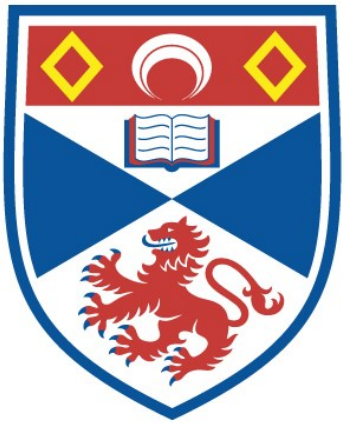
LIPASE PRODUCTION IN 'CANDIDA LIPOLYTICA 1055'

Alexandra Amorim Salgueiro

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



1994

Lipase Production

in

Candida lipolytica 1055

Alexandra Amorim Salgueiro

Submitted in accordance with the requirements for the degree of Doctor of Philosophy

School of Biological and Medical Sciences
University of St. Andrews

March 1994

DECLARATION

I, Alexandra Amorim Salgueiro hereby certify that this thesis has been composed by myself, that it is a record of my own work, and it has not been accepted in partial or complete fulfilmemt of any other degree or professional qualification.

The research was conducted in the Division of Biochemistry, School of Biological and Medical Sciences, University of St. Andrews, under the direction of Dr. W. M. Ledingham.

Signature

29/04/94
Date

CERTIFICATE

I hereby certify that Alexandra Amorim Salgueiro has spent nine terms engaged in research work under my direction and that she has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Doctor of Philosophy.

Signature of Supervisor Date

28 April 1994.

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ACADEMIC RECORD

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

I graduated with the degree of Master in Biochemistry in 1983 from the Biochemistry Department of the Federal University of Pernambuco, Recife, Brazil.

I was admitted to the School of Biological and Medical Sciences, University of St. Andrews, under the Ordinance General No. 12 and as a candidate for the degree of Ph.D. in February 1991.

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ABBREVIATIONS

α	alpha
a priori	first concern
ß	beta
BSA	bovine serum albumin
°C	degree centigrade
cm	centimetre
Co.	Company
D	dilution rate
D_M	best dilution rate
D_C	critical dilution rate
DNA	deoxyribonucleic acid
Δ	delta
e.g.	exempli gratia (for example)
et al.	et alia (and others)
F	flow rate
g	gram
GLC	gas liquid chromatography
h	hours
HPLC	High Pressure Liquid Chromatography
i.e.	id est (that is)
Inc.	Incorporation

K_s	saturation constant
KHz	kilohertz
l	litre
ln	natural logarithm
Ltd.	Limited
m	maintenance energy
M	molar
MW	molecular weight
mg	milligram
ml	millilitre
min	minutes
μ	specific growth rate
μ_{MAX}	maximum specific growth rate
µg	microgram
µl	microlitre
nm	nanometres
p.	page
P	product concentration
PEG	polyethylene glycol
PMSF	phenylmethylsulfonil fluoride
p-NPP	p-nitrophenylpalmitate
PVP	polyvinylpyrrolidone
R	acyl fatty acid
RNA	ribonucleic acid
rpm	revolutions per minute
S	substrate concentration
$\bar{S}$	residual substrate

S_0	substrate concentration at feed medium or limiting substrate concentration
sec	seconds
SCP	single cell protein
Sigma	Sigma Chemical Co. Ltd.
Span-80	sorbitan monooleate
t	time
t_d	doubling time
Triton X-100	octyl phenoxy polyethoxyethanol
Tween-20	polyoxyethylenesorbitan monolaurate
Tween-40	polyoxyethylenesorbitan monopalmitate
Tween-60	polyoxyethylenesorbitan monostearate
Tween-80	polyoxyethylenesorbitan monooleate
U	units
V	culture volume
v/v	volume per volume
x	biomass concentration
x_0	biomass concentration at zero time
x_{MAX}	maximum biomass concentration
x g	times acceleration due to gravity
&	and
Y	yield coefficient
$Y_{X/S}$	growth yield
$Y_{X/P}$	product yield

SUMMARY

Microbial lipases are now increasingly employed in the redesign of animal fats and vegetable oils. Commercial scale manufacture of microbial lipases, which is limited, tends to be surrounded by secrecy and much remains to be clarified in terms of optimization of fermentation conditions.

C. lipolytica metabolizes cheap carbon sources (from wastes and by-products), in particular non-carbohydrate substrates, accumulating lipid during the growth. As all potent producers of lipase are also amongst the lipid-accumulating microorganisms, this yeast shows potential as a major lipase producer. The future for lipase fermentation industries look promising due to these raw material being abundant and inexpensive.

Lipase activity (triolein as a substrate) and esterase activity (p-nitrophenylpalmitate as a substrate) were measured throughout this work.

Different operational strategies (batch, fed-batch, chemostat and continuous transient technique) have

been investigated aiming lipase and esterase production by *C. lipolytica 1055*.

Nutritional parameters (carbon and nitrogen sources, limiting substrates: glucose, Tween-80 and olive oil) under steady-state conditions at different dilution rates, as well as oleic acid and olive oil square wave oscillations have been studied.

Pulsing experiments with triolein, olive oil, oleic acid and Tween-80 suggest that the oleyl residue may be responsible for the induction effect in *C. lipolytica 1055* lipase and esterase production.

However, the low values of lipase productivities (< 1 U/mg dry weight.h) under different cultivation methods appear to limit the advantages in using *C. lipolytica 1055* as a lipase producer.

Extracellular esterase productivity by *C. lipolytica 1055* reached 5.3 - 6.5 U/mg dry weight.h in the presence of Tween-80 under batch and fed-batch culture subjected to oleic acid input.

The esterase production phase under fed-batch process was prolonged suggesting that this operation mode could be a route towards obtaining esterase by *C. lipolytica 1055*. For example, a two-phase bioreactor operation may be devised with high yeast cell concentration promoted by a carbohydrate waste under batch conditions in the first stage, and a high enzyme production under fed-batch operation in the second one.

CONTENTS

1 INTRODUCTION

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3) act at a lipid-water interface when water soluble enzymes interact with the insoluble triglyceride substrate. From the biotechnological point of view, these enzymes might have an extended definition due to diversified lipase properties and applications (Vorderwulbecke et al., 1992).

Although plants, animals and microorganisms are potential sources of lipases, microbial lipases have large-scale technological advantages. Bacteria, fungi and yeasts can produce lipases cell-bound or excreted free into the growth media; their industrial applications depend on the nature of the source and the reaction conditions as well as costs involved in the process (Cowan, 1991).

1.1 LIPASE REACTIONS

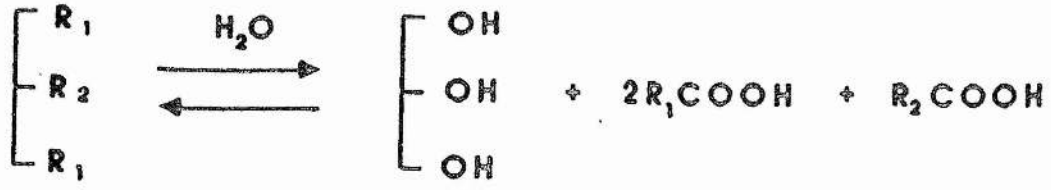
Lipases catalyse the hydrolysis of oils and fats into free fatty acids, partial glycerides and glycerol. As the reaction is reversible, these enzymes can also synthesize glycerides through esterification of alcohols with fatty acids in the presence of limited aqueous phase.

Macrae (1983) and Sztajer & Zboinska (1988) have reported two groups of lipases in function of the positional specificity. The non-specific enzymes hydrolyse the triglycerides without distinguishing the glycerol esters position giving only glycerol and fatty acids while the specific lipases catalyse reactions preferentially at the 1- and/or 3- positions of the glycerides producing 2-monoglycerides and 1,2-(2,3-)diglycerides (Scheme 1.1-a).

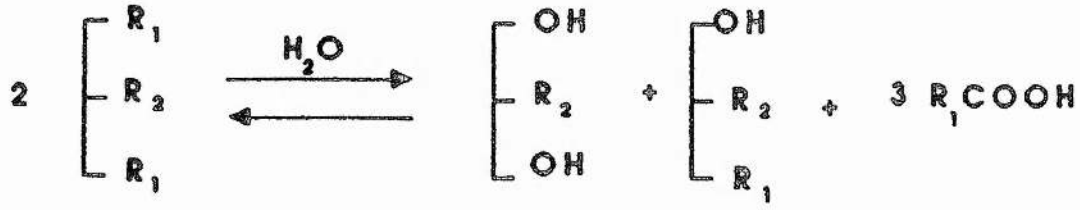
Only under condition of restricted water availability, lipases catalyse the reaction of interchange of fatty acid groups due to the reversibility of the hydrolysis and resynthesis of triglycerides. Interesterification reactions may occur between two triglycerides or between one triglyceride and a fatty acid.

Many industrially important products which can not be obtained by chemical process have been obtained by use of specific lipases in transesterification reactions (Scheme 1.1-b). However, when a non-specific lipase

(i) BY NON-SPECIFIC LIPASES

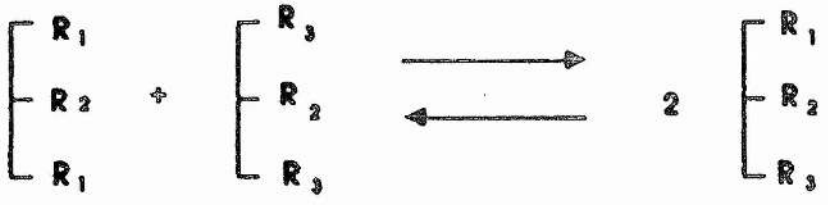


(ii) BY SPECIFIC LIPASES

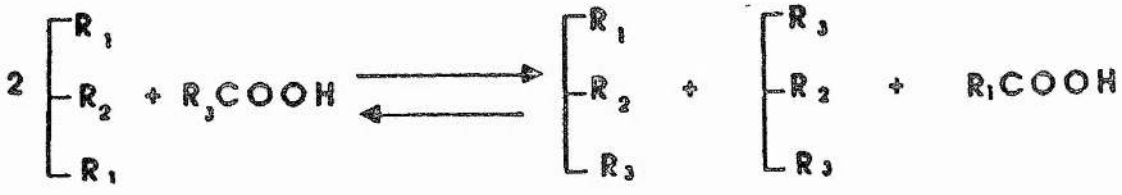


SCHEME 1.1-a HYDROLYSIS OF TRIGLYCERIDES

(i) BETWEEN TRIGLYCERIDES



(ii) BETWEEN TRIGLYCERIDE AND FATTY ACID



SCHEME 1.1-b TRANSESTERIFICATION REACTIONS BY SPECIFIC LIPASES

catalyses an interesterification, between either two triglycerides or one triglyceride and one fatty acid the mixture produced is similar to that produced by chemical processes.

Although the specific lipases are more important industrially, some non-specific lipases from *Candida cylindracea, Geotrichum candidum, Penicillium cyclopium* are currently used in industrial production (Sakaguchi et al., 1992).

Specific lipases are common amongst microbial sources. They were separated by Macrae (1985) according to their products: regiospecific lipases produce predominantely diglycerides, fatty-acid-specific lipases release a particular type of fatty acid from glycerides and stereospecific lipases which products have a predominance of one stereoisomer.

Lipases from *Penicillium simplicissimum* (Sztajer et al., 1992) and *Candida rugosa,* or *C. cylindracea,* (Macrae, 1983; Kwon & Rhee, 1984; Goldberg et al., 1990; Hadeball, 1991; Renobales et al., 1992) have been reported as non-specific enzymes that hydrolyse each of the three bonds of triglycerides.

However, it seems that different lipase preparations contain different enzymes due to some contradictory evidences about lipase specificity of *P. simplicissimum* (Stamatis et al., 1993a) and *C. rugosa* (Cambou & klibanov, 1984; Gruber-Khadjawi & Honig, 1992;

Chattopadhyay & Mamdapur, 1993). These enzymes have also been reported as stereospecific lipases.

Some microorganisms such as, *G. candidum* (Jacobsen et al., 1989b and 1990), *Mucor lipolyticus* (Macrae, 1983) and *Chromobacterium viscosum* (Aires-Barros & Cabral, 1991) have multiple forms of lipase that differ in their substrate specificities and physical properties.

G. candidum lipase has shown different fatty acid specificities in function of the strain (Baillargeon et al., 1989) and the growth conditions (Jacobsen et al., 1990). The termed lipase B from *G. candidum* (Jacobsen et al., 1990; Charton & Macrae, 1992), as well as *Galactomyces geotrichum* lipase (Phillips & Pretorius, 1991) have been very selective towards a cis-double bond in the 9 position of fatty acid.

Pseudomonas aeruginosa lipase has exhibited high enantiomeric selectivity in the hydrolysis of a diester to its S-ester acid, an important intermediate compound in pharmacological synthesis (Katz et al., 1993).

Lipase from *Candida lipolytica*, also termed *Saccharomycopsis lipolytica*, (Jonsson, 1976) has shown high specificity for oleic acid while *Candida deformans* lipase has shown more specificity for shorter and/or monosaturated fatty acids (Hadeball, 1991).

Furthermore, lipases from *Candida paralipolytica* (Ota et al., 1972) and from *Micrococcus caseolyticus* (Jonsson, 1976) have shown lipase activity with little specificity for substrate.

On the other hand, lipases from *Peniccilium cyclopium* (Macrae, 1983) and *Peniccilium camembertii* (Chen et al., 1992) hydrolyse preferentially or only partial glycerides, i.e. showing no activity toward triglycerides.

1.2 LIPASE APPLICATIONS

The uses and applications of microbial lipases have increased over the last few years expanding into new areas. Under mild conditions new products have been provided by selectivity reactions with stereo-, regio- or fatty acid lipases (Kazlauskas, 1993).

Since the beginning of the century pharmaceutical industries have used these enzymes for inclusion in digestive aids for human consumption replacing pancreatic lipase which is expensive and scarce (Aunstrup, 1979). Recently, lipases produce compounds used as intermediates in synthesis of pharmacologically active substances (Wingender et al., 1987).

In the area of nutrition lipases can be used to modify lipids by preparing mixtures of triglycerides with saturated and medium-chain fatty acids (Bjorkling et al.,

1991) or can produce omega-3-polyunsaturated fatty acid derived from fish oils (Zuyi & Ward, 1993a and 1993b). These lipids (with a well defined composition) and the polyunsaturated fatty acid are healthier and more quickly absorbed by the body. They could be an adequate supply of essential fatty acids.

Lipases hydrolyse animal fats and vegetable oils producing useful fatty acids and / or mono- and diacylglycerols for the chemical industry (Khor et al., 1986; Baillargeon & Sonnet, 1991; Renobales et al., 1992). Enzymatic splitting are moderate reactions at reduced pressure and lower temperatures, showing advantages against the chemical processes which occur under high pressure and high power-input.

Lipases have been used immobilized on macroporus particles in continuous fat/oil splitting, reducing substrate inhibition and increasing enzyme stability (Kwon & Rhee, 1984; Ruckenstein & Wang, 1993).

The importance of lipases in washing detergents has been enhanced by saving energy (washing at lower temperatures) and avoiding environmental problems since the biological detergents are biodegradable (Sztajer & Zboinska, 1988). The enzymatic hydrolysis of lipids for the production of soaps is cheaper than the conventional method while the enzymatic products have a better colour and odour (Macrae, 1983).

The use of lipases in paper manufacturing industry has improved paper quality and productivity due to less frequent machine cleaning being required and fewer chemicals used in the pre-treatment of resins (Bjorkling et al., 1991).

Lipases are being used by industry to modify fats and oils, improving some food properties such as the flavouring of dairy products. Different kinds of flavour have been developed by lipolytic activities in many manufactured cheeses (Sztajer & Zboinska, 1988; Cowan, 1991).

The interesterification of palm oil by stereospecific lipase with stearic acid or tristearine produces cocoa butter through a cheaper process (Harwood, 1989; Bjorkling et al, 1991).

Margarines with higher melting points and special low-calorie spreads could be obtained by changing certain properties of natural oils using lipases.

Powdered lipolytic enzymes have been suspended in water-immiscible organic solvent with minimal amount of water, displaying great properties such as thermostability, and catalysing unusual reactions. It has been reported that porcine pancreatic lipase in microaqueous systems (also called nonaqueous systems) exhibited a high catalytic activity at 100°C and their stability at this temperature increased to a nearly anhydrous organic media (Zaks & Klibanov, 1984).

In the presence of surfactants lipases form stable micro-emulsion droplets in organic solvent. A reversed micellar solution is a homogeneous system that allow the simulation of enzymatic kinetic studies in vivo environment (Stamatis et al., 1993b) and the selective separation and purification of lipases (Aires-Barros & Cabral, 1991).

These micelles facilitate the mass transfer providing large interface in esterification reactions with alcohols and fatty acids (Klibanov, 1989; Hayes & Gulari, 1992). Stamatis et al. (1993a) have reported that microemulsions could be an effective and fast system for esterification of menthol due to the enantioselectivity of the reaction. Moreover, high operational stability of lipase immobilized on liposome in reversed micelles was obtained in continuous glycerolysis of olive oil (Chang et al., 1991).

Furthermore, these enzymes can catalyse the synthesis of peptides in organic solvents due to their high efficient regio- and stereo-selectivities, as well as mild reaction conditions (Zaks & Russell, 1988; Macrae, 1989).

Although the synthesis of intermediate esters in enantiomerically pure form with high optical purity are not yet obtained in industrial scale, this stereospecificity of lipases in organic media have considerable interest in the manufacture of agrochemicals

such as herbicides or insecticides (Stamatis et al., 1993a), fine chemicals (Deleuze et al., 1987; Ohrner et al., 1992; Chattopadhyay & Mamdapur, 1993) and as precussor for the synthesis of natural products (Katz et al., 1993).

Another industrial lipase application is the synthesis of glucoside esters in organic solvents which have surfactant properties and could be used in cosmetics, pharmaceutics, foods and beverages, as well as in the industrial de-greasing of metals, electronics and leather, and as components of paints and varnishes (Harwood, 1989; Bjorkling et al., 1991; Oguntimein et al., 1993).

Chopineau et al.(1988) have obtained an efficient extracellular biological surfactant from sugar alcohols and vegetable oils in dry pyridine while Schlotterbeck et al.(1993) have reported that immobilized lipase from *Mucor miehei* (Liposyme, Novo Nordisk) could be used for the selective monoacylation of fructose.

Nonaqueous organic solvents have been replaced for vegetable oils as the bulk organic phase in esterification reactions. For example, Welsh et al. (1991) have reported health and safety processes with higher yields of low molecular weight ester in large scale production using vegetable oils and butter as reaction media.

Others applications of microbial lipases include: studying neutral lipids in microbial membranes (Macrae, 1983); hydrolysis of fatty acid anhydrides (Pieroni & Fourneron, 1990); intramolecular esterification when macrocyclic lactones have been produced from methyl esters of different hydroxy-acids (Zaks & Russel, 1988).

Lipase technologies have a tendency to find applications in many new areas due to the ommission of toxic catalysts, the safety of the products generated, as well as the mild and economic industrial processes. The reactions in organic solvents and the stability at high temperatures are two important future developments.

1.3 IMPORTANCE OF MICROBIAL PHYSIOLOGY IN FERMENTATION PROCESSES

Microbial physiology provides the basic knowledge enabling optimization of fermentations (biological processes). As microorganisms react to their environment, the growth of bacteria, yeasts and fungi can adjust to changing conditions by cellular modifications. This physiological adaptation could be responsible for production of some enzymes amongst other metabolites.

The relationships and interactions between the microbial metabolisms and its environment have had

important role in the Biotechnology area. The microbial physiology development as well as the use of mutants and the advance in the design and operation of large-scale bioreactors were responsible for the great improvement in penicillin production in the 1940's (Pirt, 1975).

The potential metabolic activity of a culture depends on the integrated physiological unity that changes in function of each set of condition. For example, *Escherichia coli* has utilized a mixture of xylose and glucose completely in continuous culture while a diauxic phase was present in batch mode (Barford et al., 1982).

In addition, a specific metabolite that is accumulated in a batch culture could not be obtained when the specific growth rate is adjusted to the dilution rate in a continuous procedure.

Then, the definition of physiological state in accordance with Malek has a great role in continuous culture. This dynamic concept includes not only the set of properties expressed but also the "potentialities of the culture that can be brought out" (Malek, 1976).

Nowadays more research ought to be carried out regarding the efficient use of microorganisms, understanding them at their physiological level. Lag phase has been studied by few authors (Tan & Gill, 1985b; Valero et al., 1988) and for future perspective of industrial application, this phase must be properly

understood, in order to decrease its time, or perhaps to avoid it in fermentation processes.

The physiology of microorganisms using different carbon sources others than glucose and the effect of interactions between cells and medium components are very little investigated. Few attempts have been made to understand the physiological control of protein production and microorganism growth rate, in particular its physiology at low growth rates, as well as the relationship between enzyme production, e.g. lipase, and the consumption of substrates (Del Rio et al., 1990).

Generally the proteins are synthesized at constant rates. Only some enzymes involved in cellular division and deoxyribonucleic acid (DNA) synthesis have been detected to be periodic. In the cell cycle enzymes can increase exponentially or linearly. It is possible to obtain a maximum synthesis at one specific time, as well as regular oscillations in activity during a cycle. Many microbial interactions produce oscillations probably due to different times that the metabolisms need to reach the balanced growth (Barford et al., 1982).

In terms of optimization of fermentations much remains to be clarified due to the luck of information at the microbial physiology level concerning lipase production (Sections 1.4.2, 1.5.6 and 1.6.2) although several publications and patents have appeared involving lipase purification and characterisation (Stuer, 1986;

Wingender et al., 1987; Baillargeon et al., 1989; Jacobsen et al., 1989b; Goldberg et al., 1990; Jane Gilbert et al., 1991b; Phillips & Pretorius, 1991; Sztajer et al., 1992).

1.4 BATCH CULTURE

1.4.1 Basic theory

The basic kinetic parameters for batch growth are maximum specific growth rate (μ_{MAX}), saturation constant (K_S) and yield coefficient (Y).

In exponential phase the growth is maximum and the microorganisms obey the equation:

$$dx/dt = \mu_{MAX}\ x$$

Integrating

$$\int dx/x = \mu_{MAX} \int dt$$

$$\ln x = \ln x_0 + \mu_{MAX}\ t \quad \text{(Equation 1.4.1)}.$$

Throughout the above equation the doubling time of a culture (t_d) could be determined by:

$$t_d = \ln 2 \ / \ \mu_{MAX}.$$

The microorganisms exhibit a constant specific growth rate during exponential growth while the metabolites are taken up or produced during this time.

In batch experiment the medium composition is constantly changing inducing modifications in cell composition. Thus, perfectly balanced growth can never be achieved in this process.

Monod's equation is one model for studying microbial growth under batch conditions showing the relation between specific growth rate (μ) and substrate concentration (S):

$$\mu = \mu_{MAX}\ S\ /\ (K_s + S).$$

Throughout the latter equation μ_{MAX} is the maximum growth rate achievable and the significance of the saturation constant (K_s) is the concentration of a limiting nutrient (substrate) at which the specific growth rate is half its maximum value.

The yield coefficient is a parameter used to express the efficiency of growth, i.e. the efficiency of substrate conversion. It was originally defined by MONOD in terms of mass units :

$$Y = \text{CELLS FORMED} / \text{SUBSTRATE USED}.$$

In practice :

$$Y_{x/s} = \Delta x / \Delta S$$

Or, when this principle is applied to product yields:

$$Y_{x/P} = \Delta x / \Delta P.$$

The growth yield is maintained constant when the culture conditions do not vary.

The maximum amount of cell mass formed for a given consumption of substrate in a growth-limiting substrate, tends to be constant.

As

$$Y = (x - x_0) / (S_0 - S)$$

If $S = 0$ and $x = x_{MAX}$

Thus,

$$x_{MAX} = x_0 + Y S_0.$$

1.4.2 Batch lipase production

Some nutritional factors that affect microbial lipase yield under batch conditions are summarised in the Table 1.4.2.

Lipase activity results should be carefully analysed before any comparison. A wide range of lipase activities were measured using triolein or olive oil as the substrate while some activities were expressed in function of butyric acid releases or using the ester p-nitrophenylpalmitate (p-NPP) as substrate. In addition, some authors have expressed lipase activity as the volume of the alkali used in the titration method (this volume can be converted into U/ml).

MICROORGANISM	CARBON SOURCE/INDUCER	TEMP (°C)	pH	AGITATION (rpm)	LIPASE (U/ml)	LIPASE (U/mg) °	REFERENCE
Acremonium strictum	XYLOSE & TWEEN-80	30	7.0	STATIONARY	7.45	1.46	Okeke & Okolo, 1990
Bacillus sp.	SUCROSE & TRIBUTYRIN	42	8.6	120	3.4°	-	Sztajer & Maliszewiska, 1988
Beauveria bassiana	GLUCOSE & OLIVE OIL	30	-	150	15*	-	Hegedus & Khachatouriansi, 1988
Candida deformans	CITRATE & OLIVE OIL	30	4.5	-	3.6(0.6)	-	Novotny et al., 1988
					10°(1)°	-	"
C. lipolytica	WHEY BROTH & SPAN-80	25	4.5	200	-	87°*	Kalle et al., 1972
	KEROSENE & OLIVE OIL	25	6.0	-	0.003*	0.002	Kosaric et al., 1979
C. rugosa	GLUCOSE & CHOLESTEROL	30	6.1	500	4	-	Valero et al., 1988
	OLEIC ACID	30	-	500	14.8	3.3*	Del Rio et al., 1990
	GLUCOSE & OLIVE OIL	30	-	500	5	10*	Valero et al., 1991a
	OLIVE OIL				5.2	52*	"
	OLEIC ACID	30	-	500	22	-	Valero et al., 1991b
	OLEIC ACID	30	-	500	8	3.9*	Obradors et al., 1993
	CAPRYLIC ACID				7.41	21.2*	"
Geotrichum candidum	GLYCEROL & OIL	30	7.0	300	7.3	-	Baillargeon et al., 1989
	OLIVE OIL & TWEEN-80	30	7.0	600	12	10*	Jacobsen et al., 1989a
	CITRIC ACID & OLIVE OIL	30	6.5	750	6	-	Jacobsen et al., 1989b
	OLEIC ACID	27	5.8	-	70	-	Shimida et al., 1992
Mucor javanicus	OLIVE OIL	28	-	-	19.3	-	Roblain et al., 1989
Penicillium candidum	WHEAT	29	7.0	160	19°*	-	Rivera-Munoz et al., 1991
				STATIONARY	45°*	-	"
P. citrinum	STARCH & OLIVE OIL	22	7.2	150	4.8*	-	Maliszewska & Mastalerz, 1992

Cont. ⇒

MICROORGANISM	CARBON SOURCE/INDUCER	TEMP (°C)	pH	AGITATION (rpm)	LIPASE (U/ml)	LIPASE (U/mg)°	REFERENCE
P. roqueforti	GLUCOSE	27	4.0	230	10.4	1.7*	Petrovic et al., 1990
Pseudomonas aeruginosa	TWEEN-80	37	6.5	200	-	8/1.5"	Jane Gilbert et al., 1991a
	SKIM MILK	20	-	350	35+	-	Katz et al., 1993
	PEPTONIZED MILK	25	7.0	500	37+	-	Marcin et al., 1993
Rhizopus delemar	SUN FLOWER OIL	29	7.0	150	32	1.8	Espinosa et al., 1990
	GLUCOSE & TWEEN-80	29	7.0	150	27	4.4	"
	OLEIC ACID	30	-	150	-	(990)	Chen & McGill, 1992
R. oligosporus	TWEEN-80 & SOYBEAN	25	6.5	-	1*	1.2*	Hanes, 1988
Rodotorula glutinis	PALM OIL	30	8.0	250	30.4	1*	Papaparaskevas et al., 1992
Saccharomyc. lipolytica (*C. paralipolytica*)	'NUTRIENT BROTH'	30	-	-	39	-	Jonsson & Snygg, 1974
	" & OLIVE OIL	30	8.0	500	20.1	-	Jonsson, 1976
	GLUCOSE & SOYBEAN	30	-	-	11(19)	-	Ota et al., 1978
	GLUCOSE & OLEIC ACID	30	-	180	.24(.5)*°	-	Ruschen & Winkler, 1982
	GLUCOSE & OLIVE OIL	30	7.0	300	2.5	-	Gomi et al., 1986
Staphylococcus carnosus	'BRAIN-HEART-INFUSION'	30	7.4	50-300	2.5/2.7"	1.1	Falk et al., 1991
Streptomyces	STARCH & SOYBEAN	28	-	100	0.2*	0.1*	Sztajer et al., 1988
Trichosporon fermentans	TUNG OIL	30	5.5	120	146	3.5*	Chen et al., 199.
Yarrowia lipolytica	CITRATE & OLIVE OIL	30	4.5	-	6.2(0.6)	-	Novotny et al., 1988
					13°(1)°	-	"

N.B. One lipase activity unity corresponds to the enzyme activity that liberates 1 µmole of oleic acid per minute (triolein/olive oil as substrate); or, (°)for tributyrin or (") for p-NPP as substrates.
(°) Specific lipase activity: U/mg dry weight. (+) ml of 0.05 M NaOH spent in titration method.
(*) Calculated results. The activity values in brackets correspond to cell-bound enzymes.

TABLE 1.4.2 BATCH LIPASE PRODUCTION

Lipase assays with a specific substrate carried out under different conditions are difficult to be compared itself because the activity depends on the available surface area which properties change in function of the physicochemical conditions. Furthermore, some experiments do not have defined conditions.

Different carbohydrates were used as carbon sources. Some researchers have obtained high lipase activities in the presence of xylose (Okeke & Okolo, 1990), sucrose (Sztajer & Maliszewska, 1988), citrate or succinate (Novotny et al., 1988), glycerol (Baillargeon et al., 1989), and starch (Mailszewska & Mastalerz, 1992).

Despite the fact that Baillargeon et al. (1989), Nahas (1988), Chen et al. (1992) have noticed that glucose supressed the lipase formation, good lipase yield with this carbohydrate in the presence of an inducer were obtained by Hegedus & Khachatourians (1988) and Espinosa et al. (1990).

In addition, Kalle et al. (1972) have reported that lipase from *C. lipolytica* was not repressed when glucose was the carbon source in the absence of Span-80, the inducer.

As many of the enzymes used commercially are inducible research has been developed showing the stimulation of lipase production by a greater number of natural oils, such as olive oil (Hegedus &

Khachatourians, 1988; Novotny et al., 1988; Valero et al., 1991a; Jacobsen et al., 1989a and 1989b; Maliszewska & Mastalerz, 1992; Jonsson, 1976; Gomi et al., 1986), triglycerides of palmitic and stearic acids (Kalle et al., 1972), as well as cholesterol (Valero et al., 1988). It is relevant to take in consideration that fats and oils are metabolized and used for growth by lipolytic microorganisms (Tan & Gill, 1984 and 1985a).

Olive oil was the best carbon source for growth and lipase production (Valero et al., 1991a; Roblain et al., 1989) despite some publications reporting its negative effect on lipase production (Petrovic et al., 1990) and on growth of some lipase sources (Espinosa et al., 1990).

There is some contradictory evidence about the effect of the surfactants. Hegedus & Khachatourians (1988) have noticed that Tween-80 inhibited an extracellular lipase production from *Beauveria bassiana* possibly through a mechanism of competitive inhibition, blocking the enzyme from the substrate.

On the other hand, Okeke & Okolo (1990), Jacobsen et al. 1989a), Jane Gilbert et al. (1991a), Espinosa et al. (1990) and Nahas (1988) have noticed that Tween-80 is an effective inducer in lipase synthesis, increasing the release of lipase from the cells and thus, stimulating lipase synthesis.

Hegedus & Khachatourians (1988), Okeke & Okolo (1990) and Jane Gilbert et al. (1991a) have reported that lipase production was repressed by free fatty acids including unsaturated with long chains.

There is a correlation between fatty acid chain length or degree of saturation and lipase production. Obradors et al. (1993) have reported that some saturated fatty acids induced lipase activity of *C. rugosa*. Maliszewska & Mastalerz (1992) have noticed that lauric acid inhibited *P. citrinum* lipase which activity was stimulated in the presence of unsaturated fatty acids.

Different nitrogen sources were studied such as ammonium ion and organic complex material aiming increase lipase activity. Good results were obtained with peptone (Okeke & Okolo, 1990), peptone or urea (Novotny et al., 1988), soybean meal extract (Nahas, 1988; Sztajer & Maliszewska, 1988) and yeast extract (Espinosa, 1990).

Significant improvements in enzyme production could be obtained by modification in metal ion concentrations, releasing enzymes that remain attached to the cell walls.

The effect of metal ions on lipase production has been studied by Hegedus & Khachatourians (1988), Kasuto et al. 1989), Petrovic et al. (1990), Jacobsen et al. (1990). Lipase has been stimulated by Ca^{++} and Mg^{++} ions and inhibited by Fe^{++}, Cu^{++} and Hg^{++} ions. Ions such

as Na^+ ,K^+ and Mn^{++} have shown positive and negative effects in function of their concentration in media.

Some physical parameters such as pH and temperature are also shown in table 1.4.2. Depending on the strain, *Ps. aeruginosa* produced maximum lipase at neutral pH and at 20°C (Katz et al., 1993), 25°C (Marcin et al., 1993), or 37°C (Jane Gilbert et al., 1991a).

S. lipolytica has shown optimum temperature at 30°C and pH 7.0 - 8.0 (Jonsson, 1976; Gomi et al., 1986) while others correlated yeasts, *Yarrowia lipolytica* and some *Candida* species have shown optimum acid pH (4.5) desirable for industrial applications since the bacteria would be inhibited and no expensive sterilization would be required (Kalle et al., 1972; Novotny et al., 1988).

As the melting point of fat can be higher than the optimum temperature of mesophilic lipases, investigations have been carried out on lipase activity of thermophilic sources (Liu et al., 1973a and 1973b; Silva et al., 1991; Sigurgisladottir et al., 1993).

In biological processes the agitation influences the availability of nutrients and the removal of metabolic products as well as the dispersion of biomass. Then, mass transfer and heat transfer, two limiting factors in production of microbial cells and metabolites, depend on the agitation which is responsible

for maintaining homogeneous the chemical and physical conditions (Pirt, 1975).

In the presence of lipid material must have sufficient agitation to ensure that these insoluble compounds are extensively dispersed, offering large surface area (Tan & Gill, 1985a).

Table 1.4.2 shows that some microorganisms have been grown under strong agitation aiming lipase production. On the other hand, *A. strictum* (Okeke & Okolo) and *P. candidum* (Rivera-Munoz et al., 1991) have shown high lipase production in stationary state.

Aerobic microorganism growth depends on sufficient dissolved oxygen in submerged cultures. The maximum oxygen transfer rate stimulates the growth but restricts enzyme production (Fowler, 1988).

Aeration has stimulated *R. oligosporus* growth restricting its lipase production (Nahas, 1988). In addition, *Ps. aeruginosa* has supported optimal lipase production when was cultivated in a dissolved oxygen limited culture (Marcin et al., 1993).

