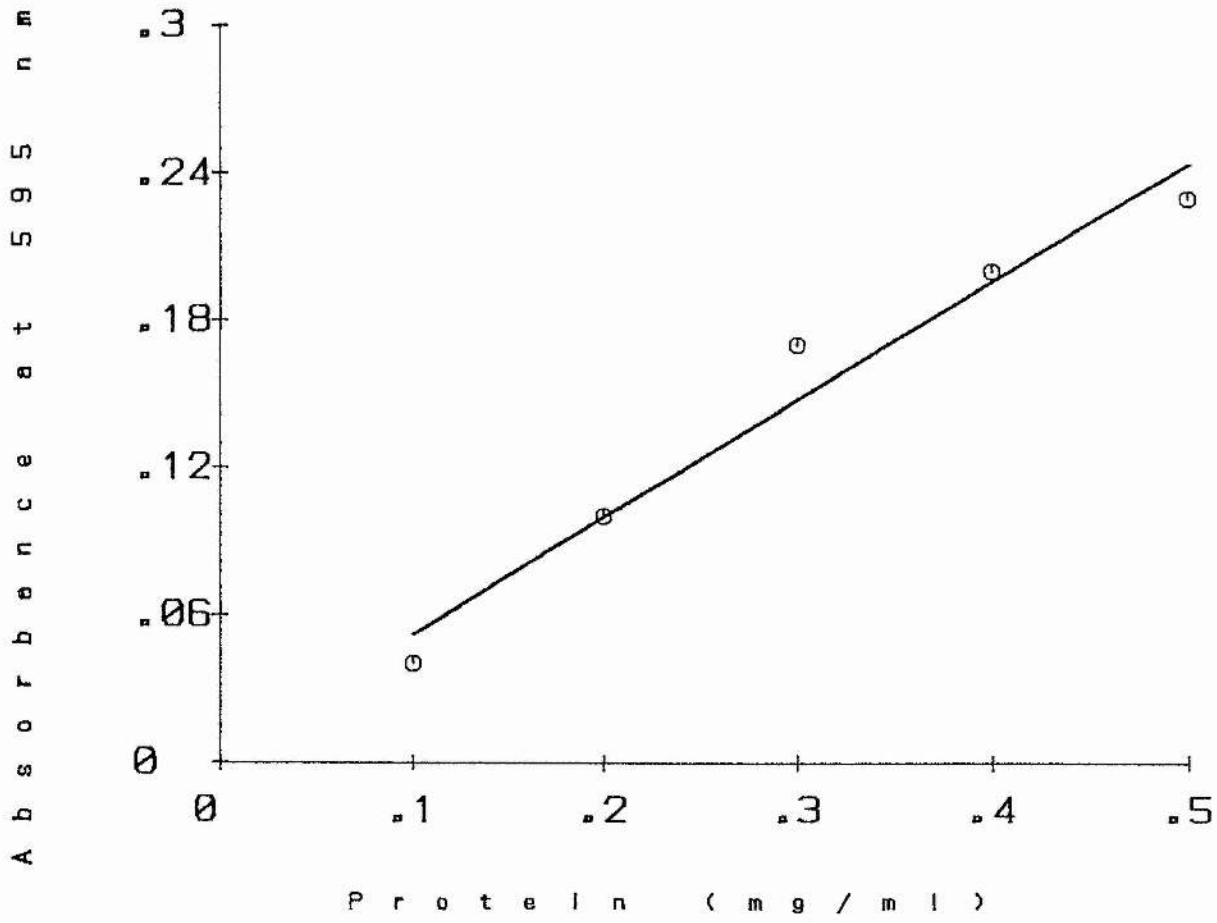


Figure 2.7 : Protein calibration plot.

2.8 Glutamate and Glutamine Measurement

The enzymatic determination of glutamate and glutamine using Glutamate dehydrogenase was carried out as described by Bergmeyer (52).

2.8.1 Reagents

1 - Acetate buffer (0.5 M, pH 5.0)

2 - Glutamine (2 mM)

3 - Glutaminase (10 kU/L) (from *E.coli*, essentially free from glutamate decarboxylase, grade V, product nº G 8880 from Sigma).

4 - Tris/hydrazine buffer (Tris 0.1 M; EDTA 2 mM; hydrazine 0.63 M : 1.2 g Tris plus 74 mg EDTA.Na_2 plus water up to 60 ml. Add 5 ml of hydrazine hydrate 62% (w/v) and adjust pH to 9.0 with HCl 5 M.)

5 - NAD (30 mM) (from yeast, grade III, product nº N 7004 from Sigma)

6 - ADP (100 mM) (sodium salt grade III, product nº A 2754 from Sigma)

7 - Glutamic acid (0.2 mM)

8 - Glutamate dehydrogenase (GlDH 1200 kU/L, from beef liver, commercial preparation from Boehringer Mannheim)

9 - Perchloric acid (10% (v/v))

10 - KOH (20% (w/v))

2.8.2 Preparation of extracts

Aliquots of cell culture (3 ml when the medium was C limited and 12 ml when it was N limited) were rapidly centrifuged (Eppendorf 3 min/13,000 rpm) and the pellets pooled in one tube and ressuspended in 1 ml of perchloric acid. Glass beads (0.45 - 0.5 mm) were added up to approximately the same volume as the pellet. Cells were broken with a vortex mixer for 3 min. Supernatants were removed after centrifugation (Eppendorf 3 min/13,000 rpm) and neutralized with 20 % (w/v) KOH , following a pre-constructed titration curve .(Table 2.8.2, fig. 2.8.2).

2.8.3 Assay conditions

2.8.3.1 Enzymatic hydrolysis

For each series of measurements the following were run :

1 - A blank reagent using 0.5 ml water instead of cell extract or standard.

2 - A glutamine standard using 0.1 ml glutamine solution (sol. 2) and 0.4 ml water, giving a final concentration of 0.4 mM.

Procedure

The following reagents were pipetted into small stoppered tubes :

Acetate buffer	0.2 ml
Water	0.1 ml
Glutaminase	0.2 ml
Sample cell or standard	0.5 ml

The assay mixtures were incubated for 1 h at 37° C. 0.5 ml of each tube were taken for assay of glutamate.

2.8.3.2 Determination of Glutamate

For each series of measurement the following were run :

1 - A blank reagent using 0.5 ml water instead of extract cells or standard.

2 - A glutamate standard using 0.5 ml glutamate standard solution (sol. 7).

Procedure

The following reagents were pipetted into cuvettes :

	A	B
Tris/hydrazine buffer	1.0 ml	1.0 ml
NAD solution	0.1 ml	0.1 ml
ADP solution	0.01 ml	0.01 ml
Water	0.39 ml	0.39 ml
Sample after hydrolysis	0.5 ml	-
Sample before hydrolysis	-	0.5 ml
Mix and read absorbance A_1 (339 nm against distilled water).		
GlDH solution	0.02 ml	0.02 ml
The assay mixtures were incubated for 40 min at room temperature and then the absorbances A_2 were measured at the same conditions described before.		

For calculation, ΔA was defined as $A_2 - A_1$, correcting for reagent blank.

2.8.3.3 Calculation

$$1 - \quad C_1 = \frac{\Delta A_A \times 2 \times 2.2 \text{ (total volume)}}{6.22 \times 0.5 \text{ (extract or standard vol.)}} \text{ umol/ml}$$

$$2 - \quad C_2 = \frac{\Delta A_B \times 2.2 \text{ (total volume)}}{6.22 \times 0.5 \text{ (extract or standard vol.)}} \text{ umol/ml}$$

$$3 - \quad C_3 = C_1 - C_2 \text{ umol/ml.}$$

C_1 = Glutamate + Glutamine

C_2 = Glutamate umol/ml

C_3 = Glutamine umol/ml.

The values obtained were corrected for the effect of deproteinisation.

Table 2.8.2 - Titration curve of Perchloric acid (10% v/v) with KOH (20% w/v)

Perchl.acid ml	KOH ml	pH
10	--	1.1
"	1.0	1.2
"	2.0	1.2
"	3.0	1.2
"	4.0	1.3
"	4.5	1.5
"	4.7	2.2
"	4.75	7.3
"	4.8	11

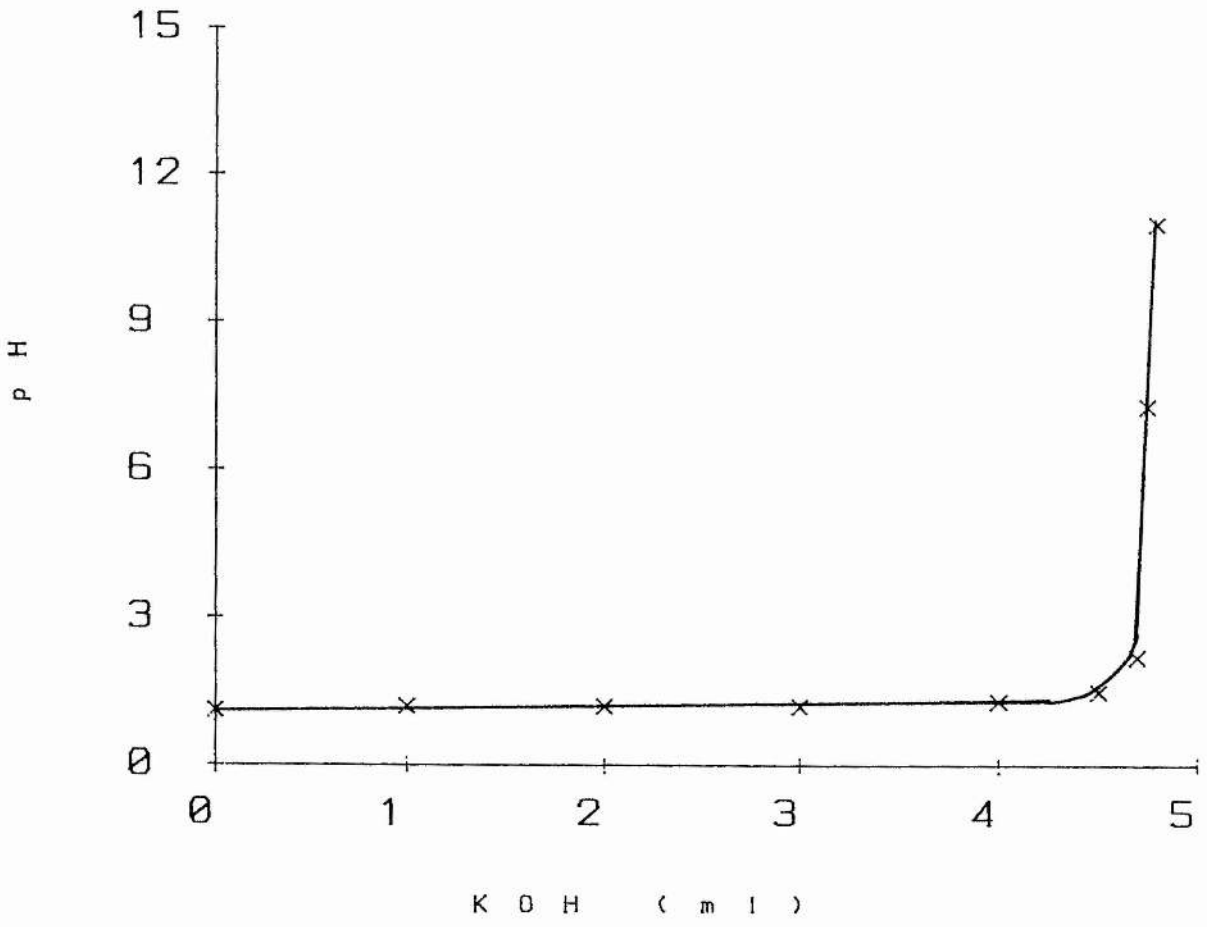


Figure 2.8.2 : Titration curve of Perchloric acid (10% v/v) with KOH (20% w/v).

2.9 Ammonia Measurement

Ammonia was measured utilizing Kit Nº 170 - A from Sigma.

2.9.1 Preparation of extracts

Aliquots of cell culture (3 ml when the medium was C limited and 12 ml when it was N limited) were centrifuged (Eppendorf 3 min/13,000 rpm) and the pellets pooled in one tube and ressuspended in 1 ml of TCA 10% (w/v). Glass beads (0.45 - 0.5 mm) were added up to approximately the same volume as the pellet. Cells were broken with a vortex mixer for 3 min. Supernatants were removed after centrifugation (Eppendorf 3 min/13,000 rpm) and neutralized with Potassium hydrogen carbonate ($KHCO_3$)(20% w/v) following the pre-constructed titration curve . (Table 2.9, fig. 2.9).

Table 2.9 - Titration curve of TCA (10 % w/v) with $KHCO_3$ (20 % w/v)

TCA ml	$KHCO_3$ ml	pH
10	--	1.1
"	1.0	1.2
"	2.0	1.3
"	3.0	2.2
"	4.0	6.8
"	4.1	7.1
"	4.2	7.2
"	4.3	7.4

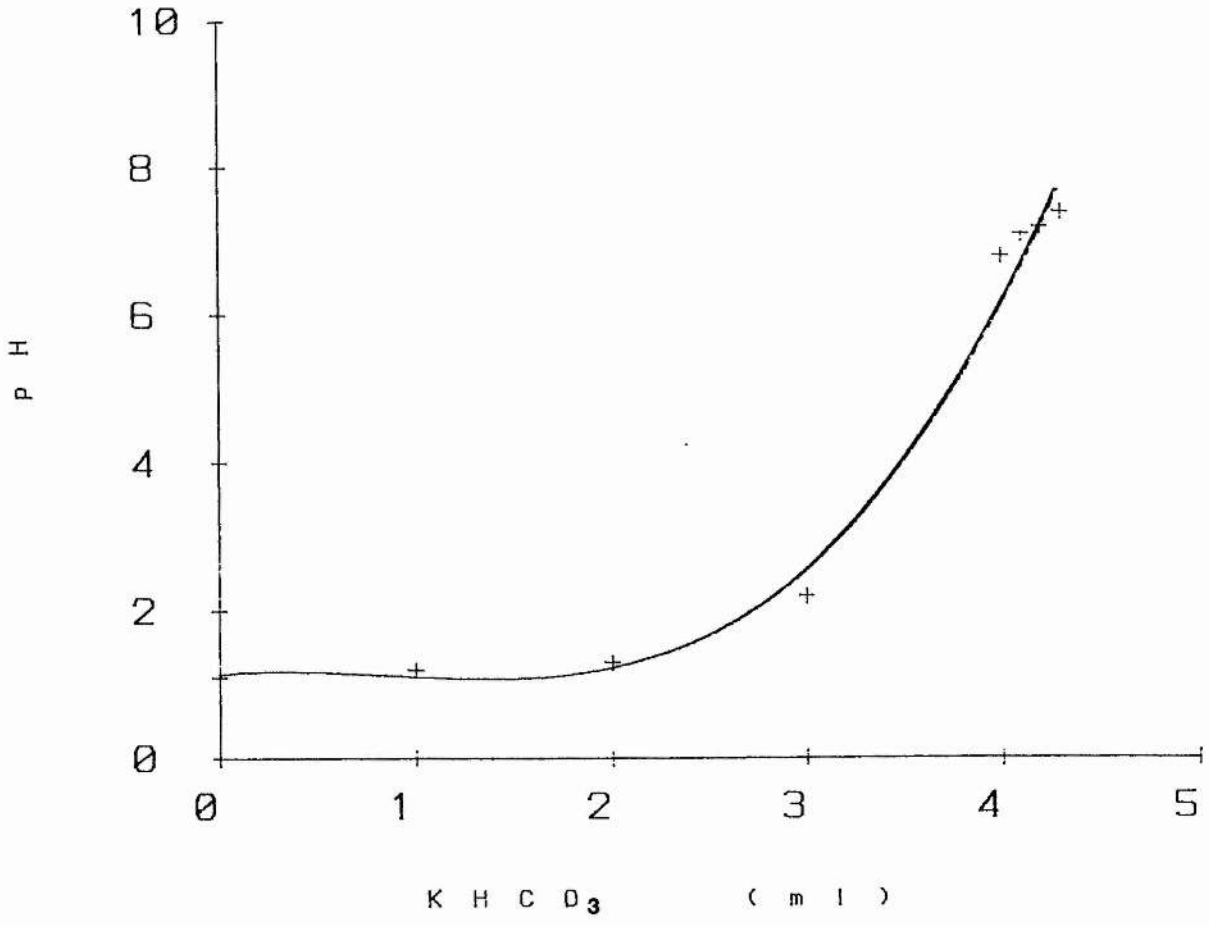


Figure 2.9 : Titration curve of TCA (10% w/v) with $KHCO_3$ (20% w/v).

2.10 Glucose Measurement

Glucose was measured by the Nelson's colorimetric modification of Somogy's method (53).

2.10.1 Reagents

1 - Low alkalinity copper reagent :

Sodium tartrate (12 g) and anhydrous sodium chloride (24 g) were dissolved in 250 ml water. A solution of 4.0 g of cupric sulphate pentahydrate in water was added with stirring, followed by 16 g of sodium hydrogen carbonate . A solution of 180 g of anhydrous sodium sulphate in 500 ml of water was boiled to expel air then the two solutions were combined and diluted to 1 litre.

2 - Arsenomolybdate reagent :

To 25 g of ammonium molybdate in 450 ml of water was added 21 ml of 96% sulphuric acid, followed by 3 g of disodium hydrogen arsenate heptahydrate dissolved in 25 ml of water. The mixed solution was incubated for 24 h at 37° C and stored protected from light in a brown bottle.

2.10.2 Glucose assay conditions

To 1-5 ml of sugar solution containing not more than 0.6 mg of glucose or its equivalent an equal volume of low-alkalinity copper reagent was added. Samples and blanks (with water instead of sugar solution) were heated for 10 min in a vigorously boiling water-bath and then cooled. 2 ml of arsenomolybdate reagent was then added with caution and the tubes were carefully mixed with glass rods to dissolve the cuprous oxide. The solutions were then diluted to 20 ml and allowed to stand for 15 min after which absorbances were measured at 500 nm.

A calibration plot was constructed utilising glucose solutions with known concentrations. (Fig. 2.10).

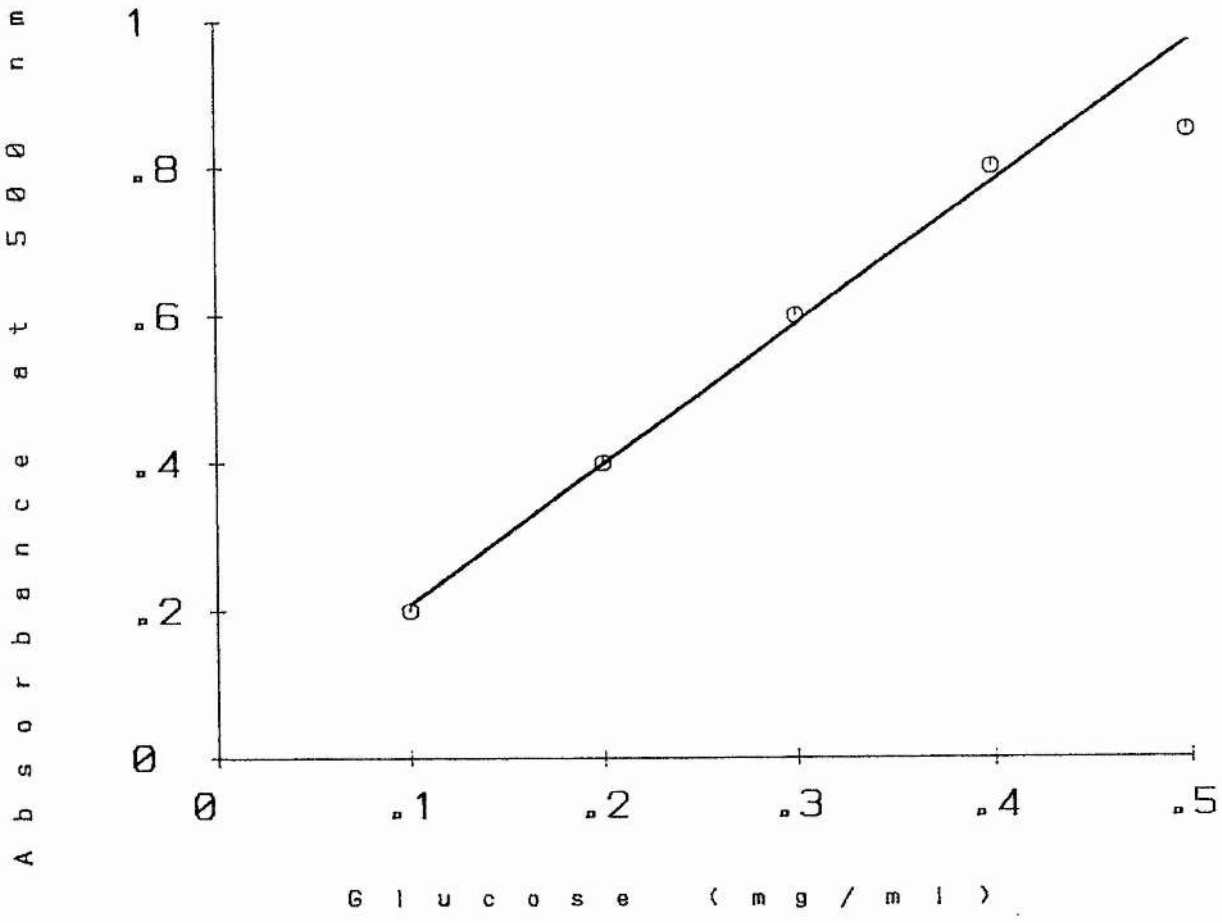


Figure 2.10 : Glucose calibration plot.