However, some aerobic organisms have produced maximal lipase activities in presence of sufficient or elevated dissolved oxygen in submerged cultures (Jacobsen et al., 1989a; Jane Gilbert et al., 1991a). Moreover, Valero et al. (1991a) have reported that oxygen deficiency restricts lipase production by *C. rugosa*.

In summary, microbial lipase and /or esterase production are strongly affected by the microorganism species, the medium composition and the physical conditions of the processes.

1.5 CONTINUOUS CULTURE

Continuous culture technique allows microbial growth under balanced conditions.

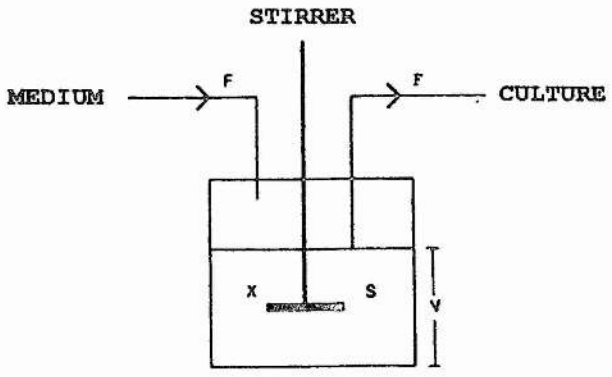
Growing cultures under steady-state condition, the concentration of any metabolic variable is constant and the microorganism grows at a predetermined rate in a constant and controlled environment.

Despite the problem of contamination of continuous culture in large-scale production, optimization of physical parameters and nutrient concentrations, as well as the control of synthesis of metabolic products, can be simply investigated by chemostatic cultures. Thus, continuous culture is a fundamental tool in Microbial Physiology.

In industrial scale continuous fermentation processes of bakers' yeast and beer are technically feasible although these processes confront economic factors concern the equipment required. Waste-water treatment and microbial protein production (SCP) are two important industrial application of continuous culture (Atkinson & Mavituna, 1991).

In the chemostat (below schematic representation) the culture of microorganism is well-mixed while fresh medium is continuously added and the product is havested at the same rate. Biomass (x) and substrate concentration (S) are constant through time due to the material added is homogeneously dispersed throughout the culture. The culture volume (V) is constant and the flow of medium into the reactor (F) per unit volume is known by the term dilution rate (D) and defined as:

$$D = F / V.$$



1.5.1 Biomass balance

In the chemostat the microbial population has an equal probability of growth and of being washed out within a given time.

The maximum growth rate is described by the equation :

$$dx / dt = \mu_{MAX}\, x.$$

The washout rate, i.e. the rate at which cells would be washed out at a constant flow rate is given by the equation :

$$- dx / dt = D\, x.$$

Under continuous culture conditions, a priori, the specific growth rate is less than the maximum specific growth rate. Then, the biomass balance depending on the difference between growth and washout can be expressed by:

$$dx / dt = \mu\, x - D\, x$$

$$dx / dt = x\, (\mu - D).$$

If (i) $\mu > D$ then, dx / dt has a positive value, and the biomass concentration increases in the reactor;

(ii) $\mu < D$ then, dx / dt has a negative value, the biomass concentration decreases with time and the culture may be washed out from the reactor;

(iii) $\mu = D$ then, $dx / dt = 0$; under this condition, called steady-state, the biomass concentration is constant with the time.

Thus, the specific growth rate of the organism in the reactor is exactly equal to the dilution rate under steady-state conditions.

1.5.2 Substrate balance

Considering that :

S_0 = substrate concentration at feed medium;

S = substrate concentration in the reactor.

The net change in substrate concentration depends on the input, output and consumption:

$$dS / dt = D S_0 - D S - \mu x / Y$$

Where the growth is expressed by μx and the cellular yield, by Y.

Rearranging the above equation:

$$dS / dt = D(S_0 - S) - \mu x / Y .$$

At steady-state condition:

(i) $dS / dt = 0$

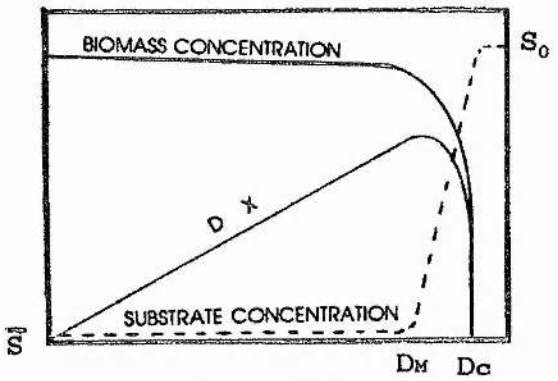
(ii) $\mu = D$.

Then, the biomass concentration depending on the substrate concentration at the feed medium, could be described by the equation :

$$x = Y (S_0 - S).$$

1.5.3 Ideal chemostatic behaviour

The growth of *Candida* in continuous culture under fully aerated conditions and sugar limitation is shown as an ideal chemostatic behaviour (Barford, 1987):



The graph above shows the effect of dilution rate on the biomass concentration (x), limiting substrate concentration (S_0), residual substrate ($\bar{S}$) and yield production (D x) in a chemostat. The best dilution rate (D_M) is obtained for the maximum biomass output (D x) and at the critical dilution rate (D_C) the culture is washed out, i.e. the biomass concentration is zero.

Thus, the critical value of the dilution rate is of great practical importance because steady-state conditions can not be obtained at dilution rates close to the maximum specifix growth rate (D_C).

For each dilution rate the culture reaches a steady-state of cells which biomass concentration depends on a single limiting factor that could be a nutrient concentration or a physical parameter.

When the microorganism grows under limiting substrate a high carbon efficiency is obtained and another kinetic constant can be investigated : a maintenance energy requirement (m). This coefficient depends on the conditions of growth and results show that the medium pH has a big effect while the temperature causes small variation (Pirt, 1975).

1.5.4 Enzyme regulation

In continuous enzyme production the dilution rate as well as the type and concentration of the limiting factor affect the enzyme levels.

In chemostatic cultures the regulation of enzyme synthesis has been shown in function of the dilution rate. The enzyme activity can pass through a maximum/minimum at a particular dilution rate, and can rise/drop with increasing dilution rate. Constant enzyme activity has been reported over restrict range of dilution rate which results do not support the many changes in cell composition and morphology by varying dilution rate (Melling, 1977).

Arvidson et al. (1976) have reported the influence of different dilution rates on some extracellular enzymes of *Staphylococcus aureus*; when the culture was grown glycerol-limited, phosphatase and protease showed maximum activities at the dilution rate of 0.4 h^{-1} while staphylokinase activity (staphylococcus enzyme involved in tissue infection) increased with increasing dilution rate from 0.3 to 0.6 h^{-1}.

MELLING (1977) has reported that ß - galactosidase production by *Escherichia coli* and amidase production by *Ps. aeruginosa* declined with increasing dilution rate. At high dilution rates the amount of cell protein is lower in function of the incresing content of storage compounds and nucleic acids. The most suitable operation region is at low dilution rates where biomass yield is maximum without the decreasing influence of maintenance energy requirements (Dean, 1972).

The effect of growth-limiting nutrient on regulation of enzyme synthesis at steady-state conditions has been reported for different cultures. For example, the level of enzymes responsible for glucose take up and metabolism in *Ps. aeruginosa* depends upon the limiting-concentration of carbon and nitrogen present in the culture (Melling, 1977).

The production of certain enzyme may rise or fall during steady-state due to any typical variation explained by genetic modifications in microbial

population. A high mutation rate was reported by Ricci & Tell (1988) growing *C. utilis* under nitrogen-limited continuous culture.

Studying regulation of enzyme synthesis under balanced condition, well-defined and reproducible variations could be imposed in the environment and consequently, it is possible to achieve mechanisms for the production of microbial enzymes.

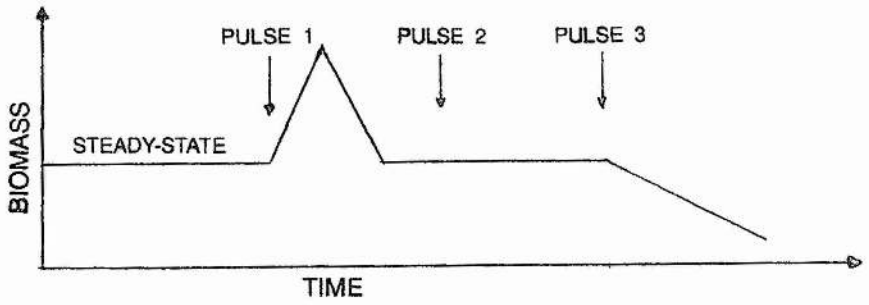
1.5.5 Delta pulse technique

The delta pulse involves an abrupt change on a physical or a chemical parameter imposed to a chemostatic culture under steady-state conditions.

During steady-state conditions the biomass and nutrient concentrations are constant. If only one nutrient concentration is changed while all the others have their concentration in excess, and the physical parameters necessaries to grow are present, the response of the culture can be interpreted.

The unbalanced growth which immediately follows the delta pulse imposed, disrupts the continuous steady-state culture, i.e. the culture will no longer maintain balanced growth.

The following schematic representation shows the responses of three different pulses (indicated by arrows) in chemostatic culture:



Pulse 1 - Response of a limiting nutrient due to the increase in biomass concentration after the input;

Pulse 2 - Response of a non-limiting nutrient because the biomass concentration is constant after the input;

Pulse 3 - Response of a toxic nutrient in function of the decrease in biomass concentration after the input.

Sometimes the cultures respond for a imposed input with a diauxic growth. The interpretation of the response culture could be difficult due to the presence of a competitive inhibitor of substrate taken up, or any nutrient limitation immediately after the abrupt elevated concentration of one specific nutrient in the pulse.

The chemostatic pulse and medium-shift technique are simple and efficient experimental tools to aid optimization of the microorganism growth conditions (Goldberg & Er-el, 1981).

Research has been done developing synthetic medium for some microorganisms by analysis of culture response to shifts in nutrient concentrations. Martin et al. (1992) using the pulse and medium-shift technique have replaced yeast extract and tryptone with a mixture of aminoacids and vitamins in the cultivation of *B. stereothermophilus*.

Nagai et al. (1968) have investigated the metabolic activity of growth and enzyme synthesis in a chemostatic culture of *Azotobacter vinelandii* by a glucose pulse technique. These authors noticed that under glucose limitation protein fraction decreased exponentially after the glucose input.

On the other hand, Harvey (1970) has shown that protein concentration increased when a chemostatic culture of *E.coli* was subjected to a pulse with glucose in a limited-glucose culture.

1.5.6 Continuous lipase production

There is little published information about continuous lipase production.

Dean (1972) has reported that lipase activity from *Anaerovibrio lipolytica* reached two maxima at dilution rates of 0.08 and 0.135 h^{-1} under carbon-limitation.

Good results were obtained by Jane Gilbert et al. (1991a) growing *Ps. aeruginosa* at low dilution rate in the presence of Tween-80 as limiting carbon. Under these conditions the culture showed approximately five-fold more lipase activity than in batch experiment.

In addition, Person et al. (1990) have investigated lipase production by *Ps. fluorescens 378* in the presence of different nutrient limitations under continuous culture.

Research has been done with some *Candida* strains, mainly *C. utilis* in continuous culture under carbon limitation, aiming only single cell protein, SCP (Ricci & Tell, 1988; Maugeri-Filho, 1988; Nunez & Callieri, 1989; Lucca et al., 1991).

Further investigation ought to be made into continuous lipase production considering the potencial advantages of continuous culture in microbial enzyme synthesis.

1.6 TRANSIENT STATE CULTURE

1.6.1 General features

Transient state cultures operate under imposed environmental conditions that can change periodically or continuously over a long time. The stimulus must be carefully defined for either chemical or physical parameters. The most frequently used are changes in substrate concentration, rate of supply, nature of substrate, as well as changes in temperature which is the physical parameter most common due to its importance in process optimization (Heitzer et al., 1990).

Improvement in yield and selectivity of intermediate products in chemical reactors at unsteady-state operations have been reported by several authors and reviewed by Douglas (1972) and Bailey (1973). Sometimes the periodic operation is superior to the optimum steady-state system respectively (Pickett et al., 1979b).

Growing microorganisms in dynamic environments, the production of secondary metabolites including enzymes has been shown as an advantageous process (Pickett et al., 1979a). However, little has been reported on the effect of this type of growth on the metabolism of organisms. In addition, oscillation culture "deserves further investigation in order to prove its technological

potential for attaining higher productivity in enzyme production" (Martini et al., 1989).

Unfortunately, some research about transient operation technique concerns unidentified cultures and/or undefined medium generating results that cannot be interpreted or generalized (Borzani et al., 1976; Vairo et al., 1977).

In nature the microorganisms are able to adapt continuously to varying conditions. For example, microorganisms are exposed to oxygen during the daytime and anaerobic conditions at night. In addition, hypersaline and marine environments are characterized by step gradients of oxygen and sulphide. Then, the study of microbial ecosystem behaviour throughout the transient operation is of great ecological interest (Wit & Gemerden, 1987).

The processes for wastewater treatment and water renovation occur at unbalanced growth in biological industrial reactors. Considering ecological problems of effluents with flow rate and composition extremely variables, research has been stimulated in this dynamic operation area (DeLorme & Kapuscinski, 1990).

Large-scale industrial culture of microorganisms results in the formation of gradients in environmental parameters throughout the vessel, imposing transient states due to insufficient mixing and mass

transfer. The feed medium is introduced at high concentration often at only one point while the medium might be exhausted in other parts.

There are few papers recording applications of transient cultures; the production of penicillin and chephalosporin C were carried out in repeated fed-batch cultures with high values of productivities for the two different antibiotics (Pirt, 1974; Pickett et al.,1979b).

In industrial scale the application of dynamic culture is well known in the production of compressed and active dry yeast by fed-batch culture.

New fed-batch control systems based on different strategies for regulating the feed have been developed for baker's yeast (Porro et al., 1991) and for heterologous protein production (Lee & Parulekar, 1993).

1.6.2 Fed-batch-type culture

Fed-batch-type culture, i.e. repeated fed-batch, operates as a semi-continuous process where a complete medium feed continuously a batch culture while the same volume is pumped out at preset time intervals. Thus, the biomass has unbalanced growth.

As the volume increases over the operation, this mode of culture faces certain barriers in industrial scale. On the other point of view, filamentous fungi and

plant cells can be easily cultivated under this operation. These cultures under chemostatic techniques have confronted some problems due to the difficulty in maintaining constant volumes (Goldberg & Er-el, 1981).

Some fed-batch operations can present different feeding strategies. They have been termed "semibatch" (Yamane & Tsukano, 1977) and "extended" (Edwards et al., 1970).

One of the advantages of repeated fed-batch is the improvement in yield of up to 160 % concerning the production of intermediate compounds (Pickett et al., 1980).

Fed-batch culture increases some enzyme production. For example, the production of ß - galactosidase by *B. subtilis* is a process fed by a controlled glucose addition enhancing the specific enzyme expression (Park et al., 1992).

In addition, the extracellular protease produced by *Streptomyces C5-A13* in fed-batch process has exhibited 9-fold greater activity than the enzyme produced under simple batch cultivation (Gibb et al., 1989).

Fed-batch cultures have been applied to growing microorganisms which metabolism is inhibited for some nutrient (Yasouri & Foster, 1992; Park et al., 1992).

One important aspect for lipase production throughout this technique is that glucose could be added gradually during the process, avoiding the repression of lipase production noticed by some authors (Boing, 1982; Nahas, 1988; Baillargeon et al., 1989).

Ishirara et al. (1989) have reported that lipase from *Ps. fluorescens* can be effective produced by semi-batch culture with turbidity-dependent feeding of olive oil and Fe^{++} ions.

Moreover, extracellular esterase production by a genetically engineered strain of *B. brevis* under fed-batch culture (with single and multi-substrate feedings) in presence of glucose, reached eight times higher than in batch mode (Tulin et al., 1992).

As batch culture has been the most common biochemical engineering operation in industrial processes and fed-batch can be easily adapted with modifications which do not involve expensive costs, this mode of transient culture could have large application considering the advantages of this oscillation process.

1.6.3 Continuous transient culture

In continuous transient culture the oscillation of any chemical or physical parameter at different cycle

times or different cycle amplitudes should occur after a steady-state condition.

Comparing a chemical transient reactor with a periodic biological reactor, the latter has a short life cycle excluding systems with immobilized enzymes. A process life cycle is the long-term operation of a reactor from start-up until regeneration, including the operation and the shutdown.

In accordance with Bailey (1973) the dynamic process can be classified in four ways one of which is the process life cycle, while the intrinsic perturbed process was classified in three different classes (Figure 1.6.3). Relaxed steady-state is the operation where the system is subjected to rapid cycling while the reactor output response becomes invariant after a time. Quasi-steady periodic operation occurs when the system yields a steady-state relationship with the input at any time; this operation is considered under time-invariant conditions. Intermediate periodic operation deals with a system where the response time is of the same order as the imposed functions cycle time.

In general, biological reactors and natural oscillations are classified as intermediate periodic operations whose class lies between the extremes of quasi-steady and relaxed operation (Pickett et al., 1979b).

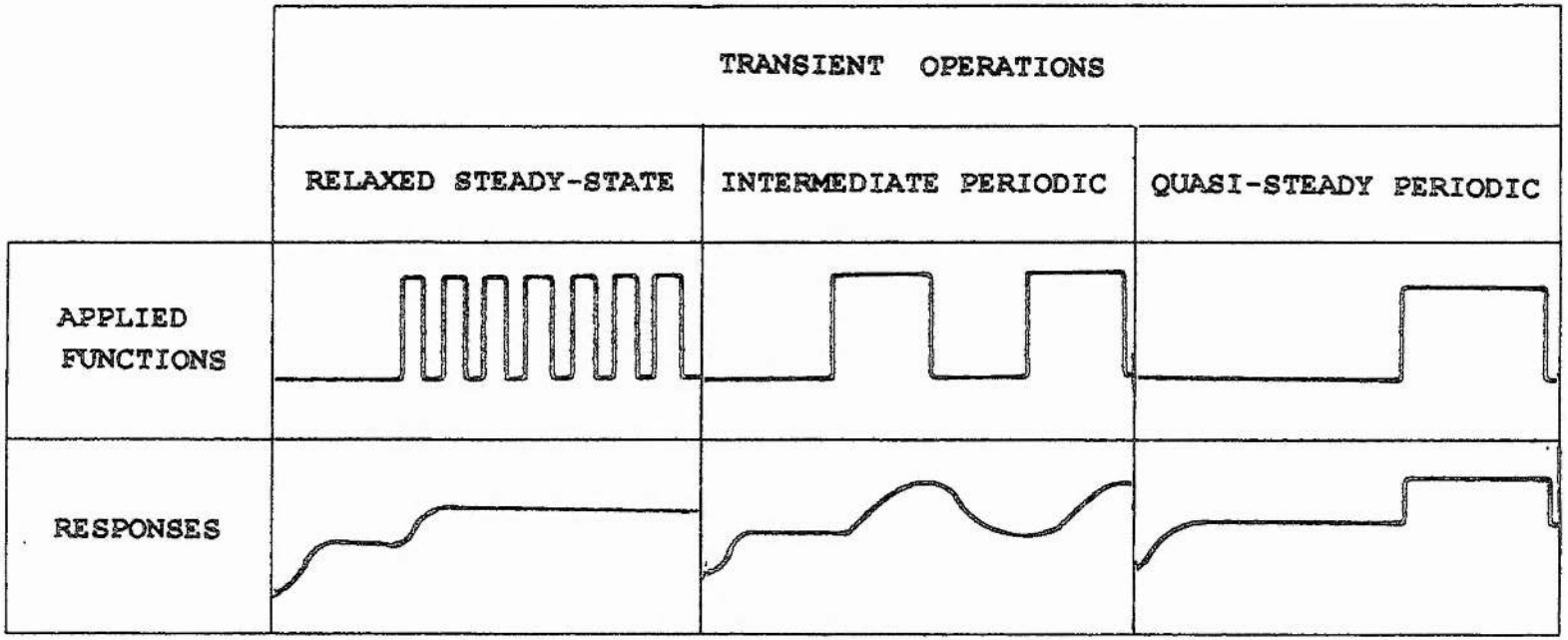


FIGURE 1.6.3 CLASSES OF CONTINUOUS TRANSIENT OPERATION TECHNIQUE WITH SCHEMATIC REPRESENTATIONS OF THREE DYNAMIC OSCILLATIONS AND THEIR RESPECTIVE OUTPUT RESPONSES

The comprehension of transient phenomena was developed with the hypothesis that the ribonucleic acid (RNA) is the macromolecule which exerts predominant control over transient responses in the cells. However, some results have been shown that, even though RNA is responsible for the protein synthesis, this nucleic acid could be disconnected with cellular constituents such as protein and polysaccharide (Nagai et al.,1968; Barford et al., 1982; Daigger & Leslie Grady, 1992).

The growth activity of microorganisms at unbalanced growth depends on the hierarchy of cell control that establishes the process priority concerning RNA and DNA synthesis, as well as protein synthesis and nuclear and cell division (Barford et al., 1982).

Daigger & Leslie Grady (1992) have reported that only some microbial cultures are indeed RNA-limited. They have also shown that the nature of a transient response depends upon the specific growth rate at which the culture was growing prior to the transient.

When a culture is transferred from one environment to another, lag times have been noticed between stimulus and organism response (Pickett et al., 1979a and 1980; Barford et al., 1982; Heitzer et al., 1990). Pickett et al. (1980) have reported that 'lag phases' were independent of cycle amplitude over most of the range examined of *Escherichia coli* chemostatic cultures subjected to square wave oscillations.

In transient state greater protein yield has been reported, reflecting an increase in metabolic activities of the cells. For example, growing *E. coli* under continuous culture with square wave variations in glucose supply at high cycle amplitudes, the protein concentration increased linearly up to 4.0 g/l (Pickett et al., 1979a).

Douglas (1972) has reported that large input variations of square wave function gives better time average performance concerning protein production than other oscillations.

Martini et al. (1989) have reported significant improvements in the production of specific ß - galactosidase activity at transient operation technique, subjecting the culture to square waves in the incoming limiting substrate concentration at 90 and 180 min cycle times.

Increase in protein content has also been obtained under unsteady-state culture of *Oscillatoria brevis* subjected to the transition from nitrogen to light limited growth (Naes & Post, 1988).

In addition, Vrana & Sobotka (1989) have obtained a higher degree of culture synchronizations in *C. utilis* transient culture subjected to the interuption of the air supply, while Minkevich et al. (1990) have reported partial cell synchrony under periodic change of limiting nutrients.

When the transient operation technique is analysed for engineering significance, mathematical modelling has been proposed (Nagai et al., 1968; Pirt, 1975; Barford et al., 1982; Pickett, 1982; Minkevich et al., 1990). The outcome of the experimental research expressed by models and the definition of the physiological state might enable the simulation of the process and consequently, the elucidation of the time response of a specific metabolism.

The investigation of microorganisms in dynamic environments attempts to detect and to understand metabolic pathways, as well as to optimize the process and to obtain important ecological information.

1.7 *Candida lipolytica* AS LIPASE PRODUCER

Bacteria, yeast and fungi have been grown in submerged and stationary cultures in different types of fermentations producing lipase amongst other important commercial enzymes (Brockman, 1984).

Over the past few years screenings of lipase producers have been the subject of many investigations (Sztajer & Maliszewska, 1988; Chakrabarti et al., 1990; Silva et al., 1991; Chen et al., 1992; Petruccioli & Federici, 1992; Katz et al., 1993; Kaur et al., 1993; Sigurgisladottir et al., 1993). Thus, different lipase

activities have been discovered in a wide variety of microorganisms. However, lipase production is subject to commercial secrecy and little is known about optimum culture conditions.

The great potential of yeasts as a major industrial organism is well known, as well as their high resistance to vigorous agitation.

The most important lipases from yeasts have been reviewed by Hadeball (1991) where *Candida, Saccharomyces, Sacharomycopsis, Torulopsis* and *Yarrowia* are the typical yeast lipase producing genera.

Moreover, considering high lipolytic activities produced by some *Candida* strains (Falk et al., 1991), this genus was choosen for lipase producer in this work.

Important aspects of yeast taxonomy have been discussed involving *C. lipolytica, S. lipolytica, C. deformans, C. paralipolytica* and the recently known *Yarrowia lipolytica* (Ota et al., 1978; Hadeball, 1991). Hadeball in his review suggested that the strains belong to one genus.

Under other point of view there are large amounts of waste waters from oil refinary plants and from food industries as potential lipid substrate available whose carbon sources could be superior to carbohydrates for fungal growth (Spencer & Spencer, 1990).

Some *Candida* species can metabolise cheap carbon sources, in particular non-carbohydrate substrates as a carbon and energy source.

For example, *C. lipolytica* can grow on waste product from manufacture of raw material for nylon-6, removing a hazardous waste (Cartledge, 1987), on by-products of fish meal (Rydin et al., 1990), as well as on oil distillates (Dostalek & Munk, 1969).

In addition, the basic requirements for growth of *S. lipolytica* on fatty wastes and on animal fats have also been reported (Tan & Gill, 1985a). As these raw materials are abundant and inexpensive the future for lipase fermentation industries looks promissing.

Although *C. lipolytica* has been identified as one of the most important yeasts to produce lipase (Roblain et al., 1989; Falk et al., 1991), only a few published papers deal with this microorganism's growth (Tan & Gill, 1984 and 1985a) and its lipase production (Kalle et al., 1972; Jonsson & Snygb, 1974; Jonsson 1976, Kosaric et al., 1979).

Walsh et al. (1989) have reported the mild pathogenicity to human, caused by certain strains of *C. lipolytica*. This is due to the presence of a foreign body that caused fungemia in all cases of infection or colonization involving this microorganism.

Nevertheless, for future applications in manufacturing processes of microbial food enzymes, safety is essential. The microorganism should therefore be Generally Regarded As Safe (GRAS) while the production needs to follow the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and Food Chemicals Codex (FCC) (Jensen & Eigtved, 1990).

C. lipolytica 1055 was chosen as a promising lipase-producing Brazilian strain. Before commercial use of this strain, however, its pathogenicity would have to be checked.

1.8 AIMS

The general purposes of this project are to acquire theoretical knowledge and practical ability in different operational strategies to obtain lipase production.

In order to identify the maximum extracellular lipase yield by *C. lipolytica 1055* I shall be using the following techniques:

(i) Batch culture;

(ii) Fed-batch culture;

(iii) Chemostatic culture including delta pulsing;

(iv) Continuous transient culture subjected to different oscillations varying frequency and amplitude.

2 MATERIAL AND METHODS

2.1 MICROORGANISM

C. lipolytica 1055 was obtained from the Mycology Department of the UFPE, Recife, Brazil.

The morphological properties of this yeast include cylindrical vegetative cell shape and true hyphae and pseudomycelium.

In glucose agar media the colonies were white to cream colour and wrinkled after 72 h at 30°C probably due to the high lipid content in their cells (Davenport, 1980).

The purity of the culture was checked by simple staining (methylene blue) of microscopic examinations.

2.2 MAINTENANCE OF THE STRAIN

All microbiological media were prepared with distilled water and sterilised by autoclaving at 120°C.

The composition of the maintenance medium was:

glucose	20 g/l
$(NH_4)_2\ SO_4$	4 g/l
yeast extract	2 g/l
agar	12 g/l
pH 6.0.	

The microorganism was subcultured from the original strain in agar slant tubes with the maintenance medium, incubated at 30°C for 72 h and subsequently stored at -15°C.

From one freeze slant culture *C. lipolytica 1055* was subcultured in the same medium and incubated at 30°C for 24 h before starting each experiment.

2.3 BATCH CULTURE MEDIA

For batch growth (including continuous culture start-up) the medium composition for glucose as carbon source was:

glucose	20.0 or 2.0 g/l
yeast extract	5.0 or 0.5 g/l
antifoam A	0.1 ml/l
pH 6.0.	

The antifoam A used throughout the experiments is a 30 % aqueous emulsion of antifoam A concentrated that contains 100 % of active silicone polymer and non-ionic emulsifier (Sigma Chemical Company Ltd.).

In the investigation of antifoams the following antifoams were tested (as well as antifoam A) at different concentrations (0.05, 0.1, 0.2 and 0.5 ml/l):

(i) Antifoam RD Emulsion, a silicone product (Dow Corning);

(ii) Antifoam 289 Mixed containing silicone and non-silicone defoams (Sigma);

(iii) Antifoam 204 Organic, a mixture of non-silicone defoams in a polyol dispersion (Sigma).

The effect of various carbon sources was tested in the following medium:

liquid carbon source	4.0 ml/l[1]
yeast extract	1.0 g/l
Na_2 H P O_4	2.0 g/l
stock salt solution	10.0 ml/l
antifoam A	0.1 ml/l
pH 7.0 - 8.0.	

[1]The carbon sources were measured in volume because of lack of specific density information of some commercial oils

Triton X-100, Tween-80 (polyoxyethylene sorbitan monooleate), oleic acid, triolein, olive oil, palm oil, sunflower oil, corn oil and cod liver oil were tested as carbon source. The oils were emulsified with gum arabic at 1 g/l.

The stock solution of salts was made according to Rosenberger & Elsden (1960) excluding Fe^{++} and Cu^{++} ions. The composition per litre of this stock solution is specified below:

Mg O	10.0 g
$Ca\ CO_3$	2.0 g
$Zn\ SO_4 . 7\ H_2O$	1.44 g
$Mn\ SO_4 . 4\ H_2O$	1.11 g
$Co\ Cl_2 . 6\ H_2O$	0.179 g
$H_3\ BO_3$	0.062 g.

50 ml concentrated HCl was added to approximately 200 ml distilled water, in order to dissolve the Mg and Ca compounds in that order followed by the others components.

This solution was sterilized separately, cooled and then added to the basal medium thus avoiding precipitation problems.

When the effect of nitrogen sources on the lipase and esterase activities was studied, 4 g/l Tween-80 was the carbon source and all the other components remained as specified except that yeast extract was

replaced by peptone, ammonium sulphate, urea and potassium nitrate with double concentration.

2.4 CONTINUOUS CULTURE MEDIA

When glucose was the carbon source the medium composition in continuous experiments was the same as in batch experiments with this carbohydrate.

For carbon limiting experiments with non-carbohydrate carbon sources the microbiological media were as follows:

(i) Tween - 80 4.0 g/l
$(NH_4)_2 SO_4$ 2.0 g/l
$Na_2 H PO_4$ 2.0 g/l
stock salt solution 10.0 ml/l
antifoam A 0.1 ml/l
pH 7.2.

(ii) olive oil 3.2 g/l
yeast extract 0.5 g/l
antifoam A 0.1 ml/l
pH 7.5.

The olive oil (Carbonell & Co. de Cordoba S.A., Spain) was sterilized separately and pumped to the reactor at constant flow rates. The limiting olive oil concentration was calculated considering the medium and olive oil flow rates.

2.5 INOCULUM

From an agar slant subcultured for 24 h the cells were grown in 125 ml Erlenmeyer flasks which ones contained the same medium that used for the final growth. These cultures were incubated at 30°C in an orbital shaker until the late exponential growth phase.

Each inoculum was added in amount corresponding to 10 % of the medium.

Experiments were carried out aiming at:

(i) inoculum standardization;

(ii) investigation of inoculum size in microorganism growth and lipase and esterase activities;

iii) investigation of inoculum age in microorganism growth and lipase and esterase activities.

Concerning the important aspect of lag phase shortness, large inoculum in fresh exponential phase was used. Before start the cultivation processes the levels of esterase activity and biomass concentration were checked.

2.6 MICROBIAL GROWTH SYSTEMS

2.6.1 Batch experiments

Batch experiments for the study of antifoam effect and carbon and nitrogen source investigations were carried out in 1 000 ml flasks (working volume 600 ml) with a very strong magnetic agitation in a controlled water bath at 30°C.

Under aseptic conditions samples were removed, centrifugated at 10 000 x g for 15 min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge. The supernatants were incubated in ice until enzymes were assayed and then stored at -15°C for further analysis.

In all batch investigations at least two sets of independent experiments were carried.

2.6.2 Fed-batch experiments

Fed-batch experiments were carried out by pulse procedures at the end of exponential phase in batch cultures.

In the experiments with Tween-80 as carbon source, sterilized oleic acid was aseptically added into the fermenter, providing the system with a sudden change in the amount of this fatty acid.

The same procedure was applied when olive oil was the carbon source. Single and multi-perturbed olive oil inputs were carried out in the presence of double nitrogen source concentration.

2.6.3 Chemostatic experiments

All continuous cultivations were carried out in 1 litre bench top-fermenter (F. T. Scientific, model Biolab) with a stainless-steel top-plate and working volume 620 ml (Figure 2.6.3).

These experiments were preceded by batch growth and the medium flow was started after the culture achieved the exponential phase.

The temperature was automatically controlled at 30°C by a sensor (PT 100 Resistance Thermometer RS 344-625) linked to a heater. The temperature was also checked by a thermometer.

Six flat-blade disk impellers were used for mixing the culture at constant agitation (500 rpm).

In order to avoid oxygen limitation air filtered to 0.2 μm membrane was introduced through a sparger at the bottom of the fermenter at constant air flow rate of 600 cm^3 / min, approximately equal to 1 vvm, i.e. 1 volume of air per volume of medium per minute.