3 RESULTS AND DISCUSSION

3.1 Measurement of maximum specific growth rate (u_m) of *S.cerevisiae* (wild type, AR2 and AR5)

3.1.1 Specific Growth Rate Measurement by Batch Culture

The maximum specific growth rate for the three strains was determined as described in 2.5.1.

Figures 3.1.1 to 3.1.1b show the growth curves for the three different strains of *Saccharomyces cerevisiae* used in our experiments. Two of them, AR2 and AR5, are genetically engineered strains, and the ≤1278b is a wild type used as control. Table 3.1.1 shows the maximum specific growth rate for the same strains. The wild type showed the higher value of u_m : 0.27/h. The values founded for AR2 and AR5 were 0.20 and 0.21/h, respectively.

The maximum specific growth rate obtained for the wild type is the same reported by Racher (54) utilising similar medium, and other published values (55).

AR2 and AR5 derived from *Saccharomyces cerevisiae* BC55 which is *gdh⁻ leu⁻* . Both AR2 and AR5 carry the plasmid pCYG4 which encodes the gene for NADPH-GDH activity (about 10-fold higher than the level of

NADPH-GDH activity found in wild type cells (54)) and also the genes for Penicillinase (marker) and leucine production . The absence of leucine in the medium is a selective pressure.

It has been reported that cells without plasmid may grow faster than those bearing plasmid (56). Lee et al (57) found that *S.cerevisiae* strain YNN 27 carrying the plasmid pMHBS, which encodes the gene for HBsAg (hepatitis B virus surface antigen), showed a specific growth rate of 0.39/h. The strain without plasmid had shown a specific growth rate of 0.42/h under the same growth conditions (10 g/l yeast extract, 20 g/l pepton and 20 g/l dextrose). The extra plasmid products (like proteins or enzymes involved in aminoacid biosynthesis) associated with the number of plasmid per cell may cost the plasmid-bearing cells a reduction in growth rate compared to cells without plasmid.

Caulcott et al (58) working with *E.coli* HB101 carrying pCT70, pCT66 or pCT54 (plasmids related to the expression of the Met-prochymosin gene), found that cells bearing a plasmid which does not express Met-prochymosin at high levels have a significant growth advantage over those cells which synthesize large amounts of the protein. The u_m of *E.coli* bearing pCT70 , plasmid which shows a high expression level, was reduced by 30-35% as compared with the plasmid-free cells. However, the presence of pCT54 or pCT66, both plasmids of low

expression level, did not reduce the growth rate of the host organism. They concluded that the demands made on the cellular protein synthetic machinery, (including ribosomes and tRNA pools) could be related to the reduction in the growth rate

The slow growth of AR2 and AR5 compared to the wild type ≤1278b can be related to the added metabolic loads due to the plasmid encoded genes (Penicillinase, enzymes of leucine biosynthesis).

AR5, however is a double mutant with very low GOGAT activity compared to the parenteral strain (BC55) (54). Although the GS-GOGAT is a more energetically expensive pathway for ammonia assimilation, the absence of this pathway could be related to the slightly difference in the specific growth rates between AR2 and AR5 .

Table 3.1.1

Batch culture of three strains of *Saccharomyces cerevisiae*

Strain	Vector	umax (/h)
W.type		0.27
AR2 (GOGAT$^+$)	pCYG4	0.20
AR5 (GOGAT$^-$)	pCYG4	0.21

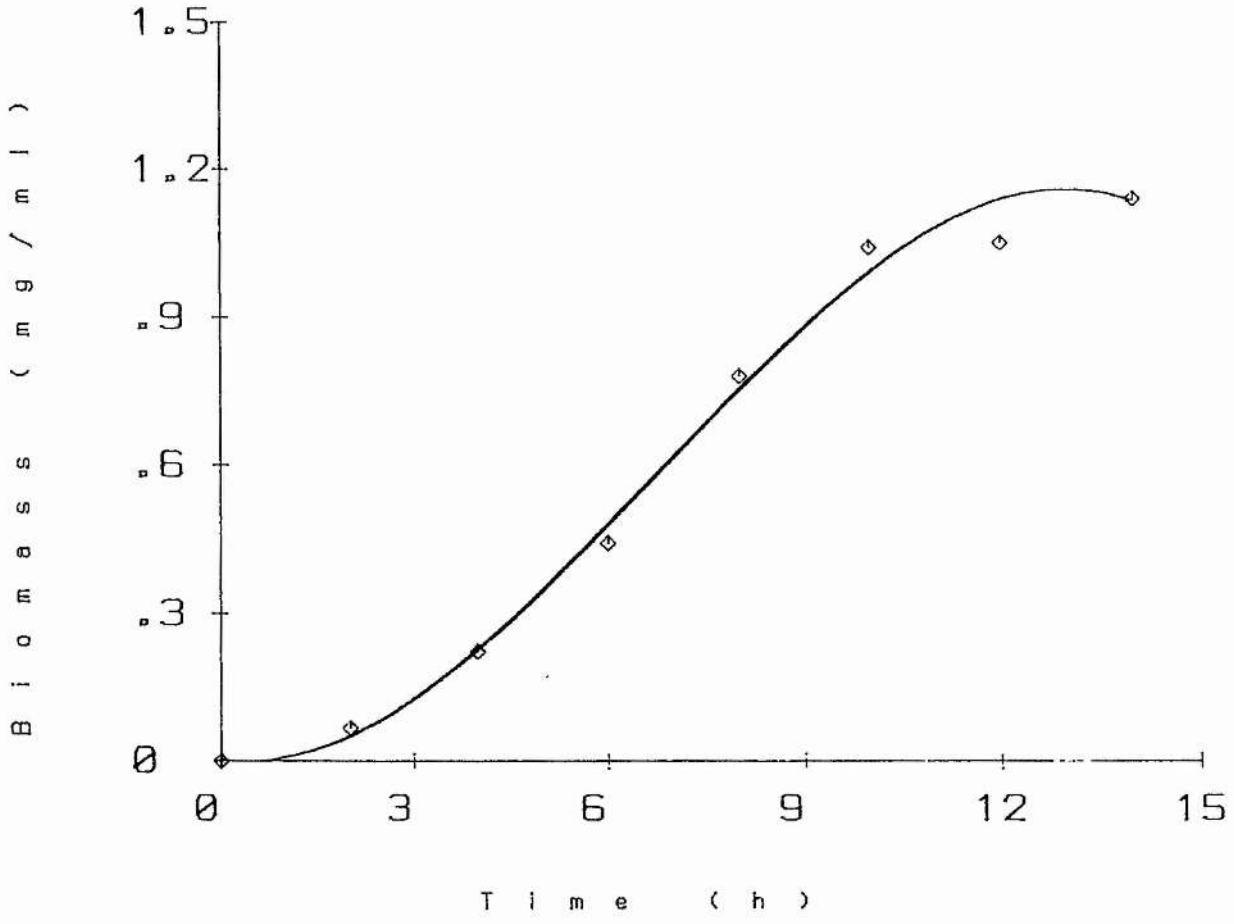


Figure 3.1.1 : Growth curve for AR2 strain growing on MM + 20 mM Ammonium Sulphate at 30° C.

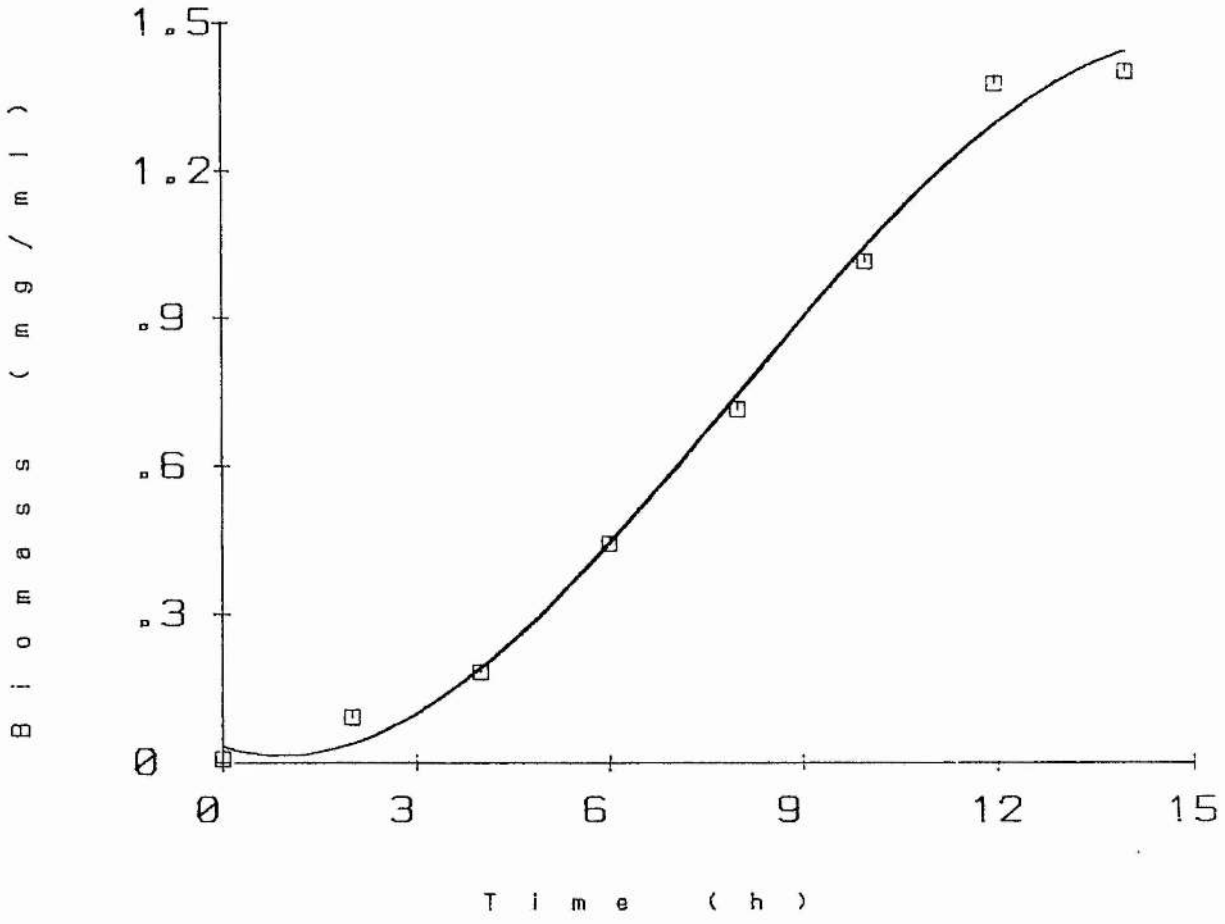


Figure 3.1.1a : Growth curve for AR5 strain growing on MM + 20 mM Ammonium Sulphate at 30° C.

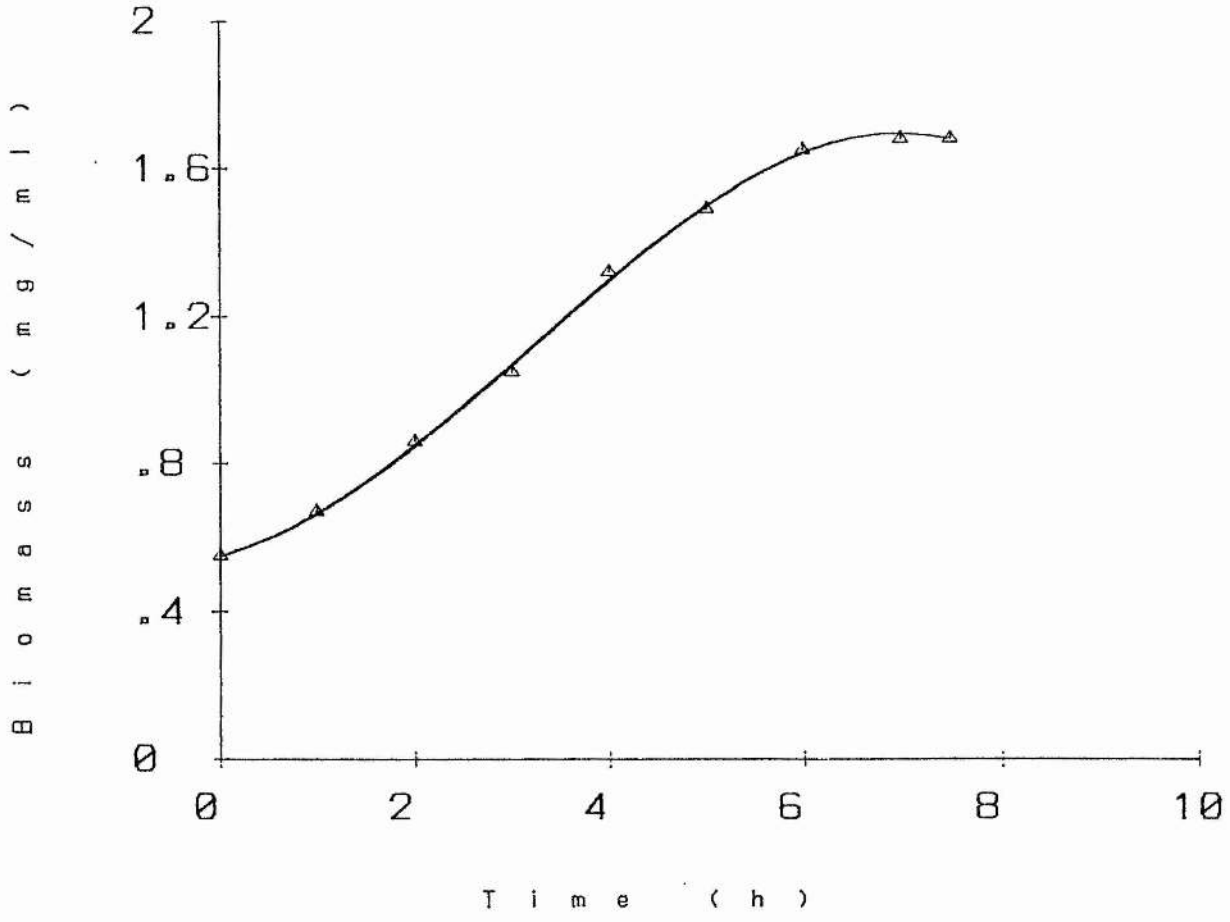


Figure 3.1.1b : Growth curve for ≤1278b wild type strain growing on MM + 20 mM Ammonium Sulphate at 30° C.

3.1.2. Specific Growth Rate Measurement by Chemostatic Culture

Maximum specific growth rates for AR2, AR5 and the wild type ≤ 1278b were determined by washout kinetics as described in 2.5. 2. with a dilution rate of 0.35/h under C limited medium following maintenance of steady state for approximately 48 hours.

As can be seen in the Table 3.1.2, the wild type showed again the highest value of u_m, 0.26/h, with values of 0.18 and 0.19/h for AR2 and AR5 respectively. The presence of a plasmid and its effects in the growth rate of an organism have already been discussed (3.1.1). However, there was a slightly difference in the maximum specific growth rates for the same strains determined in batch mode (Table 3.1.1).

Although during the exponential phase in batch culture growth should occur at the maximum rate, the microorganisms are subjected to continuous changes in its environment. The substrate concentration is constantly decreasing and inhibitory metabolic products will accumulate (45). The growth with excess of substrate is followed rapidly by nutrient starvation.

The determination of maximum specific growth rate by washout with continuous culture requires a dilution rate which is higher than the critical dilution rate (D>Dc). Obviously a steady state will not

be maintained, but there will be neither starvation nor accumulation of possible inhibitory products (e.g. ethanol). It seems then that results obtained by continuous culture experiments are more accurate than those obtained by batch mode (45).

The continuous culture was chosen for the subsequent experiments because this cultivation technique enables an analysis of physiological states of organisms under steady state conditions where parameters like pH and nutrient concentration remains constant for a high number of generations.

Continuous culture have been used mostly in the development of batch and fed-batch production optimization . However, because the environmental conditions that prevail in a continuous flow system are quite different from those in closed system cultures, microorganisms may express properties that they do not elaborate in batch culture, so that new and unexpected features of microbial behaviour may be exploited (59).

Also the usefulness of continuous flow systems to study the stability of genetically engineered strains cannot easily be overestimated.

Plasmids are finding widespread use as vectors for cloning DNA . However, the potential of this technique would be highly decreased if the recombinant

plasmid exhibit either structural or hereditary instability. Furthermore, for plasmids present at low copy number the frequency of plasmid-free cells is expected to be high. Also the probability of producing a plasmid-free cell at every division remains unchanged, and plasmid-free cells will be produced at constant rate.(60).

The requirement for an analysis of the performance of recombinant organisms under simulated processes may be of crucial importance for the industrial application of genetically engineered strains. The only alternative technique to study the plasmid stability is the repeated sub-culture of plasmid-containing strains. But in this case, cells change between conditions of nutrients excess and nutrients starvation, which might induces a variation in the degree of competition between plasmid-bearing and plasmid free cells. Also the determination of the number of generations is not accurate and the risk of contamination is increased.

Table 3.1.2

Maximum specific growth rates determined during chemostatic experiments under C limited medium at D = 0.35/h

Strain	Vector	umax (/h)
W.type	_____	0.26
AR2 ($GOGAT^+$)	pCYG4	0.18
AR5 ($GOGAT^-$)	pCYG4	0.19

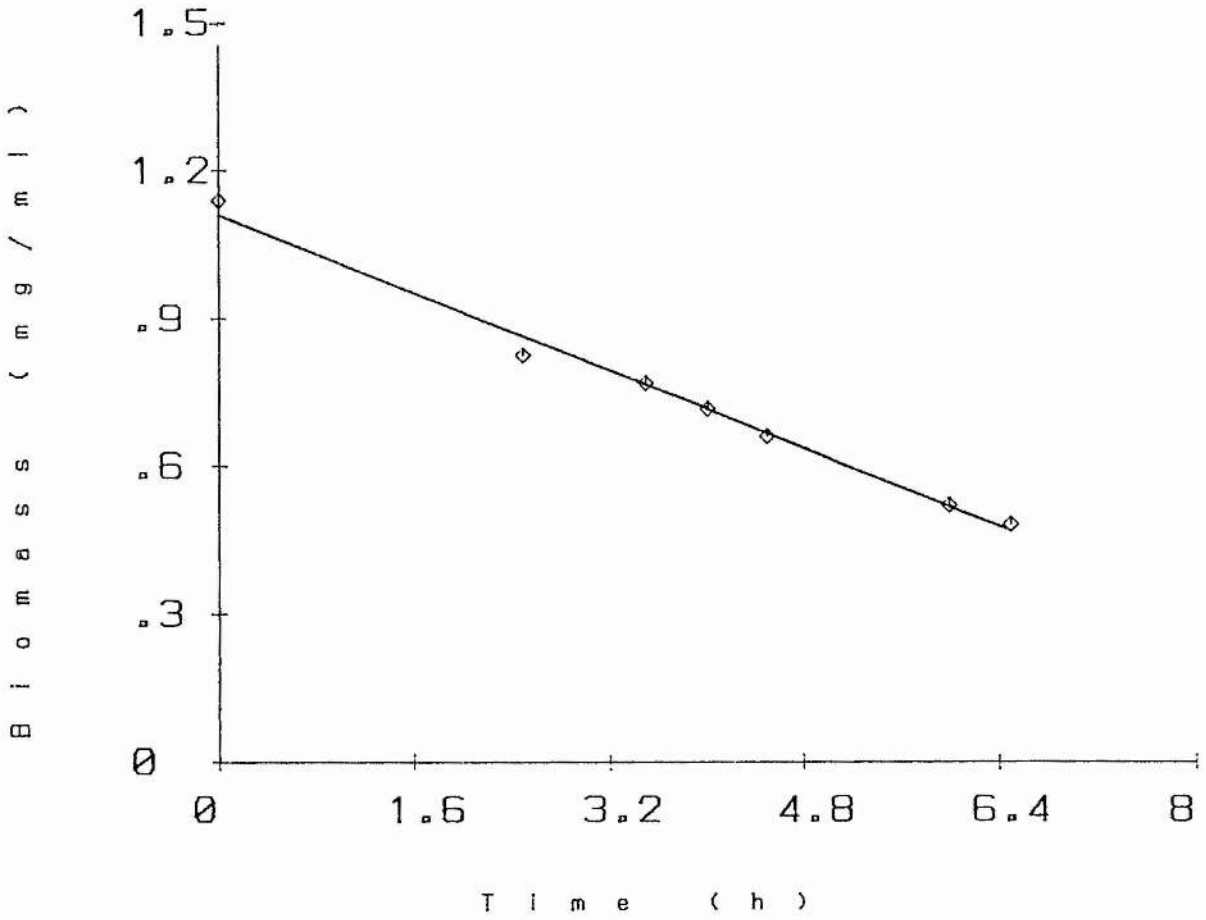


Figure 3.1.2 : Wash out of AR2 cells growing on carbon limitation.

D = 0.35/h.

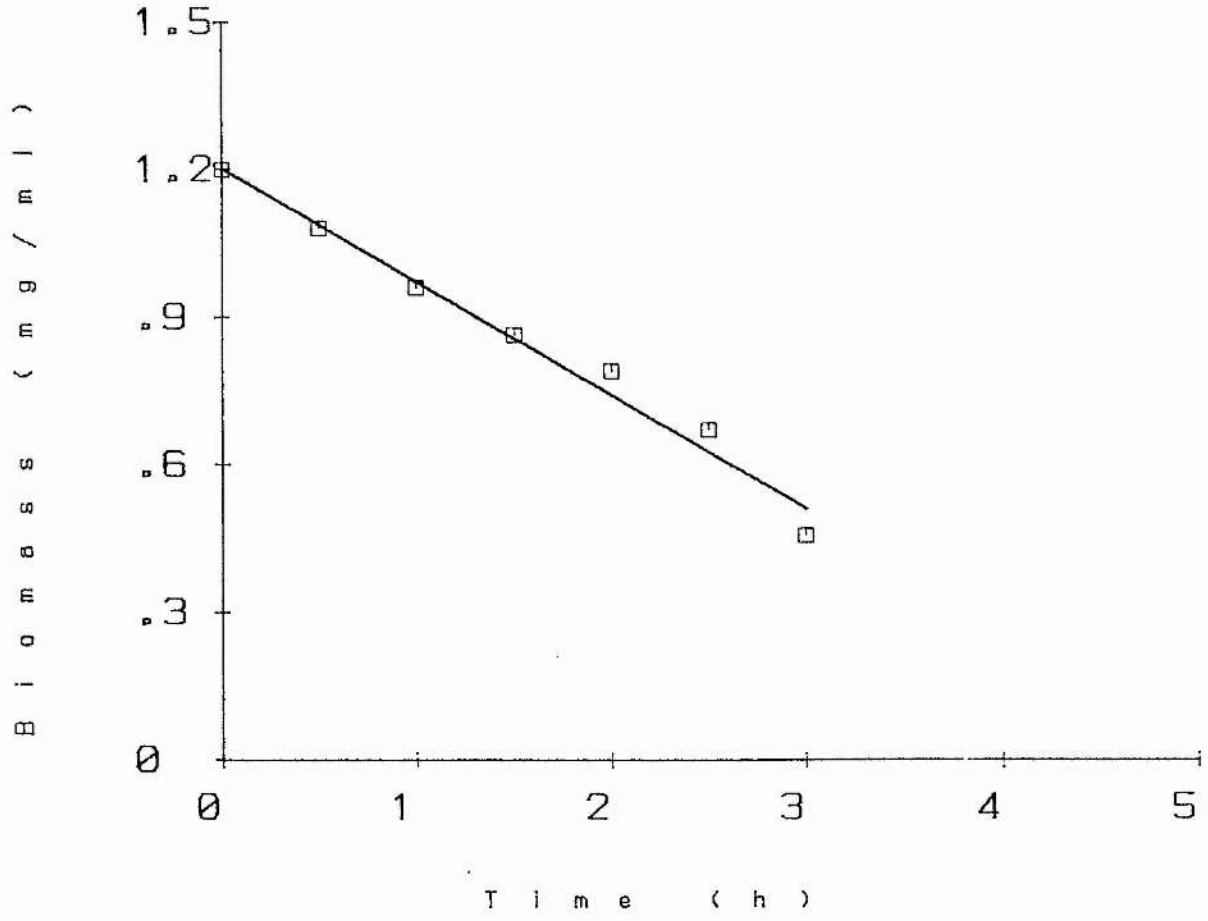


Figure 3.1.2a : Wash out of AR5 cells growing on carbon limitation.

D = 0.35/h.

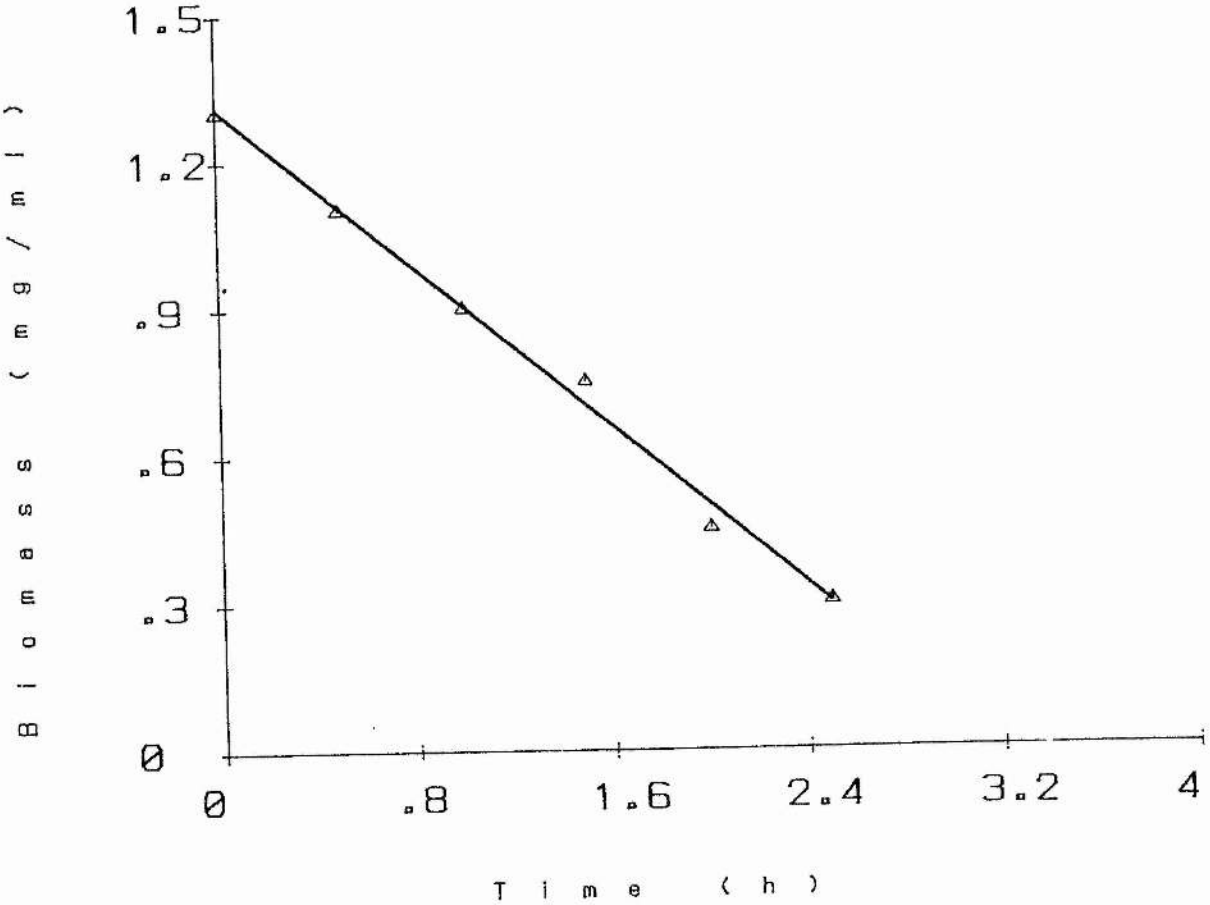


Figure 3.1.2b : Wash out of ≤1278b cells growing on carbon limitation.

D = 0.35/h.

3.2. Plasmid Stability

Plasmids are autonomously replicating genetic elements which confer new phenotypes on their host cells. They can be categorised on the basis of their being maintained at a high copy number per cell (relaxed plasmids) or as a low number of copies per cell (stringent plasmids).

If the plasmid-born genes are to remain in the genetic pool there must be an efficient mechanism for segregation of plasmids at cell division. In the absence of an active mechanism the probability P(0) of daughter cell failing to inherit a plasmid is given by the binomial distribution :

$$P(0) = 2(1/2)^c$$

where c is the number of plasmid per cell at division (61). Obviously, the fraction of plasmid-free cells relates directly to the copy number. For high number of copies, a low frequency of plasmid-free cells is expected.

Stability may be defined as the ability of plasmid-bearing cells to maintain plasmids unchanged during their growth, manifesting their phenotypic characteristics . The stability or instability of plasmid may be affected by several factors such as growth rate, genetic characteristics, environmental conditions, etc. (62) , (63) , (64) .

Horn et al (65), working with *E.coli* K12 harbouring the plasmids pUR 290 and FB 99 obtained a high segregational stability (nearly 100%) using fed batch cultivation.

One way of maximizing the density of plasmid containing cells is to assure growth advantages to them (66). If the host cell bears a defective gene, the product of this gene (complementing product) in the plasmid would function as a pressure factor in a selective medium. Cells without complementing product would not grow in a selective medium (that is, without the complementing product) (67).

Oscillations were present in the NADPH-GDH activities for AR2 and AR5 strains growing under C and N limited media (Figs.3.2.2 a to 3.2.2o). They are probably due to segregational instability of the plasmid pCYG4 in these microorganisms.

In continuous culture, at steady state, the specific growth rate is determined by the dilution rate ($u = D$) (45). In our experiments the oscillations were more evident at high values of D ($D = 0.15/h$). Kleinman et al. (68) working with *Saccharomyces cerevisiae* carrying the plasmid pJDB248 found that the plasmid was more stable at higher growth rates than it was at lower growth rates. But it has been shown that for different plasmids and host cells, the plasmid copy

number can increase or decrease with temperature, dilution rate and other parameters (69). Impoolsup et al (70) reported that the stability of the plasmid pLG668-z in *Saccharomyces cerevisiae* decreased with increasing growth rate in continuous culture. These researchers found that the major effect of the growth rate difference is that once the plasmid free cells are formed by segregational failure at cell division, they tend to dominate the population due to their higher growth rate. The excess metabolic load due to the requirement to replicate and express the extra DNA results in a slower specific growth rate of plasmid containing cells. They also suggest that as the cells are growing faster, they make more mistakes during each cell division with less time to correct them. That would result in higher levels of segregational instability at higher growth rates.

According to Satyagal et al.(71), the plasmid concentration could be determined through the following equation :

$$p_s = \frac{V^0}{u + K_h} - K_r$$

Where :

p_s = plasmid concentration

V^0 = maximal rate of plasmid synthesis

K_r = saturation constant (since the plasmid replication is a cellular enzymatic reaction)

K_h = a measure of the dependence of the plasmid on the host cell for replication (large values for this parameter meaning greater requirement of host functions for replication).

The model predicts that the plasmid concentration decreases with an increase in the specific growth rate. The model was applied to date reported by Engberg and Nordstrom (72) who cultivated *E.coli* cells harbouring the plasmid R1 -drd 19 and R1-drd 19B2 in batch cultures with different media resulting in different growth rates. The results predicted by the model were very closed to those reported by the researchers. However,, in continuous culture the nutritional condition of the environment becomes a very important determinant of cellular behaviour. Then, the effect of nutrient limitation has to be taken in account. The authors proposed the following equation to be used under continuous culture :

$$p_s = \frac{V^0 \; u^n}{(u + K_s)\; u_m{}^n} - K_r$$

Where n indicates the dependence on the limiting substrate.

The model was applied to date reported by Koizumi and coworkers (64) who cultivated *B.stearothermophilus* harboring plasmid pLP11 in continuous culture at different dilution rates and at different temperatures. It was observed that the value of the exponent *n*

increases with an increase in temperature. Also, the plasmid concentration was higher for higher values of dilution rate. They found that at 44° C the plasmid content was 0.2 mg/mg cells at a dilution rate of 0.5/h, and nearly 0.4 mg/mg cells at a dilution rate of 1/h. Our experiments showed higher NADPH-GDH activities at higher dilution (growth) rates in agreement with Koizumi et. al. AR2 and AR5 have plasmid-born NADPH-GDH activity. Both strains growing either on C or N limitation showed enzymatic activities which were nearly 2 times higher (about 12 U/mg) at dilution rate of 0.15/h than those activities at D = 0.05/h.

It has also been reported that even if the replication of the plasmid DNA and its metabolic loads does not confer growth disadvantages for the plasmid-carrying cell, the gene expression may do it. A wild type strain of *Pseudomonas putida* PPK1 carrying a non-conjugative TOL plasmid (which carries genes for the catabolism of toluene , *m*- and *p*-xylene and their intermediates) showed stable maintenance of the plasmid up to 600 h under succinate limitation. In these conditions the enzymes of the aromatic pathways are not induced . The same plasmid was lost during benzoate limited growth. In this case, the plasmid was more stable when the catabolic genes were not expressed . (73) .

Environmental conditions may emphasize any growth rate advantage possessed by plasmid-free or plasmid-bearing cells. AR2 and AR5 derived from *Saccharomyces cerevisiae* BC55 which is *leu*r (see 2.1). The medium utilized in the continuous culture experiments did not contain any aminoacids. Consequently, cells without the plasmid pCYG4 (with gene for leucine, the complementing product) would not grow . However, at high dilution rate at C or N as limiting growth, the activity of NADPH-GDH and Penicillinase rises, suggesting an increase of plasmid copy number in the cells. As a consequence the complementing product concentration will increase too, followed by an increase in the population of plasmid free cells, which can grow faster. That will lead to a decrease in the concentration of leucine. As it was said previously, cells without plasmid cannot grow in the absence of leucine, therefore the population of plasmid bearing cells tend to increase again.

Oscillations have also been reported in chemostat cultures of *Saccharomyces cerevisiae* bearing no chimeric plasmids . Porro et al (74) reported that the period of the oscillations seemed to be related to the mass doubling time, showing a relationship with the parent cells and daughter cells generation time . However, in that research, appart from the stirring there was no control of the oxygen supply. The authors

concluded that the oscillations were related to a condition of growth that does not allow a fully respiratory metabolism of glucose during specific phases of cell cycle, such as the bud emergence .The complex changes of the cell population were demonstrated by continuous and periodic modification of cell volume and protein distribution.

However,in our experiments the oxygen concentration was kept constant at 30% during the whole time of the experiments. Also,the oscillations detected for NADPH-GDH were "in phase" with oscillations in Penicillinase activity, which is also plasmid encoded (See Figs. 3.2 and 3.2a). They were far more evident than oscillations related to biomass . This would indicate that the enzymatic oscillations are better explained by the increase of the concentration of cells with or without plasmid following the availability of the complementing product in the medium. The amplitude of the oscillations may be a consequence of an inverse relation between growth rate and plasmid stability , probably due to segregational instability.

The plasmid seems to be more stable in AR5 at high values of dilution rate than in AR2. It may be due to the absence of the second pathway for ammonia assimilation (GOGAT pathway) in the first strain.

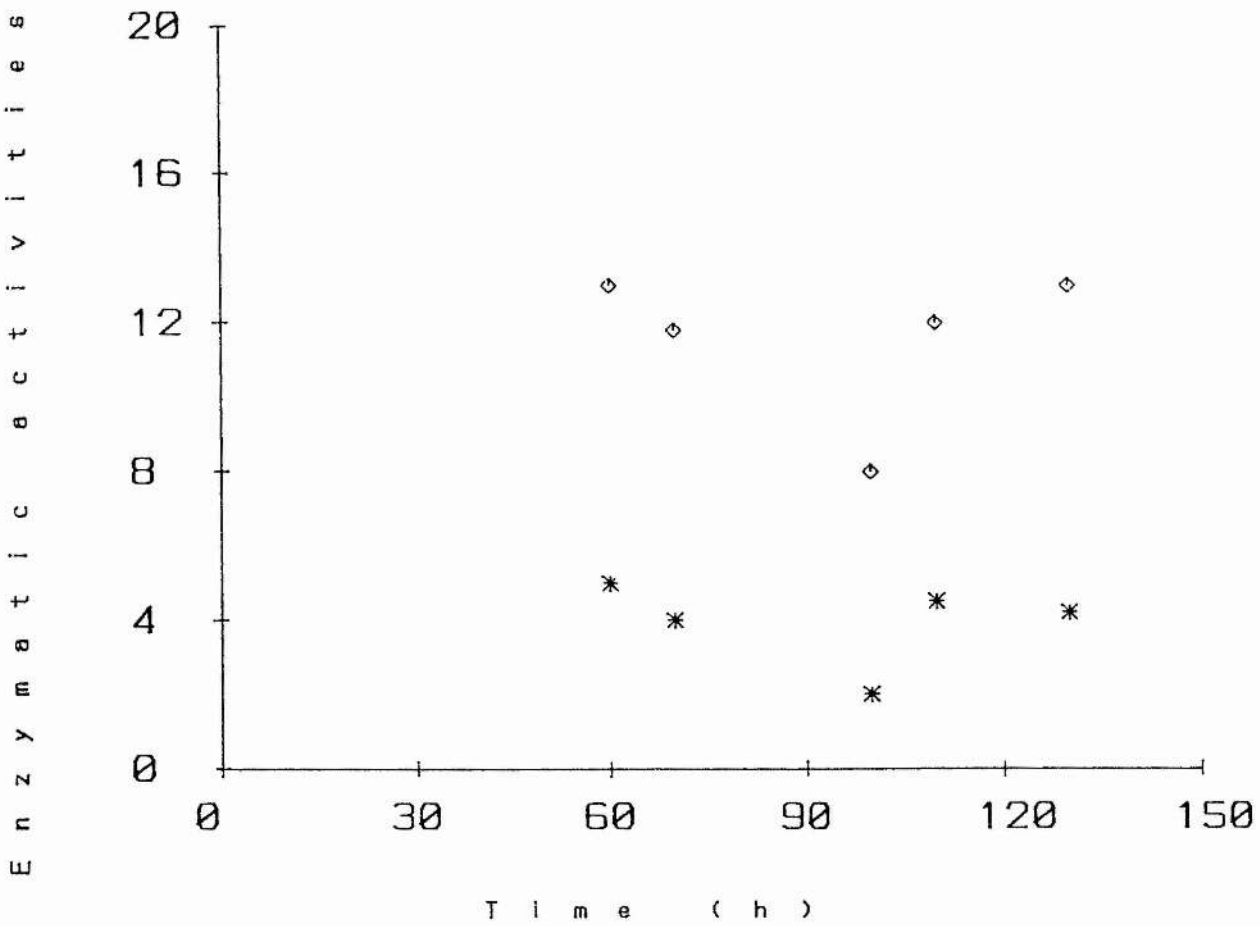


Figure 3.2 : Penicillinase (mU/ml) (✳) and NADPH-GDH (U/mg) (◇) activities of AR2 cells growing on carbon limitation. D = 0.15/h.

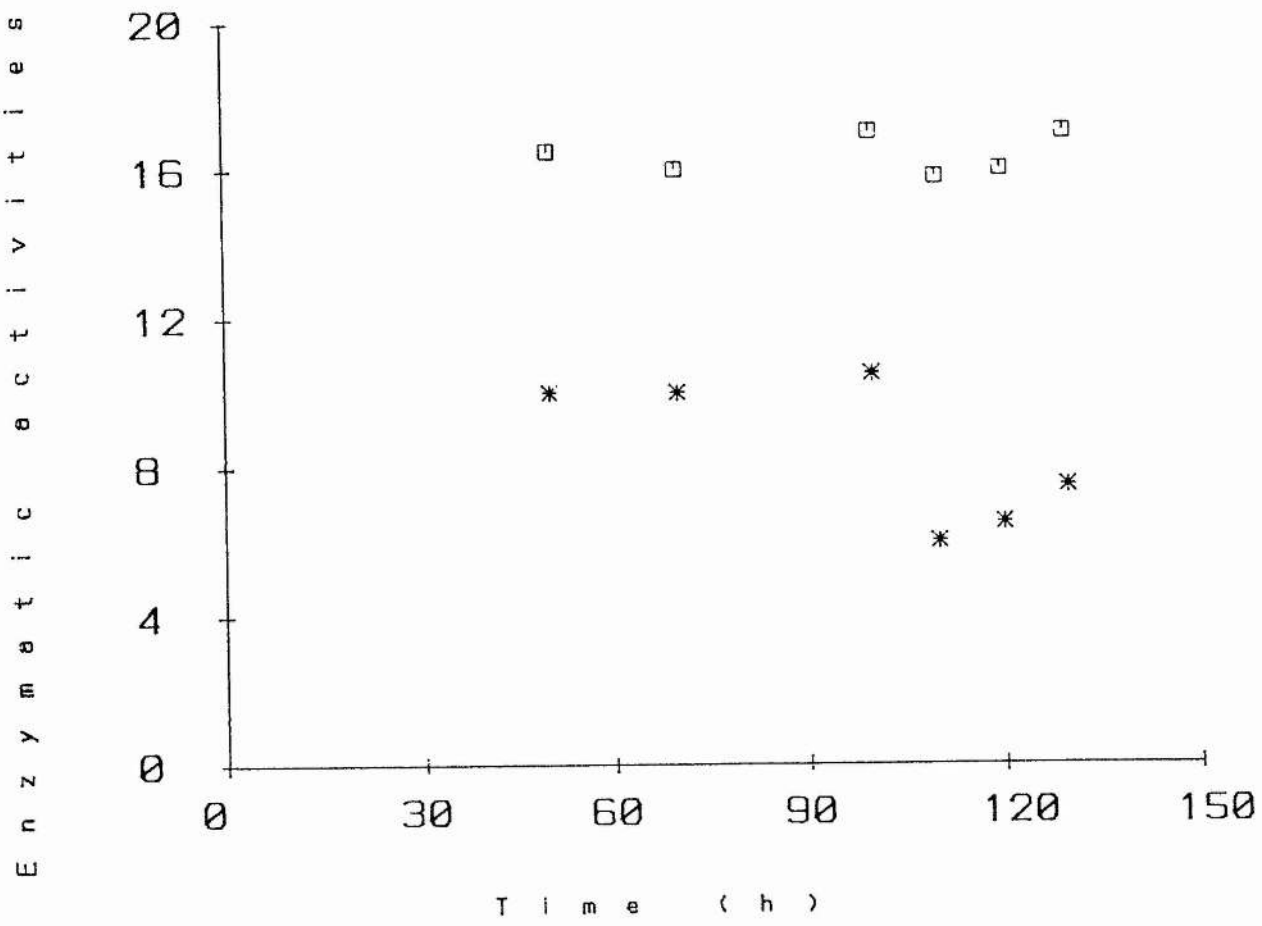


Figure 3.2a : Penicillinase (mU/ml) (✳) and NADPH-GDH (U/mg) (□) activities of AR5 cells growing on carbon limitation. D = 0.15/h.