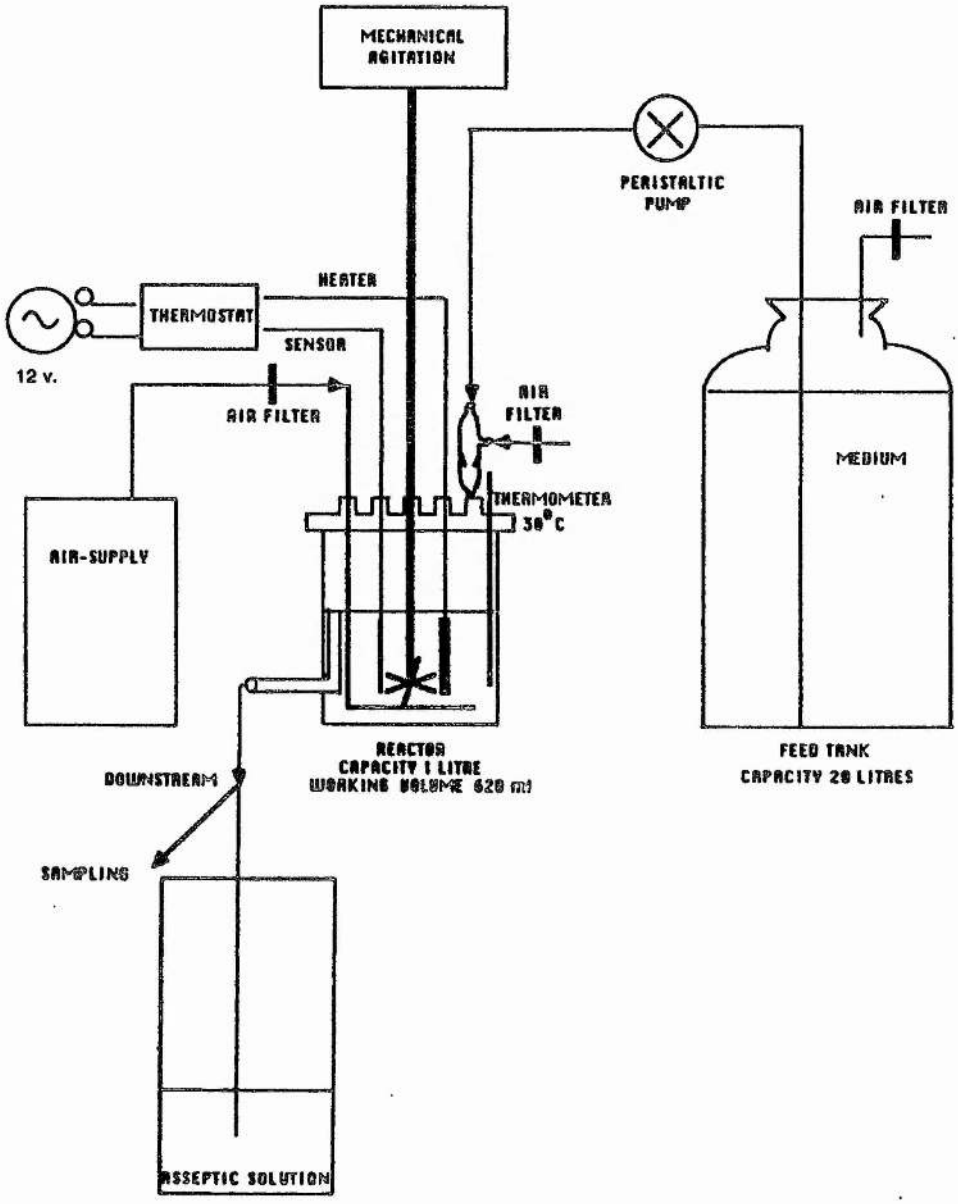


FIGURE 2.6.3 EXPERIMENTAL SET UP FOR CHEMOSTATIC CULTURE OF *C. lipolytica 1055*

Medium feed was carried out at a constant flow rate by peristaltic pump (Watson-Marlow 101 U) while the product was harvested at the same rate by an overflow pipe inserted through a side arm in the fermenter. Due to some aerosol problem the feed medium was carried out through a connection at the top of the fermenter feeding the culture drop by drop.

In order to prevent contamination in the continuous culture the outflow tube was dipped into an antimicrobial disinfectant (2 % Hycolin - William Pearson Ltd.) and the culture was periodically checked by microscopic examinations.

From the washout the flow rate was measured by the time to fill a volumetric flask with culture. As steady-state was assumed after the culture reached at least 10 generations, the Table 2.6.3 shows some parameters for chemostatic experiments.

The generation times (or doubling times) were calculated through the equation:

$t_d = \ln 2 / \mu$ where $\mu = D$ (Pirt, 1975).

During the steady-state the sampling was made asseptically from time to time at pertinent intervals to determine the biomass concentration, the residual carbon source and the enzyme activities.

DILUTION RATE (h^{-1})	GENERATION TIME (h)	STEADY-STATE* (days)
0.02	35.0	14.0
0.04	17.0	7.0
0.06	11.6	5.0
0.08	8.7	3.6
0.10	7.0	3.0
0.12	6.0	2.4
0.16	4.3	1.8
0.20	3.5	1.4
0.24	2.9	1.2
0.36	1.9	0.8
0.48	1.4	0.6
0.57	1.2	0.5

* Minimum time necessary for the culture to reach steady-state conditions (10 generation times).

TABLE 2.6.3 PARAMETERS FOR CHEMOSTATIC CULTURE EXPERIMENTS

2.6.4 Continuous transient experiments

In all experiments of transient operation technique, the data at zero cycle times were obtained from a steady-state culture fed with the basic medium continuously.

In the presence of Tween-80-limited culture, experiments were carried out subjecting the *C. lipolytica 1055* system to oleic acid square wave oscillations with different amplitudes and cycle times of 8 h at a fixed value of dilution rate.

Olive oil square wave oscillations were carried out during a specific period of 8 h in the presence of olive oil-limited cultures. The system was studied at a dilution rate of 0.09 h^{-1}.

Oscillations were achieved by connecting the reactor to two nutrient reservoirs (Figure 2.6.4-a), one containing the basic medium (feed tank A) and another small one with only sterile oleic acid / olive oil (feed tank B).

At pre-established time intervals, a timer system switched the feed tank containing the oleic acid / olive oil while the basic medium was constantly pumped (Figure 2.6.4-b).

For each set of conditions, samples were collected at regular intervals during the course of a

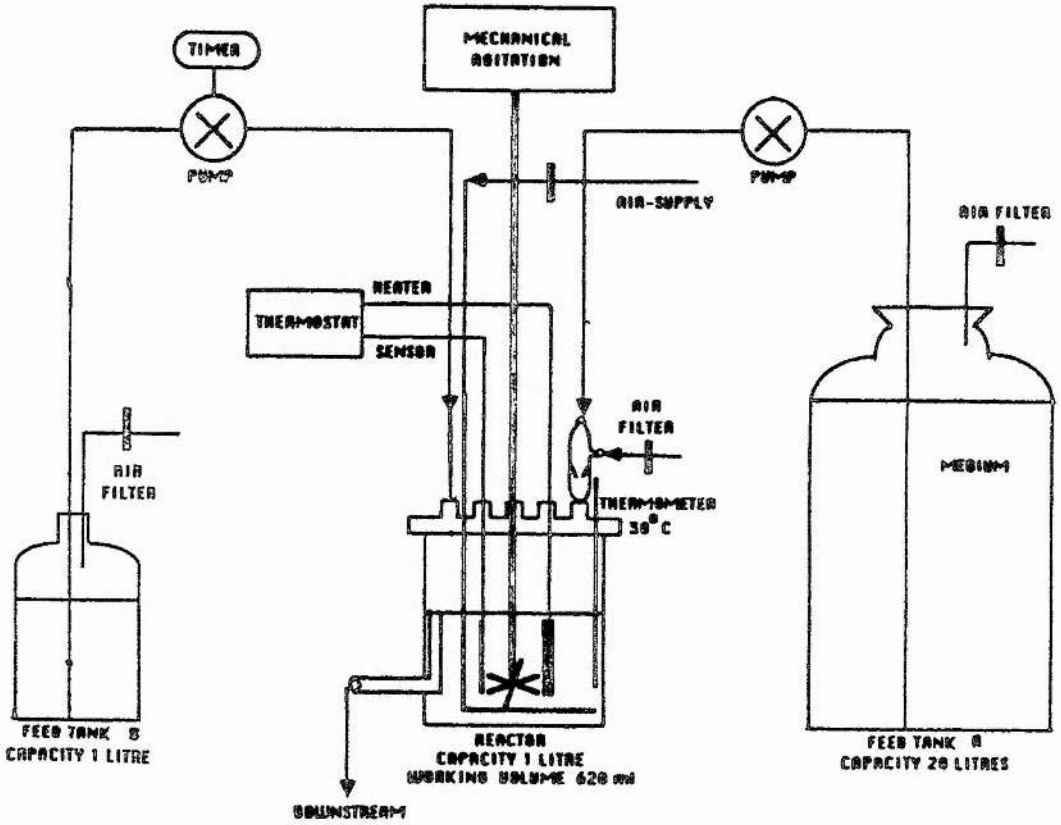


FIGURE 2.6.4-a EXPERIMENTAL SET UP FOR *C. lipolytica 1055* GROWTH UNDER CONTINUOUS PERIODIC OPERATION

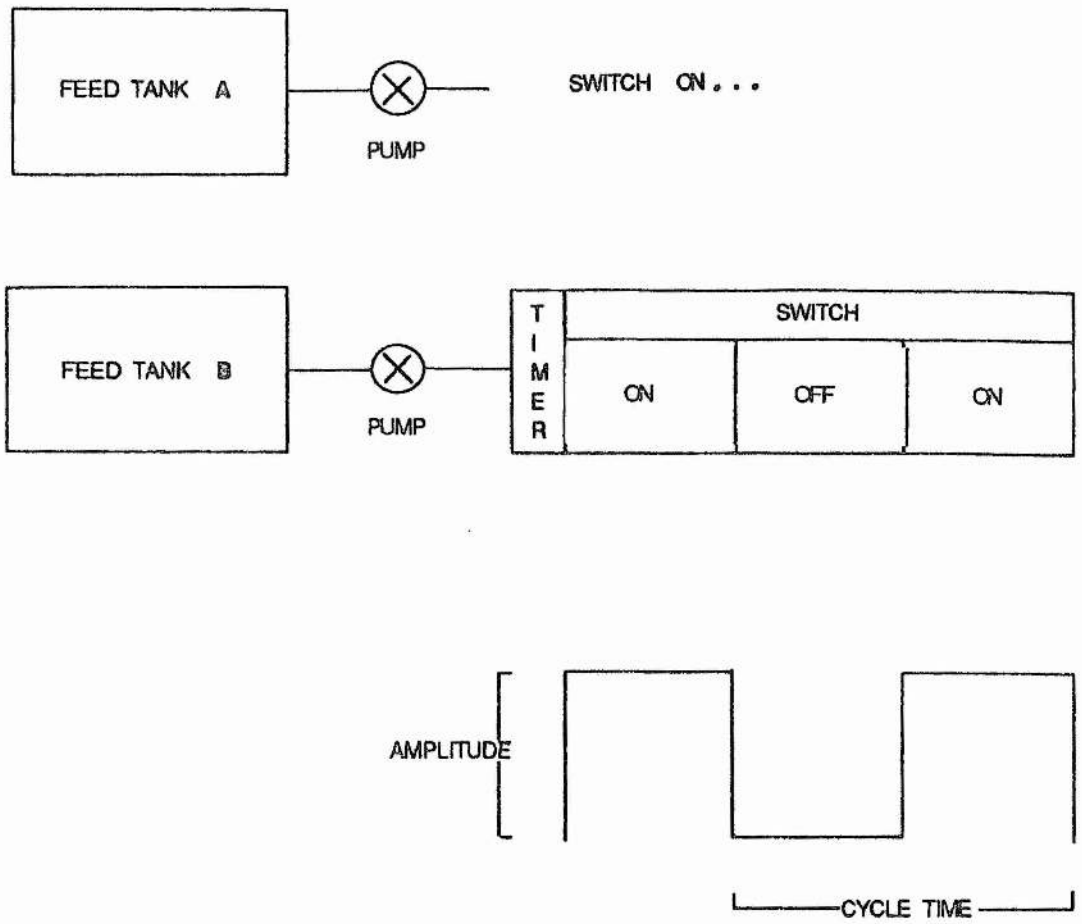


FIGURE 2.6.4-b SCHEMATIC REPRESENTATION OF THE PUMP-TIMER SYSTEM IN A CONTINUOUS PERIODIC OPERATION TECHNIQUE SUBJECTED TO SQUARE-WAVE INPUTS

nutrient cycle in order to analyse the intracycle behaviour, and continuously after regular and repetitive variations.

2.7 DELTA PULSE PROCEDURE

Delta pulsing was performed at a fixed dilution rate under steady-state chemostatic culture.

All the inputs were carried out under asseptic conditions introducing a volume less than 2 % of the working volume (approximately 10 ml).

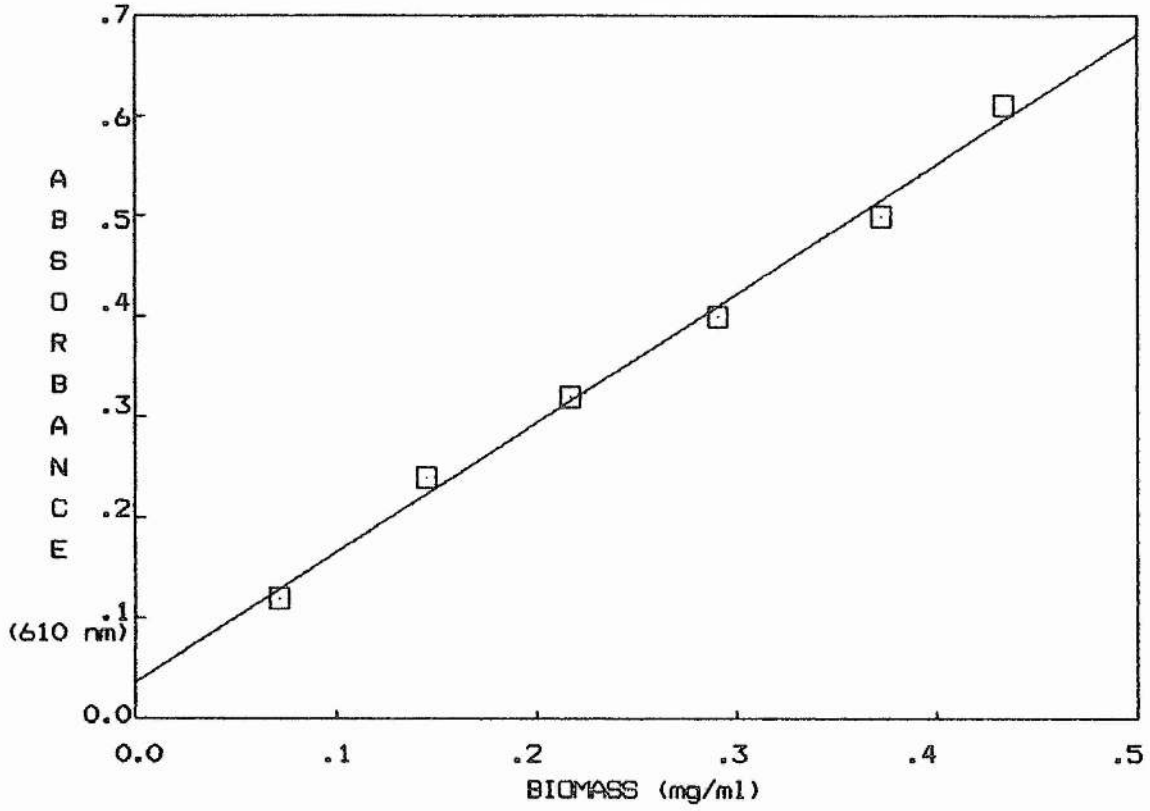
Some pulsing experiments were carried out involving the idea to check whether the carbon source / another nutrient was or not the growth-limiting factor in a continuous culture.

2.8 BIOMASS MEASUREMENT

Biomass concentration was estimated through absorbance at 610 nm with a PYE SP 600 series 2 Spectrophotometer (Unicam Instruments).

A Calibration Plot (Figure 2.8) was constructed from dry weight (100°C) samples of *C. lipolytica 1055* grown under batch conditions.

In all experiments with oils and oleic acid the biomass concentration was determined by filtration of 5-



Slope	1.289207
Y intercept	3.625214E-02
Correlation coefficient	0.9970
Standard error	1.530338E-02

FIGURE 2.8 CALIBRATION PLOT FOR BIOMASS CONCENTRATION MEASUREMENTS WITH ITS LINEAR REGRESSION

10 ml culture through a 0.45 µm nylon membrane filter - Nylaflo from Gelmon. The filters were washed with a mixture of dioxan-propionic acid (1:1) and distilled water. Then, they were dried at 85°C to constant weight.

2.9 GLUCOSE MEASUREMENT

Glucose concentration was determined as a reducing sugar by SOMOGYI-NELSON method (Nelson, 1944; Somogyi, 1945).

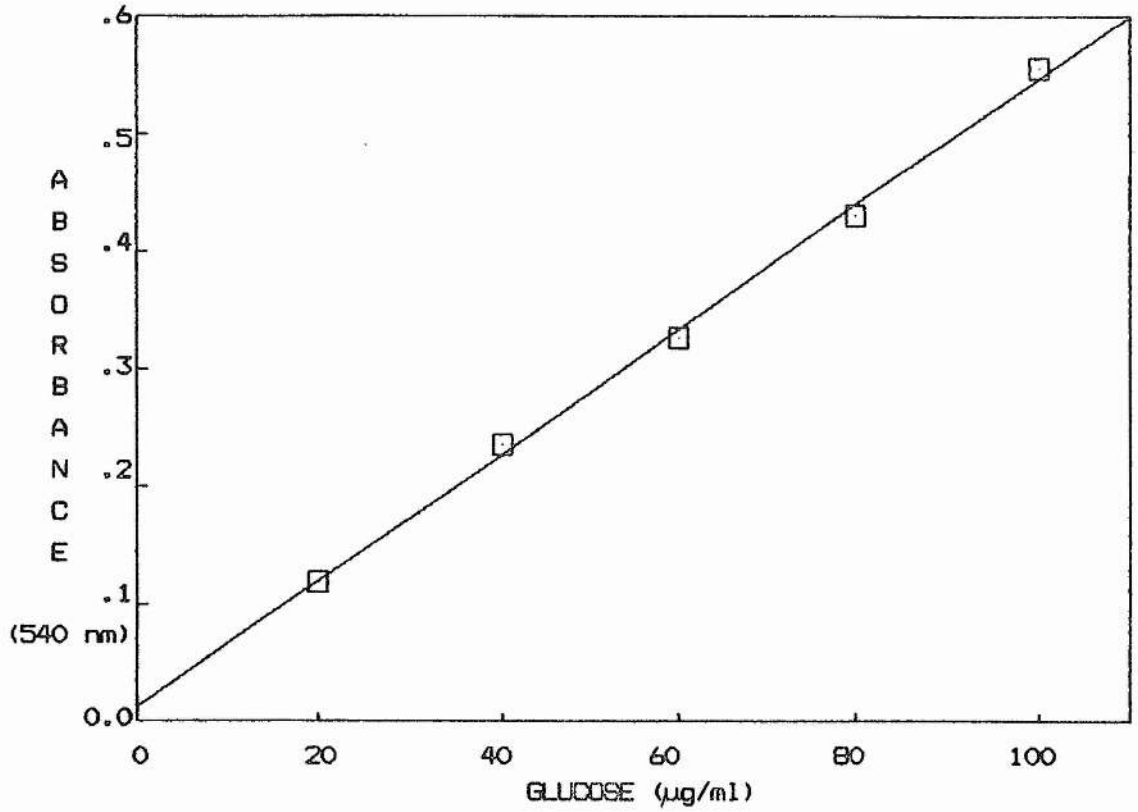
The Calibration Plot (Fgure 2.9-a) was constructed using different concentrations of a standard glucose solution against absorbance readings at 540 nm.

In all continuous culture experiments with glucose as carbon source, glucose-oxidase enzymatic method (Sigma) was carried out as procedure for the estimation of glucose due the fact that Somogyi-Nelson method is not specific for glucose.

The Calibration Plot for glucose determination by glucose-oxidase method is shown in the Figure 2.9-b.

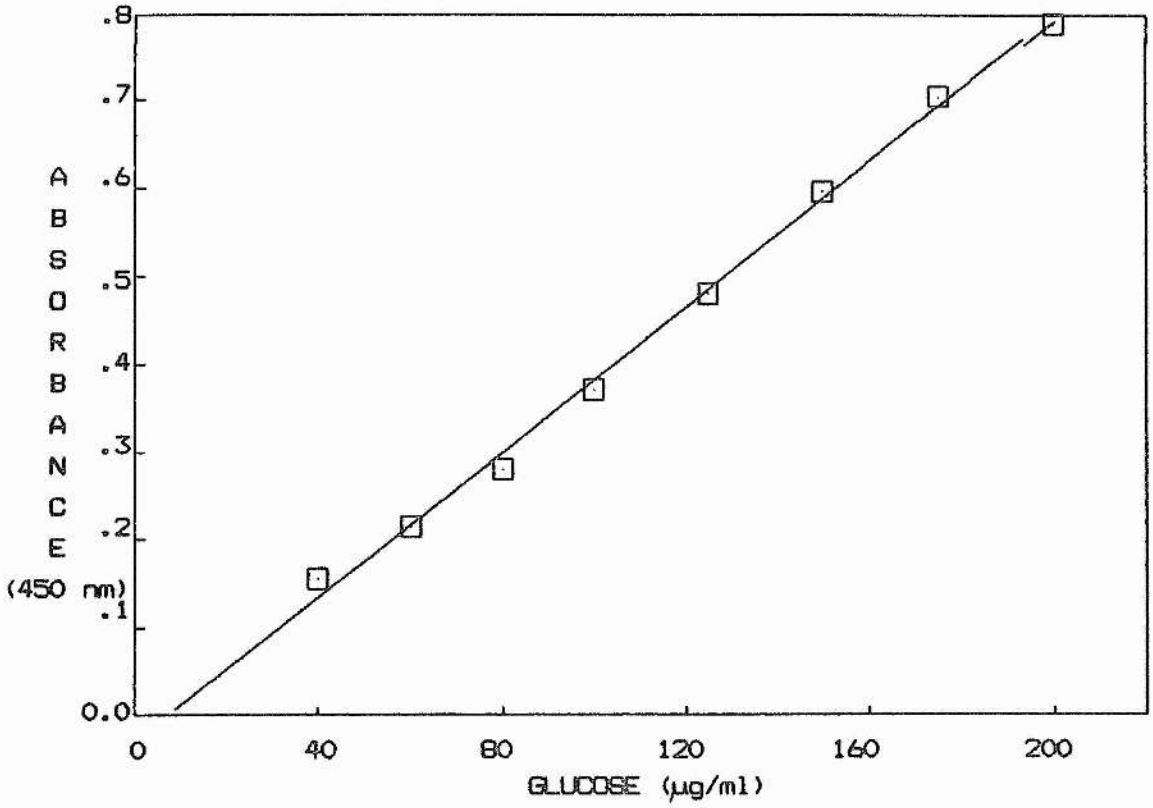
2.10 PROTEIN MEASUREMENT

Protein concentration was assayed using the Coomassie Brilliant Blue G 250 Method according to Bradford (1976). Utilising the principles of protein-dye



Slope	5.335003E-03
Y intercept	1.289983E-02
Correlation coefficient	0.9987
Standard error	9.88433E-03

FIGURE 2.9-a CALIBRATION PLOT FOR GLUCOSE CONCENTRATION MEASUREMENTS WITH ITS LINEAR REGRESSION BY SOMOGYI-NELSON METHOD



Slope	4.11439E-03
Y intercept	-3.017282E-02
Correlation coefficient	0.9985
Standard error	.0135763

FIGURE 2.9-b CALIBRATION PLOT FOR GLUCOSE CONCENTRATION MEASUREMENTS WITH ITS LINEAR REGRESSION BY GLUCOSE-OXIDASE METHOD

binding, this rapid method is very sensitive for the measurement of microgram quantities of protein.

As the reagent is unstable, every two weeks it was refiltered and a new calibration plot constructed (Figure 2.10) using a standard solution of bovine serum albumin (BSA).

2.11 OLEIC ACID MEASUREMENT

The determination of oleic acid was carried out by gas liquid chromatography (GLC) following methylation, being used reagents with highest grade of purity available (HPLC).

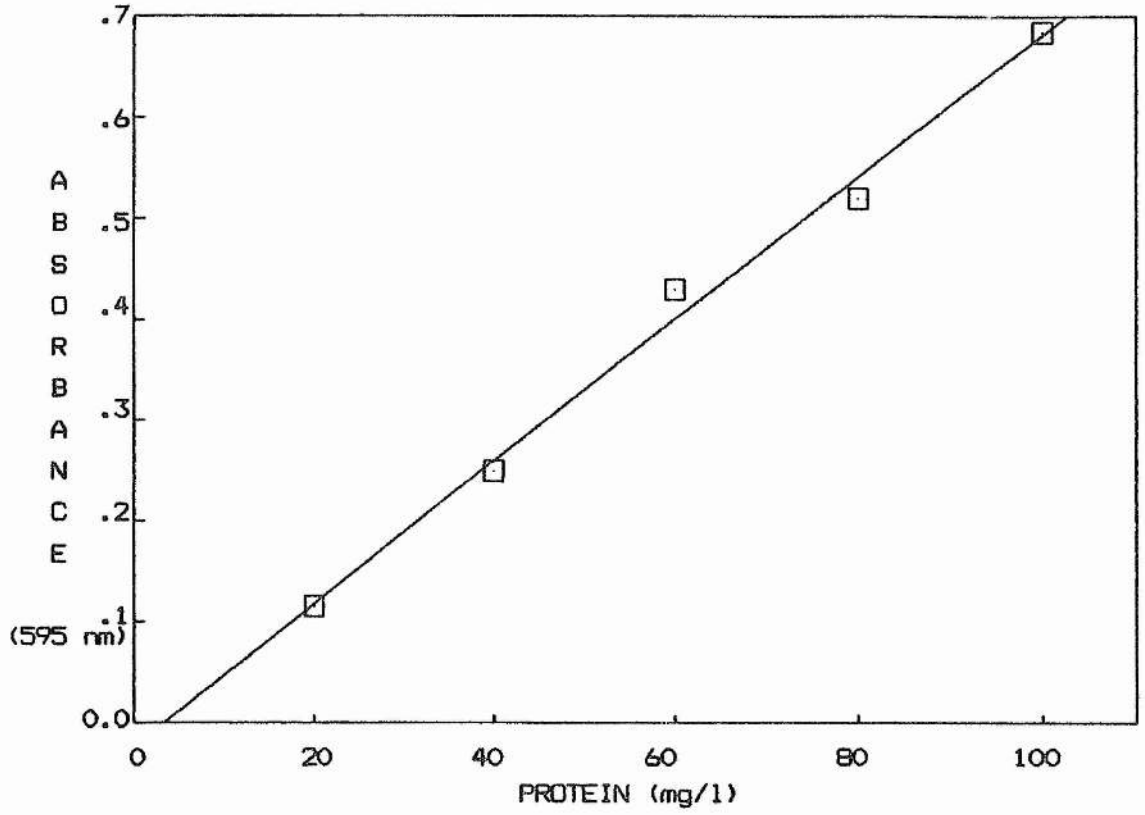
2.11.1 Methylation process

In accordance with the below reaction the free fatty acids were esterified by heating with a solution of 1 % (v/v) concentrated sulphuric acid in methanol (Christie, 1989):

$$RCOOH + CH_3OH \xrightarrow{H_2SO_4} RCOOCH_3 + H_2O.$$

Procedure:

- Sample (3 - 5 ml of culture supernatant) was dried (Freeze Drier) and dissolved in toluene (1 ml toluene per 50 mg lipid);



Slope	7.049999E-03
Y intercept	-2.299995E-02
Correlation coefficient	0.9964
Standard error	2.190892E-02

FIGURE 2.10 CALIBRATION PLOT FOR PROTEIN CONCENTRATION MEASUREMENTS WITH ITS LINEAR REGRESSION

- The reagent (2 ml) was added and the reaction was carried out at temperature below reflux for 2 h;

- In order to avoid the formation of any emulsion, 5 ml of a saturated solution of sodium chloride was added and the methyl esters were extracted twice with 5 ml hexane using Pasteur pipettes to separate the layers;

- Samples were dried over anhydrous sodium sulphate.

If necessary:

- Solvent was evaporated (5 ml) under reduced pressure in a rotary film evaporator and the residue was redissolved in 0.5 ml hexane; or,

- Sample was diluted with hexane.

2.11.2 Gas Liquid Chromatography

Methylated products were separated by gas liquid chromatography in a Phillips GLC apparatus (PYE Series 104) at 195°C isothermally on a packed column (EG SS - X 20 % on Chromosorb wax, 100-120 mesh from Jones Chromatography) with nitrogen at flow rate of 20 ml/sec as the carrier gas.

The eluted compounds were identified by comparing the retention times with a standard mixture of methyl esters with 18 carbons (Sigma). Methyl oleate was

quantified using heptadecanoic methyl ester as internal standard.

A Calibration Plot was constructed plotting the ratios of peak height between methyl oleate and methyl heptadecanoate against the equivalent methyl oleate concentration (Figure 2.11.2).

2.12 TRIGLYCERIDE MEASUREMENT

Triglyceride concentration was assayed by Gottfried & Rosenberg (1973) using a standard solution of triolein to construct a Calibration Plot (Figure 2.12).

Procedure:

(i) Extraction

Add: - Sample.....0.5 ml
- Heptane.....2.0 ml
- Isopropanol.....3.5 ml
- Sulphuric acid (0.04 M).....10 ml;

This was mixed for 30 s by vortex;

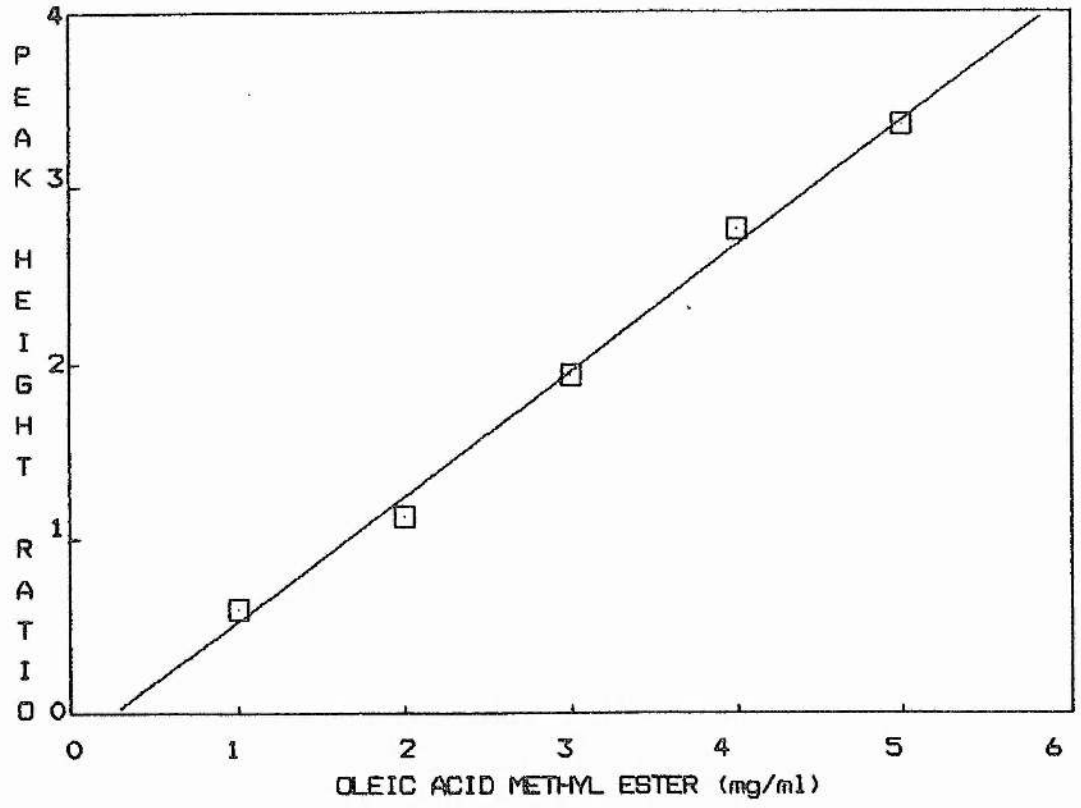
(ii) Saponification reaction

Add to the upper layer.....0.4 ml
- Isopropanol.....4.0 ml
- KOH.....4 drops;

This was mixed well by vortex and incubated at 70°C for 10 min;

(iii) Oxidation and color development

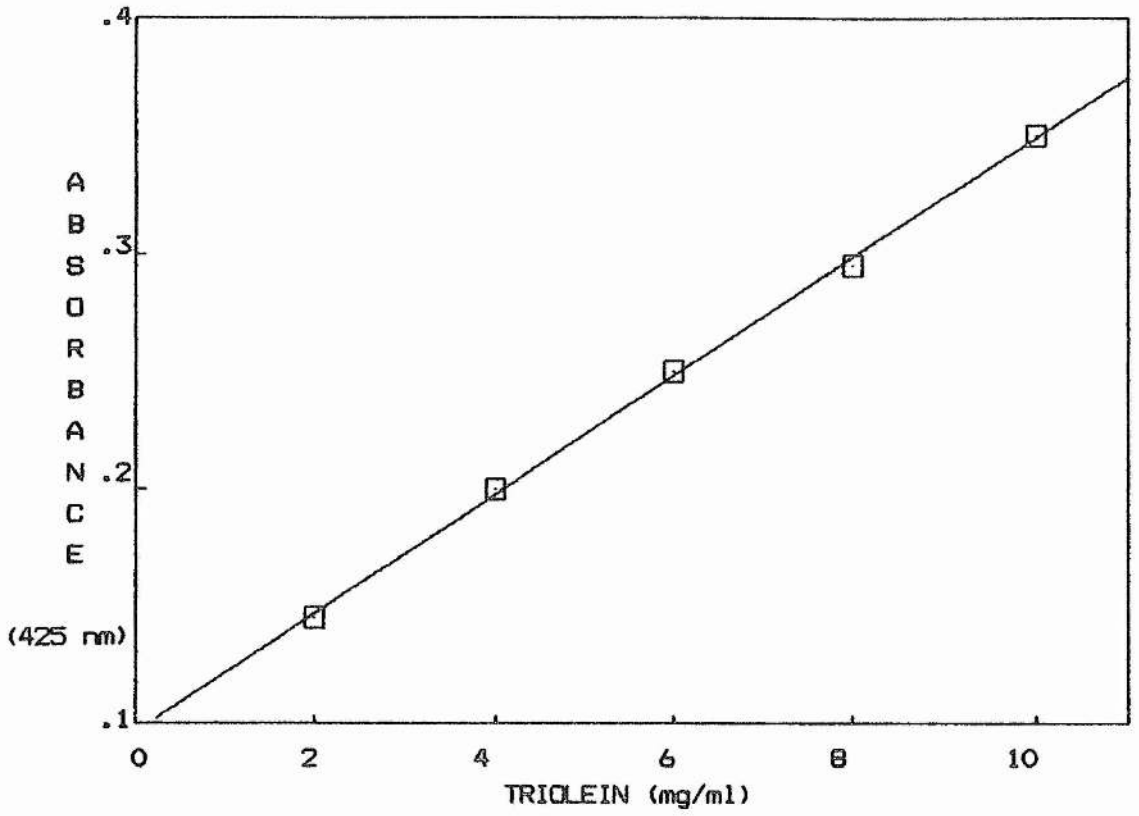
Add: - Sodium metaperiodate (0.6 %)...0.2 ml



N.B. Peak height ratios were measured in the chromatograms between methyl oleate of the samples and internal standard (methyl heptadecanoate).

Slope	.7129002
Y intercept	-.1829006
Correlation coefficient	0.9972
Standard error	.0978616

FIGURE 2.11.2 CALIBRATION PLOT FOR OLEIC ACID CONCENTRATION MEASUREMENTS WITH ITS LINEAR REGRESSION



Slope	2.524999E-02
Y intercept	9.650004E-02
Correlation coefficient	0.9995
Standard error	3.027661E-03

FIGURE 2.12 CALIBRATION PLOT FOR TRIGLYCERIDE CONCENTRATION MEASUREMENTS WITH ITS LINEAR REGRESSION

- Acetylacetone (7.5 %).....10 ml;

This was mixed well by vortex and incubated at 70°C for 10 min.

The concentration of the colored compound produced was measured at 425 nm.

2.13 LIPASE ASSAY

Lipase activity was estimated by titration method using triolein as a substrate at 10 % (v/v).

The substrate was emulsified in the presence of polyvinylpyrrolidone (PVP, 1 mg/ml) at the proportion 1:1 in vortex for 30 sec. The reaction system contained 3 ml of this resulting emulsion mixed with the sample and distilled water in vortex for more 30 sec, totalizing a volume of 10 ml.

The temperature was controlled at 30°C by a water bath and the emulsion system was maintained stable under constant magnetic agitation at 750 rpm.

Lipase assay was investigated in order to enhance lipolytic activity of *C. lipolytica 1055*.

In the presence of PVP, solutions of calcium acetate were added to the reaction system at 0.005 M and 0.05 M as well as Na^+ ions at 0.01 and 0.1 M as sodium chloride.

In addition the influence of sodium deoxycholate at 1.0 and 2.0 mg/ml and BSA at 0.1 and 0.5 mg/ml were also investigated.

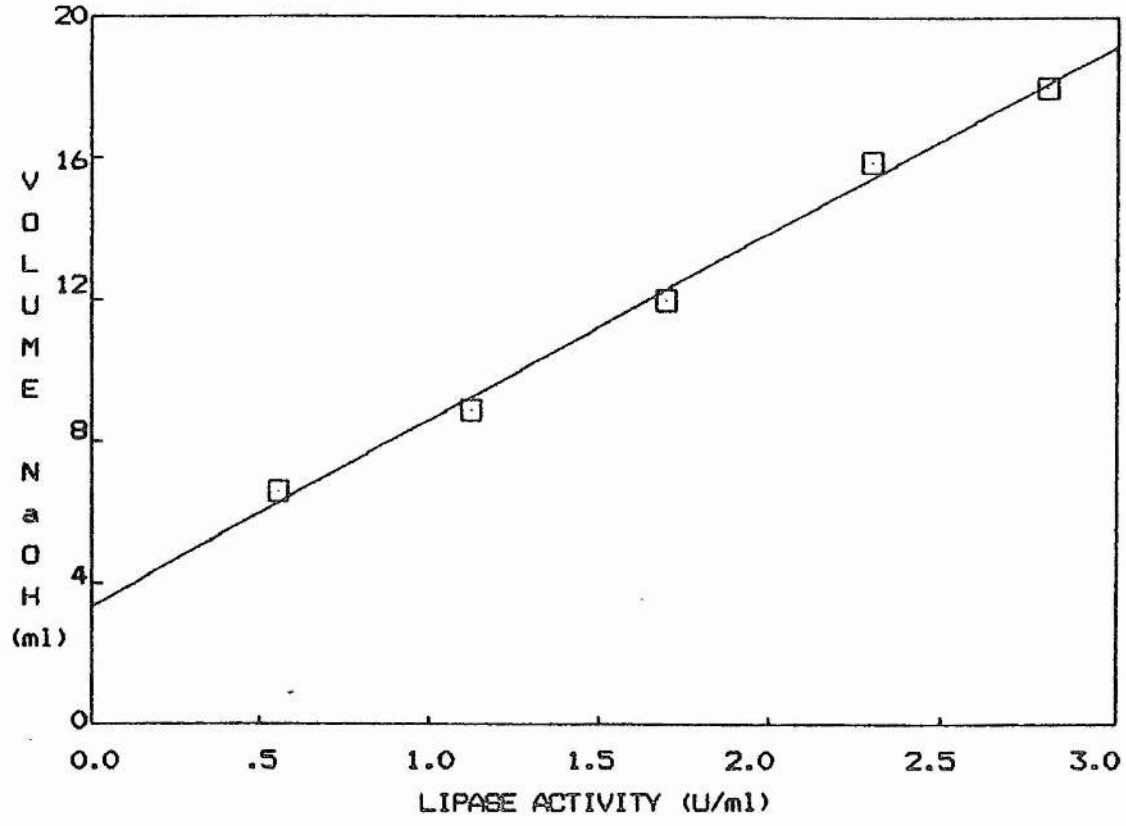
As neither these metal ions, nor sodium deoxycholate, nor albumin bovine showed any positive effect in lipase activity by *C. lipolytica 1055*, this assay was carried out only in the presence of olive oil and PVP.

The enzyme reaction was developed for 3 min and the activity was stopped by the addition of 20 ml of a cold acetone-ethanol mixture (1:1 v/v). The reaction rate was determined in linear phase before any limitation by the reactants and /or any inhibition by the product.

The fatty acids released were titrated at once to pH 10.5 against 0.05 M NaOH in the presence of thymolphthalein using a pH meter. All the results were subtracted from blanks (samples previous boiled for 3 min).

A standard curve was constructed using lipase from *C. cylindracea* (Sigma) relating the volume of NaOH added to lipase activity (Figure 2.13).

Considering the problem of low lipase activity, samples were concentrated by membrane systems, Centriprep 10 000 MW cut-off ultrafilter (Amicon).



Slope	5.271374
Y intercept	3.339749
Correlation coefficient	0.9972
Standard error	.4098396

FIGURE 2.13 CALIBRATION PLOT FOR LIPASE ACTIVITY WITH ITS LINEAR REGRESSION

One Unit (U) of lipase activity was defined as the amount of enzyme which liberated 1 µmole of free oleic acid per minute under test conditions.

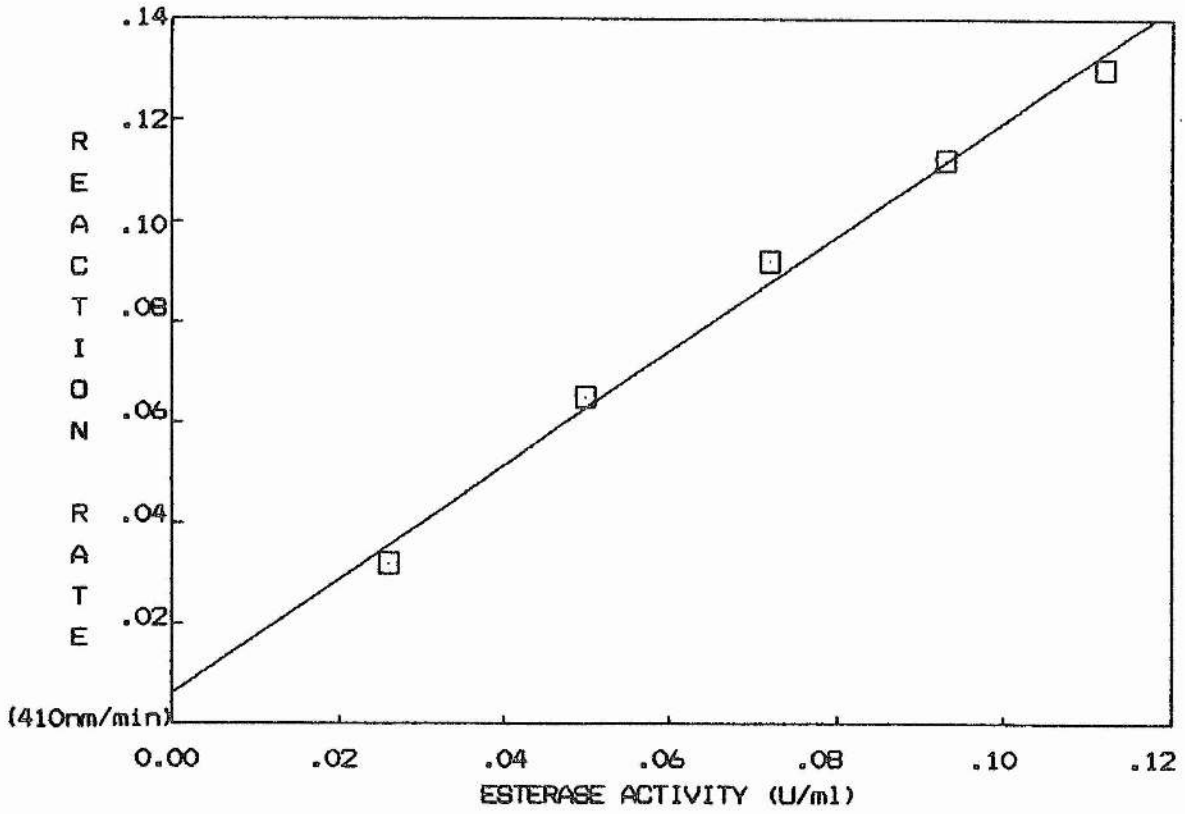
2.14 ESTERASE ASSAY

Esterase activity was estimated according to Winkler & Stuckmann (1979) using p-nitrophenylpalmitate (p-NPP) as the substrate.

A Calibration Plot (Figure 2.14) was constructed measuring the absorbances at 410 nm of the p-nitrophenyl in a Beckman DU-Series 60 spectrophotometer against an enzyme-free control.

The fresh substrate emulsion was made with 30 mg p-NPP dissolved in 10 ml isopropanol, and mixed with 207 mg sodium deoxycholate and 100 mg gum arabic in phosphate buffer 0.05 M at pH 7.2 totalizing a volume of 200 ml. In the reaction system 0.1 ml of the sample was added to 2.5 ml of substrate emulsion in the presence of 0.1 ml of phenylmethylsulfonil fluoride (PMSF, 0.001 M) to inhibit protease activity.

One Unit (U) of esterase activity was defined as the amount of enzyme which hydrolysed 1 µmole of palmitate per minute at pH 7.2 and 37°C.



Slope	1.134349
Y intercept	6.11493E-03
Correlation coefficient	0.9962
Standard error	3.89905E-03

FIGURE 2.14 CALIBRATION PLOT FOR ESTERASE ACTIVITY WITH ITS LINEAR REGRESSION

2.15 PROTEASE ASSAY

Protease activity was estimated according to Pejhan (1984) using α - casein 1 % as the substrate in phosphate buffer 0.05 M at pH 7.4.

Each sample (1 ml) was mixed with 1 ml substrate and incubated in a controlled water bath at 37°C.

The reaction was stopped after 1 and 60 min by adding 10 % trichloracetic acid (TCA) at the proportion 1:1. The one minute sample served as a blank for the 60 min sample.

The precipitate was removed by centrifugation and the proteins in TCA soluble fraction were measured according to Section 2.10.

2.16 ULTRASONIC DISRUPTION

For extraction of cell-bound enzymes, samples from exponential phase (1.5 ml) were centrifuged at 10 000 x g for 2 min; cells were washed with cold distilled water and resuspended in the same volume of phosphate buffer 0.05 M pH 7.2.

Ultrasonic disruption was carried out by high frequency vibration via a titanium probe operating at a nominal frequency of 23 KHz. The amplitude was monitored continuously by a meter (MSE Soniprep 150 Ultrasonic Desintegrator). The samples were kept in ice and

submitted to the frequency for 0.5 to 5.0 min. The Figure 2.16 shows the highest esterase activity at 1.5 min and a high denaturation rate after 2.0 min.

For lipid extraction, cells were resuspended in the same volume of isopropanol; after the cells disruption by ultrasonic, the supernatants were used for triglyceride assay (Section 2.12).

2.17 CALCULATION OF THE MAXIMUM SPECIFIC GROWTH RATE

The maximum specific growth rate of *C. lipolytica 1055* was estimated by batch experiment; the slope of the straight line from the plot of natural logarithm (ln) of biomass in exponential growth against time, gives the value of μ_{MAX}, according to the Equation 1.4.1.

By the washout method of Pirt & Callow (Pirt, 1975) this kinetic constant was estimated in chemostatic culture at high values of dilution rates when the biomass concentration decreases.

Considering the biomass balance in chemostat:

$$dx / dt = x (\mu - D)$$

Integrating

$$\int dx / x = (\mu - D) \int dt$$

Thus,

$$\ln x - \ln x_o = (\mu - D)\ t$$

Where x_0 is the biomass concentration at zero time.

By the condition that substrate concentration is greater than saturation constant ($S >> K_s$), through the Monod's equation the specific growth rate reaches its maximum value ($\mu = \mu_{MAX}$).

Then, the later equation can be given in function of the maximum specific growth rate:

$$\ln x - \ln x_o = (\mu_{MAX} - D)\ t$$

$$\ln x = (\mu_{MAX} - D)\ t + \ln x_o.$$

Therefore, according to the above equation, the slope of ln x versus time gives the value of μ_{MAX}.

3 RESULTS AND DISCUSSION

3.1 BATCH CULTURE

3.1.1 Growth of *C. lipolytica 1055* in the presence of glucose as carbon source

C. lipolytica 1055 growth in batch culture using glucose (20 g/l) as carbon source at 30°C, pH 6.0, 500 rpm mechanical agitation and 1 vvm aeration rate can be seen in Figure 3.1.1-a.

Following the initial exponential growth phase the microorganism reached a biomass of 3.5 mg/ml with approximately 50 % of the initial glucose remaining in the broth. The microorganism continued to grow very slowly until the carbon source was totally metabolized after 4 days.

The reduced rate of glucose consumption after 12 h of culture may suggest that an inhibitor factor is present in the broth.

It is important to emphasize that the ratio between carbon and nitrogen sources was 4:1 on *C. lipolytica 1055* batch cultures in presence of glucose.

Kosaric et al. (1979) have reported two different growth rates for a strain of *C. lipolytica*

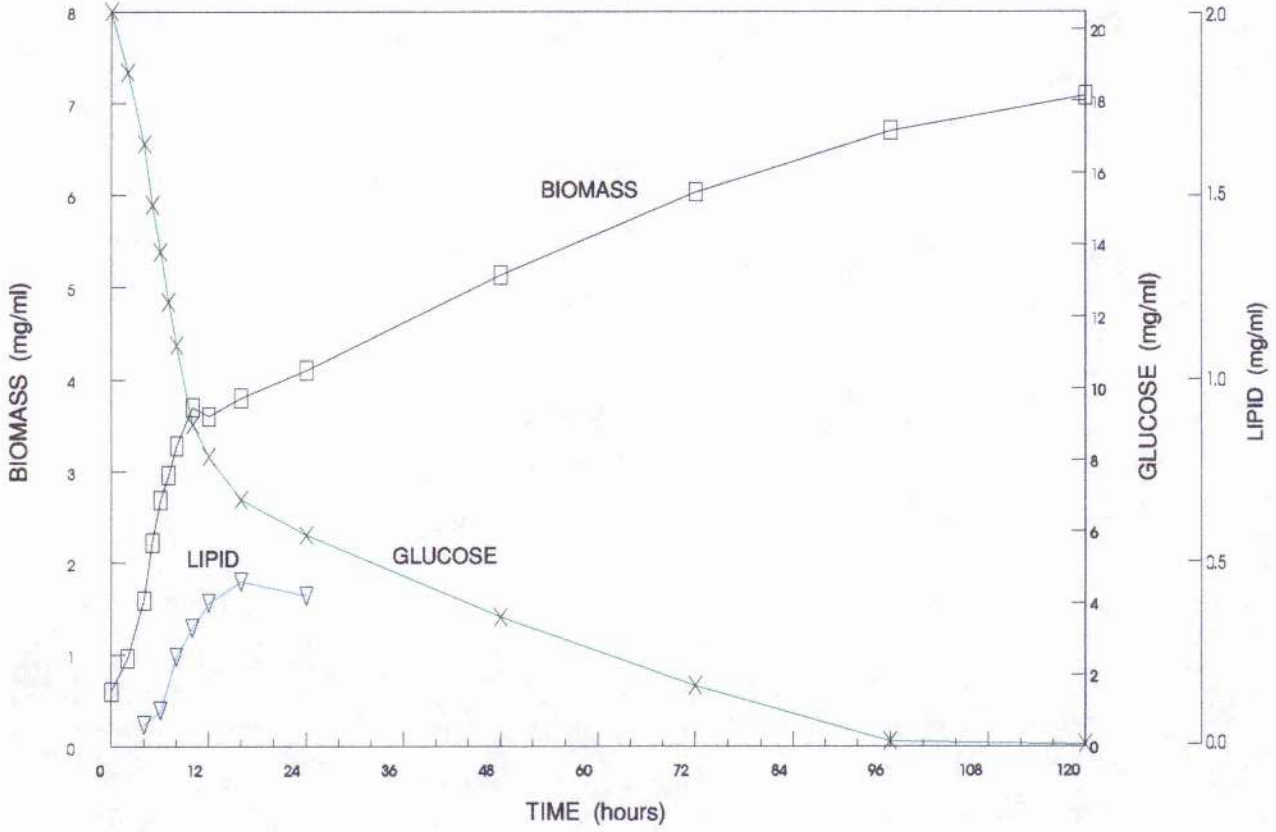


FIGURE 3.1.1-a BATCH CULTURE OF *C. lipolytica 1055* GROWN IN 20 g/l GLUCOSE AND 5 g/l YEAST EXTRACT MEDIUM AT 30°C

growing in presence of 'kerosene'. The microorganism showed a rapid increase in biomass about the first 72 h of growth although the maximum biomass was reached only after 150 h of growth.

Klasson et al. (1989) have reported that 65 % glucose concentration was still present after 5 days of *C. lipolytica NRRL Y-7576* cultivation. The *Candida* was grown in presence of glucose (130 g/l), ammonium chloride (1.3 g/l), yeast extract (0.05 g/l), as well as mineral salts and thiamine-HCl. This microorganism showed a rapid growth followed by ammonia exhaustion and subsequently citric acid accumulation at stationary phase being metabolised less than 50 % of the glucose.

In yeast growth, toxic effects of some metabolites have been published by some authors (Pichard et al., 1985; Valero et al., 1991a).

Valero et al. (1991a) have noticed that *C. rugosa* grown on glucose as sole carbon source, accumulated ethanol, requiring more than 3 days for glucose consumption.

Figure 3.1.1-a also shows that *C. lipolytica 1055* accumulated intracellular triglyceride as storage material.

Tan & Gill (1984) have reported that *S. lipolytica* in presence of olive oil under nitrogen- and oxygen-limited cultures accumulated lipids.

Moreover, a *C. lipolytica* strain has produced a relatively small amount of extracellular lipid in the presence of 'kerosene' as sole carbon source (Kosaric et al., 1979).

It is relevant to consider that all potent producers of lipase are capable of lipid accumulation in a carbon rich medium (Boing, 1982) whereas a complete exhaustion of nitrogen or metal ions is required for higher level production of lipid (Granger et al., 1993).

Glucose is a good carbon source for most microorganism growth and in particular for *C. lipolytica* C_1B (Kalle et al., 1972). However, the inhibitory effect of high glucose concentration in lipase production has been reported by Nahas (1988) and Chen et al. (1992).

Thus, the behaviour of *C. lipolytica 1055* in the presence of 2 g/l glucose was investigated. The Figure 3.1.1-b shows that the strain in study reached a stationary growth phase after 8 h and the carbon source was totally metabolized about 16 h.

Although the pH was not controlled throughout the experiment a decline of the initial pH was observed from 6.0 to 4.5 at the late exponential phase (Figure 3.1.1-b). As this pH decline coincided with an increase in biomass, the change in pH might have resulted from the metabolic production of organic acids.

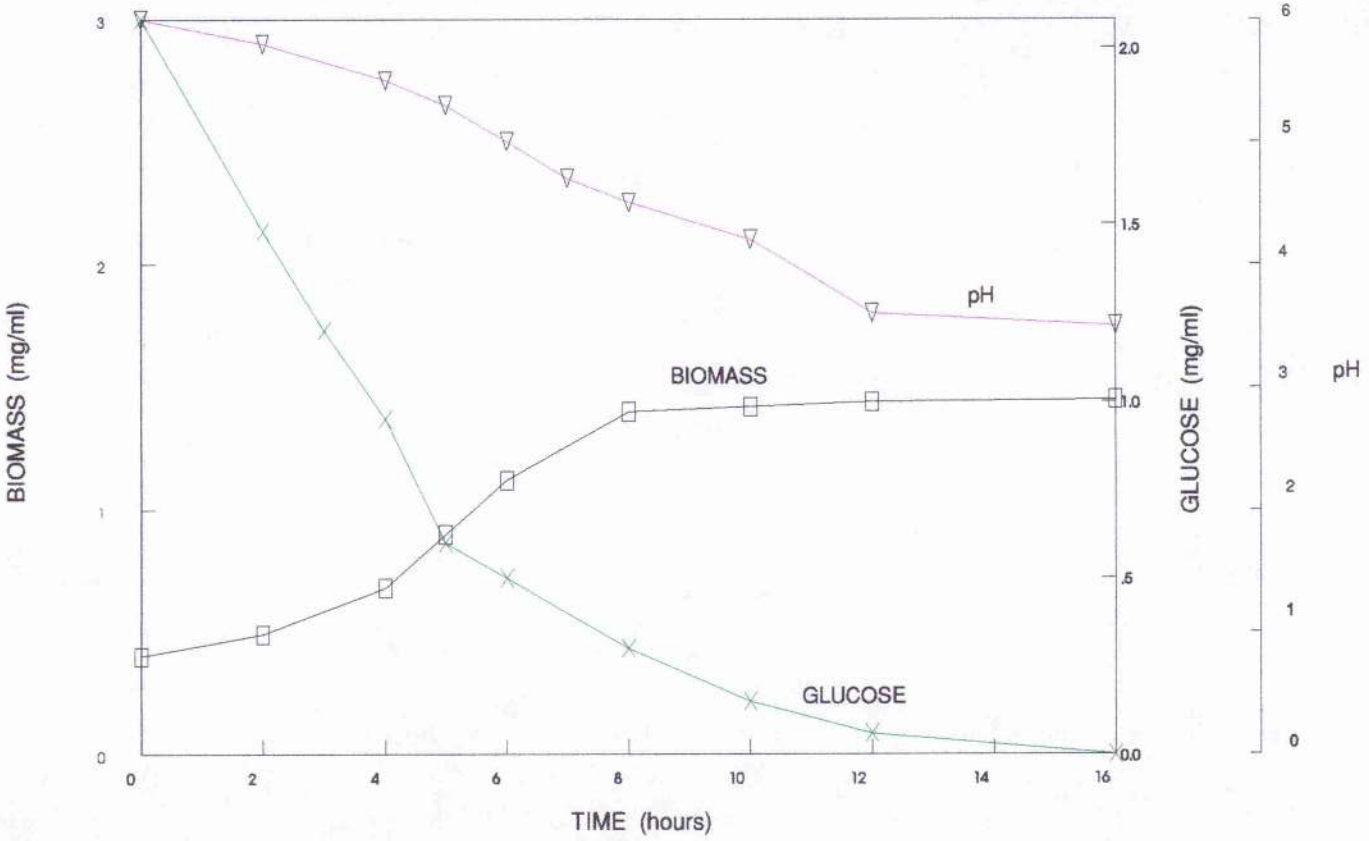


FIGURE 3.1.1-b BATCH CULTURE OF *C. lipolytica 1055* GROWN IN 2 g/l GLUCOSE AND 0.5 g/l YEAST EXTRACT MEDIUM AT 30°C

In *C. lipolytica 1055* growth curves (Figures 3.1.1-a and 3.1.1-b), lag phases were not apparent because 10 % inocula were used from logarithmic phase and the cultures were grown on the same medium.

Lack of lag phases is consistent with the presence of metabolically active cells from the exponential growth phase, not involving induction of new enzymes. Furthermore, Tan & Gill (1984) have reported that washed cells of *S. lipolytica* grown on glucose medium showed a short lag phase in the presence of olive oil.

3.1.2 Lipase and esterase activities

According to the Sections 2.14 and 2.15, enzyme activity estimated with olive oil was designated as lipase whereas that estimated using p-NPP (substrate) as esterase.

Extracellular and cell-bound lipase and esterase activities from *C. lipolytica 1055* were studied in a 1 litre stirred bioreactor under batch conditions in the presence of glucose (20 g/l).

The highest values for both hydrolase activities were reached at approximately 8 h of culture when the microorganism was at the late logarithmic growth phase (Figure 3.1.2).

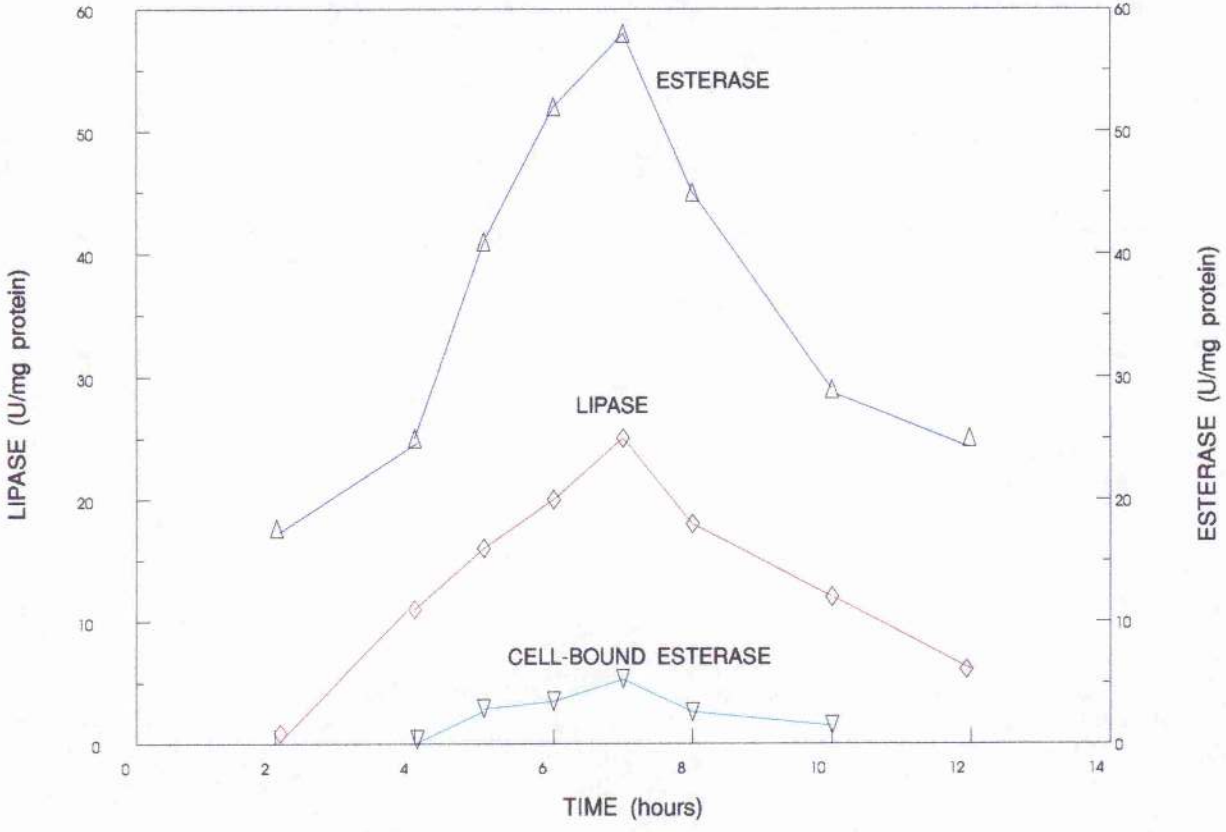


FIGURE 3.1.2 EXTRACELLULAR AND CELL-BOUND SPECIFIC LIPASE AND ESTERASE ACTIVITIES OF C. lipolytica 1055 IN PRESENCE OF 20 g/l GLUCOSE AT 30°C

Protein levels detected in supernatant were very low reaching as maximum value 80 mg/l at the late exponential phase. The specific extracellular hydrolase activities by *C. lipolytica 1055* reached high values: 58 U/mg protein as maximum esterase activity and 25 U/mg protein as maximum lipase activity.

Jane Gilbert et al. (1991a) have reported that lipase activity was not detected and very low esterase activity was obtained during stationary phase by a lipase-producing bacteria, *Ps. aeruginosa AF2* in presence of glucose. However, *P. roqueforti* has shown a maximum lipase activity of 10 U/ml in presence of this carbohydrate (Petrovic et al., 1990).

As cell-bound lipase activity was not detected and only approximately 10 % of the total esterase activity was detected in cell-bound subjected to ultrasonic (Section 2.16), *C. lipolytica 1055* produces lipase and esterase largely as extracellular enzymes (Figure 3.1.2).

Certain *Candida* strains and *Y. lipolytica* (Novotny et al., 1988; Valero et al., 1988), as well as *Ps. aeruginosa AF2* (Jane Gilbert et al., 1991a) have exhibited lipase / esterase predominante extracellularly.

Kosaric et al. (1979) have reported that a strain of *C. lipolytica* revealed a negative cell-bound lipase activity despite the fact that Kalle et al. (1972) have reported that the lipase from *C. lipolytica* C_1B was

a cell-bound constitutive enzyme. Although reports of constitutive lipase exist lipase is generally inductive.

Furthermore, lipase from *S. lipolytica* was detected in cell-free medium when the cell-bound lipase started to disappear (Ruschen & Winkler, 1982).

Cell-bound lipase have been also reported for *S. lipolytica* (Ota et al., 1978) and for *R. delemar* (Chen & McGill, 1992).

Esterase activity from *C. lipolytica 1055* could be detected in samples kept at -15°C for 1 month while lipase activity was considerable more labile losing approximately 50 % of achieved after approximately 1 week at -15°C.

3.1.3 Antifoam investigation

In microbial processes proteins secreted by cells can form stable foams, mainly depending on composition and pH of the medium. Foams can cause several problems (e.g. output blockage) and on industrial scale the medium may be lost, reducing the productivity.

Moreover, cell cultivation is influenced by not only the kind of antifoam present in the medium, but also by its concentration (Schugerl, 1985).

Antifoam agents inhibit foam formation by destroying the surface elasticity / viscosity of foam system.

The effect of four different antifoams on *C. lipolytica* 1055 growth and lipase and esterase activities was investigated for the following reasons:

(i) Submerged cultures of *C. lipolytica 1055* with mechanical agitation and air flow-rate of 1 vvm produced excessive foaming and wall growth;

(ii) Nutrient availability is enhanced (especially lipid based) upon vigorous agitation of the culture; Tan & Gill (1985a) have reported that sufficient agitation of the medium is one of the major factors required for *S. lipolytica* growth on fats.

Furthermore, aerated submerged cultures are important for *Candida* genus growth as well as advantageous to lipase production (Jacobsen et al., 1989a; Valero et al., 1991a).

The Figures 3.1.3-a and 3.1.3-b show, respectively, esterase activity and growth effects of some antifoams tested at different concentrations in batch experiments.

Esterase production from *C. lipolytica 1055* was stimulated by low concentrations of all the four antifoams investigated. However, high concentrations (> 0.1 ml/l) of silicone product (Dow Corning) and antifoam 289-mixed (Sigma) inhibited esterase activity (Figure 3.1.3-a), as well as microorganism growth. Biomass concentration decreased around 50 % in the

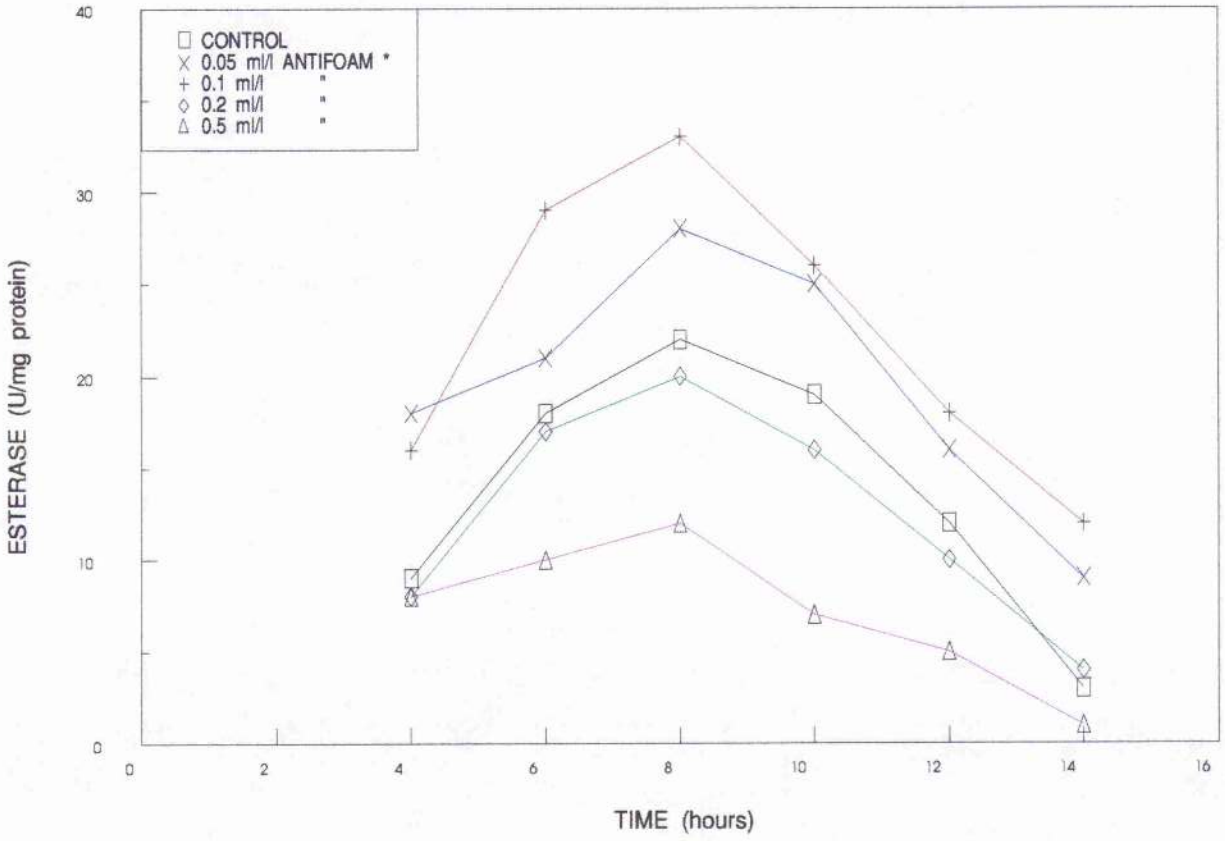


FIGURE 3.1.3-a EFFECT OF SILICONE PRODUCT ON SPECIFIC ESTERASE ACTIVITY BY *C. lipolytica 1055*.