3.3 NADPH-GDH , GOGAT and GS activities under carbon limitation

NADPH-GDH activity was tested at four different dilution rates (0.05, 0.10, 0.15 and 0.20/h) for AR2 and AR5 strains. The activity increased proportionally to the dilution rate for both strains (Figs.3.3 to 3.3g). These results agree with Caulcott (75), who pointed out that the activity of NADPH-GDH in yeast increases with increasing dilution rate, which corresponds to increasing in the specific growth rate. It was found that under carbon limitation, the activity was maximal when the dilution rate was approximately 0.15/h, which was also detected in our experiments.

When the dilution rate was approximately 0.2/h, it was too closed to the umax detected in batch experiments (Table 3.1.1), and even above the values detected by chemostatic mode (Table 3.1.2). In that case, the system was unstable, and although the activities were high at the beginning, there was washout of cells with consequent loss of NADPH-GDH activities..

Oscillations were detected for NADPH-GDH activities of AR2 and AR5, and as it has already been discussed (3.2.2) they are probably due to plasmid-bearing and plasmid-free cells competition.

Taking average values of the AR2 and AR5 NADPH-GDH activities under 0.05, 0.10 and 0.15/h, no

considerable differences were detected. Lomnitz et al, working with *Neurospora crassa* (wild type and a GOGAT$^-$ mutant) under ammonia excess conditions, detected only a slightly increase in the enzymatic activity of the mutant compared to the wild type (76).

. NADPH-GDH activity for wild type was tested for dilution rate of 0.15/h only (See fig.3.3h). As it was expected, under the same conditions AR2 and AR5 showed activities which were nearly 5-fold higher , due to the presence of pCYG4 plasmid in these strains.

GOGAT activity was about 8% of the NADPH-GDH detected for the wild type (Fig.3.3h),in agreement with Roon et al (25) who observed that levels of GOGAT are approximately 10-fold lower than the maximum level of activity for NADPH-GDH

As the presence of the plasmid pCYG4 in AR2 increases about 5-fold times its NADPH-GDH activity (but not the GOGAT one), for every tested dilution rate the levels of GOGAT activity were less than 1% of the detected NADPH-GDH (Fig. 3.3 to 3.3.b).

GOGAT activities for AR2 did not show the same level of oscillations detected for NADPH-GDH. As GOGAT activity in this strain is not plasmid encoded,the oscillations are probably related to oscillations in biomass . It has already been demonstrated that factors

like the bud emergence might affect the stability of the biomass, with a marked degree of synchronization between the budding index and the oscillatory cycles (74),(77) (78).

GOGAT activity was not detectable in AR5 growing under carbon limited cultures.

Although GS is the only way to synthesize glutamine, and is also an important element of nitrogen repression in *Saccharomyces cerevisiae* , GS activities were below 1 mU/mg for AR2 and the wild type, and pratically undetectable for AR5 under carbon limitation. These data are in agreement with Thomulka and Moat (79), who found GS activity minimal in *Saccharomyces cerevisiae* cells grown with ammonia 4 mM, and markedly lower than the NADPH-GDH activity.

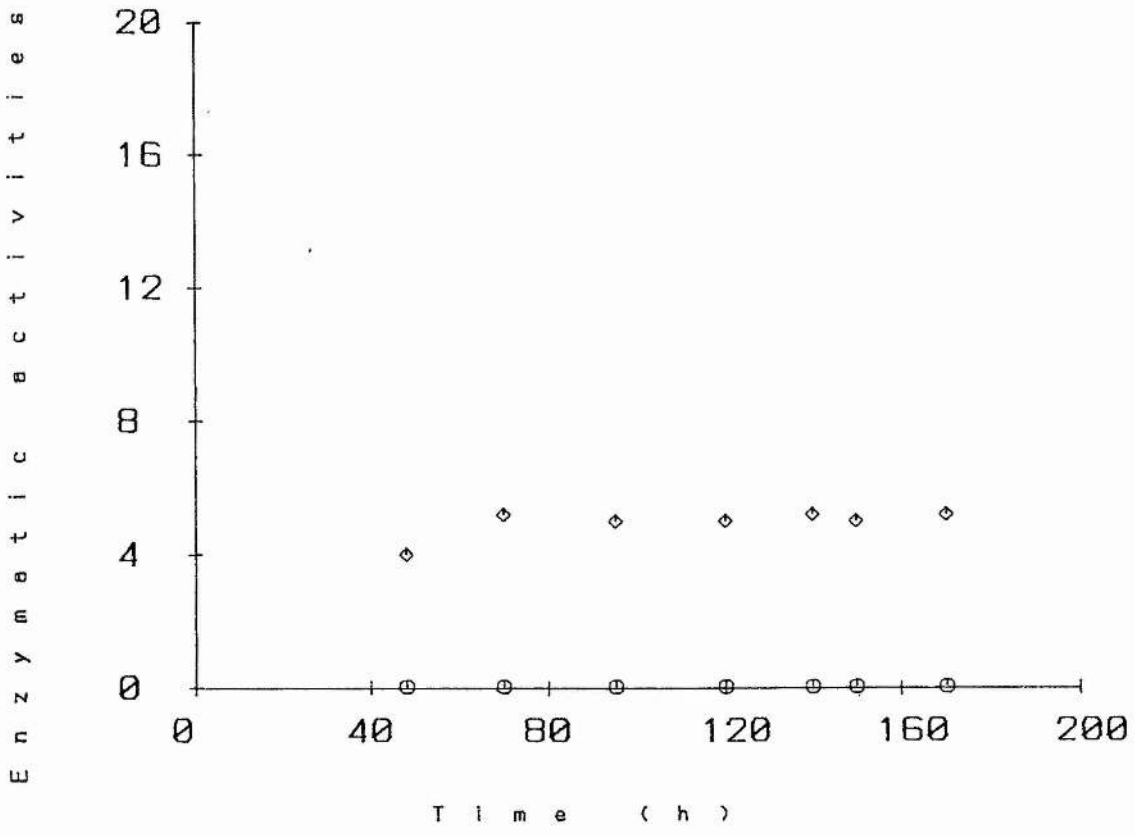


Figure 3.3 : NADPH-GDH (◇) and GDGAT (○) activities of AR2 cells growing under carbon limitation. D = 0.05/h.

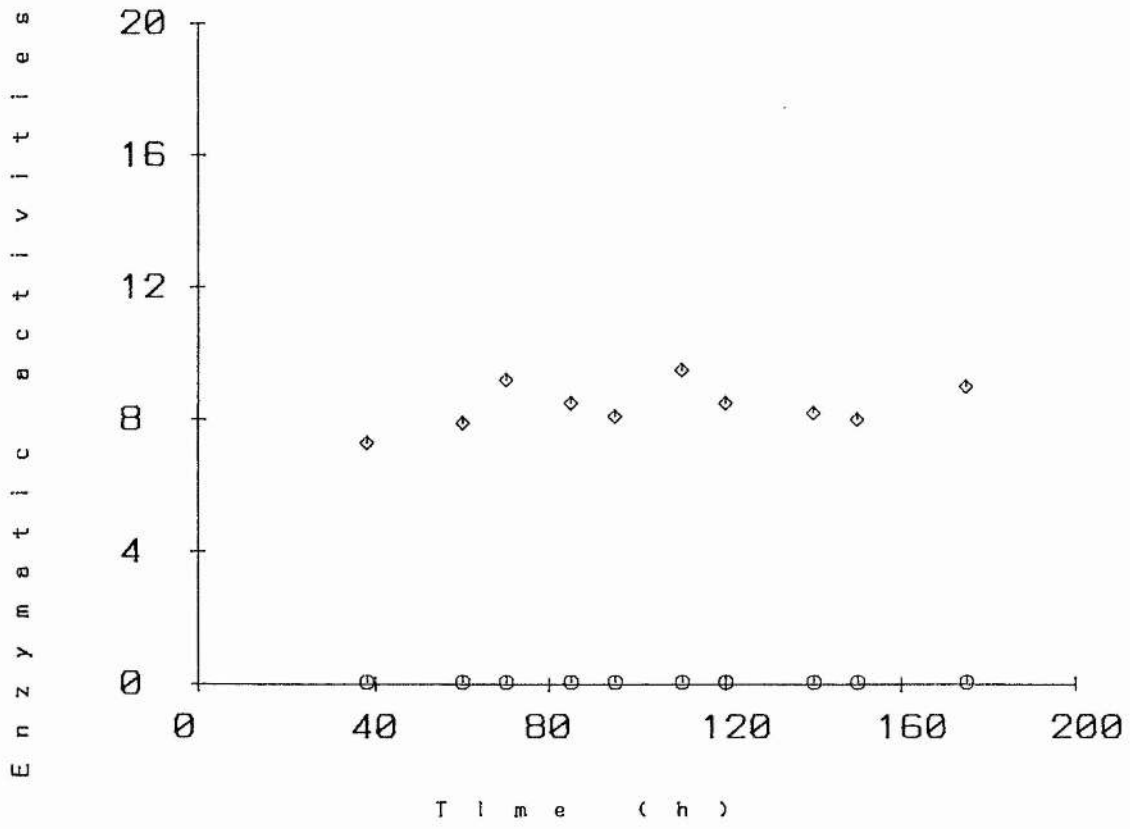


Figure 3.3a : NADPH-GDH (◇) and GOGAT (○) activities of AR2 cells growing under carbon limitation. D = 0.10/h.

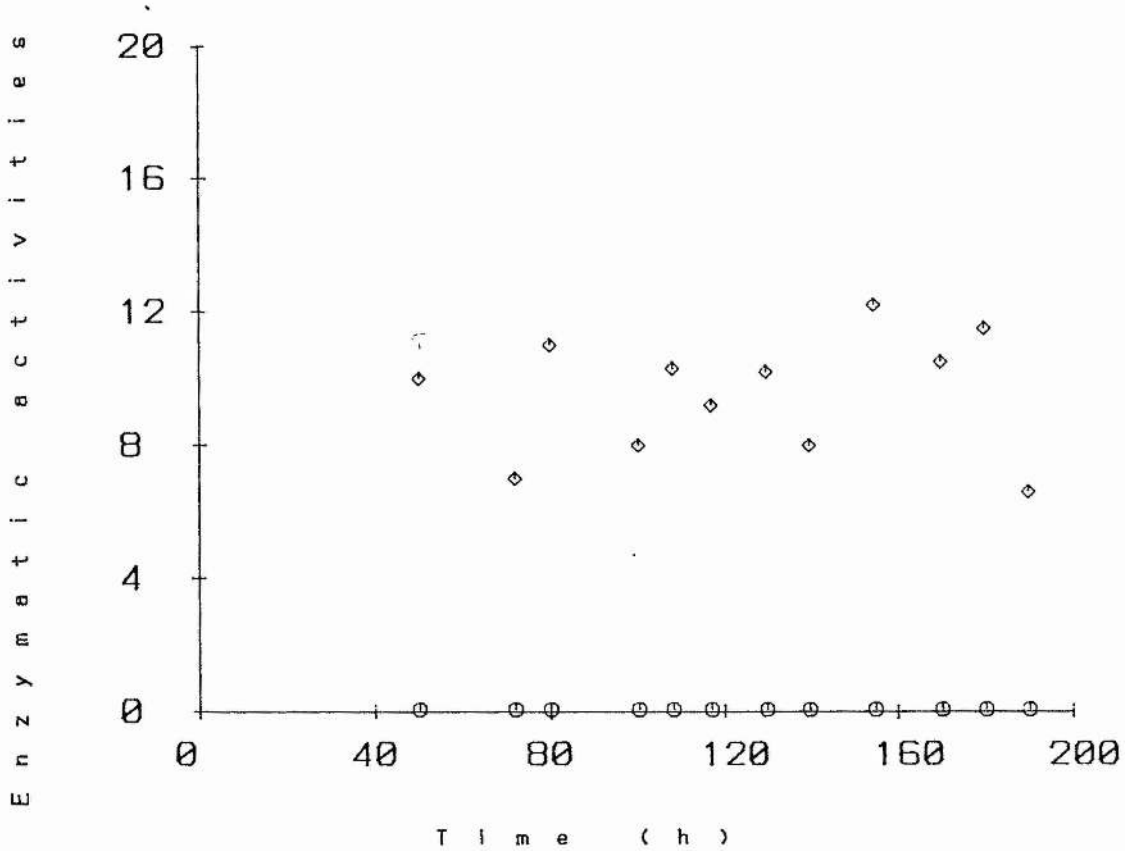


Figure 3.3b : NADPH-GDH (◇) and GOGAT (○) activities of AR2 cells growing under carbon limitation.

D = 0.15/h.

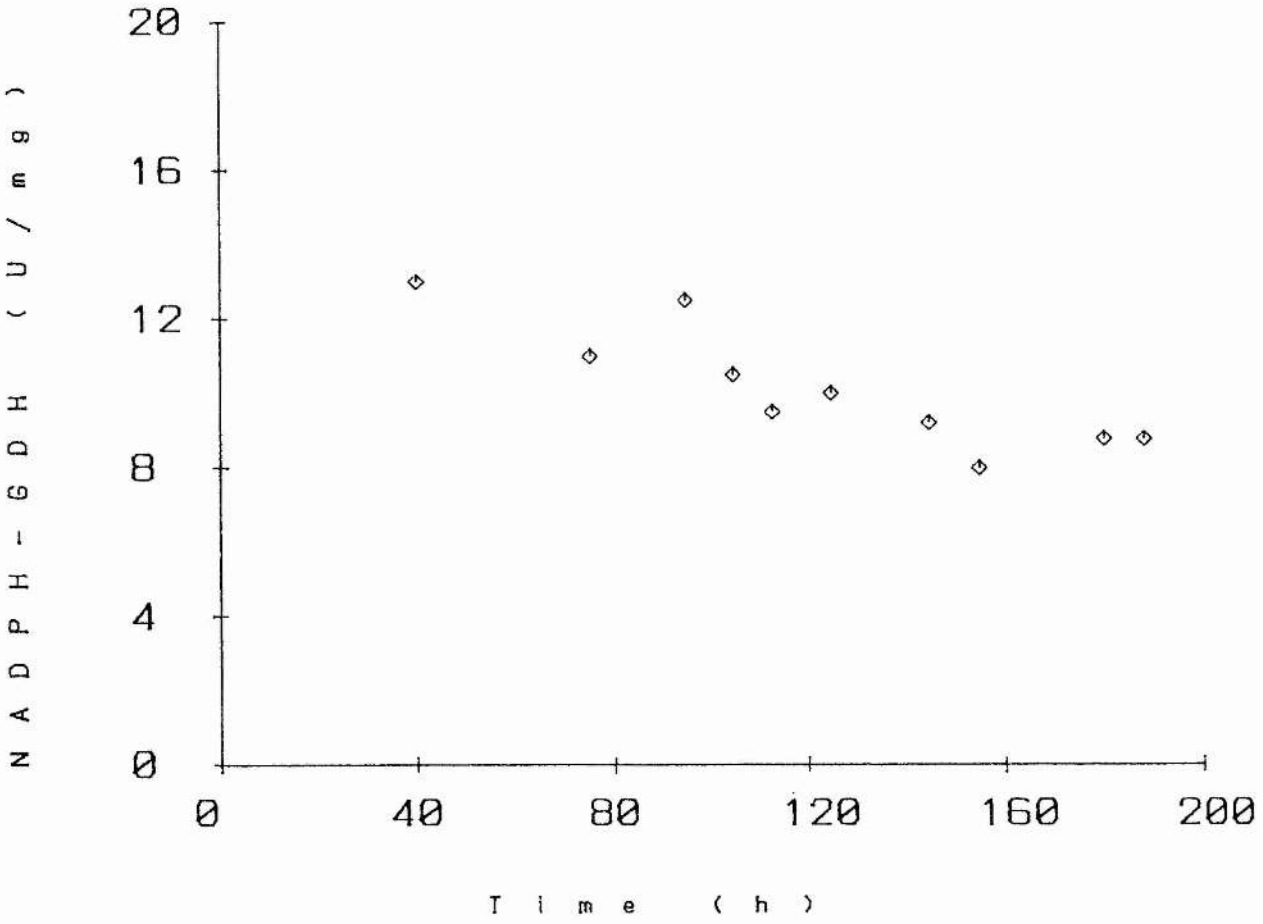


Figure 3.3c : NADPH-GDH activity of AR2 cells growing under carbon limitation. D = 0.20/h.

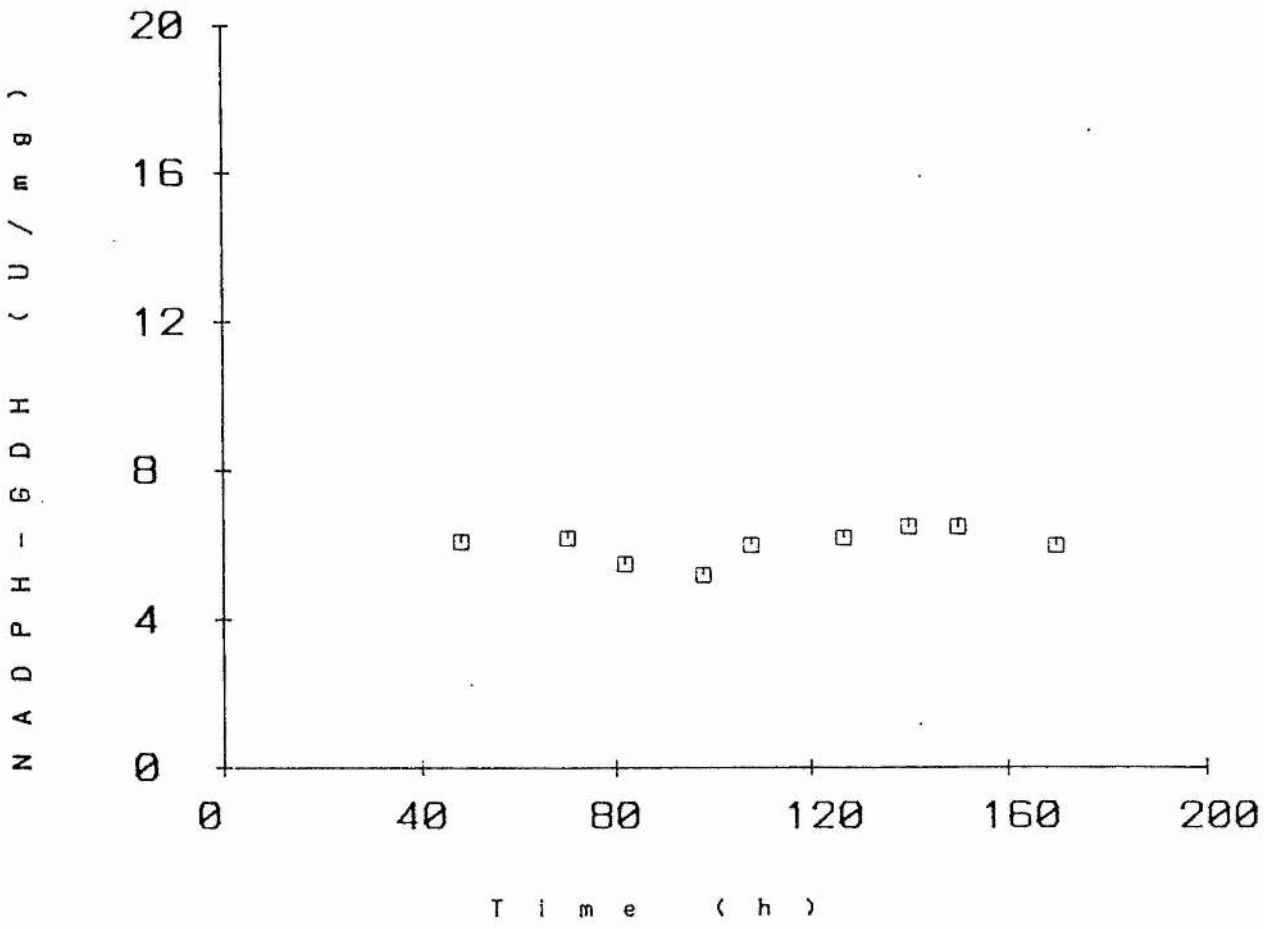


Figure 3.3d : NADPH-GDH activity of AR5 cells growing under carbon limitation. D = 0.05/h.