* 2 SILICONE PRODUCTS, DOW CORNING/SIGMA ANTIFOAM 289-MIXED, WERE TESTED GIVING SIMILAR RESULTS

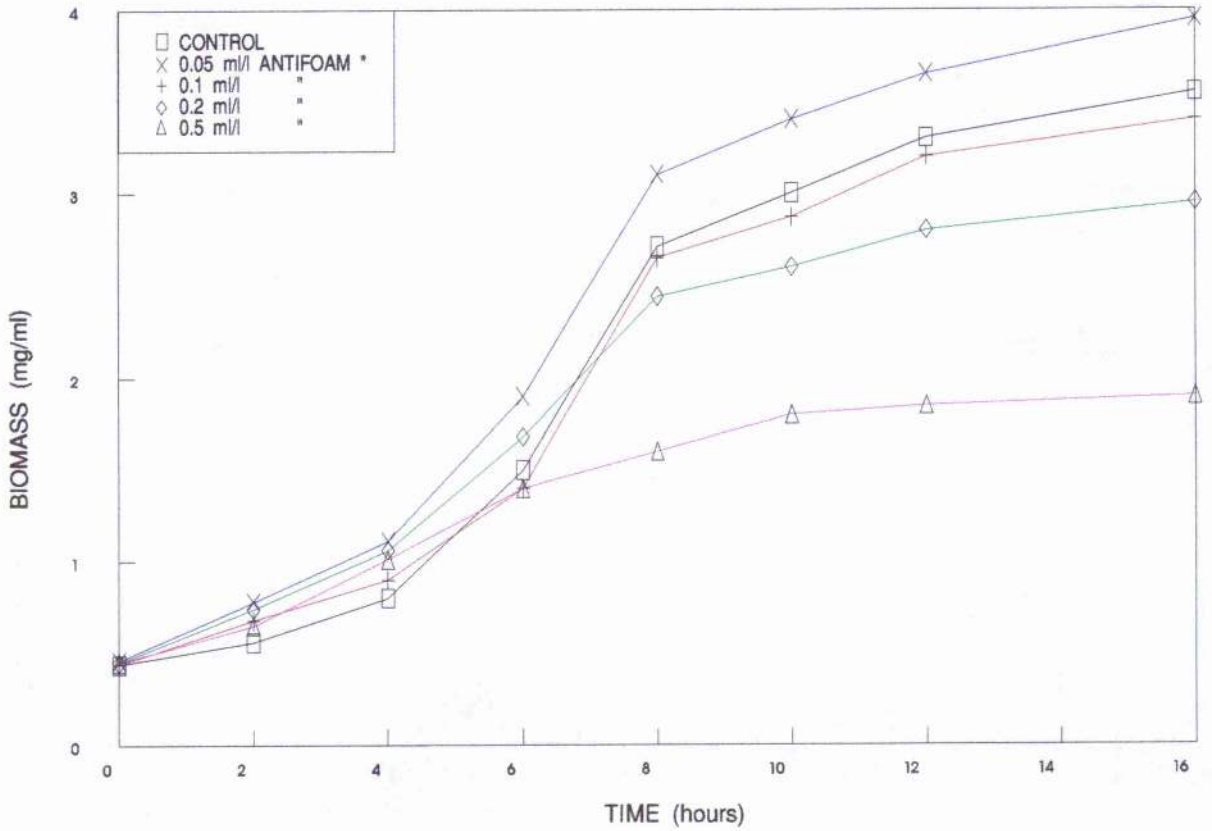


FIGURE 3.1.3-b EFFECT OF SILICONE PRODUCT ON *C. lipolytica 1055* GROWTH

* 2 SILICONE PRODUCTS, DOW CORNING/SIGMA ANTIFOAM 289-MIXED, WERE TESTED GIVING SIMILAR RESULTS

presence of 0.5 ml/l of these latter antifoams tested (Figure 3.1.3-b).

On the other hand, a neutral growth effect was noticed with antifoam 204-organic and antifoam A (Sigma). Despite the fact that the antifoam A (Sigma) contains silicone, its composition also includes non-ionic emulsifier while the antifoam 204-organic (Sigma) is a mixture of non-silicone de-foaming agents in a polyol dispersion.

Best results were obtained with antifoam A at 0.1 ml/l (Figure 3.1.3-c) whose antifoam was selected for all development studies. Higher antifoam concentrations did not enhance esterase production while at lower concentrations foams appeared in the culture after 12 h of growth.

The antifoam A (Sigma) also increased *C. lipolytica 1055* lipase (Figure 3.1.3-c). The maximum value was near two-fold when compared with the control experiment.

These results were similar to those observed by MARCIN and co-workers (1993) who have reported a screen for three basic types of antifoams. The majority of silicone products increased lipase production by *Ps. aeruginosa MB 5001* at the concentration of 5 ml/l under batch condition.

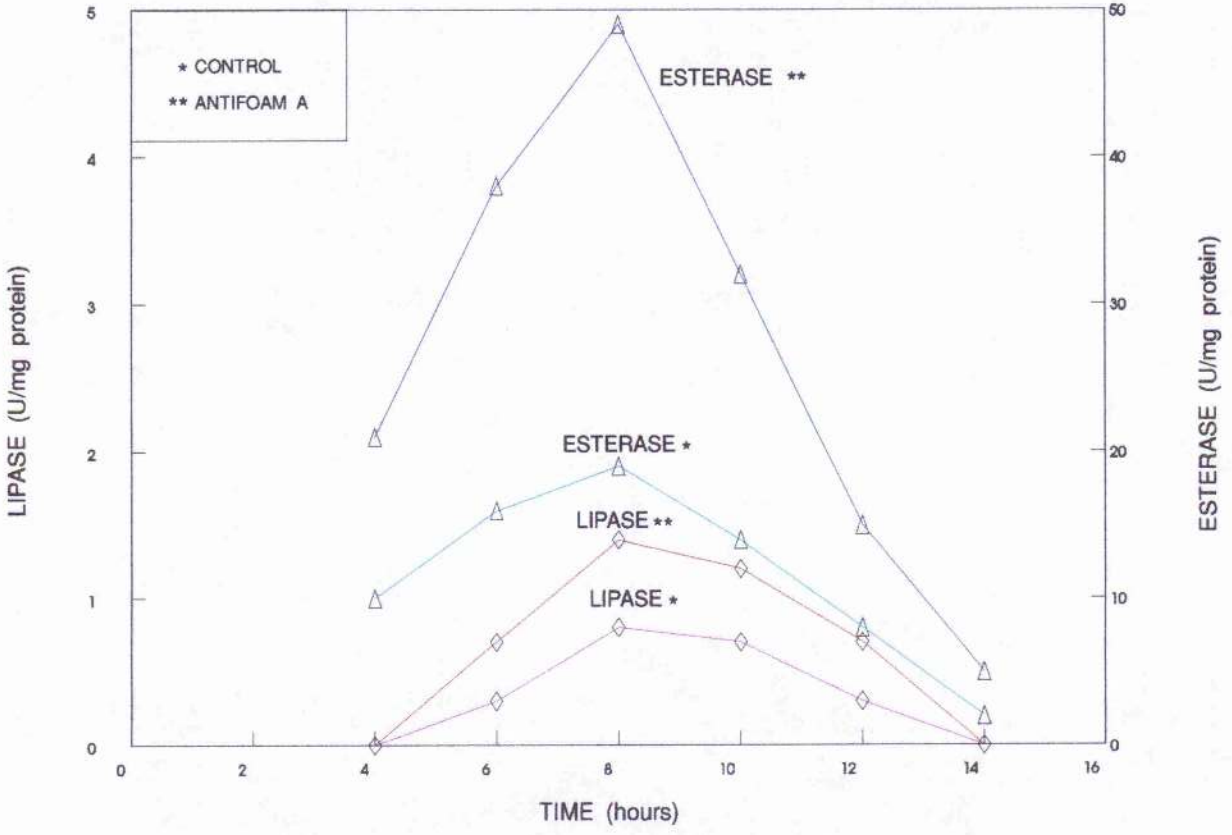


FIGURE 3.1.3-c EFFECT OF ANTIFOAM A (SIGMA) AT 0.1 ml/l ON SPECIFIC EXTRACELLULAR LIPASE AND ESTERASE ACTIVITIES BY C. lipolytica 1055

3.1.4 Effect of different carbon sources on specific lipase and esterase activities

The choice of carbon source is important for both lipase and/or esterase activities, as well as for yeast growth (Tan & Gill, 1984; Novotny et al., 1988; Hadeball, 1991).

Table 3.1.4-a shows different levels of specific extracellular lipase and esterase activities (expressed in U/mg dry weight) from *C. lipolytica 1055* batch cultures in the presence of various carbon sources.

Considering that enzyme assays measure only the activity under specific conditions, it was not possible to distinguish if the growth condition affected the synthesis or also the excretion of the enzymes. The techniques are not sufficiently sensitive to distinguish difference between the enzyme activation and the enzyme synthesis.

C. lipolytica 1055 showed the best lipase and esterase activities when Tween-80 was used as the sole carbon source. Surface-active compounds such as Tween-80, decrease the medium superficial tension, increasing proteins released from the cell wall and consequently, extracellular enzyme production by microorganisms is stimulated.

This result agrees with the results presented by several authors in which Tween-80 has supported good

CARBON SOURCES (4 ml/l)	LIPASE (U/mg)*	ESTERASE (U/mg)*
TWEEN - 80	2.5	42.0
TRITON X - 100	N.D.	N.D.
OLIVE OIL	2.2	16.5
CORN OIL	< 1.0	1.0
SUNFLOWER OIL	< 1.0	1.1
PALM OIL	N.D.	< 1.0
COD LIVER OIL	< 1.0	< 1.0
TRIOLEIN	1.2	1.1
OLEIC ACID	1.8	1.5

* SPECIFIC ENZYME ACTIVITY: U/mg dry weight
N.D. = NOT DETECTABLE

TABLE 3.1.4-a EFFECT OF CARBON SOURCES ON SPECIFIC LIPASE AND ESTERASE ACTIVITIES BY *C. lipolytica 1055*

growth and enzyme production in *Rhizopus oligosporus* (Nahas, 1988), *G. candidum* (Jacobsen et al., 1989a), *R. delemar CDBB H313* (Espinosa et al., 1990; *Ps. aeruginosa AF2* (Jane Gilbert et al., 1991a), *P. citrinum* (Maliszewska & Mastalerz, 1992). However, Hegedus & Khachatourians (1988) have reported that Tween-80 inhibited lipase production by *Beauveria bassiana*.

The exact mechanism for lipase and esterase activity stimulation by Tween-80 is not known. As its chemical nature, ester, is similar to some esterase substrates, it could act as an inducer for this kind of hydrolytic enzyme.

Jane Gilbert et al. (1991a) have reported that lipase production by *Ps. aeruginosa AF2* was strongly induced by Tween-20, -40, -60 and -80 (Table 3.1.4-b).

In contrast, *C. lipolytica* 1055 exhibited neither lipase nor esterase activity in the presence of Triton X-100, a detergent containing octyl phenoxy polyethoxyethanol (Union Carbide Chemicals and Plastics Co., Inc.).

Table 3.1.4-c shows average values of fatty acid compositions of some natural oils (Padley et al., 1986).

Amongst various kind of natural oils tested, olive oil containing approximately 70 % oleic acid, was the most effective for both lipase and esterase. On the

COMMERCIAL NAME	CHEMICAL NOMENCLATURE	FATTY ACID COMPOSITION
TWEEN - 20	POLYOXYETHYLENESORBITAN MONOLAURATE	LAURIC ACID 55 %
TWEEN - 40	POLYOXYETHYLENESORBITAN MONOPALMITATE	PALMITIC ACID 90 %
TWEEN - 60	POLYOXYETHYLENESORBITAN MONOSTEARATE	STEARIC ACID 55 %
TWEEN - 80	POLYOXYETHYLENESORBITAN MONOOLEATE	OLEIC ACID 75 %

N.B. All detergents (Sigma) were sterilised before use.

TABLE 3.1.4-b BIOLOGICAL DETERGENTS AND THEIR COMPOSITIONS

FATTY ACID	OLIVE OIL	CORN OIL	SUNFLOWER OIL	PALM OIL	COD LIVER OIL
MYRISTIC ACID	-	-	-	3.2 %	4.4 %
PALMITIC ACID	14 %	13.5 %	6.5 %	45.5 %	14 .1 %
PALMITOLEIC ACID	2 %	-	-	-	12.0 %
STEARIC ACID	2 %	2.2 %	5.5 %	5.0 %	2.6 %
OLEIC ACID	70 %	44 %	46 %	39.5 %	22.4 %
LINOLEIC ACID	12 %	65 %	48 %	9.5 %	41.4 %*

* This value includes all long-chain fatty acid unsaturated.

TABLE 3.1.4-c FATTY ACID COMPOSITION OF OILS (Padley et al., 1986)

other hand, in the presence of triolein (Sigma) as carbon source these hydrolase activities reached around 1 U/mg dry weight while the technical oleic acid (BDH Chemicals Ltd.) with only 70 % of purity was as effective as olive oil concerning lipase production.

The differences in enzyme levels between corn oil (44 % oleic acid and 65 % linoleic acid) and sunflower oil (46 % oleic acid and 48 % linoleic acid) were not significants (< 15 %) despite these oils have different proportions of unsaturated fatty acids.

While lipase activity of *C. lipolytica 1055* was not detectable in presence of palm oil (45.5 % palmitic acid and 39.5 % oleic acid), esterase activity reached only 0.2 U/mg dry weight.

These results could be justified through the fact that the major fatty acid in this carbon source is saturated and lipolytic microorganisms preferentially consume unsaturated fatty acid (Tan & Gill, 1985a). On the other hand, *Rhodotorula glutinis* has exhibited the best lipase activity (30.4 U/ml) in presence of palm oil and ammonium phosphate at 35°C and pH 7.5 (Paparaskevas et al., 1992).

Cod liver oil, consisting predominantly of long-chain unsaturated fatty acid and 14 % palmitic acid (Table 3.1.4-c), showed small positive effects on the production of these extracellular enzymes by *C. lipolytica 1055*.

3.1.5 Effect of different nitrogen sources on specific lipase and esterase activities

The effect of organic and inorganic nitrogen sources on specific lipase and esterase activities can be seen in Table 3.1.5.

Ammonium ion and yeast extract were found as the best nitrogen sources for extracellular production of lipase and esterase by *C. lipolytica 1055* in the presence of Tween-80 as carbon source.

Microorganism growth was more stimulated in the presence of yeast extract. The maximum specific growth rate with yeast extract, μ_{MAX} = 0.27 /h was slightly higher than that with ammonium sulphate, μ_{MAX} = 0.14 /h (appendix 1).

Yeast extract provides a variety of organic nitrogeneous constituents and it also contain vitamins, mineral salts and most of the organic growth factors, likely to be required for microorganisms.

As specific lipase and esterase activities (U/mg dry weight) were highest with ammonium sulphate, this nitrogen source was chosen to study *C. lipolytica 1055* behaviour in presence of Tween-80.

Despite good results in lipase production with peptone (Okeke & Okolo, 1990), this enzymatic hydrolysate from proteins containing only peptides and aminoacids,

NITROGEN SOURCES	LIPASE (U/mg)*	ESTERASE (U/mg)*
PEPTONE	< 1.0	< 1.0
YEAST EXTRACT	2.3	37.0
UREA	N.D.	< 1.0
AMMONIUM SULPHATE	3.2	65.0
POTASSIUM NITRATE	N.D.	N.D.

*SPECIFIC ENZYME ACTIVITY: U/mg dry weight
N.D. = NOT DETECTABLE

TABLE 3.1.5 EFFECT OF NITROGEN SOURCES ON SPECIFIC LIPASE AND ESTERASE ACTIVITIES BY *C. lipolytica 1055* IN PRESENCE OF TWEEN-80 AS CARBON SOURCE

stimulated neither lipase nor esterase activities by *C. lipolytica 1055*.

Low levels of biomass concentration and esterase activity were obtained in presence of urea while lipase activity was not detected although Paparaskevas et al. (1992) have reported that this nitrogen source stimulated the growth and lipase activity by *Rodotorula glutinis*.

In addition, Novotny et al. (1988) have reported that some organic nitrogen sources: peptone, urea and soy flour are more efficient than ammonium ion in both lipase and esterase production.

C. lipolytica 1055 did not grow in presence of potassium nitrate in spite of the fact that good lipase results were obtained with *Ps. aeruginosa AF2* in presence of nitrate (Jane Gilbert et al., 1991a). On the other hand, only few yeast genera such as *Hansenula*, are capable of nitrate assimilation while both nitrate-positive and nitrate-negative species are found in *Candida* genus (Rose, 1987).

3.1.6 Behaviour of *C. lipolytica* 1055 in the presence of Tween-80 and olive oil as carbon sources

It has in fact been demonstrated that extracellular lipase/esterase production is influenced by culture conditions. Hadeball (1991) has published a

review about the most important lipase-producing yeasts as well as the main factors affecting lipase yield.

Sztajer & Maliszewska (1988) have noticed that acid reaction media reduced lipolytic productivity for some strains of *Bacillus, Streptomyces and Pseudomonas,* while Baillargeon et al. (1989) have obtained similar results for *G. candidum*.

Furthermore, *C. lipolytica* (termed *S. lipolytica*) has shown optimum neutral pH for growth (Tan & Gill, 1984 and 1985a) and pH 9.5 for maximum lipase activity (Jonsson & Snygg, 1974).

On the other hand, Kosaric et al. (1979) have reported that a strain of *C. lipolytica* has shown a optimum pH between 6.0 and 6.5 for biomass and lipase production with a rapid decline in enzyme productivity at higher and lower pH values.

As the pH declined to a value of 3.5 during *C. lipolytica 1055* growth in presence of glucose (Figure 3.1.1-b), the microbial behaviour was investigated in presence of disodium hydrogen orthophosphate (medium pH 7.2) when Tween-80 was the carbon source.

Figures 3.1.6-a and 3.1.6-b show the growth curves of *C. lipolytica 1055* batch cultures using Tween-80 and olive oil as carbon sources, respectively.

Metabolism of these substrates is associated with lipase and/or esterase activities. Thus, *C. lipolytica 1055* produces lipase and/or esterase

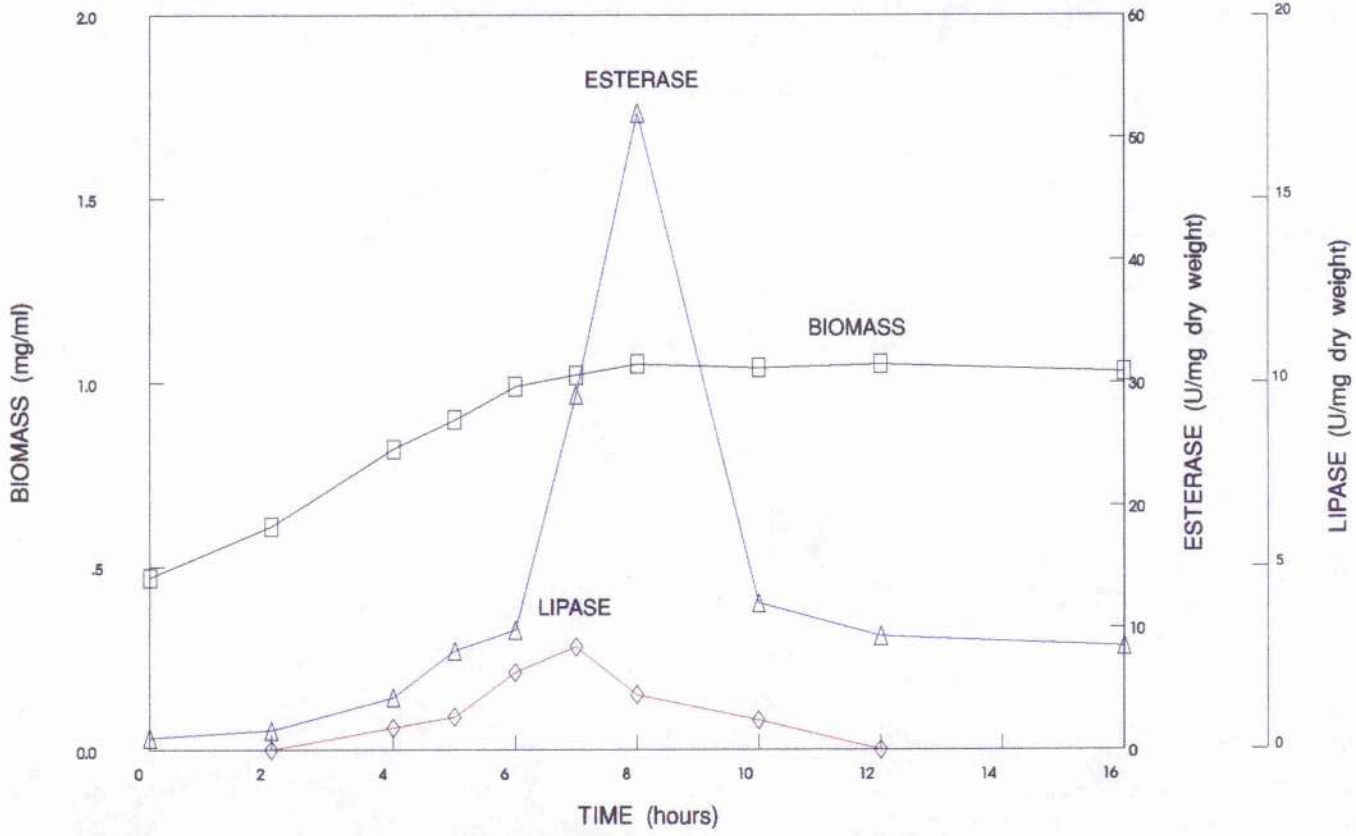


FIGURE 3.1.6-a BATCH CULTURE OF *C. lipolytica 1055* GROWN IN 4 g/l TWEEN-80 AND 2 g/l AMMONIUM SULPHATE MEDIUM AT 30°C

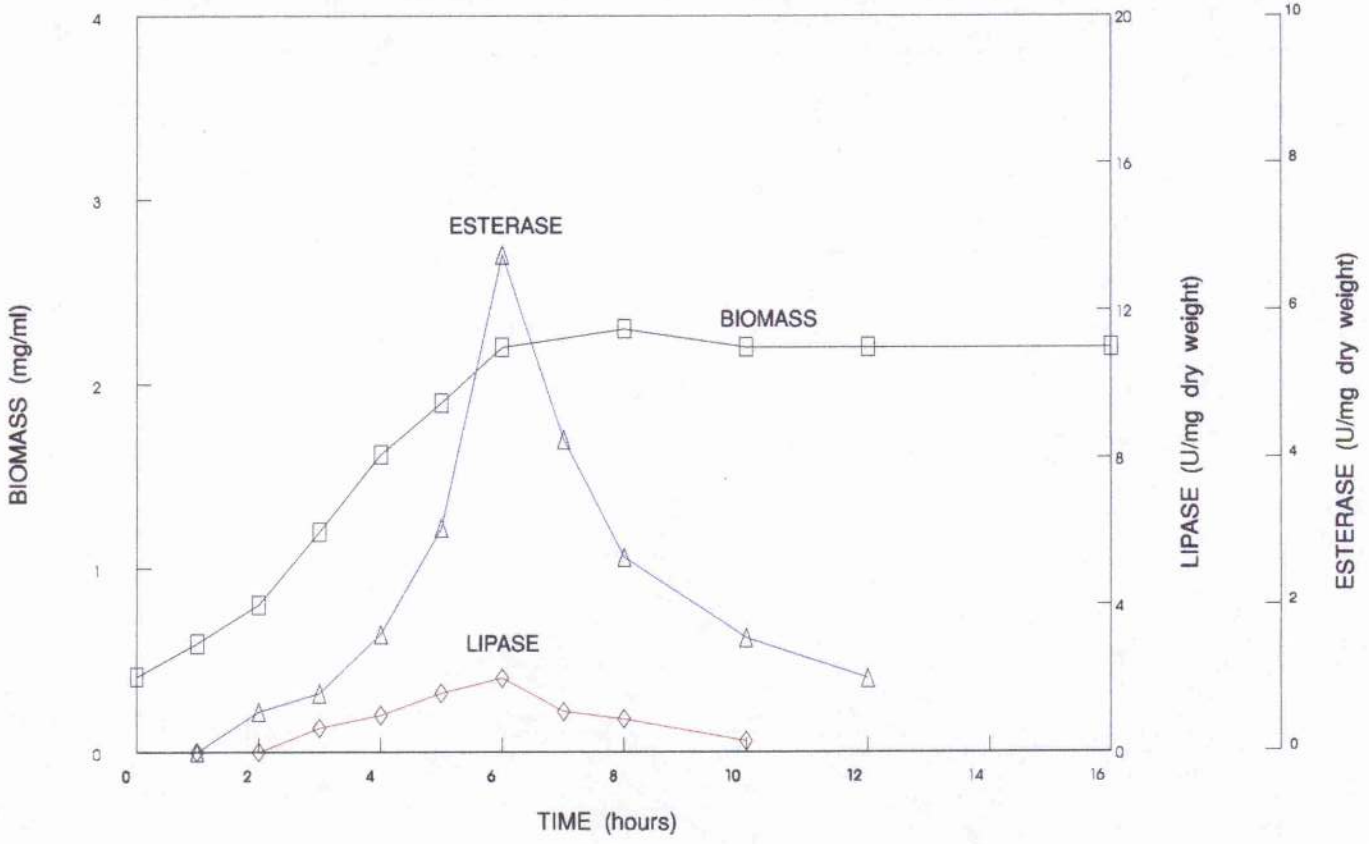


FIGURE 3.1.6-b BATCH CULTURE OF *C. lipolytica 1055* GROWN IN 3.2 g/l OLIVE OIL AND 0.5 g/l YEAST EXTRACT MEDIUM AT 30°C

activities, releasing predominantly oleic acid from Tween-80 and olive oil batch cultures.

In the presence of Tween-80 free oleic acid concentration in the reactor increased during exponential phase attained 0.80 mg/ml as maximum value.

Olive oil was utilized completely in the presence of yeast extract by *C. lipolytica 1055* after 6 h of cultivation. Although ammonium ion is to be preferred as the nitrogen source to facilitate the analysis of the results, yeast extract was choosen due to the fact that lipase production in complex medium is much higher than on defined medium (Ota et al., 1978; Baillargeon et al., 1989; Marcin et al., 1993).

Three additional experiments were carried out to further understand the microbial physiology of *C. lipolytica 1055* in presence of Tween-80 and olive oil. Using polyethylene glycol (PEG-200) and glycerol as carbon sources in presence of 1 g/l yeast extract, this microorganism could gain energy and promote growth whose maximum specific growth rates were 0.13 and 0.12 h^{-1}, respectively (Appendix 1).

However, *C. lipolytica 1055* also exhibited a 0.12 h^{-1} maximum specific growth rate in the presence of 1 g/l yeast extract and antifoam (Appendix 1). Hence, yeast extract supports microorganism growth as carbon and nitrogen sources.

Lipase and esterase activities reached their maxima in the transient between exponential and stationary phases of Tween-80 and olive oil growths (Figures 3.1.6-a and 3.1.6-b).

Despite the growth of *C. lipolytica 1055* on olive oil, only low level of lipase was detected. On the other hand, esterase activity showed very sharp graphs on Tween-80 and on olive oil, remarking that proteolytic activity was detected in both cultures.

Protease activity has been detected in microbial growth for lipase/esterase productions (Jonsson, 1976; Kalle et al., 1972; Espinosa et al., 1990) while maximum lipase level decreased rapidly during the growth for different microorganisms (Rivera-Munoz et al., 1991).

On the other hand, Jonsson & Snygg (1974) have reported a decrease of lipase activity while proteolytic activity remained zero for *S. lipolytica* growing on 'nutrient broth' with emulsified olive oil.

It is important to emphasize that some compounds have been reported as inhibitors of lipases: carbon dioxide (Pichard et al., 1985); glycerol tributyrate (Jacobsen et al., 1990); glycero phospholipids (Sugiura et al., 1975) ; fatty acids (Hegedus & Khachatourians, 1988; Okeke & Okolo, 1990; Jane Gilbert et al., 1991a).

The stimulation of *C. lipolytica 1055* growth in presence of olive oil while only low level of lipase and esterase activities were detectable (Figure 3.1.6-b) may indicate that the excretion of these hydrolases was not directly related to biomass production.

On the other hand, olive oil can stimulate lipase production by increasing growth. This is consistent with the results reported by Valero et al. (1991a) for *C. rugosa*.

In addition, high lipase/esterase productivity was positively correlated with the cells throughout the growth period for *C. lipolytica* C_1B (Kalle et al., 1972). However, olive oil has stimulated *C. lipolytica* lipase production only at low concentration while cell growth was favored at high olive oil concentrations (Kosaric et al., 1979).

3.1.7 Parametric data

The Table 3.1.7-a shows some important parametric data of *C. lipolytica 1055* under different batch conditions.

The maximum specific growth rates were obtained through the plot of the natural logarithm of biomass values against time from exponential phases (Appendices 2, 3, 4 and 5).

CARBON SOURCES	PARAMETERS					
	μ_{MAX} (h^{-1})	$Y_{X/S}$ (mg cells/mg substrate)	LIPASE (U/mg dry weight)*	LIPASE (U/mg dry weight.h)°	ESTERASE (U.mg dry weight)*	ESTERASE (U.mg dry weight.h)°
GLUCOSE (20 g/l)	0.29	0.36	0.28	0.04	1.0	0.13
GLUCOSE (2 g/l)	0.27	0.70	2.2	0.31	5.1	0.73
TWEEN-80 (4 g/l)	0.14	0.28	2.6	0.32	52.0	6.50
OLIVE OIL (3.2 g/l)	0.34	0.72	2.0	0.33	7.3	1.20

μ_{MAX}: MAXIMUM SPECIFIC GROWTH RATE
$Y_{X/S}$: GROWTH YIELD
* SPECIFIC ENZYME ACTIVITY
°MAXIMUM PRODUCTIVITY

TABLE 3.1.7-a PARAMETRIC DATA FROM *C. lipolytica 1055* BATCH GROWTH

The specific growth rate of *C. lipolytica 1055* in glucose at 30°C and pH 6.0 (0.27 - 0.29 h^{-1}) was unaffected by variation of sugar concentration in the range of 2 to 20 g/l.

Not surprisingly some bacteria such as *Ps. aeruginosa AF2* showed higher specific growth rates (0.48 - 0.56 h^{-1}) with glucose as carbon source in presence of metal ions (Mg^{++} and Ca^{++}) and trace elements, although this bacteria attained 0.27 - 0.37 h^{-1} as maximum specific growth rates in presence of Tweens and Spans (Jane Gilbert et al., 1991a).

While *C. lipolytica 1055* exhibited only 0.14 h^{-1} as a maximum specific growth rate in the presence of Tween-80 and ammonium sulphate, the maximum specific growth rate attained 0.27 h^{-1} when yeast extract replaced ammonium ion (Appendix 1).

The positive effect of yeast extract in *C. lipolytica 1055* growth was already discussed in Section 3.1.6.

Maximum specific growth rate in the presence of olive oil was not significantly higher, 0.34 h^{-1}, when compared with glucose and Tween-80 as carbon sources in presence of yeast extract.

Tan & Gill have reported that *S. lipolytica* grown in presence of olive oil (1984) and different animal fats (1985a) at pH 7.0 and 30°C showed 0.28 - 0.32 h^{-1} as maximum specific growth rate.

The Table 3.1.7-a also shows that glucose was good carbon and energy sources for *C. lipolytica 1055* growth. A high yield coefficient of 0.7 mg cells/mg substrate was obtained in a batch culture containing 2 g/l glucose.

Moreover, only 3.2 g/l olive oil was converted into 2.3 mg / ml biomass. Then, a high yield coefficient was also reached (Y = 0.72 mg biomass / mg substrate) in presence of this commercial oil.

For each carbon source tested esterase production by *C. lipolytica 1055* was higher than lipase production (Table 3.1.7-a). Novotny et al. (1988) have obtained similar results for different *Candida* strains and *Y. lipolytica* that have been grown on complex media in presence of various carbon and nitrogen sources.

High glucose concentration inhibited lipase and esterase production by *C. lipolytica 1055*. In the presence of 2 g/l glucose lipase activity reached nearly 10 times more, while esterase activity reached 5 times more, in comparison with the activities in the presence of 20 g/l glucose.

Some authors have reported that glucose suppressed the lipase production by *R. oligosporus* (Nahas, 1988), *G. candidum* (Baillargeon et al., 1989) and *Trichosporon fermentans* (Chen et al., 1992).

Lipase production by *C. lipolytica 1055* reached maximum value, 2.6 U/mg dry weight, in presence of Tween-80 as carbon source.

It has been reported some contradictory evidences of lipase production in presence of olive oil. Lipase increased significantly when this oil was the sole carbon source for *G. candidum* (Jacobsen et al., 1989a) and *P. citrinum* (Maliszewska & Mastalerz, 1992). However, this lipid supressed lipase by *S. lipolytica, B. licheniformis, Micrococcus caseoliticus* and *Staphylococcus sp.* (Jonsson & Snygg, 1974), as well as by *P. roqueforti S-86* (Petrovic et al., 1990).

Esterase production by *C. lipolytica 1055* was several times higher following growth on Tween-80 than on olive oil and on glucose. The positive effect of Tween-80 on esterase synthesis could be explained through the chemical structure of this compound (an ester).

Extracellular esterase productivity reached maximum value 6.5 U/mg dry weight.h in presence of Tween-80 while extracellular lipase productivity attained only less than 1 U/mg dry weigh.h for all carbon sources investigated.

Table 3.1.7-b shows a comparison of lipase and esterase productivities by *C. lipolytica 1055* with maximum productivities calculated with the results reported for some lipase / esterase hyperproducing microorganisms.

The highest lipase productivity, 2.5 U/mg dry weight.h, was obtained in presence of glucose and olive oil as carbon and energy source by *S. lipolytica* (Gomi et al., 1986).

Although *C. lipolytica 1055* showed around 0.32 U/mg dry weight.h as maximum lipase productivity in presence of glucose (2 g/l), Tween-80 or olive oil (Table 3.1.7-a), this microorganism showed the highest esterase productivity in presence of Tween-80, in comparison with lipase/esterase hyperproducing microorganisms (Table 3.1.7-b).

3.2 FED-BATCH CULTURES

Fed-batch cultures were run in efforts to improve lipase production by *C. lipolytica 1055* considering that some enzyme productions have been enhanced by this operation in the last few years (Gibb et al., 1989; Giesecke et al., 1991; Lee & Parulekar, 1993).

On an industrial scale, if agitation in fed-batch culture were optimized, the introduction of the feed at one specific point into the reactor would not pose a problem. Namdev et al. (1992) have reported that the feed zone, i.e. zone of concentrated nutrients, had not affected *S. cerevisiae* biomass yield in a bench-scale reactor under fully aerobic conditions even if a complex medium is used.

MICROORGANISM	CARBON SOURCE	LIPASE (U/mg.h)°	ESTERASE (U/mg.h)°	REFERENCE
C. lipolytica 1055	TWEEN-80	0.32	6.5	-
C. lipolytica C_1B	WHEY BROTH & SPAN-80	-	3.6*	Kalle et al., 1972
C. rugosa	OLIVE OIL	0.9*	-	Valero et al., 1991-a
C. rugosa	OLEIC ACID	1.0*	0.4*	Obradors et al., 1993
G. candidum	OLIVE OIL & TWEEN-80	0.37*	-	Jacobsen et al., 1989-a
Ps. aeruginosa	TWEEN-80	1.3*	0.21*	Jane Gilbert et al., 1991
P. roqueforti	GLUCOSE	0.02*	-	Petrovic et al., 1990
R. delemar	OLEIC ACID	1.0*	-	Chen & McGill, 1992
S. lipolytica	GLUCOSE & OLIVE OIL	2.5*	-	Gomi et al., 1986

°ENZYME PRODUCTIVITY: U/mg cells.h
*CALCULATED RESULTS

TABLE 3.1.7-b COMPARISON OF MAXIMUM LIPASE AND/OR ESTERASE PRODUCTIVITIES FROM *C. lipolytica 1055* WITH HYPERPRODUCING MICROORGANISMS

C. lipolytica 1055 fed-batch experiments were carried out with inputs of oleic acid and olive oil in a 1,000 ml flask containing 600 ml of working volume at 30°C and 750 rpm magnetical impellor speed.