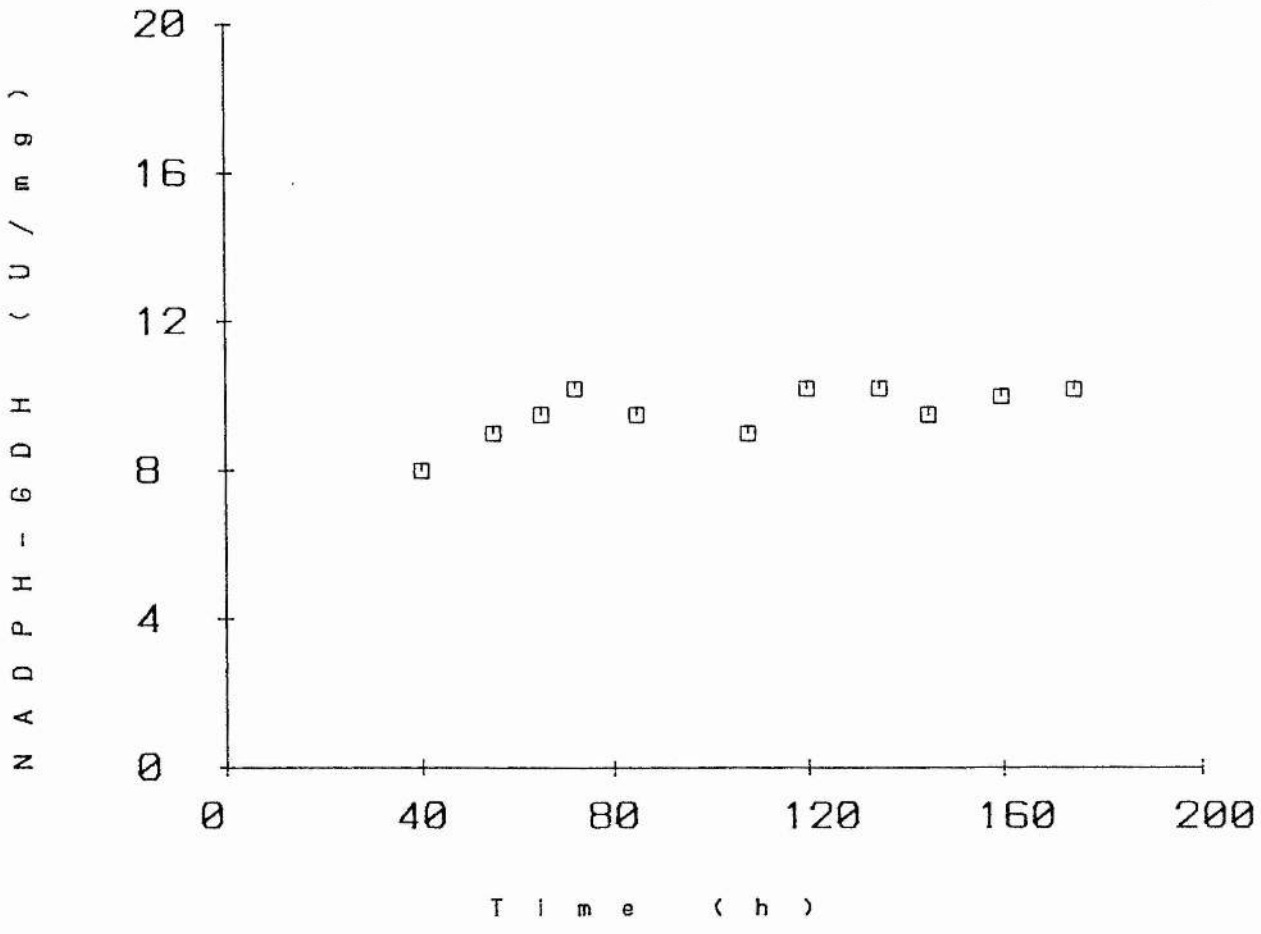


Figure 3.3e : NADPH-GDH activity of AR5 cells growing under carbon limitation. D = 0.10/h.

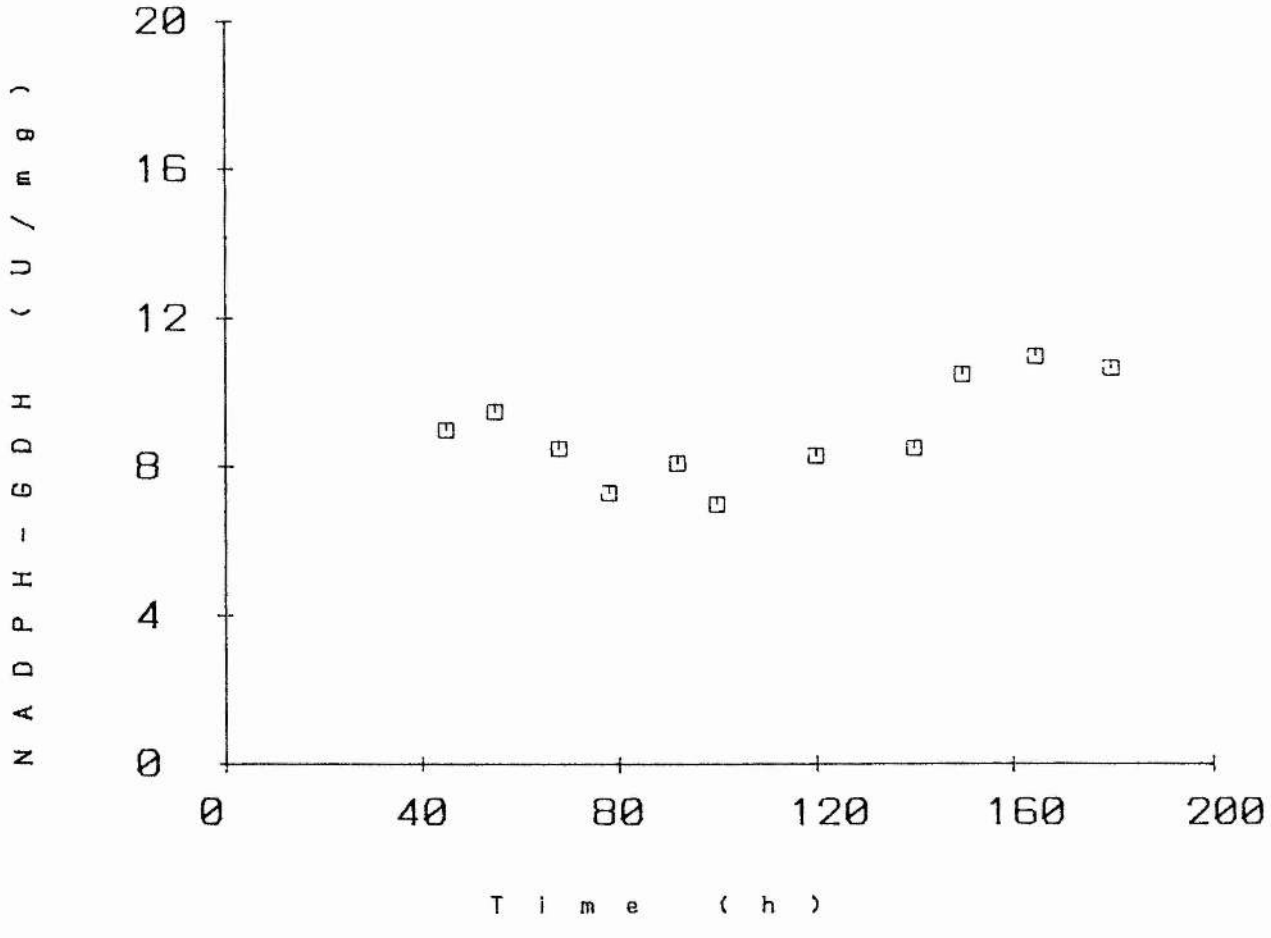


Figure 3.3f : NADPH-GDH activity of AR5 cells growing under carbon limitation. D = 0.15/h.

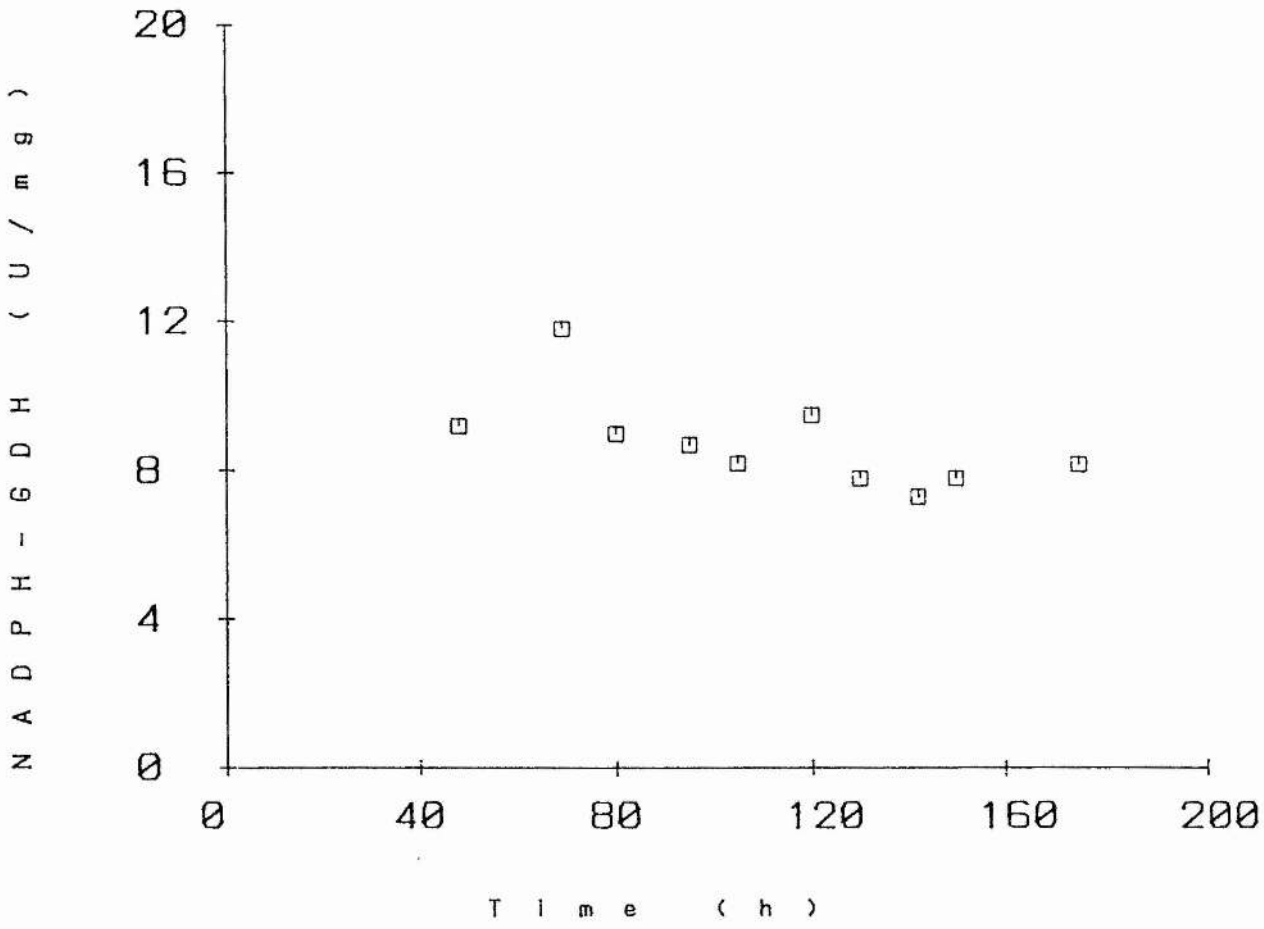


Figure 3.3g : NADPH-GDH activity of AR5 cells growing under carbon limitation. D = 0.20/h.

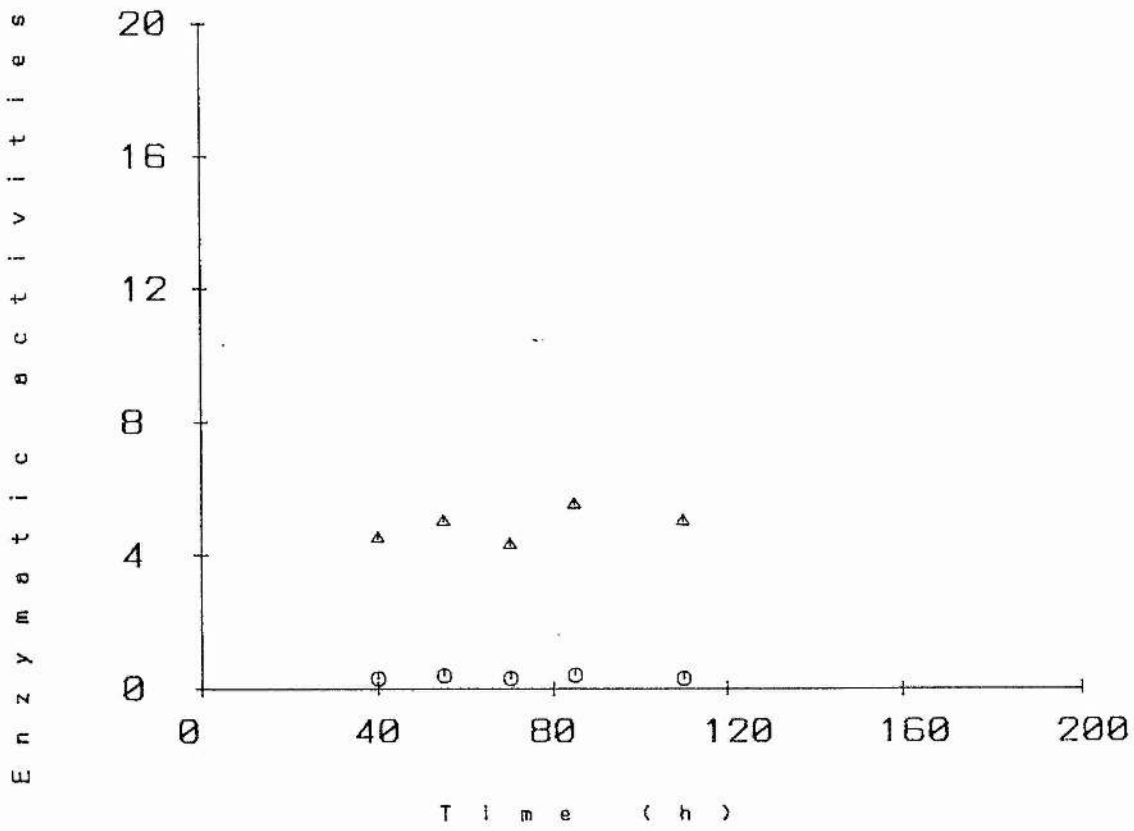


Figure 3.3h : NADPH-GDH (△) and GOGAT (○) activities of ≤1278b wild type cells growing under carbon limitation.

D = 0.15/h.

3.4 NADPH-GDH , GOGAT and GS activities under nitrogen limitation

At the three ammonia-limited dilution rates tested, the NADPH-GDH activities were slightly elevated compared to the activities detected under carbon limited cultures for both AR2 and AR5 strains (Fig. 3.4 to 3.4e). The wild type was tested only for the dilution rate of 0.15/h (Fig 3.4f). Its NADPH-GDH activity was more than 5 fold times lower than the activities for AR2 and AR5, as expected.These data constrast with Lomnitz et all (76) who found that under ammonia-limited cultures the GDH activity for a GOGAT$^-$ strain of *N.crassa* was almost twice the activity detected for the wild type . But as *N.crassa* is a filamentous fungi, metabolic differences are quite expected.

As in the experiments under carbon limitation, oscillations appeared for AR2 and AR5 NADPH-GDH activities and are probably related to the plasmid stability as it has already been discussed.

The GOGAT activities for AR2 were only slightly elevated compared to the carbon limited cultures. Obviously, the activities detected for AR2 were about 1% of the NADPH-GDH activities, due to the presence of the plasmid pCYG4.

GOGAT activity was not detectable in AR5 growing under nitrogen limited cultures.

For the wild type, GOGAT activity was found to be about 6% of the NADPH-GDH activity.

GS activities were again below 1mU/mg for AR2 and the wild type, and not detectable for AR5, as in the experiments reported in 3.2.3.

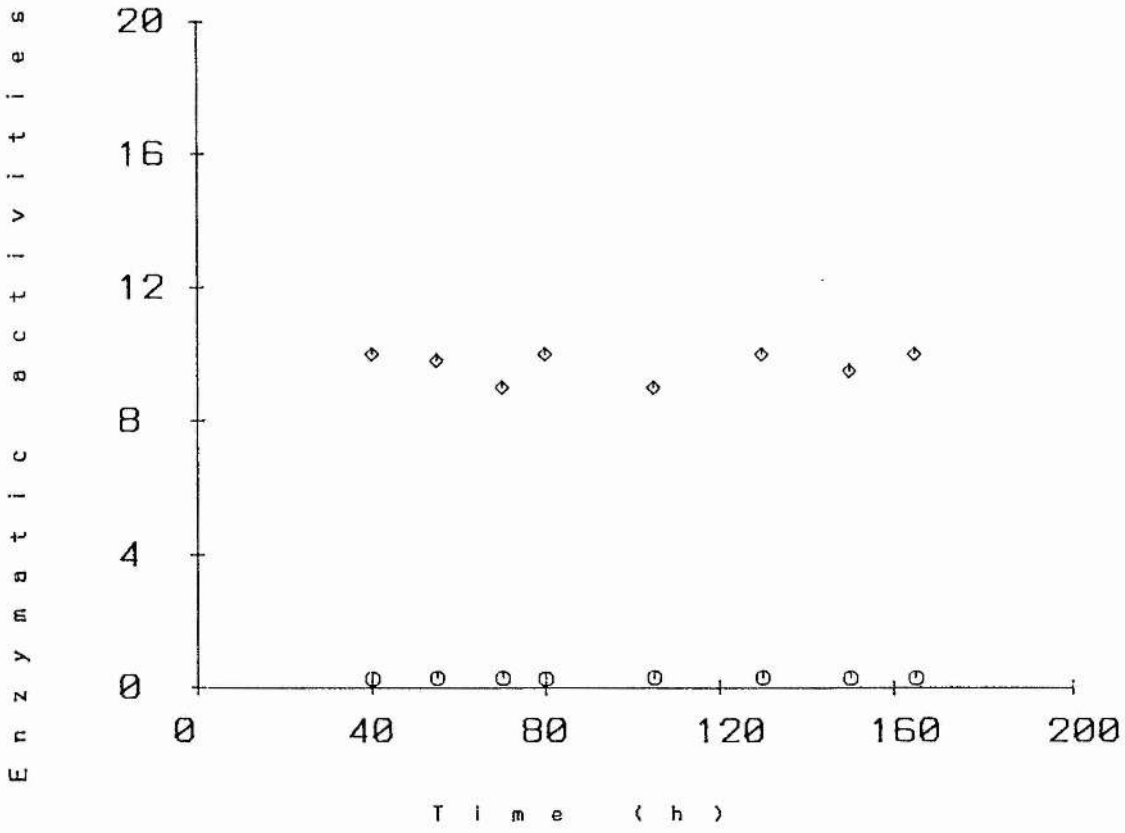


Figure 3.4 : NADPH-GDH (◇) and GOGAT (○) activities of AR2 cells growing under nitrogen limitation.
D = 0.05/h.