3.2.1 Tween-80 grown cells fed with oleic acid

Firstly, *C. lipolytica 1055* was cultivated under batch condition in presence of 4 g/l Tween-80 as carbon source until late exponential growth phase.

After lipase and esterase reached their maximum specific activities, around 8 h of batch culture, single and multi-perturbed oleic acid fed-batch experiments were carried out.

At each pulse time oleic acid was added to give a final concentration of 2 g/l.

Figure 3.2.1-a shows *C. lipolytica 1055* behaviour on Tween-80 grown cells fed with a single pertubed oleic acid pulse.

Oleic acid was found to stimulate growth of C. lipolytica 1055. Growth with Tween-80 as carbon source (μ_{MAX} = 0.14 h^{-1}) increased in the presence of oleic acid (single input) to give a μ_{MAX} = 0.31 h^{-1}.

In addition, biomass concentration was enhanced by a factor of around 2.2 in comparison to batch experiment.

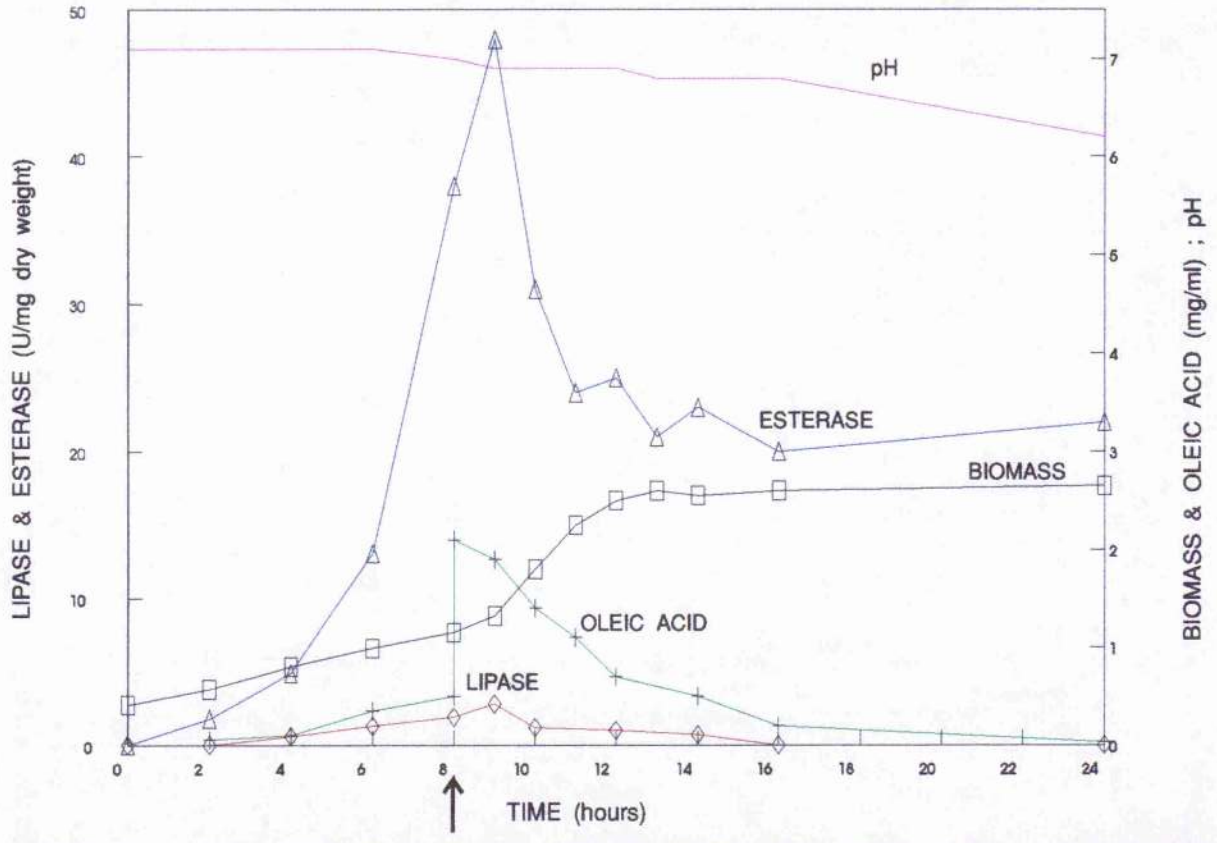


FIGURE 3.2.1-a BEHAVIOUR OF *C. lipolytica 1055* IN SINGLE-PERTURBED OLEIC ACID FED-BATCH

The arrow indicates when the pulse was introduced.

The microorganism showed a small lag phase when the fatty acid was suddenly added to the reactor. *C. lipolytica 1055* may not have cells easily adapted to oleic acid, i. e. the lipid metabolising enzymes are not available in this yeast cells.

Immediately after the oleic acid input lipase and esterase activities reached their maxima. As biomass concentration increased, specific enzyme activities per unit of cells showed sharp peaks.

C. lipolytica 1055 did not improve lipase nor esterase production by fed-batch culture in comparison to batch fermentations.

Figure 3.2.1-b shows *C. lipolytica 1055* behaviour on Tween-80 grown cells fed with multi-perturbed oleic acid fed-batch culture.

A maximum specific growth rate of 0.32 h^{-1} was attained after the first oleic acid input. This value remainined constant upon two additional oleic acid pulses.

A significantly higher biomass concentration was reached by multi-perturbed oleic acid fed-batch culture of *C. lipolytica 1055.* This value corresponds to near three-fold the biomass in the single-perturbed oleic acid fed-batch culture.

The higher biomass concentration obtained (6.1 mg/ml) gave a decrease in lipase and esterase

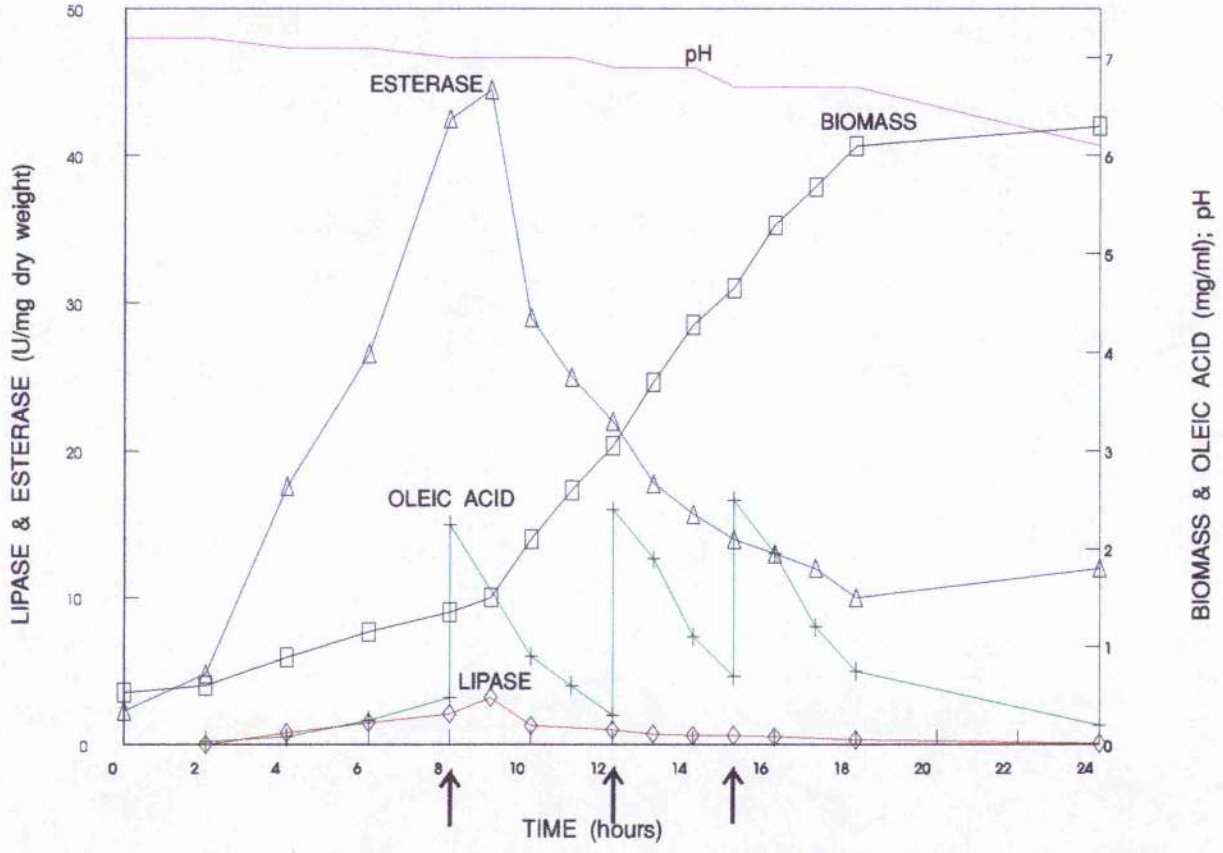


FIGURE 3.2.1-b BEHAVIOUR OF *C. lipolytica 1055* IN MULTI-PERTURBED OLEIC ACID FED-BATCH

The arrows indicate when the pulses were introduced.

activities per unit of cells. One hour after the pulse esterase reached 44 U/mg dry weight while lipase reached 3 U/mg dry weight as maximum activities.

However, volumetric esterase production was kept constant (58 - 67 U/ml) during three days. The stabilizing effect on lipase synthesis was less. Lipase was not detected nine hours after the third input.

C. lipolytica 1055 fed-batch culture was able to keep esterase production rate constant for longer than batch mode.

Similar results have been reported by Chevalot & Marc (1993) as one advantage of fed-batch operation over batch process.

Figures 3.2.1-a and 3.2.1-b show the oleic acid present in the reactor throughout the two sets of experiment. The highest values observed immediately after the fatty acid inputs decreased gradually over the time.

These Figures also show some changes in pH values. The pH decreased with approximately the same rate until 24 h in single-perturbed oleic acid input and until 48 h in multi-perturbed oleic acid input. The changes in pH might be related to the production of organic acids by yeast cells.

Porro et al. (1991) have suggested a fed-batch process for the manufacturing of baker's yeast based only on increase/decrease pH values. The authors observed

that *S. cerevisae* haploid strain S288C (mal, mel, gal2, SUC2, CUP1 and MATα) cultivated under fed-batch process, changed the pH in function of its metabolic cellular activities.

Oleic acid is known to enhance level of lipase/esterase production for *C. rugosa* (Valero et al., 1988; Del Rio et al., 1990), *P. citrinum* (Maliszewska & Mastalerz, 1992), *R. delemar* (Chen & McGill, 1992).

On the other hand, Obradors et al. (1993) have reported that lipase activity from *C. rugosa* showed an inhibitory effect in the presence of more than 1 g/l oleic acid. In addition, Jonsson (1976) has reported that *C. lipolytica* lipase was activated by oleic acid although higher amount of this fatty acid showed a negative effect on lipase production.

Fed-batch mode did not improve lipase and esterase productions by *Ps. aeruginosa EF2* (Jane Gilbert et al., 1991a). The culture was grown as for batch omitting Tween-80 from the medium which was continuously added using a peristaltic pump at 0.09 h^{-1}.

C. lipolytica 1055 oleic acid fed-batch cultures with single and multi-feedings exhibited similar levels of specific lipase and esterase. Specific enzymatic activities did not improve significantly in the multi-perturbed oleic acid fed-batch as the added oleic

acid mainly contributed to the microorganism growth (Figure 3.2.1-b).

Lee & Parulekar (1993) have reported a negative correlation between growth of a recombinant *B. subtilis* with its α-amylase production. These authors found that conditions favouring cell growth were not efficient for enzyme production.

3.2.2 Olive oil grown cells fed with further olive oil

In the experiments with olive oil grown cells fed with further olive oil, *C. lipolytica 1055* was grown in presence of olive oil as carbon source under batch conditions until the late logarithmic phase when lipase and esterase activities reached their maxima.

After each olive oil input (3.2 g/l olive oil final concentration), the microorganism responded to the nutrient rich environment by regulating its cellular metabolism.

Figures 3.2.2-a and 3.2.2-b show that the additional carbon source present in the single and multi-perturbed fed-batch enhanced microbial growth.

The maximum specific growth rate in the presence of olive oil as carbon source (μ_{max} = 0.34 h^{-1};

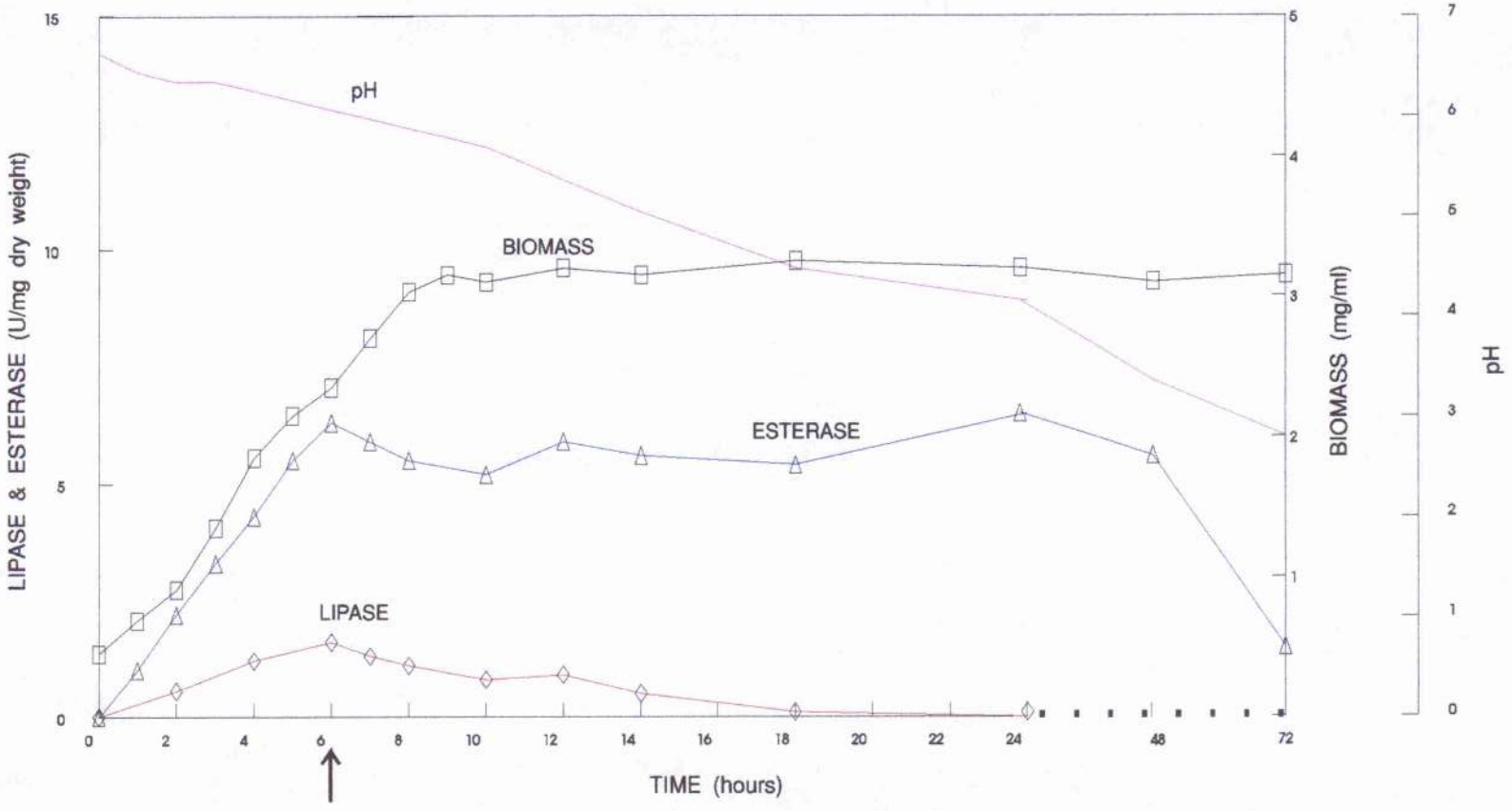


FIGURE 3.2.2-a BEHAVIOUR OF *C. lipolytica 1055* IN SINGLE-PERTURBED OLIVE OIL FED-BATCH

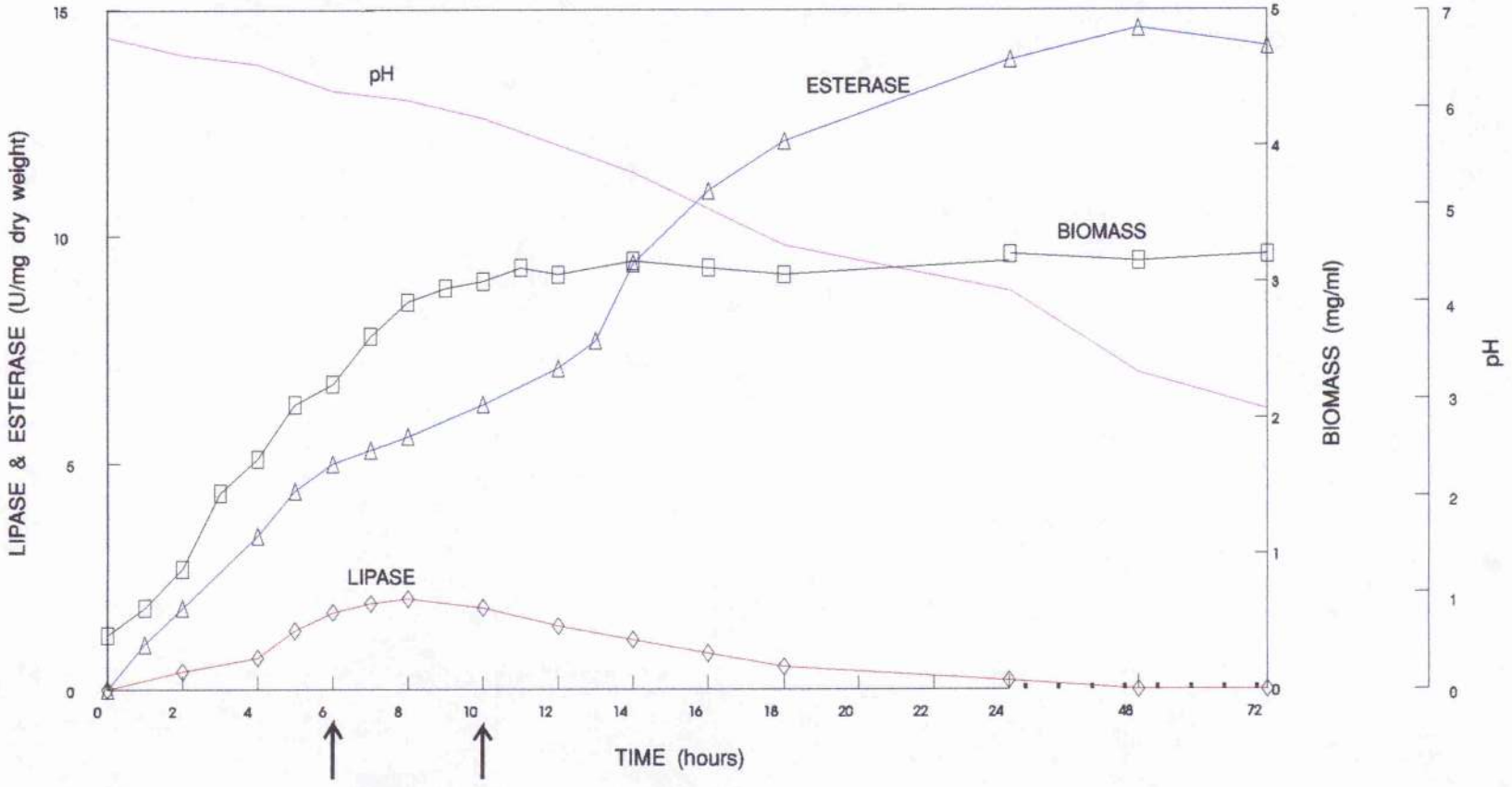


FIGURE 3.2.2-b BEHAVIOUR OF *C. lipolytica 1055* IN MULTI-PERTURBED OLIVE OIL FED-BATCH

The arrows indicate when the pulses were introduced.

Appendix 5) was maintained after the first olive oil pulse.

The presence of olive oil promoted growth although, over extended fed-batch operation, the microorganism did not exhibited the same maximum growth rate. Immediately after the second input in the multi-perturbed olive oil fed-batch culture of *C. lipolytica 1055*, the biomass concentration reached a maximum value of 3.2 mg/ml (which was not increased by further inputs of olive oil).

In this case, the added carbon source was readily metabolised until nitrogen became limiting due to the fact that a pulse experiment with yeast extract further enhanced biomas concentration.

The Figures 3.2.2-a and 3.2.2-b also show pH values throughout the two sets of olive oil fed-batch experiments. The pH decreased from a neutral value at the beginning of the experiments to an acid value (pH = 3.0) after three days of cultivation. Then, a large amount of oleic acid is produced in he presence of olive oil.

Specific lipase and esterase activities for single and multi-perturbed olive oil fed-batch experiments are also shown in the Figures.

In single pulse experiment esterase activity reached a level of 6 U/mg dry weight until 24 h while the maximum specific lipase reached at pulse time 1.8 U/mg

dry weight, maintaining the activity constant (about 1.0 U/mg dry weight) during six hours after the pulse.

In multi-perturbed olive oil fed-batch *C. lipolytica 1055* ceased growth after 10 h of cultivation while esterase synthesis occurred over an extended period with substantial production of enzyme. This activity increased from 5 to 14 U/mg dry weight and the maximum value was maintained approximately constant until 72 h of cultivation.

The maximum lipase activity reached after the first olive oil input (2 U/mg dry weight) decreased slowly until no detectable level after 48 h of cultivation.

Analyses of these results may indicate that olive oil and/or oleyl residue present in the broth prolonged lipase and esterase production suggesting that olive oil fed-batch culture was able to keep enzyme production rates constant for longer than in oleic acid fed-batch.

Valero et al. (1988) have reported that lipase production by *C. rugosa* fed-batch experiment, involving olive oil grown cells fed with olive oil, reached the same level as in batch growth.

On the other hand, higher lipase production was obtained by *C. rugosa* grown on glucose in batch culture following olive oil fed-batch input (Valero et al., 1991a).

Ishihara et al. (1989) have reported that *Ps. fluorescens* exhibited an effective extracellular lipase production by semi-batch culture. Cells grown on olive oil were subjected to turbidity-dependent automatic feeding of both olive oil and Fe^{++} ions in fed-batch operation. These authors suggested that excessive olive oil concentration in the culture suppressed lipase production.

Lipase and esterase productivities by *C. lipolytica 1055* found to be not improved by fed-batch processes in comparison to batch cultivations.

On the other hand, the enzyme production phases under multi-perturbed fed-batch was prolonged suggesting that repeated fed-batch culture could be a route towards to improve lipase/esterase production by *C. lipolytica 1055*.

3.3 CHEMOSTATIC CULTURE

3.3.1 Carbon Source: Glucose

3.3.1.1 Behaviour of *C. lipolytica 1055* at different dilution rates

C. lipolytica 1055 was grown in chemostatic culture under conditions specified in the Figure 3.3.1.1-a. The values presented are an average of two sets of

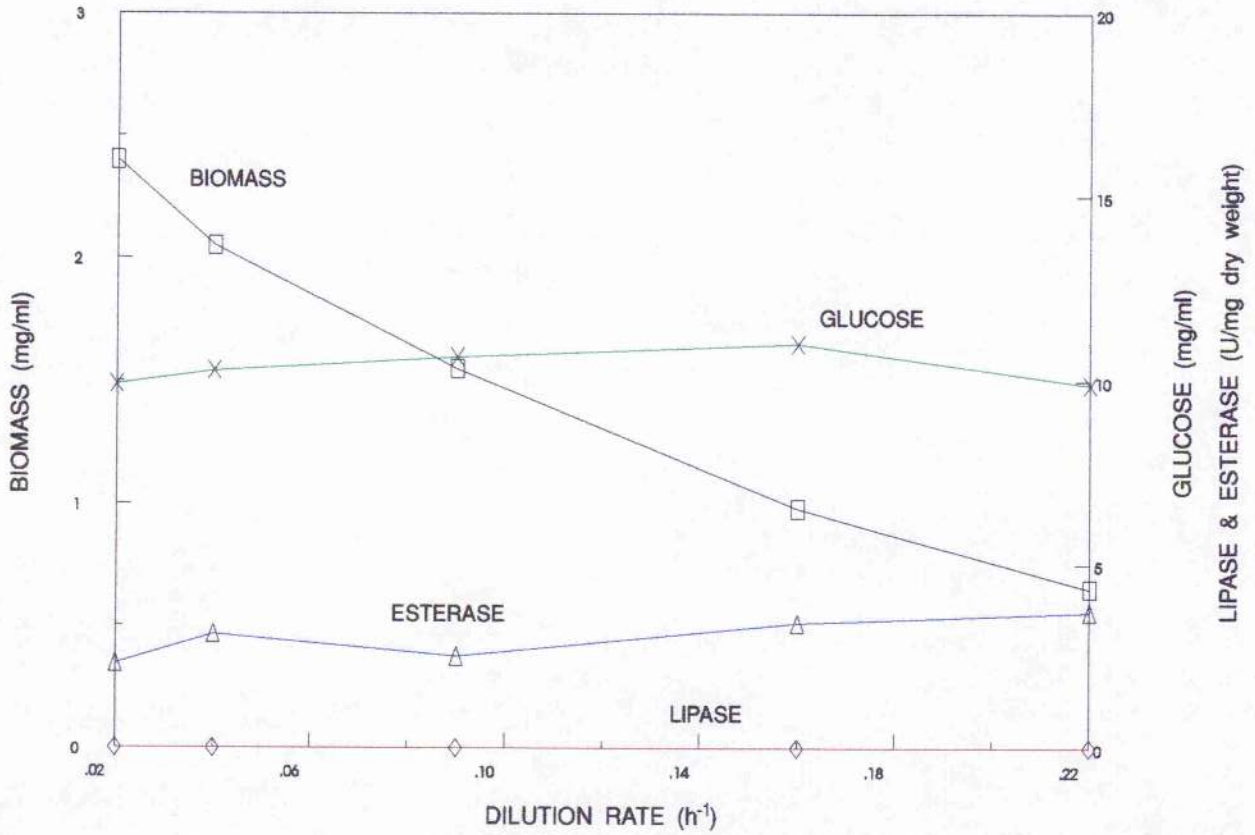


FIGURE 3.3.1.1-a CHEMOSTATIC DATA OF *C. lipolytica 1055* UNDER THE CONDITIONS:

MEDIUM: 20 g/l glucose; 5 g/l yeast extract; 0.1 ml/l antifoam A
TEMPERATURE: 30 °C
AGITATION: 500 rpm
AERATION: 1 vvm

independent experiments. The samples were analysed for biomass, glucose concentration and lipase and esterase activities during at least 10 generation times in each dilution rate investigated.

The microorganism showed deviation from ideal chemostatic behaviour due to the fact that biomass average values decreased when dilution rates increased from 0.02 to 0.22 h^{-1}.

Klasson et al. (1989) have reported that a strain of *C. lipolytica* has shown a linear inverse relationship between biomass and dilution rate in a single-stage continuous reactor in presence of glucose as carbon source for different ammonia concentrations.

A similar effect has been shown for *C. utilis* under continuous culture in presence of sulfite liquor (Camhi & Rogers, 1976) and reducing sugars and starch (Pasari et al., 1989).

The conversion of substrate into intracellular storage compounds under some limiting conditions is not accounted for in the simple Monod's theory (Monod, 1949).

As noted in the Figure 3.1.1-a, *C. lipolytica 1055* showed intracellular lipid formation during batch growth in the presence of 20 g/l glucose.

In addition, some *Candida* strains growing under glucose-limited chemostatic conditions have also accumulated lipid (Gill et al., 1977; Heredia & Ratledge,

1988). Chepigo et al. (1969) have reported that *C. lipolytica* growing in the presence of oil distillates as a feed medium, accumulated lipid as storage material.

Deviation from simple chemostatic theory is a poorly understood area. However, decrease in biomass concentration in relation to dilution rate has been shown for microorganisms capable of storing compounds under limiting conditions (Marison, 1988).

Thus, *C. lipolytica 1055* behaviour in chemostatic culture in the presence of 20 g/l glucose in the feed medium compares well with the chemostatic deviation where the biomass concentration shows a linear inverse relationship with the dilution rate.

The Figure 3.3.1.1-a also shows that residual glucose was kept at approximately 10 mg/ml at all dilution rates investigated. Therefore, glucose was only 50 % metabolized which shows that this sugar was poorly utilised by the yeast.

Dean (1972) has reported that a culture of streptococcus (strain H64) continuously fed by a complex medium containing glucose as carbon source has shown an excess of glucose in the wash out. The author suggested that the growth rate of the culture was limited by some unknown substance although the streptokinase activity (streptococcus enzyme involved in tissue infection) was constant irrespective of the dilution rate.

In continuous culture an incomplete glucose utilization by *C. utilis NRRL Y-900* could be explained by a deficient dissolved oxygen and/or an imbalanced nutritional culture in presence of cane molasses in the feed medium (Ghoul et al., 1991).

In addition, Alexander et al. (1989) have reported that the accumulation of ethanol in *C. shehatae* continuous culture caused an inhibitory effect in glucose metabolism. Anaerobic respiration has been suggested as the metabolic pathway followed by *C. rugosa* grown on glucose, yielding 9.9 g/l ethanol under batch conditions with a shortage of dissolved oxygen in the medium (Valero et al., 1991a).

C. lipolytica 1055 exhibited ideal chemostatic behaviour under carbon limitation in the presence of 2 g/l glucose and 0.5 g/l yeast extract as a feed medium, at dilution rates between 0.02 and 0.12 h^{-1} (Figure 3.3.1.1-b). Biomass concentration was maintained constant at approximately 0.7 mg/ml for all the dilution rates investigated.

In general, yeasts tolerate sugar concentrations between 0.2 and 2 % well, being inhibited at 20 %.

C. lipolytica 1055, however, totally metabolised 0.2 % glucose, being inhibited at 2 %. It follows that *C. lipolytica 1055* is 10-fold less

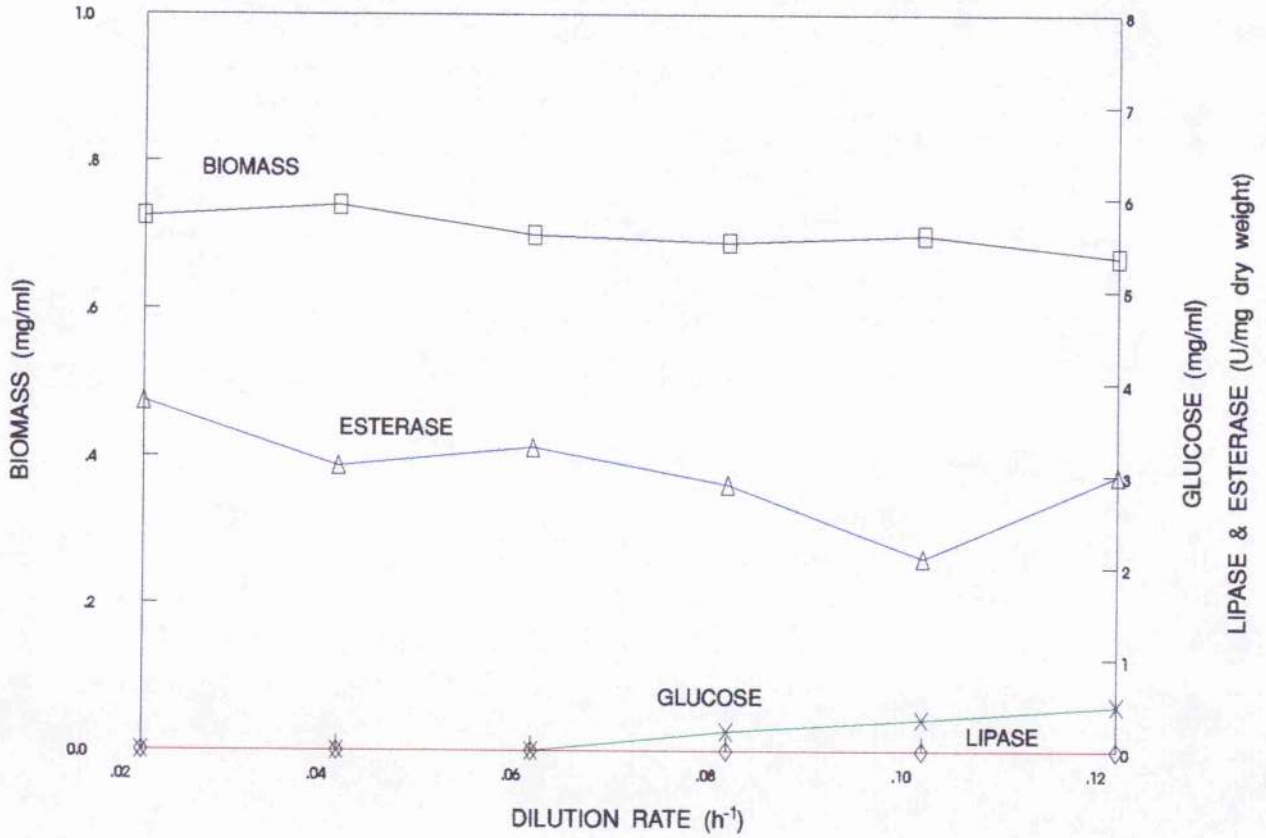


FIGURE 3.3.1.1-b CHEMOSTATIC DATA OF *C. lipolytica 1055* UNDER GLUCOSE LIMITATION:

MEDIUM: 2 g/l glucose; 0.5 g/l yeast extract; 0.1 ml/l antifoam A
TEMPERATURE: 30 °C
AGITATION: 500 rpm
AERATION: 1 vvm

efficient in its capability to metabolize glucose as carbon source than other yeasts.

The Figure 3.3.1.1-b shows that glucose was not totally metabolised at dilution rates above 0.08 h^{-1}, remaining in the reactor near 25 % of this sugar concentration at the dilution rate of 0.12 h^{-1}.

A similar effect was reported for *Klebsiella pneumoniae* grown on xylose; microorganism behaviour at steady-state conditions showed 'true limitation' only at low dilution rates. (Baloo & Ramkrislna, 1991a).

In addition, Martin et al. (1992) have reported that the residual carbon concentration in sucrose-limited growth depended on the feed medium composition; for example, when fed only with sucrose and yeast extract, *B. stearothermophilus LLD-15* consumed 92 % of the sucrose at a dilution rate of 0.2 h^{-1} while 95 % were consumed when tryptone was also present in the incoming medium.