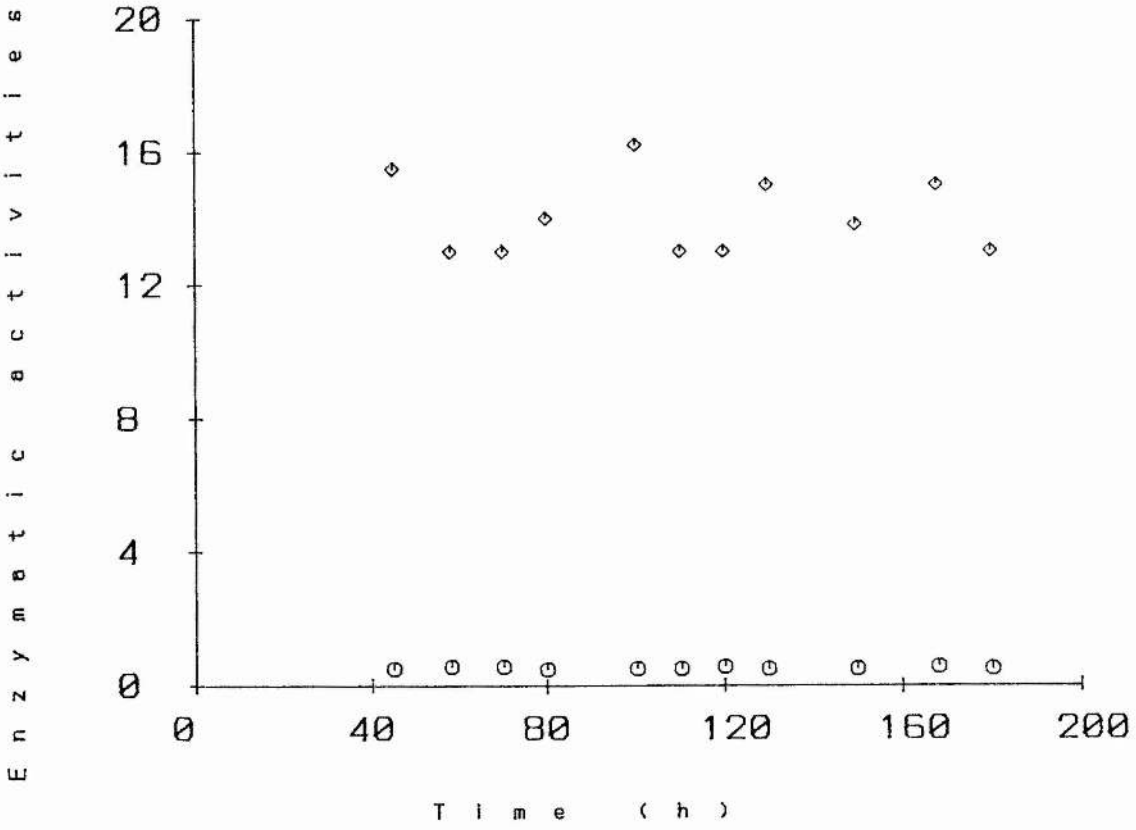


Figure 3.4a : NADPH-GDH (◇) and GOGAT (○) activities of AR2 cells growing under nitrogen limitation.

D = 0.10/h.

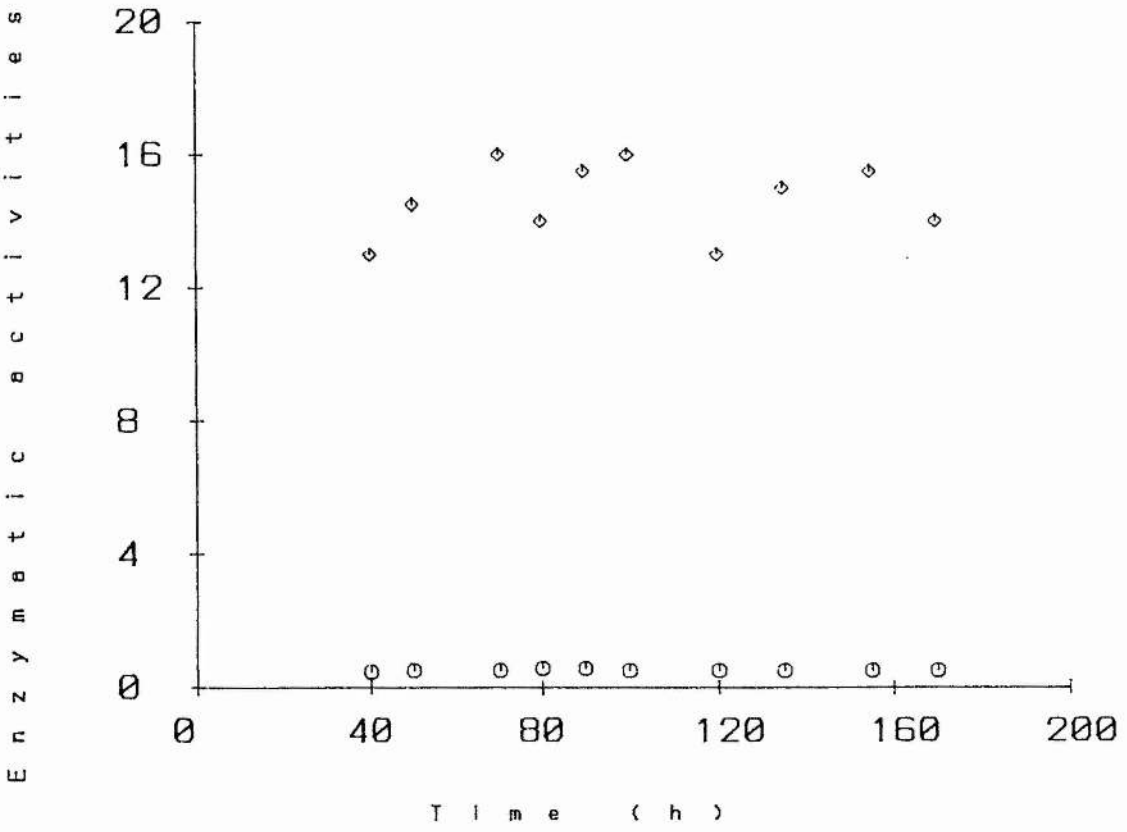


Figure 3.4b : NADPH-GDH (◇) and GOGAT (○) activities of AR2 cells growing under nitrogen limitation.

D = 0.15/h.

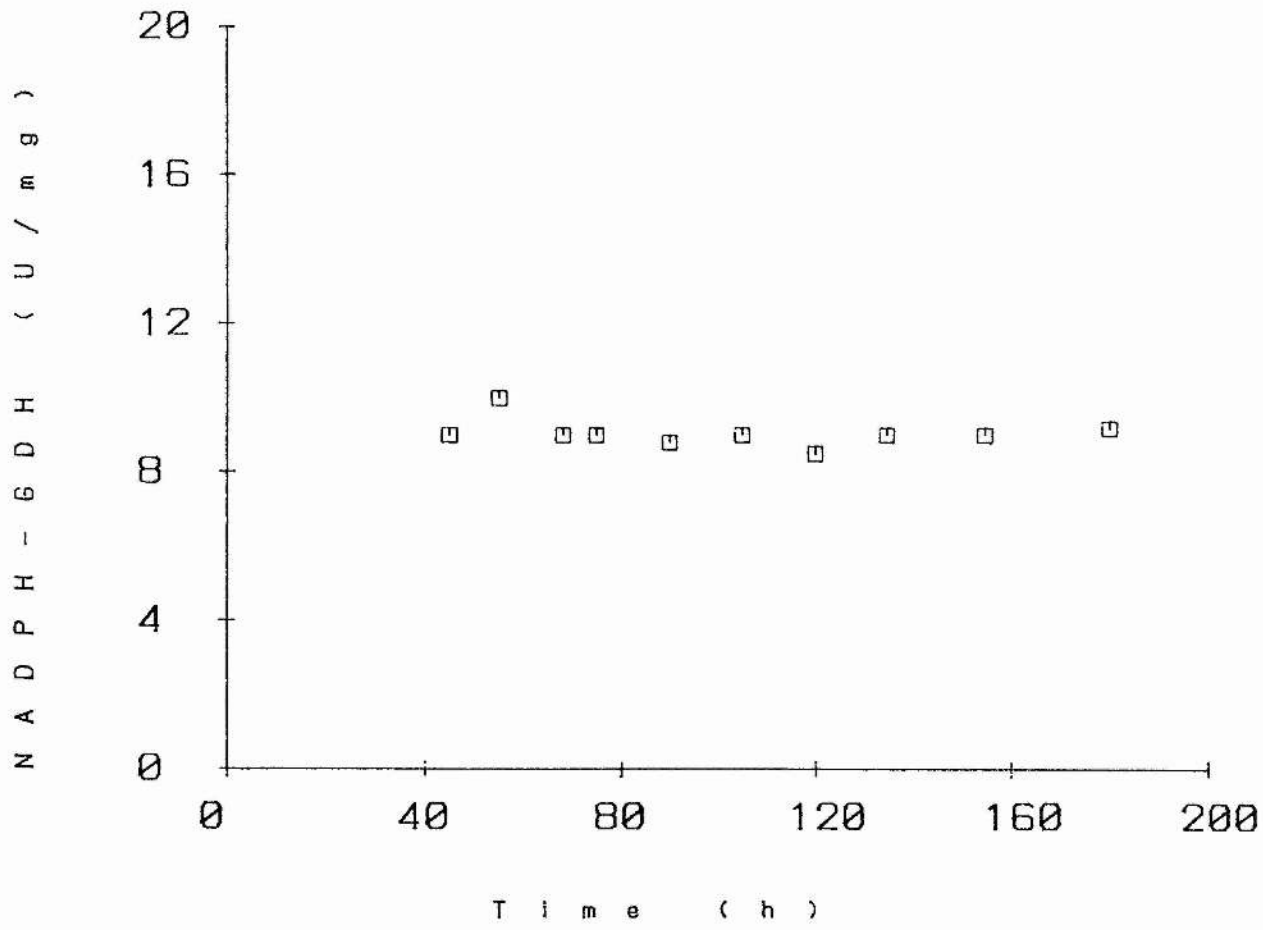


Figure 3.4c : NADPH-GDH activity of AR5 cells growing under nitrogen limitation. D = 0.05/h.

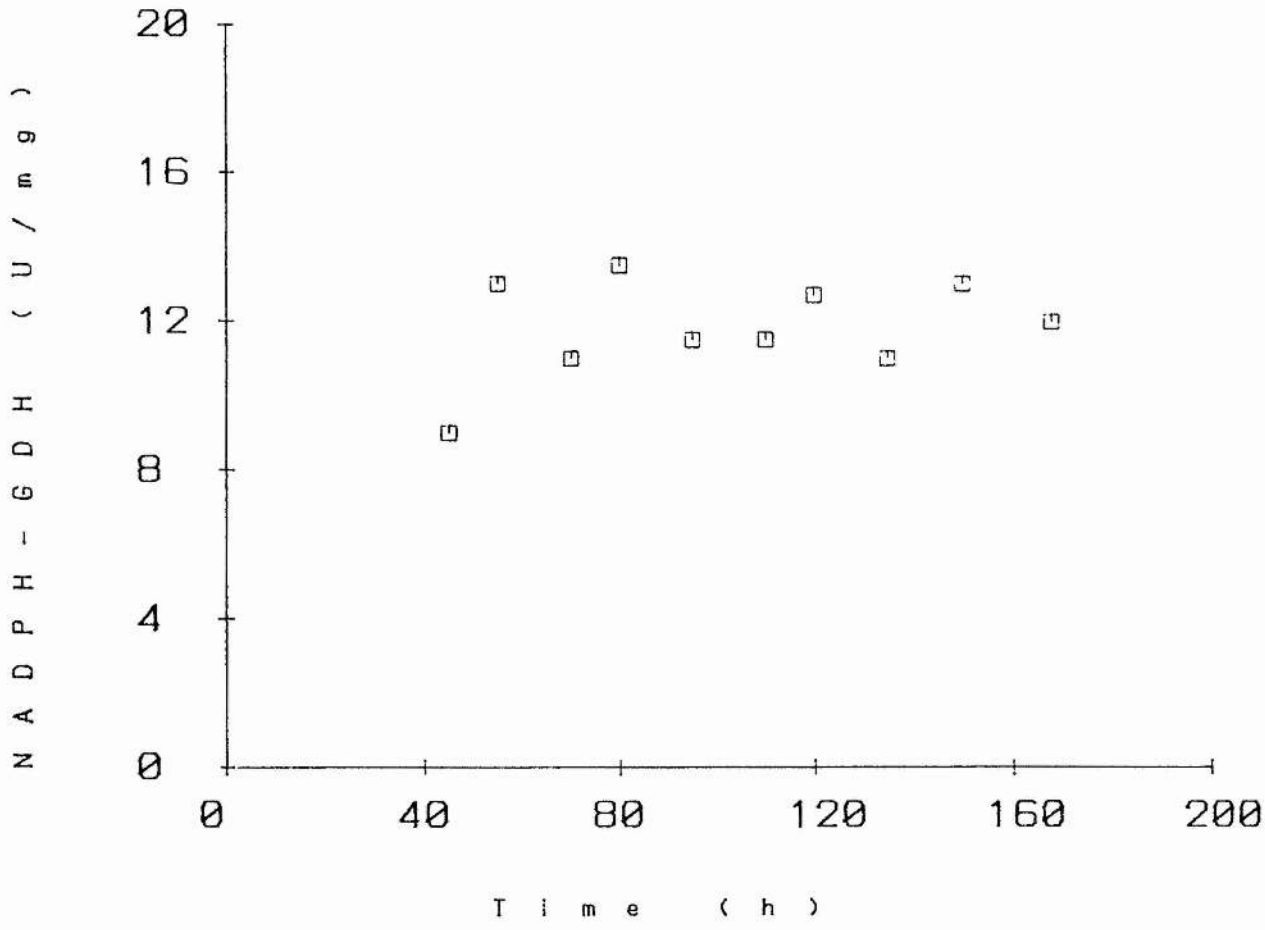


Figure 3.4d : NADPH-GDH activity of ARS cells growing under nitrogen limitation. D = 0.10/h.

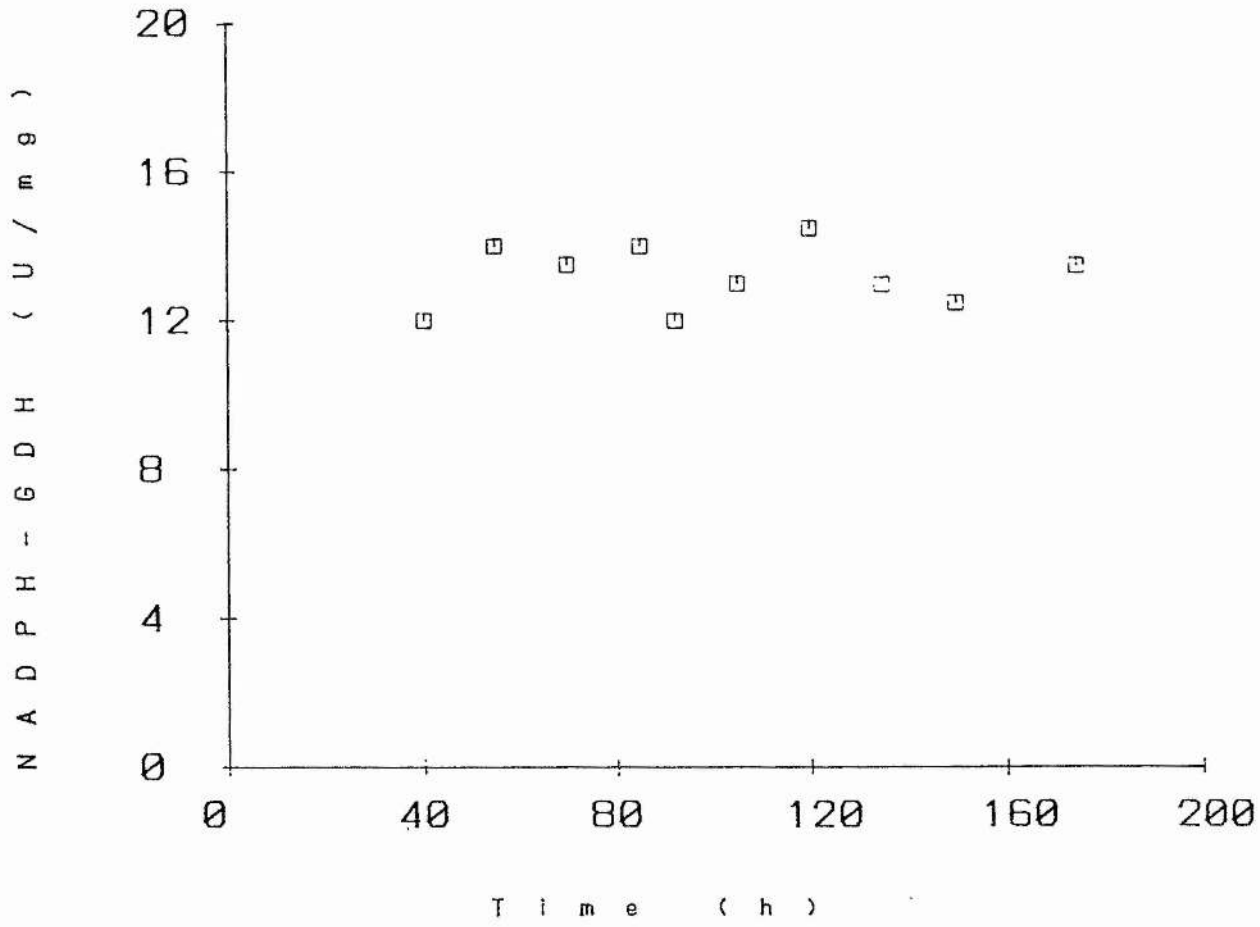


Figure 3.4e : NADPH-GDH activity of AR5 cells growing under nitrogen limitation. D = 0.15/h.

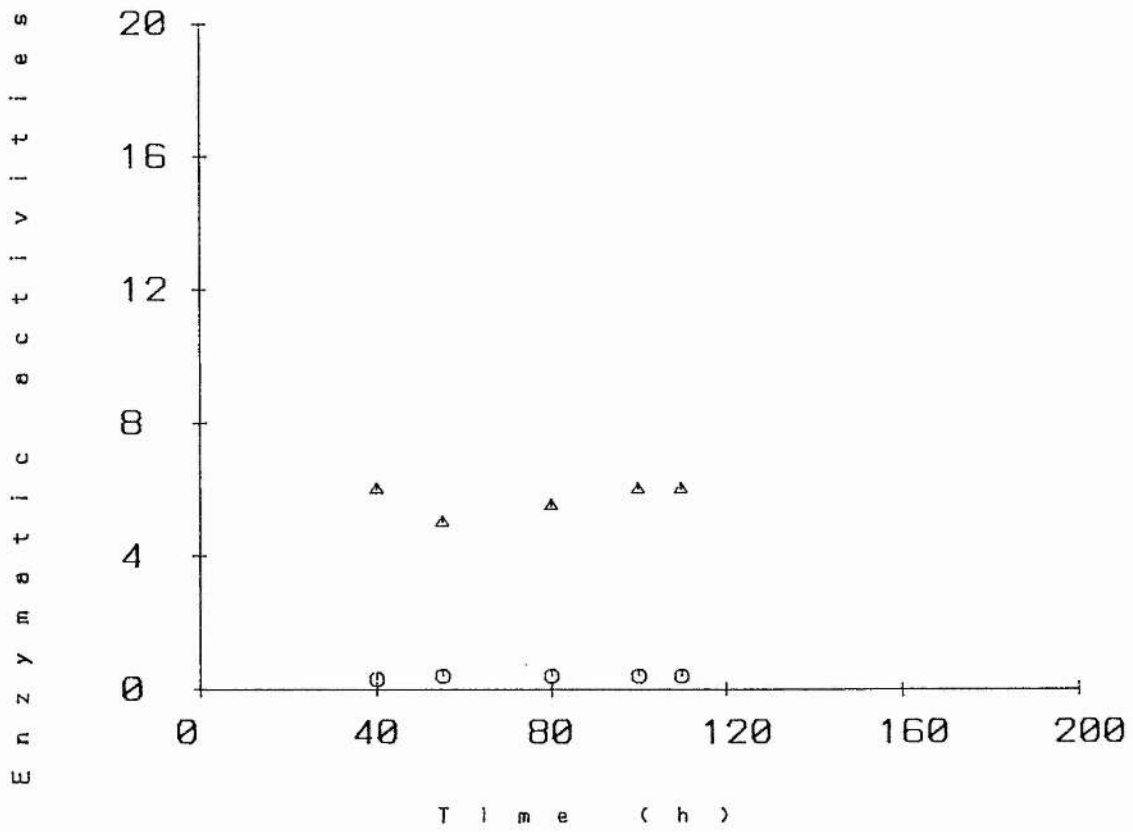


Figure 3.4f : NADPH-GDH (△) and GOGAT (◷) activities of ≪1278b wild type cells growing under nitrogen limitation.

D = 0.15/h.

3.5 Intracellular concentration of ammonia, L-glutamate and L-glutamine under carbon limitation

Table 3.5 shows the average concentrations for ammonia, glutamate and glutamine for AR2, AR5 and the wild type ξ1278b growing under various dilution rates.

Although there were no remarkable differences between the NADPH-GDH activities of AR2 and AR5, the concentrations of glutamate and glutamine for AR5 (GOGAT $^-$) were much lower than those of AR2 .

The GS-GOGAT pathway has been suggested as a scavenging pathway for ammonia assimilation under nitrogen starvation. In that case the lack of this pathway would not be expected to affect the primary products of ammonia assimilation (L-glutamate and L-glutamine) in conditions of excess of ammonia. However, in our experiments, even under such conditions, the glutamate and glutamine pools of the strain lacking GOGAT activity were only around 40% those of the GOGAT$^+$ strain (AR2).

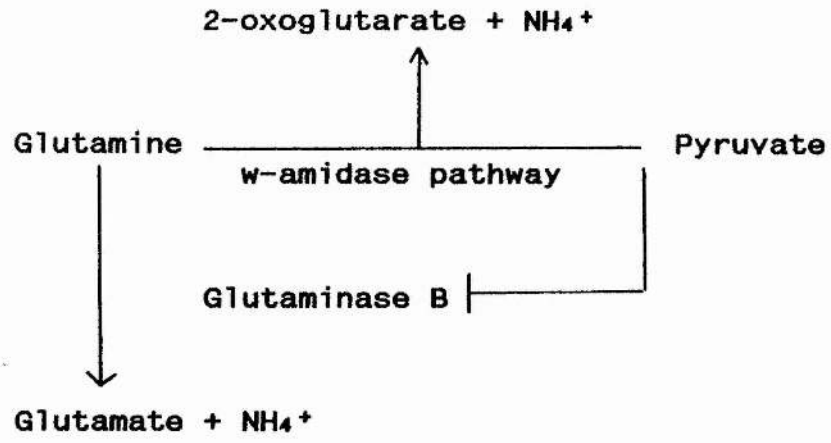
Lomnitz et al (76) working with mutants of *N.crassa* which lack GOGAT activity found that the glutamate pool of the strain was lower compared to the wild type. There was also accumulation of glutamine in the GOGAT$^-$ strain. It was proposed then that the primary

function of GOGAT in *N.crassa* is the recycling of organic nitrogen from glutamine to glutamate, ensuring substrate availability for the efficient operation of reversible transamination reactions. However, the presence of two different mechanisms of glutamine degradation (Glutaminase activity and w-amidase pathway) in *S.cerevisiae* would prevent the accumulation of this metabolite even for the GOGAT$^-$ strain.

The participation of two Glutaminases in glutamine degradation in *S.cerevisiae* has been reported by Soberon and Gonzalez (80). Glutaminase A appears to be membrane-bound, is thermostable and has pH optima at 7.5 while Glutaminase B is cytoplasmic, heat labile and has pH optima at 8.1. There are evidences that Glutaminase B plays a central role in the regulation of the intracellular pool of glutamine, since the mutant CN10 which has an alteration in this enzyme accumulates a large amount of this amino acid. The authors also suggested that Glutaminase A might deal with the degradation of exogenous glutamine, since it is membrane bound. Under microaerophilic conditions, pyruvate accumulates due to fermentative processes and Glutaminase activity is repressed (81). Then an alternative route of glutamine utilization may be activated : the w-amidase pathway.

The existence of the w-amidase pathway in *S.cerevisiae* has been confirmed by Soberon and Gonzalez

(82). In this pathway glutamine is transaminated to yield different aminoacids and 2-oxoglutaramate in a reaction catalysed by Glutamine transaminase. Then the 2-oxoglutaramate formed is hydrolysed in 2-oxoglutarate and ammonia by the action of w-amidase. Glutamine transaminase activity is high in glutamine grown cells, condition in which the intracellular glutamine pool is expected to be high. The authors proposed that under conditions in which pyruvate accumulates, the Glutaminase B is inhibited and glutamine is degraded through the Glutamine transaminase. In conditions in which accumulation of pyruvate is decreased (agitation), the enzyme participating in the catabolism of glutamine would be Glutaminase as can be seen in the diagram bellow :



Buurman et al (83) have suggested that cells of *K.pneumoniae* growing at pH values of 4.5-5 in excess of extracellular ammonia (80 mM) would show the physiology of nitrogen-limited cells. It has been shown that in this microorganism ammonia is exceedingly mobile through the membrane . Then the phenomenon could be explained by the rapid diffusion of ammonia through the cell membrane , leading to low levels of cytoplasmic ammonia. Growth at pH 8.0 resulted in high GDH and undetectable GOGAT activities but a decrease in the pH to values below 6 led to a derepression of GOGAT activity. The authors suggest that the GS-GOGAT pathway is not restricted to conditions in which the extracellular concentration of NH^+ is low.

However, it has been shown that in yeast, the ammonia transport is mediated by two active transport systems which operate over a narrow pH range (5.5 - 7.5) (84)(85). At higher values of pH ammonia uptake is essentially a diffusion process (84). Bogonez et al have demonstrated that the prevention of the pH increase in the medium results in decrease in intracellular ammonia concentration accompanied by an increase in NADPH-GDH activity (86).

In our experiments, the pH was kept constant at 5.0 by addition of 0.5M KOH , a value that would allow the active transport systems to function. Furthermore, the high levels of intracellular ammonia

concentration detected for both strains argue for the possibility of intracellular nitrogen limitation.

Although the GS/GOGAT pathway has been regarded as a scavenging pathway to work under conditions of ammonia limitation, according to our results the amount of glutamate and glutamine was higher (from 20 to 40%) for the AR2-GOGAT$^+$ strain than for the AR5-GOGAT$^-$ one.

The intracellular ammonia concentration for AR2 and AR5 were almost similar and higher than the values found for the wild type (see Table 3.2.5). These results are in agreement with Lima Filho (6) who suggested that the presence of the plasmid pCYG4 increases the amount of ammonia taken up by the cells.

Table 3.5

	Protein mg/ml	Glutamate	Glutamine umol/mg protein	Ammonia	GDH U/mg
D = 0.05/h					
AR2	0.40	0.80	0.20	4.00	6.00
AR5	0.35	0.35	0.08	4.00	6.00
D = 0.10/h					
AR2	0.40	0.70	0.20	5.00	10.00
AR5	0.35	0.36	0.04	4.20	10.00
D = 0.15/h					
AR2	0.40	1.30	0.80	5.00	11.00
AR5	0.35	0.80	0.07	5.00	10.00
Wild Type	0.33	0.33	0.11	3.8	5.00

Average intracellular metabolite concentrations and NADPH-GDH activity for AR2, AR5 and the 1278b Wild Type growing under carbon limitation (0.5% glucose), at 30° C and pH 5.0.

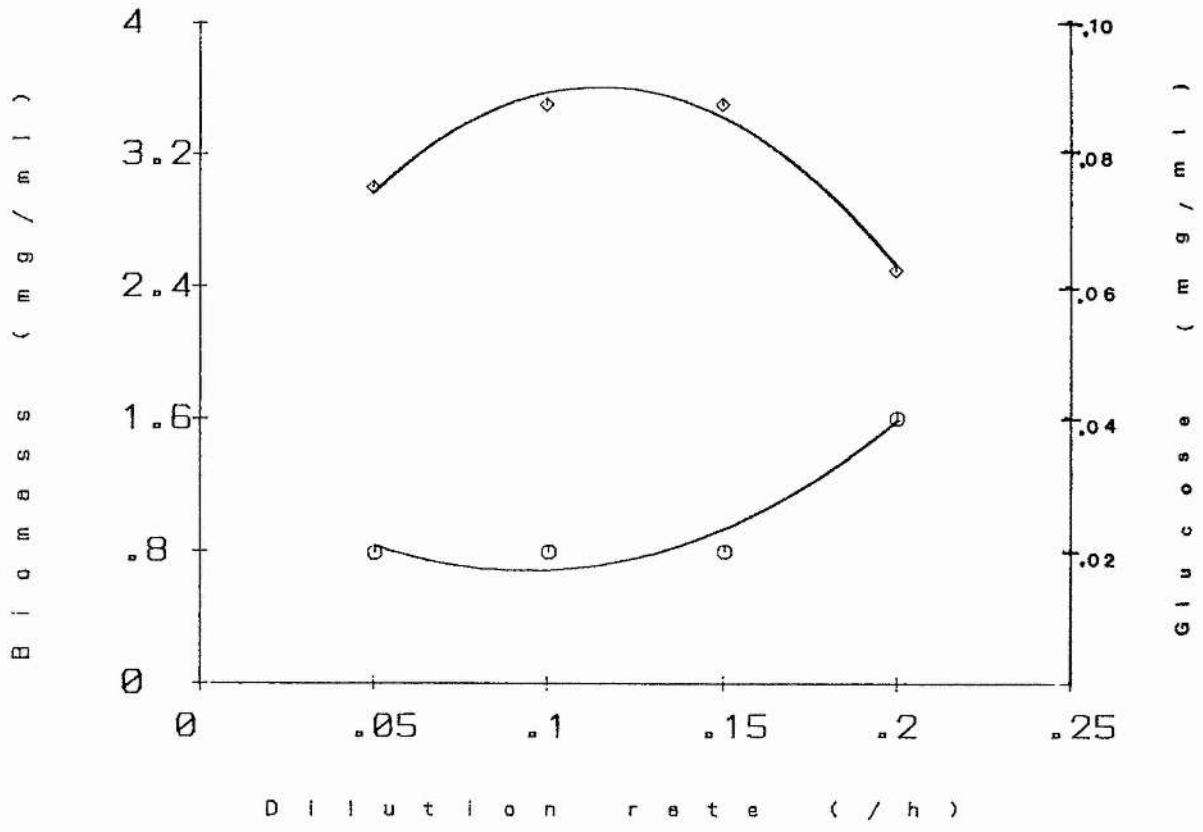


Figure 3.7.1 : Steady-state concentrations of biomass (◇) and growth-limiting substrate (○) for AR2 strain growing at different dilution rates.

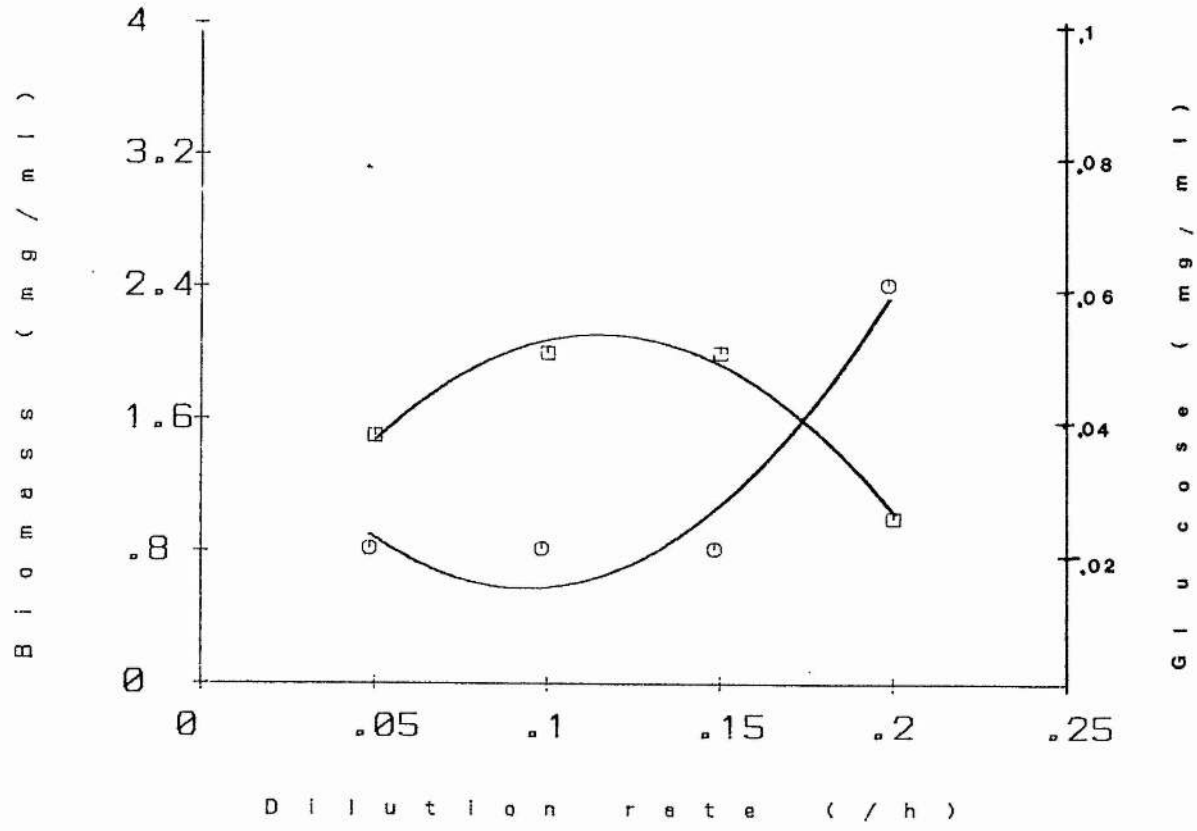


Figure 3.7.1a : Steady-state concentrations of biomass (□) and growth-limiting substrate (○) for AR5 strain growing at different dilution rates.

3.6 Intracellular concentration of ammonia , L-glutamate and L-glutamine under nitrogen limitation

Table 3.6 shows the average values for ammonia, glutamate and glutamine for AR2, AR5 and the wild type strains growing under nitrogen limitation at the dilution rates employed related to the protein concentration.

Although the amount of protein per mg of cells was lower than in the experiments under carbon limitation, the glutamate and glutamine levels were much higher for both strains.

It has been shown (87) that *S.cerevisiae* has two classes of mechanism for transporting amino acids across the plasma membrane. There is a general amino-acid permease (GAP) which transports all basic and neutral amino acids, but proline. *S.cerevisiae* can also synthesize a range of at least 11 transport systems each of which is specific for a small number of amino acids. Grenson et al. (88) showed that nitrogen starvation results in a marked increase in general aminoacid permease activity, what could explain the increased amino acid concentration we found under nitrogen limitation. Furthermore, Woodward and Cirillo (89) described the fate of amino acids accumulated under these conditions. The workers postulated the existence of a salvage pathway

originally shown to exist in cell-free extracts of *S.cerevisiae* by Sentheshan-Mughanathan (90). The pathway involves transamination of an amino acid to yield it α-keto derivative. Decarboxylation yields an aldehyde that is reduced to a primary alcohol or fusel oil in an NADH-linked reduction.

Yeast cells can employ rather drastic measures to scavenge usable nitrogen in the face of a diminished external nutrient supply, like the turnover of previously synthesised constituents or the utilisation of the cell vacuoles (87), (91), (92).

Vacuoles of *S.cerevisiae* contain a variety of hydrolases such as proteases, glycosidases, nucleases and phosphatases (93). Substantial amounts of all classes of amino acids were associated with vacuoles of *S.cerevisiae*, and the association depended upon the strain, the medium and the nutritional status of the organism. (94). The K_m for transport of amino acids by vacuolar membrane vesicles of *S.cerevisiae* is higher by two or three orders of magnitude than the corresponding plasma membrane system (95). Then, in conditions of nitrogen limitation, the use of amino acids stored in vacuoles associated to hydrolysis of non essential proteins would provide the necessary amount of amino acids for the cell to continue its vital cycle.

The amount of glutamate and glutamine for the GOGAT$^+$ strain (AR2) was again higher than for the

GOGAT⁻ one (AR5). Kusnan et al. (96) working with *A.nidulans* growing under low concentrations of ammonia (2 mM ammonium sulphate) found that about half of the glutamate was synthesized via the NADPH-GDH pathway and the other half was formed from glutamine via the glutamate synthase pathway. The authors worked with [^{15}N]ammonia, and they estimated that about 42% of the new assimilated ammonia was fixed into the amide group of glutamine. 52% of the total glutamate was formed via the GOGAT pathway from recently synthesized glutamine and 48% via the NADPH-GDH one. In our experiments we found a larger amount of glutamate and glutamine for the GOGAT⁺ strain either under carbon or nitrogen limitation. These could indicate a more important role for the GS/GOGAT pathway than a secondary pathway to function only under nitrogen starvation.

As it can be seen in tables 3.2.5 and 3.2.6, the NADPH-GDH activities for both strains were higher under nitrogen limitation than under carbon limited conditions. On the other hand, the ammonia concentrations were higher under carbon limitation than under nitrogen limitation. These results are in agreement with the findings of other researchers (86), (97), (98), that the activity of NADPH-GDH is decreased under conditions in which intracellular ammonia concentration increases. During ammonia accumulation there is a repression of synthesis with little degradation.

Table 3.6

	Protein mg/ml	Glutamate	Glutamine	Ammonia	GDH
		umol/mg protein			U/mg
D = 0.05/h					
AR2	0.08	1.60	0.25	1.50	9.00
AR5	0.08	0.50	0.14	1.40	9.00
D = 0.10/h					
AR2	0.08	1.80	0.25	1.50	14.00
AR5	0.08	0.60	0.15	1.40	12.00
D = 0.15/h					
AR2	0.08	2.00	0.25	1.60	15.00
AR5	0.08	0.65	0.18	1.60	14.00
Wild Type	0.07	1.3	0.2	1.50	6.00

Average intracellular metabolites concentrations and NADPH-GDH activity for AR2, AR5 and the 1278b Wild Type cells growing under nitrogen limited cultures (2 mM ammonia sulphate) at 30° C and pH 5.0.

3.7 Biomass Growth

3.7.1 Carbon Limitation

Under steady-state conditions, cell concentration is described by the following equation :

$$\tilde{x} = Y(Sr - \tilde{s})$$

Where :

$\tilde{x}$ = cell concentration
Sr = original substrate concentration
$\tilde{s}$ = residual substrate concentration
Y = Yield factor (a dimensionless constant).

The growth yield is expressed by the quotient : $Y = x/Sr$, where $\tilde{x}$ is the biomass concentration equivalent to the utilization of substrate $(Sr - \tilde{s})$. It is important as an expression of the quantitative nutrient requirement of an organism (45).

Table 3.7.1 shows the values of biomass for AR2 , AR5 and ξ1278b wild type growing under carbon limitation at different dilution rates. The values of glucose concentrations inside the fermenter $(\tilde{s})$ with yield at each dilution rate also appear in the Table. As

it can be seen, at every dilution rate AR5 showed a lower amount of cells per ml compared to AR2.

The lower biomass at D = 0.05/h for both AR2 and AR5 strains is explained by the requirement of an energy of maintenance which is more apparent at very low dilutions. The biomass was constant at dilution rates of 0.10 and 0.15/h, however at 0.20/h there was a decrease in biomass. This is explained because D = 0.20/h is very closed to the u_{max} of the strains ocurring wash out of cells with a rising in the residual substrate concentration ($\tilde{s}$).

It has been reported by Racher (54) who constructed the AR2 and AR5 strains, that both growing on batch culture with 20 mM ammonia had similar final densities. However, batch-growth is subjected to changes in substrate concentrations and pH, which could lead to different results from those with chemostatic cultures.

As was already pointed out in this research, the GS/GOGAT pathway has been suggested to be a scavenging pathway to function under conditions of nitrogen limitation. In that case this pathway would probably not be necessary under carbon limitation, where we find an excess of ammonia. And also the energy saved per ammonia assimilated by the GOGAT$^-$ strain could be used in the formation of more biomass. However, our results under carbon limitation showed that the GOGAT$^+$

strain (AR2) and the wild type had the same Yield factor for D = 0.15/h, and the same cell concentration., while the GOGAT$^-$ (AR5) had a lower Yield and lower cell concentration .

As has already been discussed (2.3.5), the amount of glutamate and glutamine were also lower under the same conditions for the GOGAT$^-$ strain.

According to our results, the lack of the GOGAT pathway did not have any beneficial effect on the biomass Yield under carbon limitation. On the contrary, the lack of the pathway resulted in a decrease in the intracellular concentration of glutamate and glutamine and also a decrease in the biomass concentration, compared to the wild type.

Table 3.7.1

	cells mg/ml	glucose mg/ml	Y
D = 0.05/h			
AR2	3.0	0.02	0.60
AR5	1.5	0.02	0.30
D = 0.10/h			
AR2	3.5	0.02	0.70
AR5	2.0	0.02	0.40
D = 0.15/h			
AR2	3.5	0.02	0.70
AR5	2.0	0.02	0.40
Wild Type	3.0	0.02	0.60
D = 0.20/h			
AR2	2.5	0.04	0.50
AR5	1.0	0.06	0.20

The date were obtained from experiments with the strains growing under carbon limitation (glucose 0.5%) at 30° C and pH 5.0.

3.7.1a Nitrogen Limitation

Table 3.7.1a shows the results for AR2 and AR5 strains growing under nitrogen limitation for dilution rates of 0.05, 0.10 and 0.15/h, and for the Wild Type under the same conditions for the dilution rate of 0.15/h.

As it can be seen, the biomass concentration was the same for both engineered strains for every dilution rate, and also similar to the cell concentration of Wild Type strain under D = 0.15/h.

The extracellular ammonia concentration was not detectable inside the fermenter. This together wit the high glucose concentrations in the cultures is strong evidence that ammonia is the limiting substrate.

Although the biomass level was the same for the three strains, the shape of the cells was not. Pictures 1 to 6 show cells of AR2, AR5 and the Wild Type grown under carbon or nitrogen limitation. AR2 and the Wild Type showed rounded shaped cells under both conditions. However, AR5 showed rounded cells under carbon limitation but an ellipsoidal form under nitrogen limited cultures (See pictures 3.7 to 3.7e).

Changes in the shape of the yeast cells may be related to different factors like membrane

composition, amino acid concentration, starvation of some nutrient, etc. (99), (100).