In the case of *C. lipolytica 1055* verification of the glucose limitation was provided by analysis of residual carbon concentration as well as by pulsing with sterile glucose. After a direct glucose pulse into the fermenter at a dilution rate of 0.09 h^{-1}, an increase in biomass concentration confirmed glucose limitation.

In the two sets of experiments, 2 and 20 g/l glucose as carbon source in the feed medium, the

extracellular lipase activity was not detectable while the extracellular esterase activity reached around 2 - 3 U/mg dry weight at all dilution rates studied (Figures 3.3.1.1-a and 3.3.1.1-b).

It is relevant to specify that during steady-state conditions the pH values varied between 4.6 to 5.5. This acidic range has been shown not to be ideal for lipase/esterase activities (Jonsson & Snygg, 1974; Paparaskevas et al., 1992).

Proteolytic activity might affect the stability of lipase in continuous culture in the presence of 2 and 20 g/l glucose as a feed medium. However, the absence of *C. lipolytica 1055* lipase activity during chemostatic culture could not be justified by the presence of proteases in the broth due to the fact that protease tests were negative despite the positive protease test under batch conditions (Section 3.1.6).

A factor worthy of attention concerns lipase production by *Ps. fluorescens 378* in continuous culture in the presence of succinic acid, NH_4Cl, KH_2PO_4 and ammonium citrate; lipase production was not detected under nitrogen limitation while it was lost after 8 to 15 retention times (retention time = dilution rate x time) under carbon, phosphorus or oxygen limitation. Under iron limitation and at low dilution rates lipase production was maintained for more than 30 retention times (Persson et al., 1990).

A negative effect of glucose on lipase production under batch conditions was already been discussed in Section 3.1.7. In addition, Macrae (1983) has reported that *G. candidum* lipases were synthesized after glucose has been exhausted from the medium, synthesis being repressed at high glucose concentrations.

Moreover, Falk et al. (1991) have suggested that lipase from recombinant *Staphylococcus carnosus* was inactivated by different factors that contribute to changes in protein structure.

Lipase synthesis requires further investigation. The activity of lipases depends on a complex interaction between the cell and the environment as well as between the enzyme and the environment.

3.3.1.2 Nitrogen pulses

The pulse technique as applied to chemostatic cultures is a very efficient tool for nutrient utilization studies (Goldberg & Er-el, 1981; Martin et al., 1992).

Due to the fact that only 50 % of glucose was metabolised by *C. lipolytica 1055* in continuous culture in the presence of 20 g/l glucose in the feed medium, pulsing experiments were carried out in an attempt to determine the inhibiting factor.

Figure 3.3.1.2 shows *C. lipolytica 1055* nitrogen pulses under steady-state conditions at 0.16 h^{-1} in the presence of 20 g/l glucose in the feed medium.

Yeast extract at 5 g/l (final concentration) was added to the reactor to check the system in respect of rate limiting nitrogen while the peptone pulsing (5 g/l final concentration) was carried out, considering that Okeke & Okolo (1990) have reported good results in lipase production with this enzymatic hydrolysate from protein.

However, the two different pulses showed no evident effect or disturbance of the culture despite the sudden nitrogen source addition to the chemostatic flow system.

Thus, chemostatic culture of *C. lipolytica 1055* in presence of 20 g/l glucose as a feed medium was found not to be nitrogen-limited.

3.3.1.3 Metal ion pulse

Metal ions have a very important role in the action of many enzymes, increasing their activities, particularly those involved in glucose metabolism.

Calcium ions can precipitate released fatty acids as their soaps, preventing their inhibitory effect on lipolysis while magnesium ions can release cell-wall

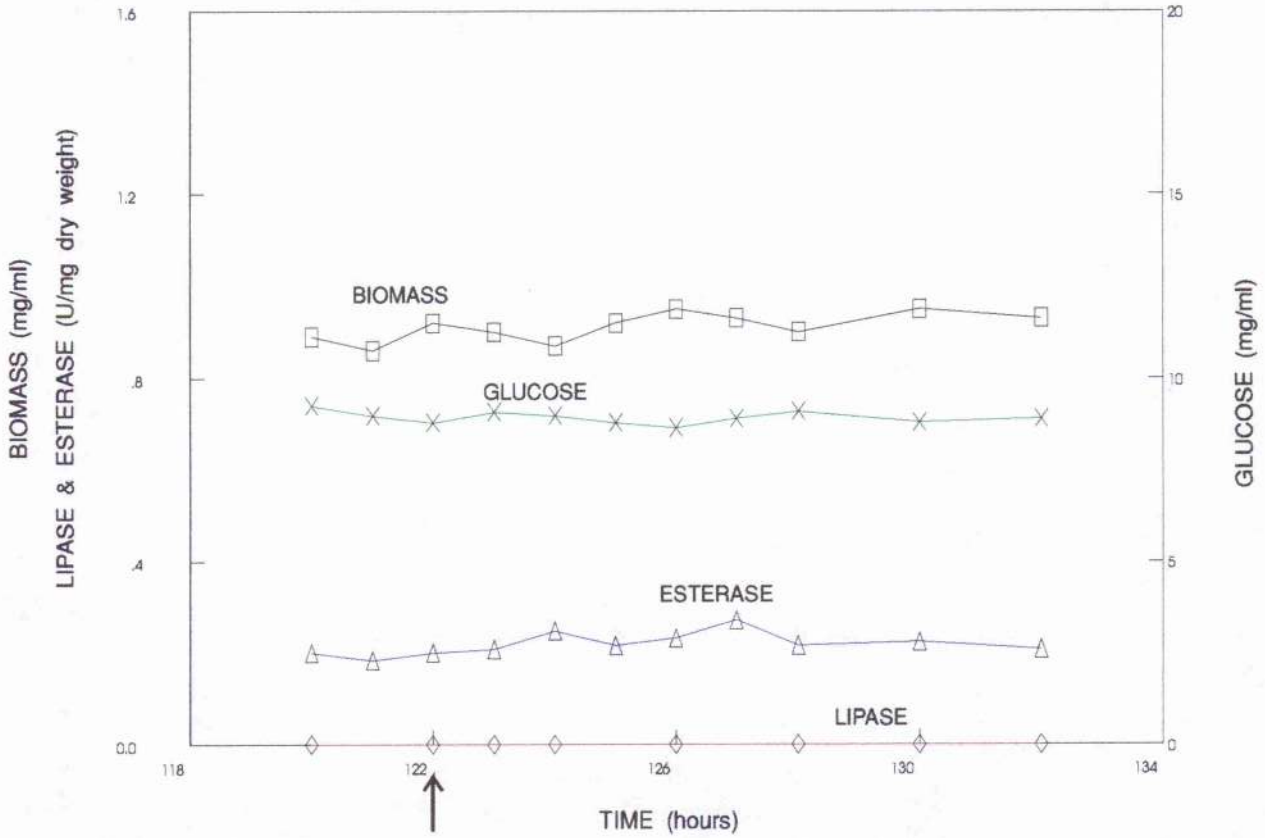


FIGURE 3.3.1.2 YEAST EXTRACT AND PEPTONE PULSINGS OF *C. lipolytica 1055* CHEMOSTATIC CULTURE CONTINUOUSLY FED BY 20 g/l GLUCOSE AT THE DILUTION RATE OF 0.16 h^{-1}

The arrow indicates when a pulse was introduced.

bound lipase, increasing extracellular lipase production (Hegedus & Khachatourians, 1988; Kasuto et al., 1989).

Goldberg & Maximum specific lipase reached 1.8 U/mg dry weight 1 h after the first olive oil input, maintaining the activity constant (about 1.0 U/mg dry weight) during six hours after the second input.

Goldberg & Er-el(1981) have shown that some *Candida* strains require Mg^{++} and Ca^{++} ions for optimum growth in continuous culture of SCP production.

A pulse with a stock solution of salts according to Rosenberger & Elsden (1960) was carried out in *C. lipolytica 1055* steady-state conditions at a dilution rate 0.16 h^{-1} in the presence of 20 g/l glucose as a feed medium. See Section 2.3 for stock salt solution composition.

As some authors have shown that Fe^{++} and Cu^{++} ions inhibited lipase production (Hegedus & Khachatourians, 1988; Petrovic et al., 1990) these metals were excluded from the stock solution.

The additional metal ion pulse did not disturb the continuous culture (Figure 3.3.1.3). The presence of metal ions such as Ca^{++} and Mg^{++} present in the stock solution did not stimulated esterase nor lipase production by *C. lipolytica 1055* chemostatic culture (20 g/l glucose).

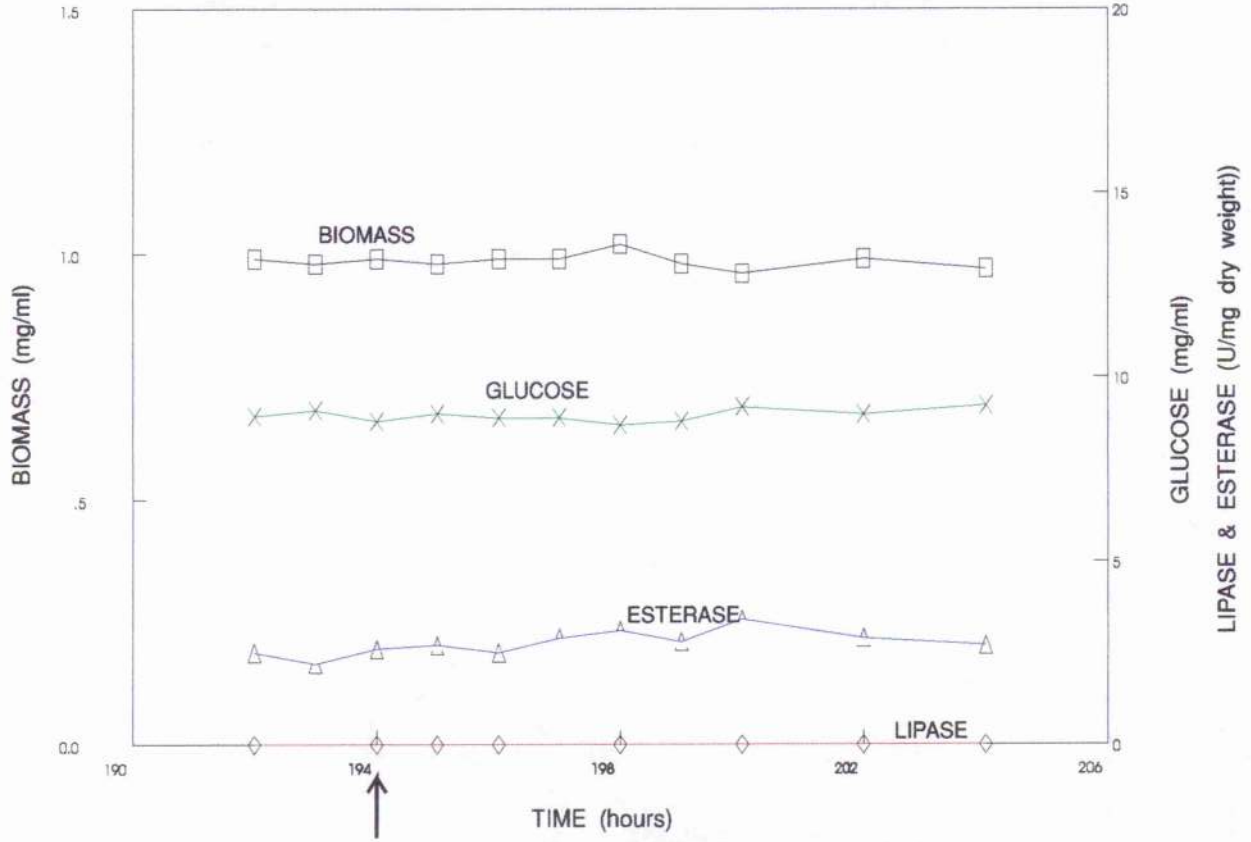


FIGURE 3.3.1.3 METAL ION PULSING OF *C. lipolytica 1055* CHEMOSTATIC CULTURE CONTINUOUSLY FED WITH 20 g/l GLUCOSE AT THE DILUTION RATE OF 0.16 h^{-1}

The arrow indicates when the pulse was introduced.

3.3.1.4 Olive oil and triolein pulses

Many researchers have contributed to the understanding of the effect of lipid material on lipase production. Espinosa et al. (1990) and Petrovic et al. (1990) have shown that some oils depress microorganism growth while lipase production is enhanced in batch cultures.

Harder & Dijkhuizen (1983) have reported that microorganism will increase the synthesis of enzymes required for the metabolism of the limiting nutrient in chemostatic cultures.

Therefore, growth and lipase/esterase activities may well be related with lipid-limited microorganisms.

C. lipolytica 1055 pulses with triolein at 1 % and olive oil at 1 % (final concentrations) can be seen in the Figure 3.3.1.4. The culture continuously fed with 20 g/l glucose, was under steady-state conditions at a dilution rate of 0.16 h^{-1} before the two different pulses were introduced into the reactor.

As noted in the same Figure the biomass concentration increased more than three fold when compared to steady-state biomass values.

As lipid material requires enzymatic hydrolysis outside the cell, lipase activity was subsequently activated. After 1 h of the two different pulses a

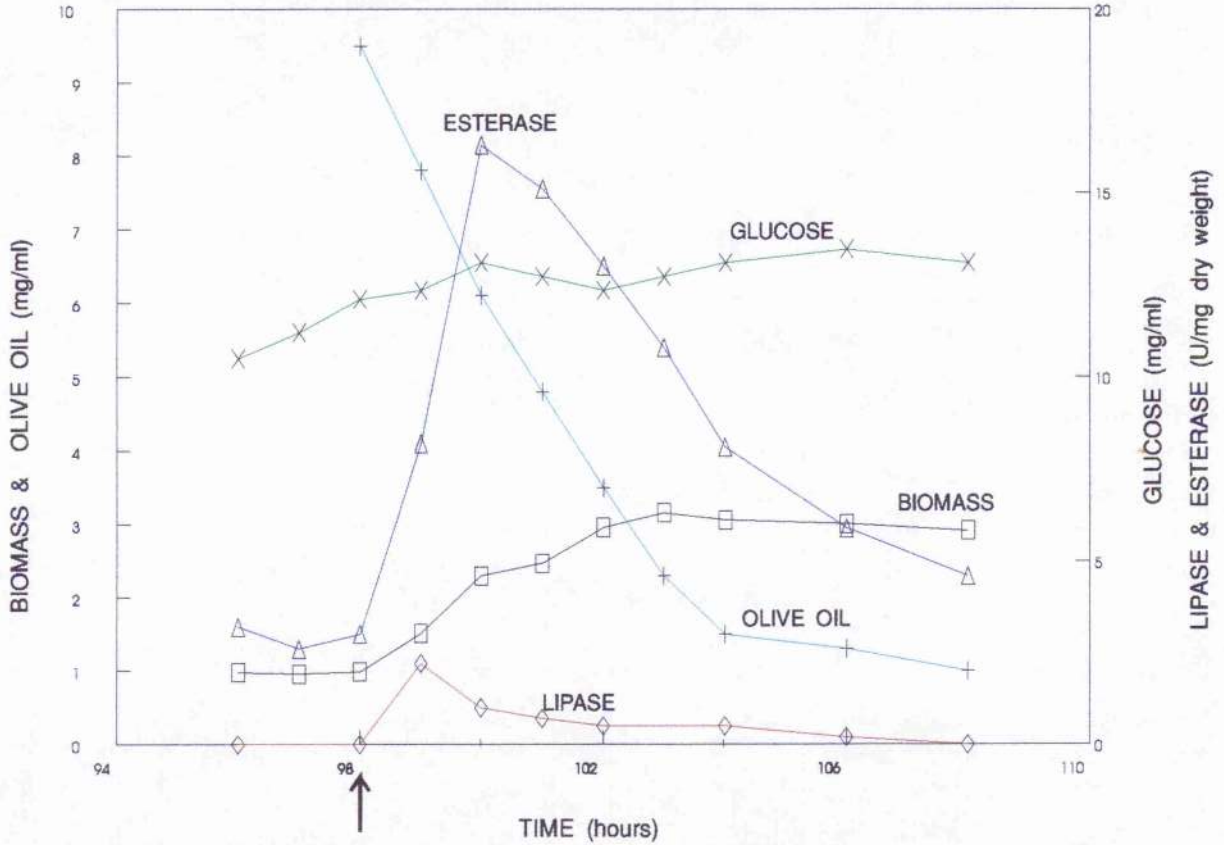


FIGURE 3.3.1.4 OLIVE OIL AND TRIOLEIN PULSINGS OF *C. lipolytica 1055* CHEMOSTATIC CULTURE CONTINUOUSLY FED WITH 20 g/l GLUCOSE AT THE DILUTION RATE OF 0.16 h^{-1}

The arrow indicates when the pulses were introduced.

maximum lipase activity of about 2 U/mg dry weight was obtained while esterase activity reached 16 U/mg dry weight.

However, these enzymes were unstable and 8 h later about 80 % of esterase activity was lost while lipase was no longer detectable.

In summary, olive oil and triolein pulsing experiments suggested that the presence of triolein and olive oil induce lipase and esterase production but some unknown substance and/or a physical parameter perhaps acts as the rate-limiting factor due to the excess of glucose in all experiments (Figure 3.3.1.4).

Considering the negative effect of glucose on lipase/esterase production as reported by several authors (Nahas, 1988; Baillargeon et al., 1989; Chen et al., 1992), lipase and esterase production by *C. lipolytica 1055* could be catabolite repressed by this sugar.

3.3.1.5 Tween pulses

Biological detergents have been shown to stimulate lipase production by *R. oligosporus* (Nahas, 1988), *G. candidum* (Jacobsen et al., 1989), *R. delemar* (Espinosa et al., 1990), *Acremonium strictum* (Okeke & Okolo, 1990).

C. lipolytica 1055 chemostatic culture at the dilution rate of 0.08 h^{-1} under carbon limitation (2 g/l glucose at feed medium) was pulsed with Tween-20, -40, -60 and -80. The different compositions of these compounds are shown in the Table 3.1.4-b.

Figure 3.3.1.5-a shows pulsing experiments with Tween-20, -40 and -60 at 1 % (final concentration).

These three different experiments had no apparent effect on biomass concentration, nor in lipase activity while esterase activity decreased immediately after the addition of these detergents. It may be suggested that lauric, palmitic and stearic acids do not stimulate *C. lipolytica 1055* lipase while esterase production was repressed under test conditions.

Figure 3.3.1.5-b shows the *C. lipolytica 1055* pulse experiment with Tween-80 at 1 % (final concentration) carried out at the dilution rate of 0.08 h^{-1} under carbon limitation.

Tween-80 containing about 75 % oleic acid (Table 3.1.4-b), increases the release of protein and exerts a stabilizing effect on the lipase production (Jacobsen et al., 1989; Espinosa et al., 1990; Jane Gilbert et al., 1991a).

The biomass concentration of *C. lipolytica 1055* increased following analyses of the transient

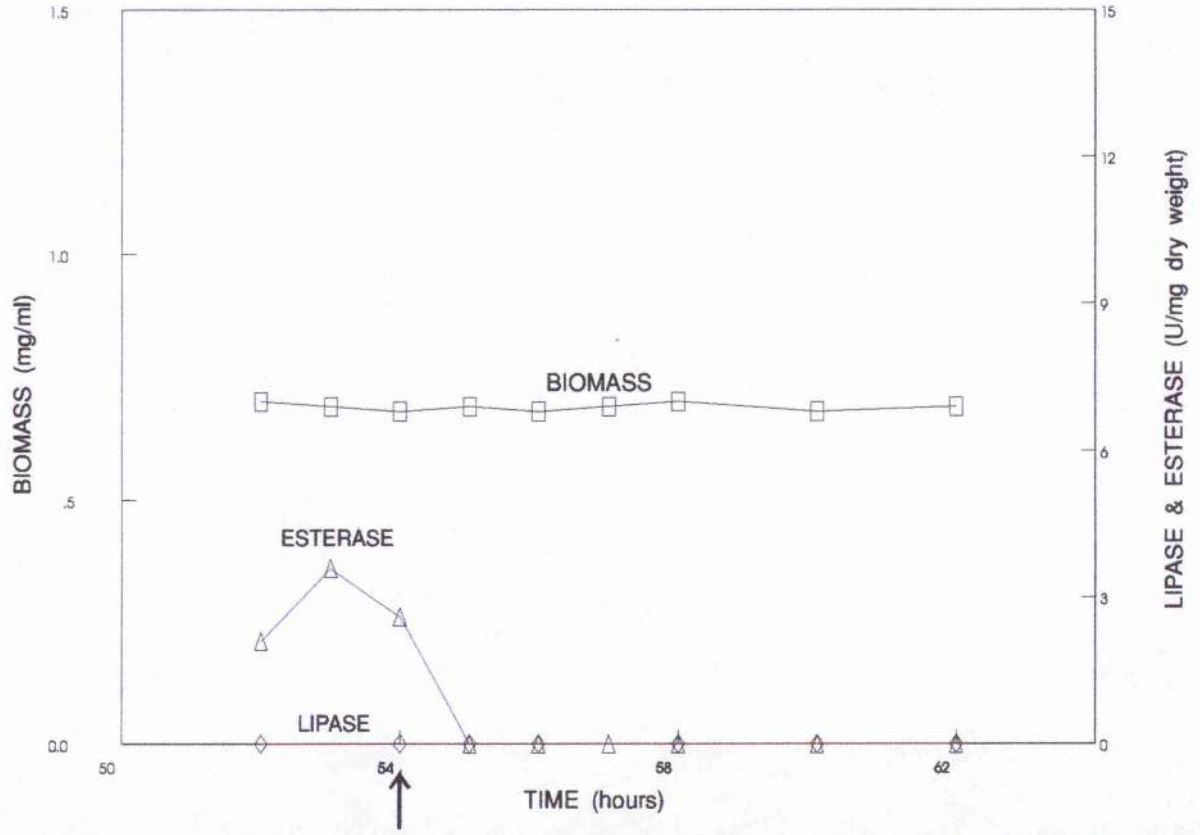


FIGURE 3.3.1.5-a TWEEN-20, 40 AND 60 PULSINGS OF *C. lipolytica 1055* GLUCOSE-LIMITED CHEMOSTATIC CULTURE AT THE DILUTION RATE OF 0.08 $h.^{-1}$

The arrow indicates when the pulse was introduced.

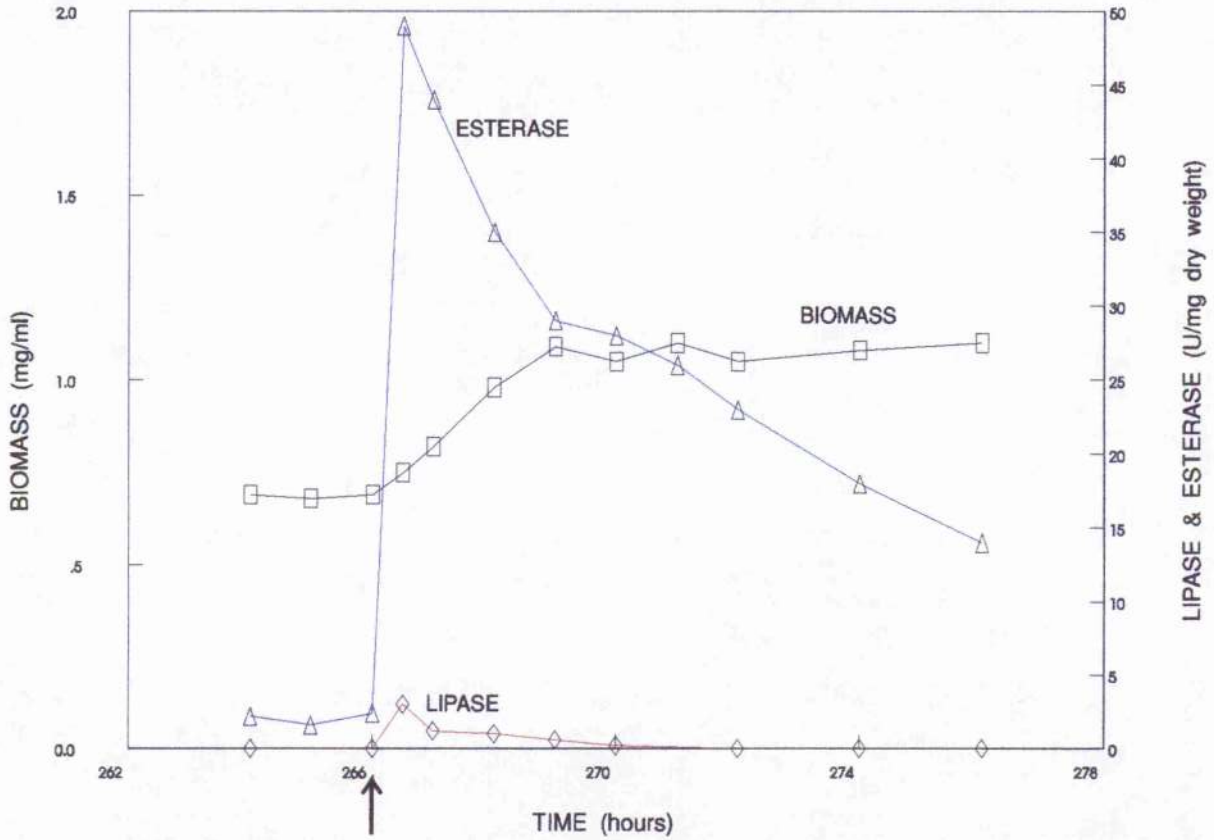


FIGURE 3.3.1.5-b TWEEN-80 PULSING OF *C. lipolytica 1055* GLUCOSE-LIMITED CHEMOSTATIC CULTURE AT THE DILUTION RATE OF 0.08 h^{-1}

The arrow indicates when the pulse was introduced.

microorganism behaviour immediately after the Tween-80 pulse.

Tween-80 increased *C. lipolytica 1055* esterase production approximately 16-fold than under steady-state conditions. However, more than 70 % of this activity was lost 10 h later.

Lipase production by C. lipolytica 1055 was induced by Tween-80 although the maximum activity reached approximately 3 U/mg dry weight which was no longer detectable (Figure 3.3.1.5-b).

3.3.2 Carbon source: Tween-80

3.3.2.1 Behaviour of C. lipolytica 1055 at different dilution rates

Figure 3.3.2.1 shows the behaviour of *C. lipolytica 1055* grown in the presence of 4 g/l Tween-80 and 2 g/l ammonium sulphate at 30°C, 500 rpm and 1 vvm under steady-state conditions.

A fair agreement with the general theory of continuous culture (Monod, 1949) could be established for *C. lipolytica 1055* on Tween-80 as carbon limiting.

Biomass concentration was kept constant at approximately 0.12 mg/ml in a range of dilution rate from 0.02 to 0.10 h^{-1}.

In all steady-state conditions at each dilution rate and during at least 10 generation times, esterase

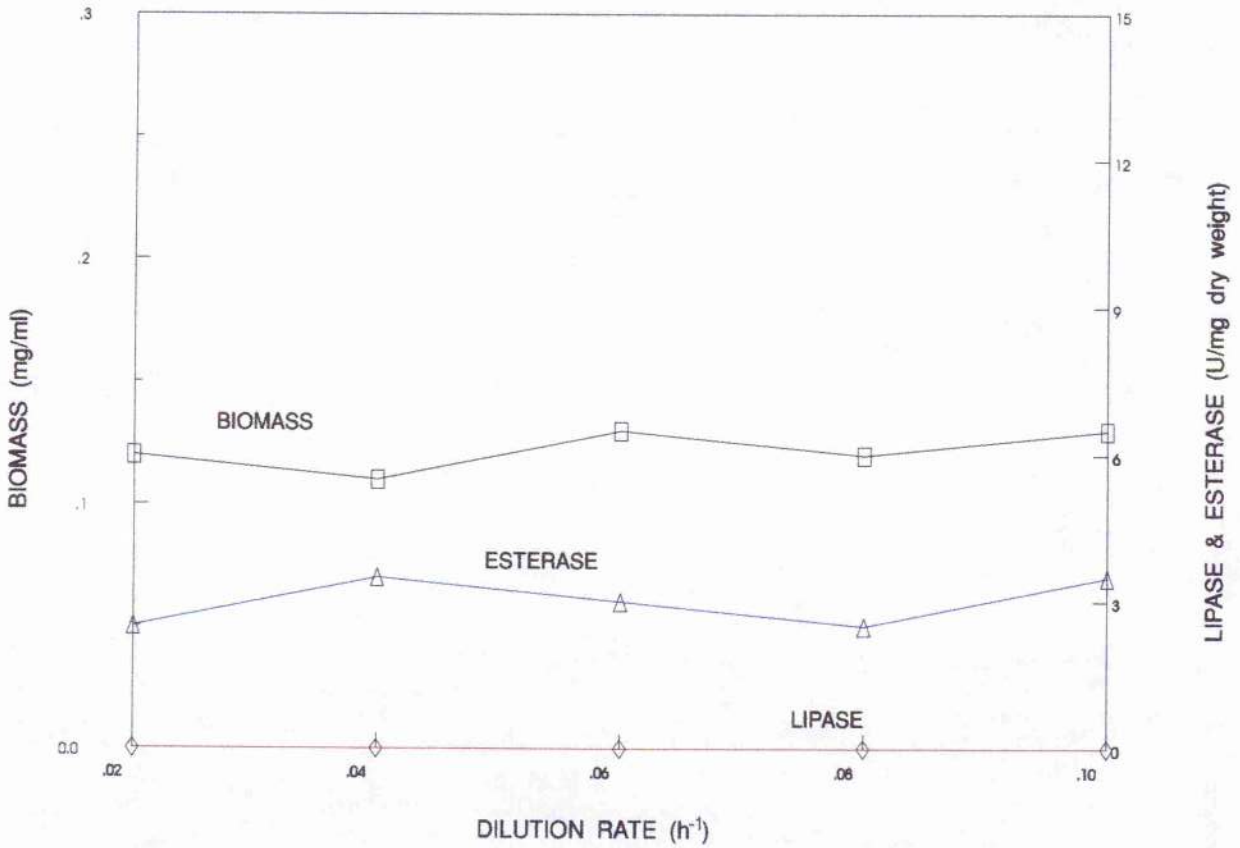


FIGURE 3.3.2.1 CHEMOSTATIC DATA OF *C. lipolytica 1055* CONTINUOUSLY FED WITH 4 g/l TWEEN-80 AND 2 g/l AMMONIUM SULPHATE AT 30 °C

activity reached only 2-3 U/mg dry weight while lipase activity was not detectable (Figure 3.3.2.1).

Meanwhile, esterase activity provides a satisfactory explanation for the observed growth.

In contrast, *Ps. aeruginosa EF2* has exhibited maximum lipase and esterase activities (27.8 U/mg cells and 5.35 U/mg cells, respectively) in the Tween-80-limited culture at the low dilution rate of 0.04 h^{-1}. Both of these activities were inversely related to the dilution rate and were approximately four-fold superior to the highest values under batch conditions in the presence of the same carbon source (Jane Gilbert et al., 1991a).

During *C. lipolytica 1055* Tween-80-limited culture at steady-state conditions the pH was kept constant at 6.8 - 7.0, probably due to the phosphate present in the medium (Section 2.4).

A pulse experiment with 4 g/l Tween-80 (final concentration in the reactor) at the dilution rate of 0.08 h^{-1} disturbed the steady-state conditions, increasing biomass. Thus, *C. lipolytica 1055* chemostatic culture was carbon-limited under the test conditions.

3.3.2.2 Oleic acid pulses

Three different pulses were carried out with oleic acid at 0.5, 2.0 and 10.0 g/l (final concentrations) in the presence of 4 g/l Tween-80 as a feed medium and steady-state conditions at the dilution rate of 0.08 h^{-1}.

Although the production of lipases by *Acremonium strictum* (Okeke & Okolo, 1990) and *Ps. aeruginosa AF2* (Jane Gilbert et al., 1991) was inhibited by oleic acid, the extracellular lipase production from *C. lipolytica 1055* was induced in the presence of this fatty acid.

However, no effect on *C. lipolytica 1055* lipase and esterase activities was detectable when 0.5 g/l oleic acid was added to the reactor system.

The induction effect on *C. lipolytica 1055* lipase and esterase activities obtained by the 10.0 g/l oleic acid pulse was not proportional to that obtained by the 2.0 g/l oleic acid input.

FIGURE 3.3.2.2. shows the maximum lipase and esterase activities obtained with the oleic acid pulse at 2.0 g/l in *C. lipolytica 1055* under Tween-80 limitation.

Maximum esterase and lipase activities were obtained after the microorganism growth ceased and when oleic acid concentration was less than 0.5 mg/ml.

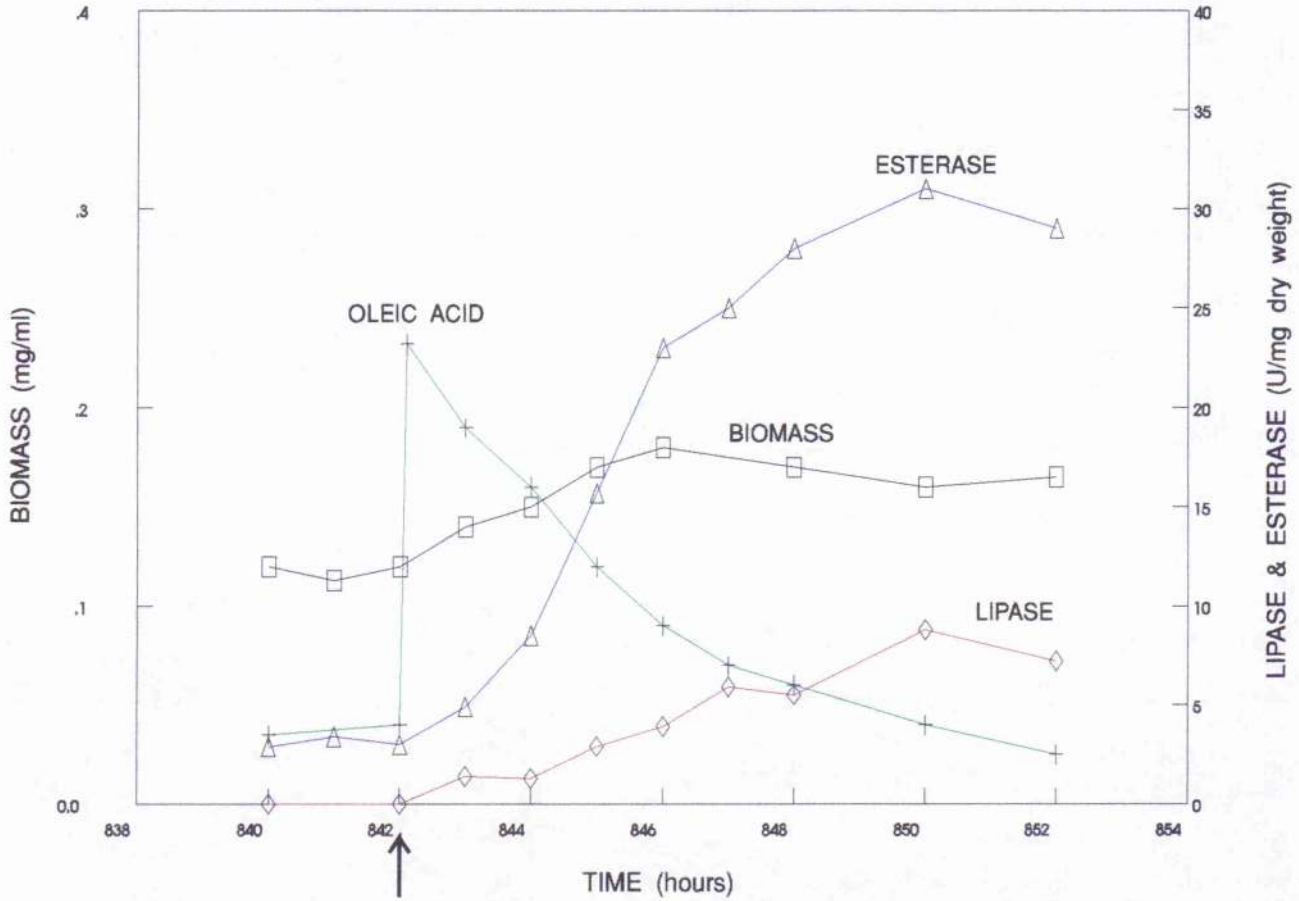


FIGURE 3.3.2.2 OLEIC ACID PULSING OF *C. lipolytica 1055* CHEMOSTATIC CULTURE IN TWEEN-80-LIMITED CULTURE AT THE DILUTION RATE OF 0.08 h^{-1}

The arrow indicates when the pulse was introduced.