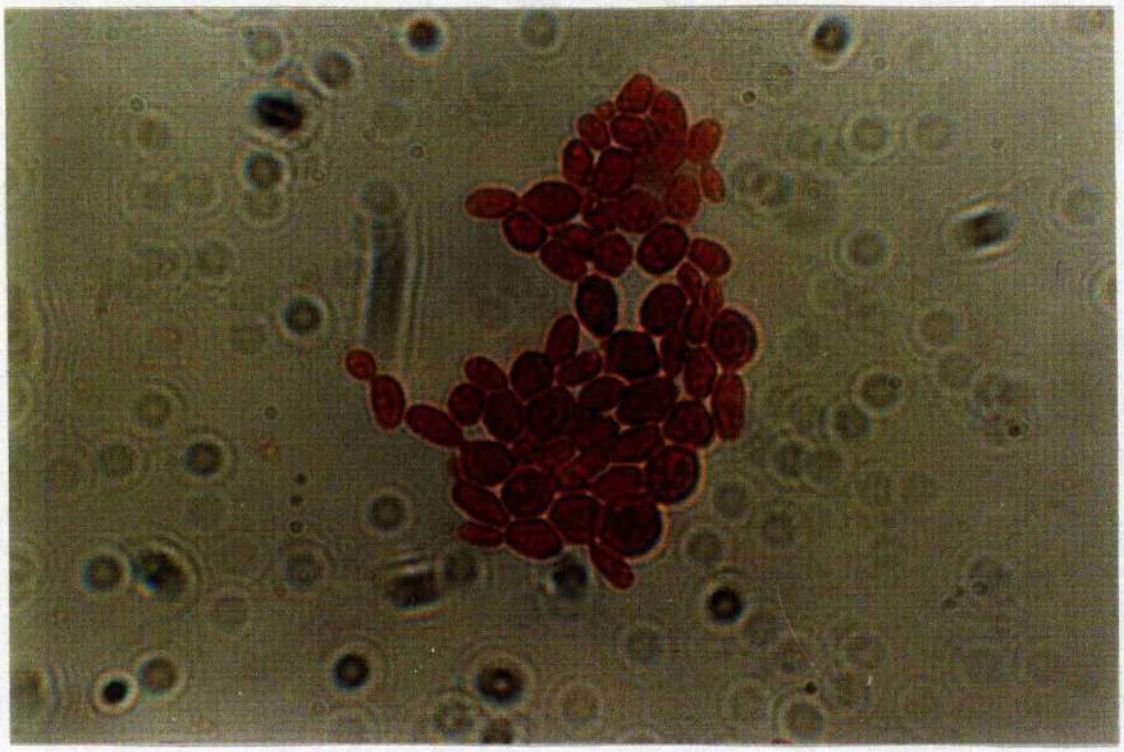
It has been reported (101) that cells of *T.variabilis* appear in a triangular form when grown in the presence of methionine but in the absence of this amino acid ellipsoidal shaped cells are produced. Cells of *S.cerevisiae* PLA 851 were founded to change their size and shape when grown under different temperatures (102).

As the enzymes of ammonia assimilation are in the interface between the carbon and nitrogen metabolism, it may be that the GOGAT pathway is related to the maintenance of any of the intermediate of the Krebs cycle, which was not tested in this research. However, the low biomass concentration of the GOGAT$^-$ strain under carbon limitation together with the change in the shape of the cells under nitrogen limitation, suggest a more important role than just a scavenging pathway for the GOGAT system.

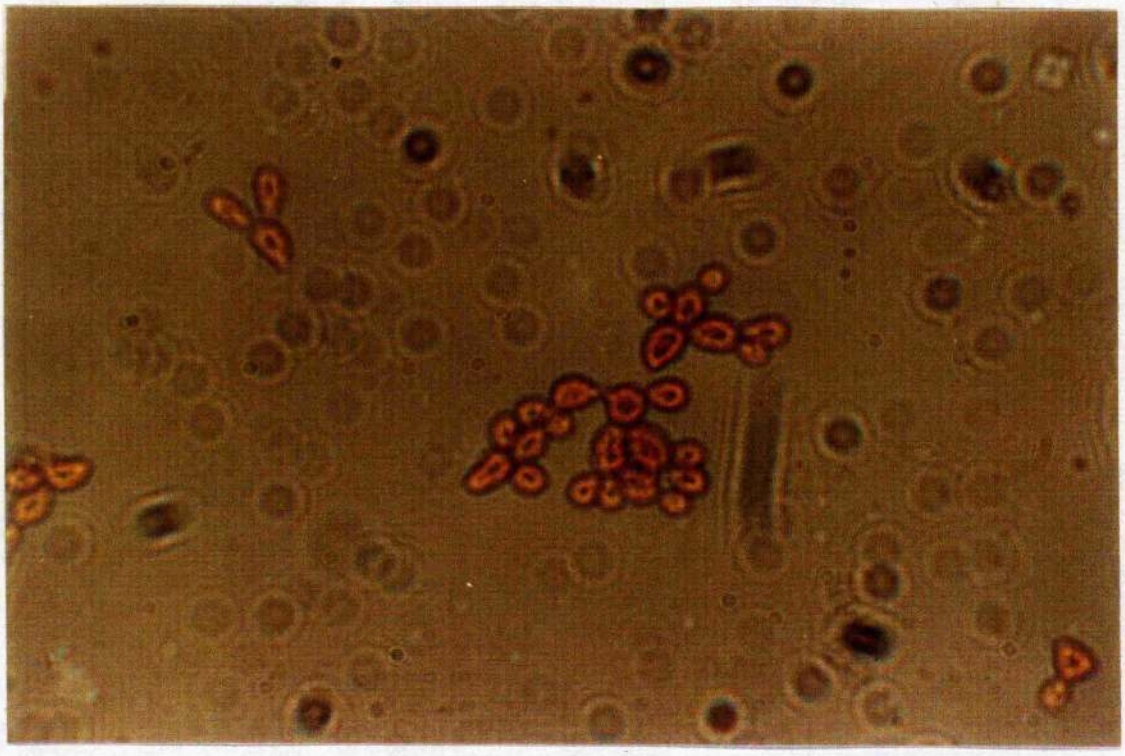
Table 3.7.1a

	cells mg/ml	glucose mg/ml	Y
D = 0.05/h			
AR2	1.4	0.2	0.07
AR5	1.4	0.2	0.07
D = 0.10/h			
AR2	1.45	0.2	0.07
AR5	1.45	0.2	0.07
D = 0.15/h			
AR2	1.45	0.2	0.07
AR5	1.45	0.2	0.07
Wild Type	1.45	0.2	0.07

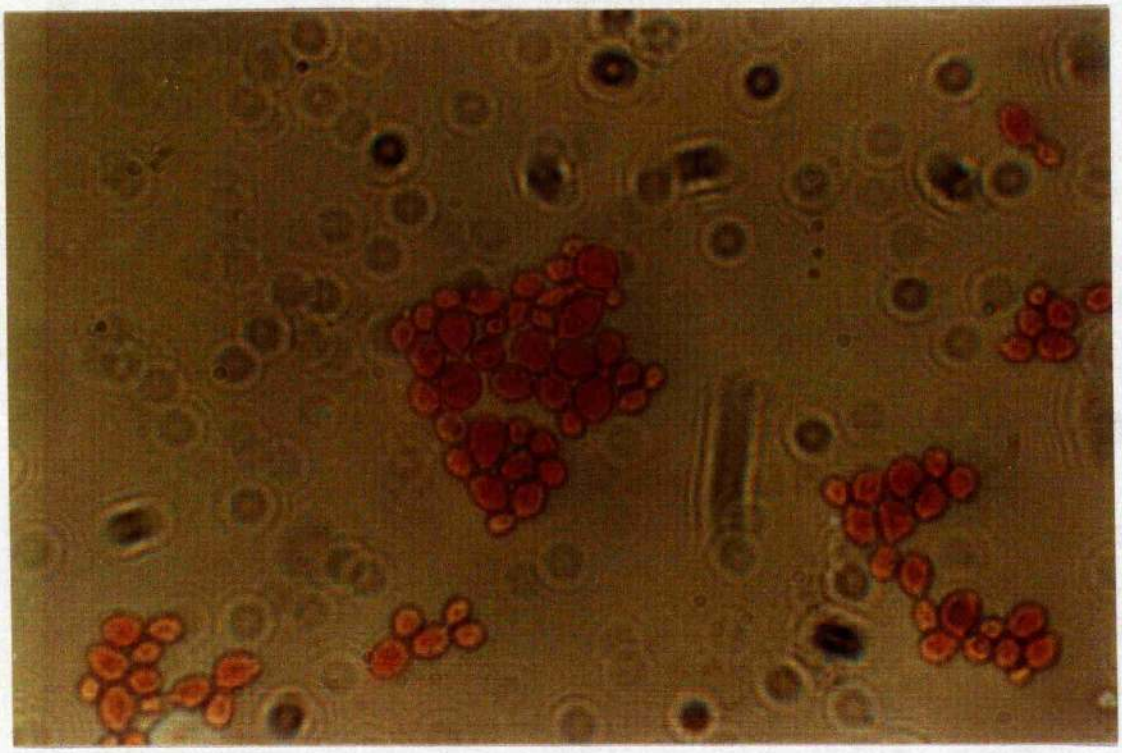
The data were obtained from experiments with AR2, AR5 and the Wild Type growing on nitrogen limitation (2 mM Ammonium Sulphate), at 30° C and pH 5.0.



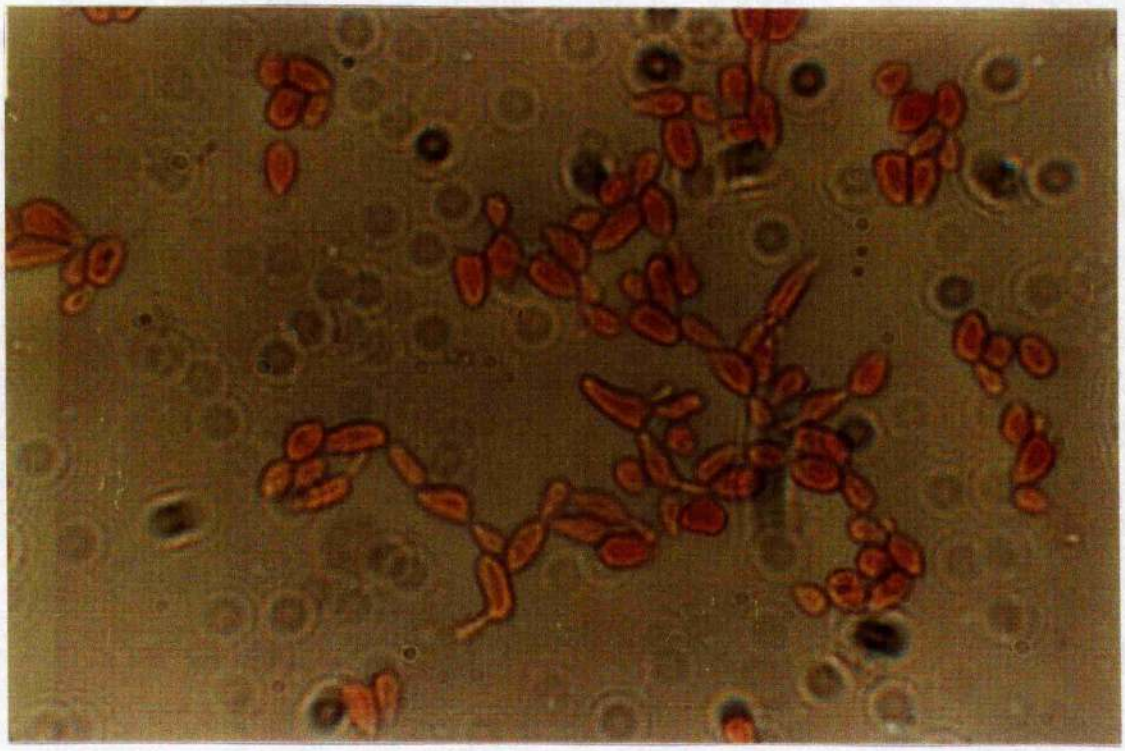
Picture 3.7 - AR2 cells growing under carbon limitation.



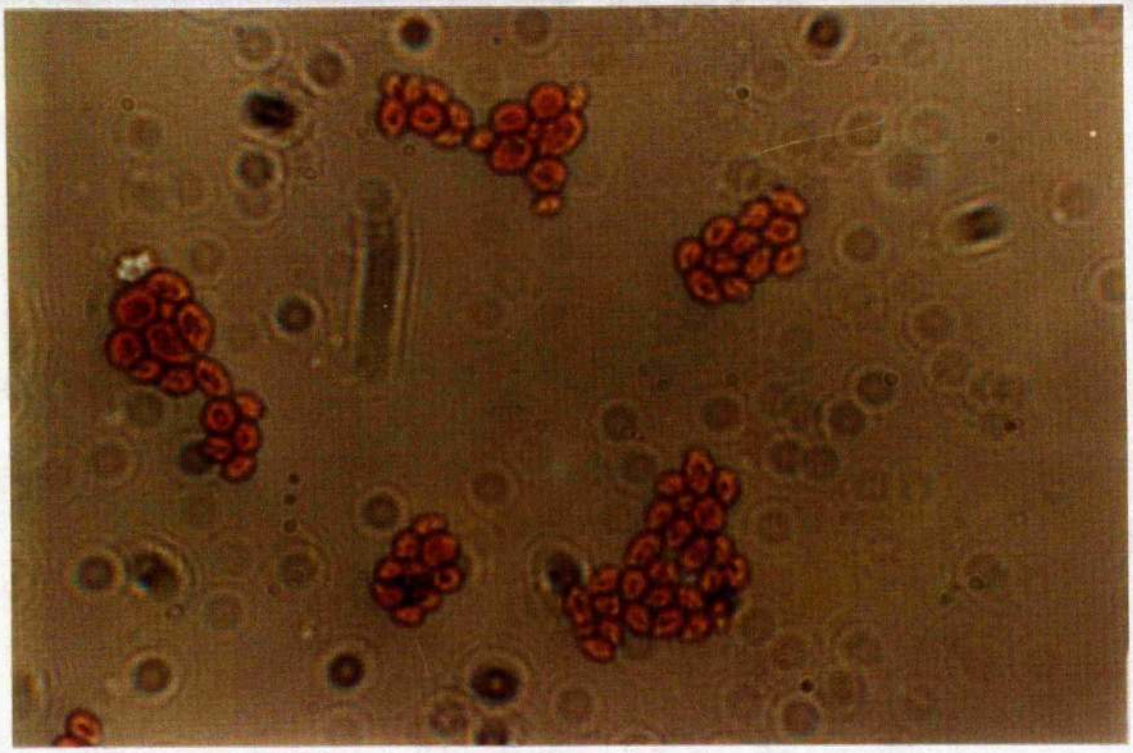
Picture 3.7a - AR2 cells growing under nitrogen limitation.



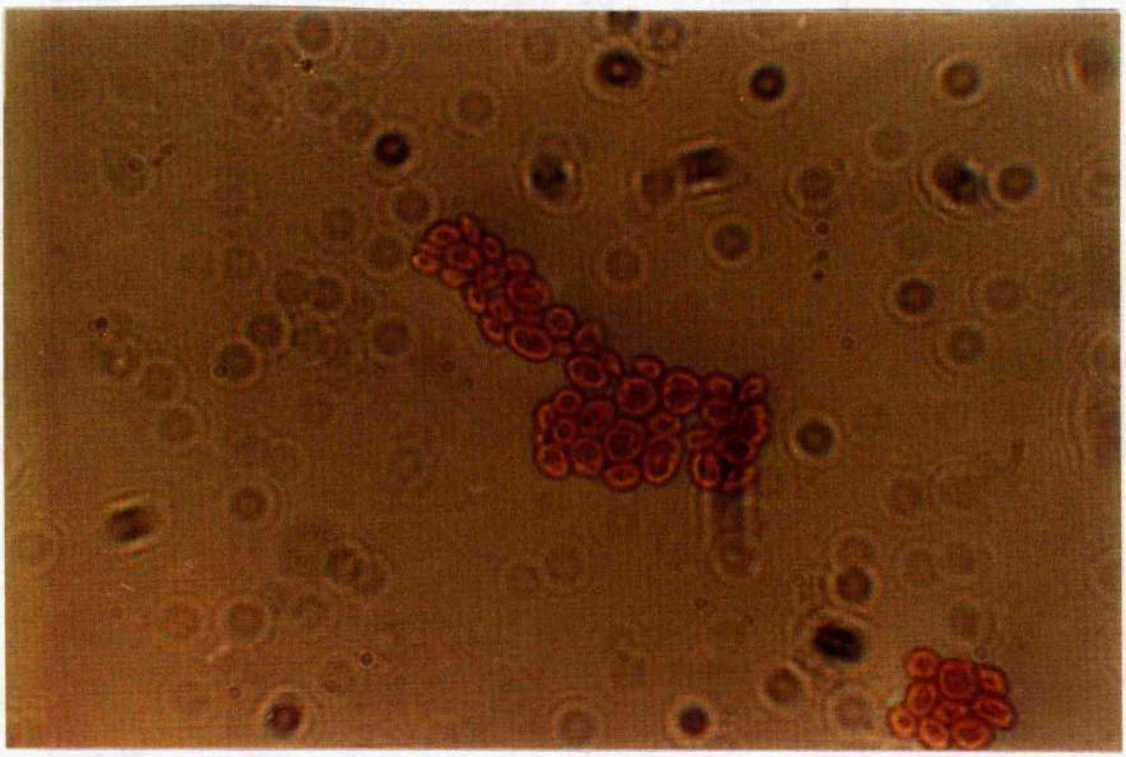
Picture 3.7b - AR5 cells growing under carbon limitation.



Picture 3.7c - AR5 cells growing under nitrogen limitation.



Picture 3.7d - ≤ 1278b wild type cells growing under carbon limitation.



Picture 3.7e - ≤ 1278b wild type cells growing under nitrogen limitation.

4. CONCLUSIONS

The specific objetives of this research were to discover whether the elimination of the GS-GOGAT pathway would result in improved yield for *S.cerevisiae* , due to the elimination of a more energetic pathway to assimilate ammonia.

The accepted view is that NADPH-GDH is the primary route for ammonia assimilation with a minor contribution of GS-GOGAT pathway, which would work mainly in conditions of nitrogen starvation. In our experiments, we found that under carbon limitation the GOGAT$^+$ strain had a higher yield for glucose than the GOGAT$^-$, resulting in higher biomass level. As the enzymes of ammonia assimilation are in the interface of nitrogen/glucose metabolism, we found evidence that this GS-GOGAT pathway has a role in the maintenance of the cellular metabolism rather than a minor role in ammonia assimilation under conditions of nitrogen starvation. Under nitrogen limitation, GOGAT$^-$ cells changed their shape, probably due to changes in the cellular membrane composition.

NADPH-GDH activity for both strains increased with growth rate. There was no inhibition of NADPH-GDH activity under nitrogen limitation.

Oscillations found in enzyme activities were a consequence of a mixed culture (the presence of cells with plasmid and cells without plasmid), confirmed by oscillations in penicillinase activity.

GOGAT activity was only slightly higher in AR2 cells growing under nitrogen limitation than carbon limited medium.

5. SUMMARY

In order to investigate the effect of the elimination of GOGAT activity in *S.cerevisiae*, the pool sizes of ammonia, glutamate and glutamine plus the specific activities of the enzymes involved in ammonia assimilation were determined for two genetically engineered strains (AR2 and AR5) and an haploid wild type (ξ 1278b). AR2 and AR5 strains carry the plasmid pCYG4 which directs about 5 fold more NADPH-GDH activity than wild type cells. AR5 strain is a double mutant, which lacks GOGAT activity.

The studies were carried out using a microprocessor-controlled fermenter (PCS) which has the following features :

- 3 Main Boards (Central Processor Board, Memory Board and Analog/Digital - ON/OFF Switch Board).
- 4 Auxiliary Boards (pH, Oxygen, Temperature and Biomass Interface Boards).
- A connection block to link the PCS with the video terminal, with sensors from the fermenter, with a control box and with other microcomputer.

AR2 and AR5 showed lower values of maximum specific growth rates than the wild type, determined either by batch mode or by washout kinetics. The reduction in the growth rate for AR2 and AR5 can be related to the added metabolic loads due to the plasmid encoded genes.

Under carbon limitation there were no remarkable differences between the NADPH-GDH activities of AR2 ($GOGAT^+$) and AR5 ($GOGAT^-$). However, the concentrations of glutamate and glutamine for AR2 were higher (from 20 to 40 %) than those of AR5. The lack of the GOGAT activity also resulted in a decrease in the biomass concentration for AR5 compared to the $GOGAT^+$ strains.

Under nitrogen limitation NADPH-GDH activities were higher and intracellular ammonia concentrations lower than under carbon limited conditions. The intracellular concentrations of glutamate and glutamine were higher for the $GOGAT^+$ strain than for the $GOGAT^-$ one. Although the biomass level was the same for the three strains, AR5 ($GOGAT^-$) cells changed from rounded to ellipsoidal form under nitrogen limited conditions.

Oscillations were present in the NADPH-GDH activities of AR2 and AR5 strains growing under carbon and nitrogen limited media. They are probably due to segregational instability of the plasmid pCYG4 in these microorganisms.

6. REFERENCES

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