These results suggest that oleic acid stimulates *C. lipolytica 1055* lipase and esterase production.

Oleic acid increases membrane permeability by releasing proteins (Espinosa et al., 1990). The same publication reported that an oleic acid concentration at 2 % stimulated *R. delemar* growth with a slight increase in lipolytic activity.

3.3.3 Carbon Source: Olive Oil

3.3.3.1 Behaviour of C. lipolytica 1055 at different dilution rates

C. lipolytica 1055 was continuously fed with 3.2 g/l olive oil and 0.5 mg/l yeast extract. Figure 3.3.3.1 shows the microorganism behaviour at a range of dilution rates between 0.09 and 0.58 h^{-1} under carbon limitation. Each dilution rate was investigated during at least 10 generation times.

Steady-state conditions were obtained at the dilution rates of 0.36, 0.48 and 0.58 h^{-1}, higher values than the maximum specific growth rate estimated from exponential batch culture (μ_{MAX} = 0.34 h^{-1}; Appendix 5).

These results may suggest that some metabolite was accumulated during the batch growth exerting an inhibitory effect on the *C. lipolytica 1055* growth-rate.

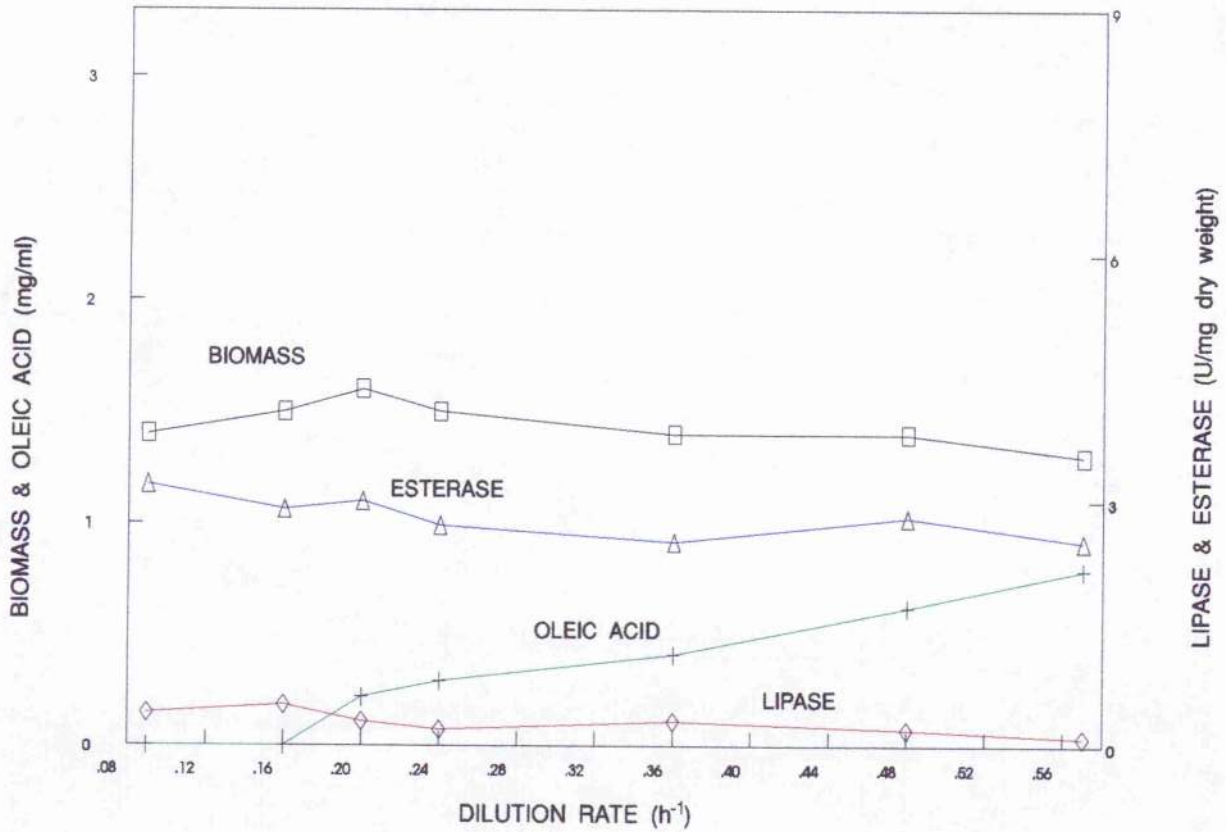


FIGURE 3.3.3.1 CHEMOSTATIC DATA OF *C. lipolytica 1055* CONTINUOUSLY FED WITH 3.2 g/l OLIVE OIL AND 0.5 g/l YEAST EXTRACT AT 30 °C

An increase in biomass after the olive oil pulse (3 g/l final concentration) at the dilution rate of 0.16 h^{-1}, confirmed that *C. lipolytica 1055* culture was olive oil-limited.

The lower biomass concentration at the dilution rate of 0.10 h^{-1} could be explained by the requirement of maintenance energy. The decrease in biomass concentration at low growth rates in function of substrate consumption for microorganism metabolism has been reported by PIRT (1975).

As is noted in the Figure 3.3.3.1-a esterase activity was approximately 3 U/mg dry weight while lipase reached 0.5 U/mg dry weight as maximum activity. Approximately 50 % of potential lipase activity points were at undetectable levels.

The results obtained from the olive oil-limited chemostatic culture may suggest that this natural oil maintains constant lipase and esterase production by *C. lipolytica 1055* at different dilution rates.

3.3.3.2 Estimation of maximum specific growth rate by the washout method

The estimation of maximum specific growth rate in olive oil-limited chemostatic culture of *C. lipolytica 1055* was carried out as described in Section 2.17.

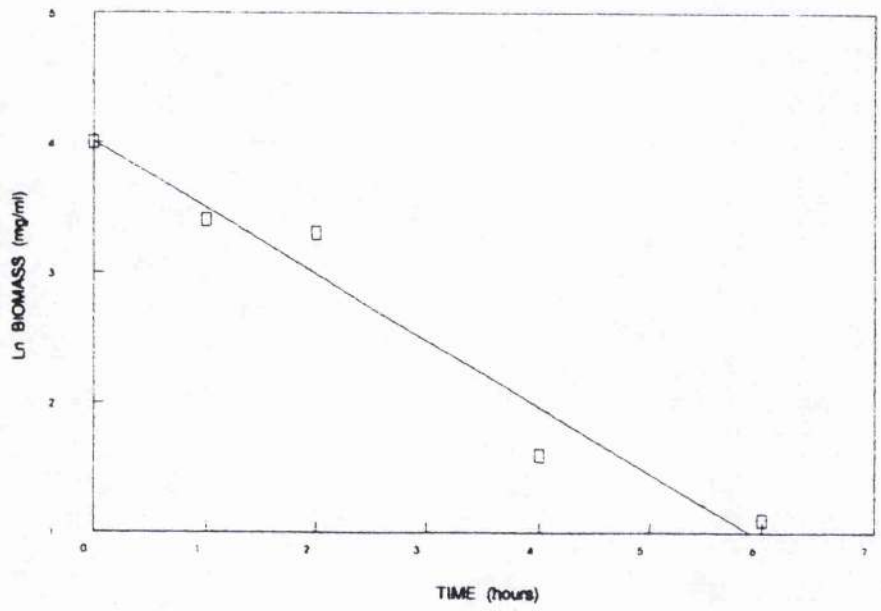
Following the establishment of a steady-state ($D = 0.58\ h^{-1}$) for 10 generation times, μ_{MAX} was estimated by washout applying a step change of $D = 0.58\ h^{-1}$ to $D = 0.70\ h^{-1}$ (Figure 3.3.3.2).

Although growth should take place at the maximum rate during the exponential phase in batch culture, the *C. lipolytica 1055* chemostatic culture with 3.3 mg/l olive oil at feed medium, exhibited a higher specific growth rate of $0.65\ h^{-1}$.

A similar effect was noticed by Preez et al. (1991) for *C. blankii* grown under batch and continuous culture. The estimated μ_{MAX} from washout was superior than through the exponential phase in batch culture. The authors have shown that accumulated acetic acid inhibited *C. blankii* growth during batch culture.

Batch culture growth is subject to nutritional imbalance between concentration increase of biomass and its nutrient environment; the substrate concentration is constantly decreasing while inhibitory metabolic products are accumulated (Pirt, 1975).

In the case of *C. lipolytica 1055*, the estimation of maximum specific growth rate at continuous culture is more accurate than at exponential phase in batch culture due to an inhibition effect in microorganism growth-rate through a metabolite accumulation, such as fatty acids.



LINEAR REGRESSION

Slope	-5.1034448E-02
Y intercept	0.4006897
Correlation coefficient	-0.9787
Standard error	2.974992E-02

FIGURE 3.3.3.2 WASHOUT PLOT OF C.lipolytica 1055 GROWN IN OLIVE OIL LIMITED CHEMOSTATIC CULTURE AT 30 °C

3.4 CONTINUOUS TRANSIENT CULTURES

Microbial enzyme production depends on many factors such as physical and chemical parameters imposed on the system, as well as genetic properties of the strain (Yamane & Tsukano, 1977; Jorgensen et al., 1990).

So far in this work, aimed at *C. lipolytica 1055* lipase production, the chemical parameters approach dealt with the variation of nutrients and other components, involving different cultivation methods: batch, fed-batch and chemostat.

Greater protein yields, e.g. ß-galactosidase from *Kluyveromyces lactis,* have been obtained in continuous transient states by changing the limiting substrate (Martini et al., 1989).

The continuous transient technique was investigated in search to improve lipase production in *C. lipolytica 1055.*

This operational strategy was carried out by subjecting the microorganism to defined oscillation conditions of growth in terms of limiting carbon sources.

3.4.1 Oleic acid square wave oscillations in Tween-80-limited culture

The oscillation of certain parameters in the continuous transient culture (for different periods and different amplitudes) should occur after a steady-state condition in order to analyse the response output.

First of all, *C. lipolytica 1055* was fed continuously with Tween-80 (4 g/l) and all other components of the medium as previously described (Section 2.4). The value of the dilution rate was fixed as 0.09 h^{-1} and after 10 generation times under steady-state conditions repetitive oleic acid inputs (3.2 and 8.1 g/l final concentrations) during 15 min were applied to the system.

C. lipolytica 1055 responded to the disturbances by showing a 'lag phase' immediately after the oleic acid inputs. The Figure 3.4.1-a shows *C. lipolytica 1055* output response after 3.2 g/l oleic acid input at a cycle time of 8 h.

Similar microorganism adaptation for imposed oscillations in chemostatic cultures have been reported by several authors (Pickett et al., 1979-a; Pickett et al., 1980; Barford et al., 1982; Heitzer et al., 1990).

It is relevant to remark the behaviour of *C. lipolytica 1055* lipase during the transition from batch (first 6 h of culture) to chemostat and from chemostat to

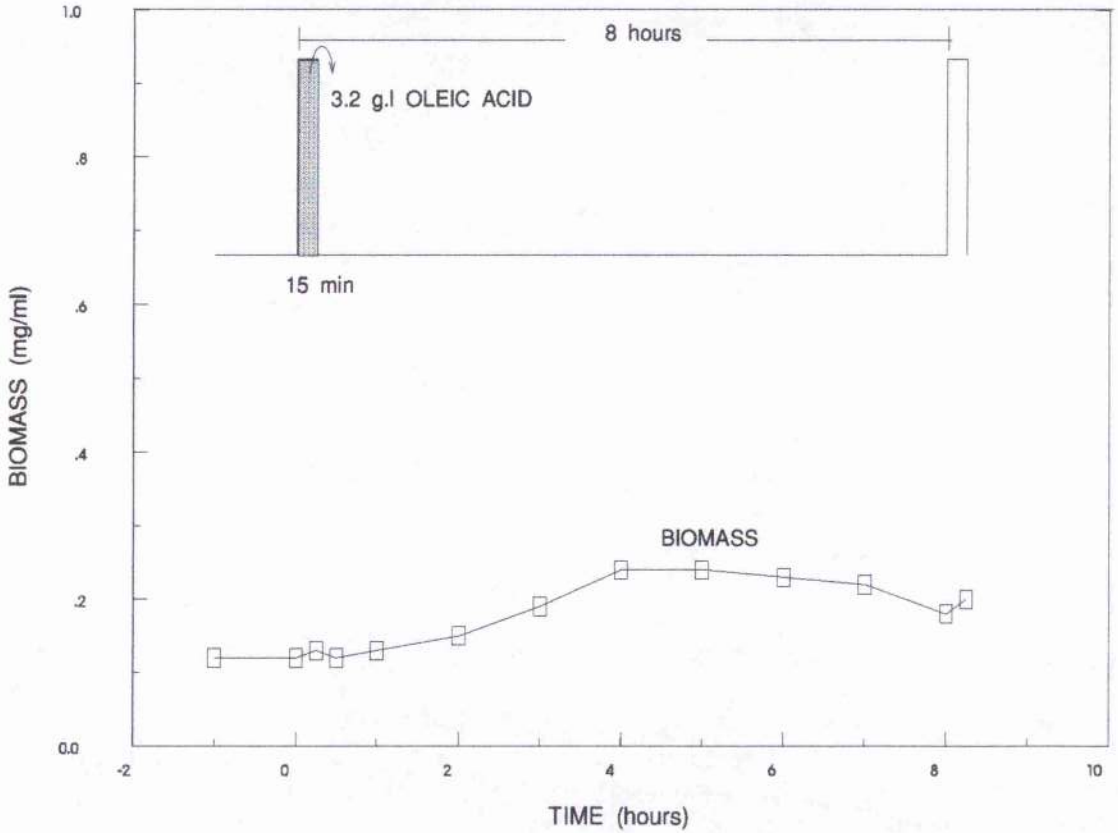


FIGURE 3.4.1-α BIOMASS OSCILLATION FOR C.lipolytica 1055 SUBJECTED TO 3.2 g/l OLEIC ACID INPUT AT PERIOD OF 8 h. The disturbance was introduced at zero time.

continuous transient culture subjected to 3.2 g/l oleic acid input and a cycle time of 8 h. The lipase activity decreased from 2 U/mg dry weight in batch culture to values below detectable levels in the chemostat.

However, the oleic acid inputs induced this enzyme, reaching steady-state levels after 10 cycle times of oleic acid square wave oscillations in Tween-80-limited culture (Figures 3.4.1-b and 3.4.1-c).

A general observation is that biomass and lipase and esterase activities were kept constant for the two transient conditions. It did not matter whether the magnitude of the difference between the two oleic acid concentration used was small (0 and 3.2 g/l) or large (0 and 8.1 g/l).

No apparent effect could be observed as the regular cyclic behaviour of *C. lipolytica 1055* tended to approach a steady-state condition suggesting that this dynamic process was under relaxed steady-state in accordance with Bailley (1973).

Relaxed steady-state is the operation where the system is subjected to rapid cycling while the reactor output response becomes invariant after a time.

However, *C. lipolytica 1055* systems were not subjected to rapid cycling although the output response became invariant after a period. Perhaps the cells were unable to respond to small changes in the growth

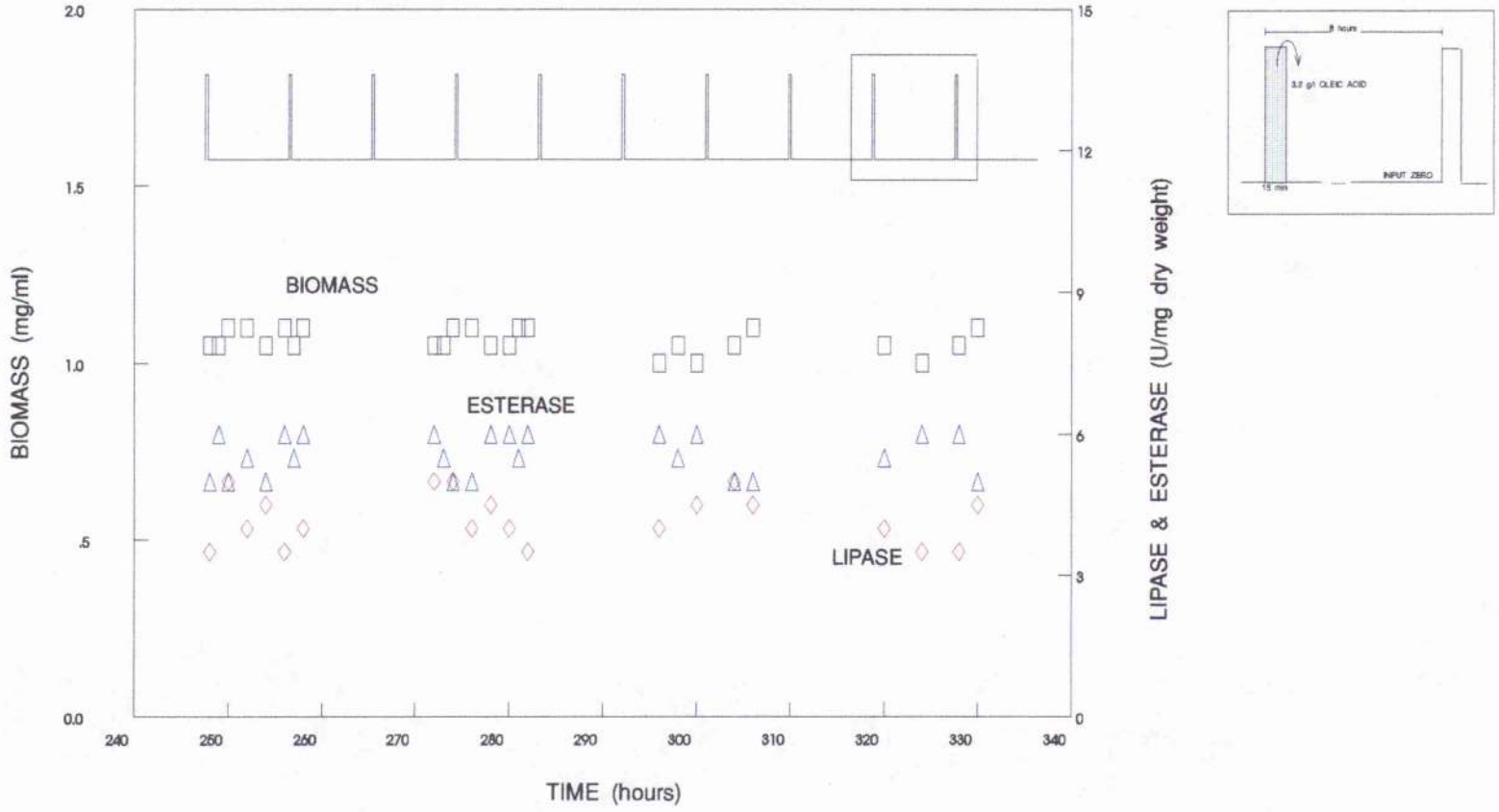


FIGURE 3.4.1-b CONTINUOUS TRANSIENT OF *C. lipolytica 1055* UNDER TWEEN-80 LIMITATION SUBJECTED TO 3.2 g/l OLEIC ACID OSCILLATIONS AT CYCLE TIME OF 8 h

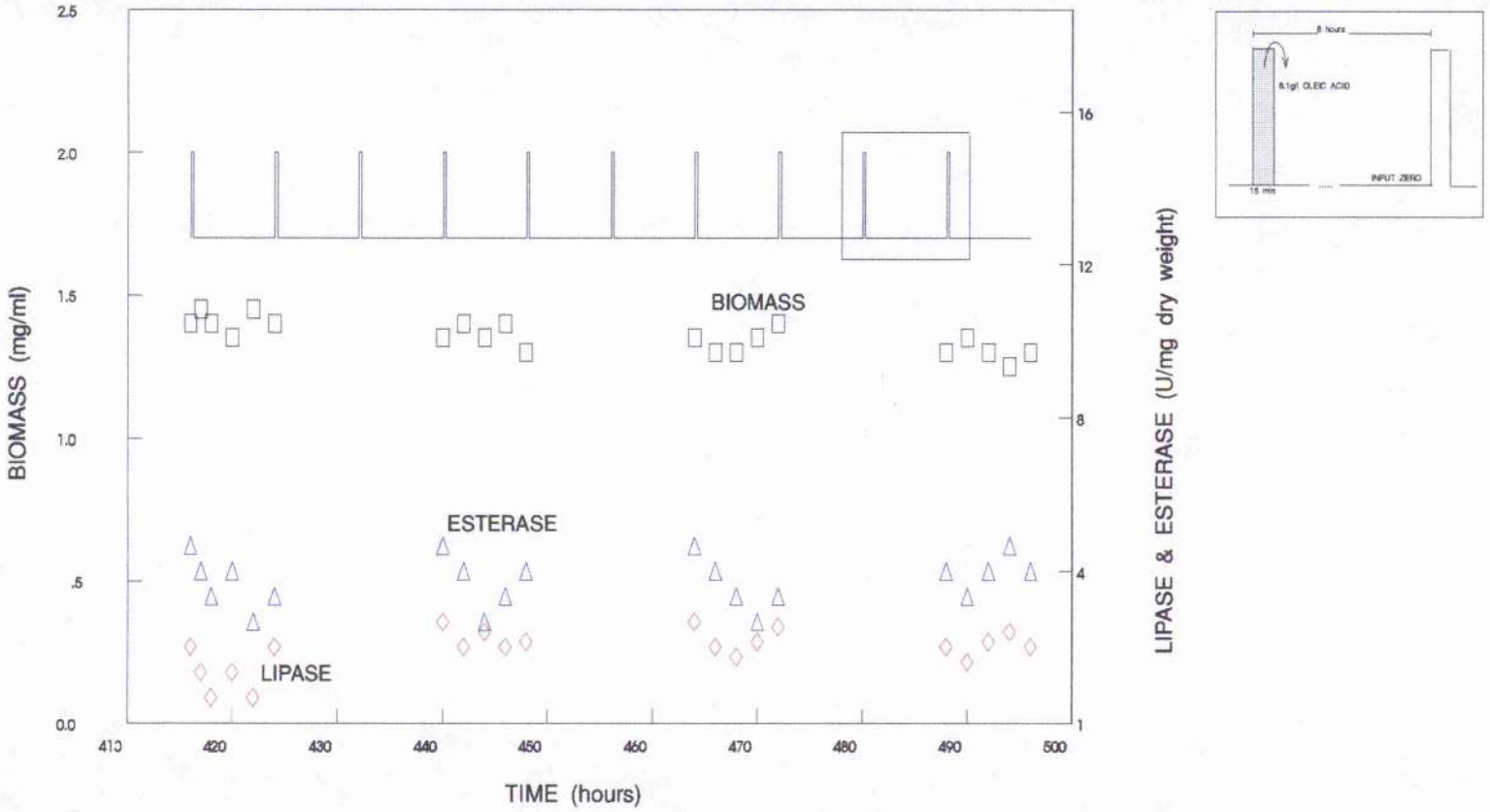


FIGURE 3.4.1-c CONTINUOUS TRANSIENT OF *C. lipolytica 1055* UNDER TWEEN-80 LIMITATION SUBJECTED TO 8.1 g/l OLEIC ACID OSCILLATIONS AT CYCLE TIME OF 8 h

environment, or the response was too small to be detected experimentally.

The average biomass values were 1.05 and 1.35 mg/ml when Tween-80 limited cultures of *C. lipolytiva 1055* were operated with continuous oleic acid oscillations of 0 - 3.2 and 0 - 8.1 g/l, respectively.

Another general comment is that oleic acid induced the *C. lipolytica 1055* hydrolases (volumetric lipase and esterase activities) at same levels despite the different amplitudes of this fatty acid imposed on the cultures.

As microbial growth was stimulated when the culture was subject to 8.1 g/l oleic acid inputs, best results of specific lipase and esterase activities were obtained when the culture was subjected to repetitive 3.2 g/l oleic acid input at a cycle time of 8 h. Lipase activity reached 3.9 U/mg dry weight while esterase activity reached 5.2 U/mg dry weight, as average values.

3.4.2 Olive oil square wave oscillations in olive oil-limited culture

The increase in protein content with higher efficiency has been achieved with large input variation of square wave rather than small input oscillations (Douglas, 1972).

Figure 3.4.2 shows *C. lipolytica 1055* behaviour under transient operation technique with large input variation of olive oil square wave oscillations. The culture was studied for 200 h, i.e. approximately 25 generation times.

The data at cycle time zero was obtained from a steady-state culture continuously fed with 1.7 mg/l olive oil. The average of biomass concentration reached 1.2 mg/ml while esterase activity reached approximately 1.5 U/mg dry weight and lipase activity 0.2 U/mg dry weight under steady-state conditions.

In this set of experiments the culture of *C. lipolytica 1055* was grown under continuous transient culture whose carbon source, olive oil, was alternatively added as square wave inputs with amplitudes of 0 and 3.4 g/l and cycle time of 8 h. The combined rate of entry of the medium with the carbon source gave a constant dilution rate of 0.09 h^{-1}.

Samples were taken each hour during the oscillation operation to investigate the intra-cyclic response of the organism to the oscillations

The Figure 3.4.2 shows an increase in biomass immediately following the step change in carbon source. *C. lipolytica 1055* behaviour may suggest an instantaneous increase in substrate metabolised by the cells causing an immediate change in specific growth rate. Then, no lag was noticed in response to the added olive oil input.

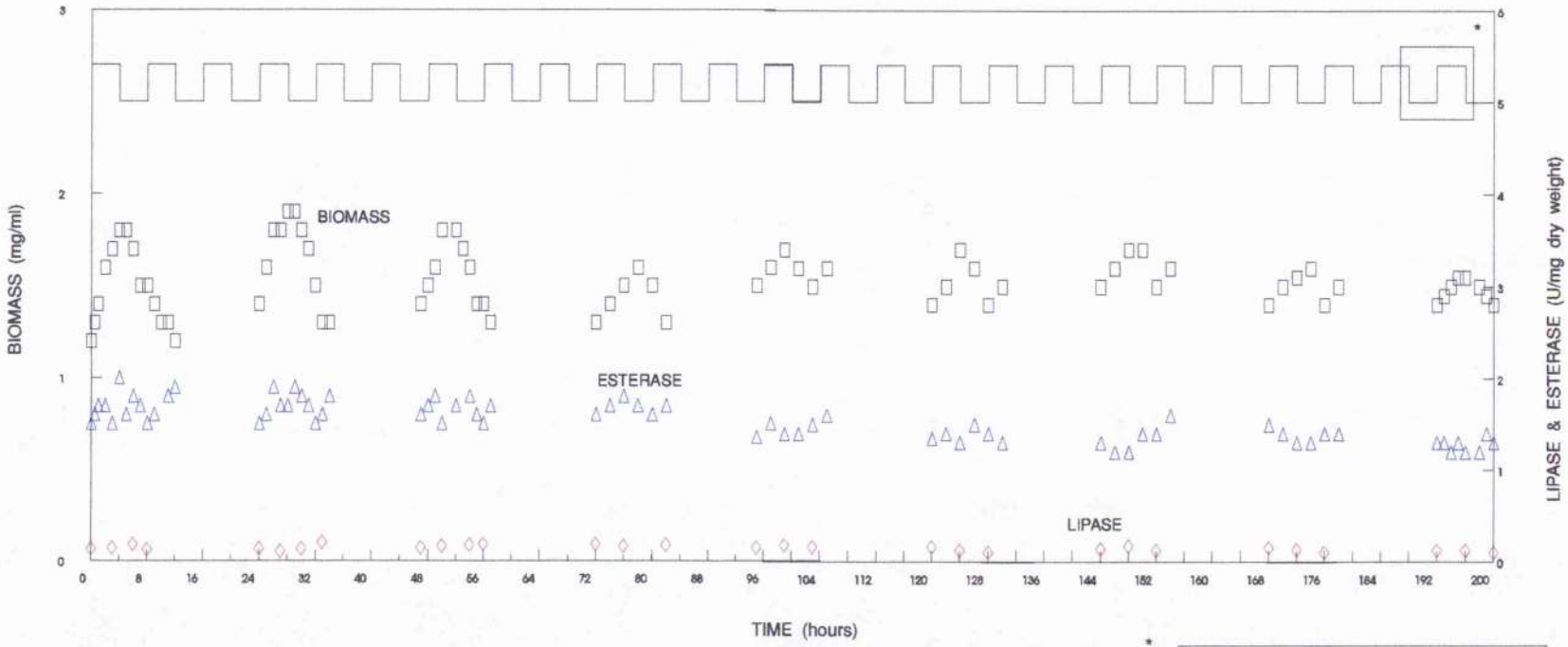


FIGURE 3.4.2 CONTINUOUS TRANSIENT OF *C. lipolytica 1055* SUBJECTED TO OLIVE OIL SQUARE WAVES (0 - 3.2 g/l) AT CYCLE TIME OF 8 h

Meanwhile a microbial response for an imposed oscillation in the environment depends on the substrate as well as the physiological state of the culture involving all the history of the culture prior to the transient condition (Chi & Howell, 1976).

After successive cycles the amplitude of the biomass oscillation had a tendency to diminish. In addition, esterase activity results tend to reach a steady-state condition while lipase activity did not show any big oscillation. However, more than 50 % of the analysed lipase samples were below the detection level.

The olive oil square wave oscillations in olive oil-limited culture appeared not to be advantageous for lipase production by *C. lipolytica 1055*.

3.5 FINAL CONCLUSIONS

Lipase and esterase production by *C. lipolytica 1055* reached maximum values when growth had nearly ceased under batch and fed-batch conditions.

The results suggest that lipase and esterase could be primary metabolites not directly linked to the growth since high lipid (oleic acid and olive oil) concentrations inhibited enzyme synthesis while stimulated growth.

Possibly, *C. lipolytica 1055* lipase and esterase production under continuous culture was limited

by the fact that these hydrolases are produced in the late exponential phase of growth while mid-log phase conditions are typical of chemostatic growth.

Extracellular lipase productivity by *C. lipolytica 1055* in the presence of olive oil as the carbon source was low with all operational strategies assessed, i.e. batch, fed-batch, chemostat and continuous transient technique (Table 3.5).

All the techniques used were quite inefficient as the lipase productivities were as low as 0.33 U/mg dry weight.h when batch culture was used, 0.30 U/mg dry weight.h for fed-batch culture with olive oil input, 0.025 U/mg dry weight.h for oil-limited chemostatic culture and 0.012 U/mg dry weight.h for continuous olive oil square wave oscillations.

In contrast, extracellular esterase productivity by *C. lipolytica 1055* reached 5.3 - 6.5 U/ mg dry weight.h in the presence of Tween-80 under batch and fed-batch subjected to oleic acid input (Table 3.5).

Fed-batch allows flexible operation and its productivity could be improved by choosing the repeated fed-batch mode. Tween-80 grown cells subjected to oleic acid pulse, prolonged esterase production phase, suggesting that fed-batch culture could be a route towards esterase production by *C. lipolytica 1055.*

OPERATIONAL STRATEGY	LIPASE (U/mg dry weight.h)	ESTERASE (U/mg dry weight.h)
BATCH		
OLIVE OIL	0.33	1.2
TWEEN-80	0.32	6.5
FED-BATCH		
OLIVE OIL	0.3	1.0
OLEIC ACID	0.31	5.3
CHEMOSTAT		
OLIVE OIL	0.025	0.28
TRANSIENT		
OLIVE OIL	0.012	0.12
OLEIC ACID	0.35	0.47

TABLE 3.5 COMPARISON OF *C. lipolytica 1055* LIPASE AND ESTERASE PRODUCTIVITIES UNDER DIFFERENT CULTIVATION METHODS

The low lipase productivities by C. lipolytica 1055 under different cultivation methods could be overcome by genetic manipulation of the microorganism.

In the last few years genetic modification of different microorganism strains involving lipase production has been reported by several authors (Nga et al., 1988; Fernandez et al., 1990; Hass et al., 1990; Jorgensen et al., 1990).

Recently, recombinant lipases from *Humicola lanuginosa, Rhizomucor miehei* and *C. arctica* are widely utilized as washing powder additives. High level expression of lipase genes was achieved with heterologous hosts, e.g. *A. oryzae,* promoted by an α-amylase gene (Sakaguchi et al., 1992).

Some suggestions for future experiments are discussed as follows:

(i) Effect of the following physical parameters on *C. lipolytica 1055* growth and lipase production:

- agitation
- dissolved oxygen concentration
- pH
- temperature;

(ii) Influence of a range of 2 to 20 g/l glucose concentrations on *C. lipolytica 1055* growth and lipase production;

(iii) Investigation of lipase production of *C. lipolytica 1055* in the presence of a protease inhibitor at neutral pH and controlled dissolved oxygen;

(iv) Influence of varying Tween-80 concentrations on the induction or possible "activation" of *C. lipolytica 1055* lipase;

(v) Investigation of the shelf-life of lipase and esterase from *C. lipolytica 1055*;

(vi) Investigation of further diauxic growth occuring when *C. lipolytica 1055* is grown on olive oil as carbon source (i.e. diauxic growth experiments with glycerol and oleic acid);

(vii) Effect of increased Tween-80 concentrations (> 4 g/l) on esterase activity by *C. lipolytica 1055* under chemostatic culture;

(viii) Investigation of *C. lipolytica 1055* lipase production in a two-phase bioreactor:

Phase 1 - Microorganism growth promoted by carbohydrate waste under batch conditions;

Phase 2 - Fed-type batch operation with inputs of an inducer compound at neutral pH and controlled dissolved oxygen aiming high enzyme production.

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APPENDIX 1

MAXIMUM SPECIFIC GROWTH RATES FOR

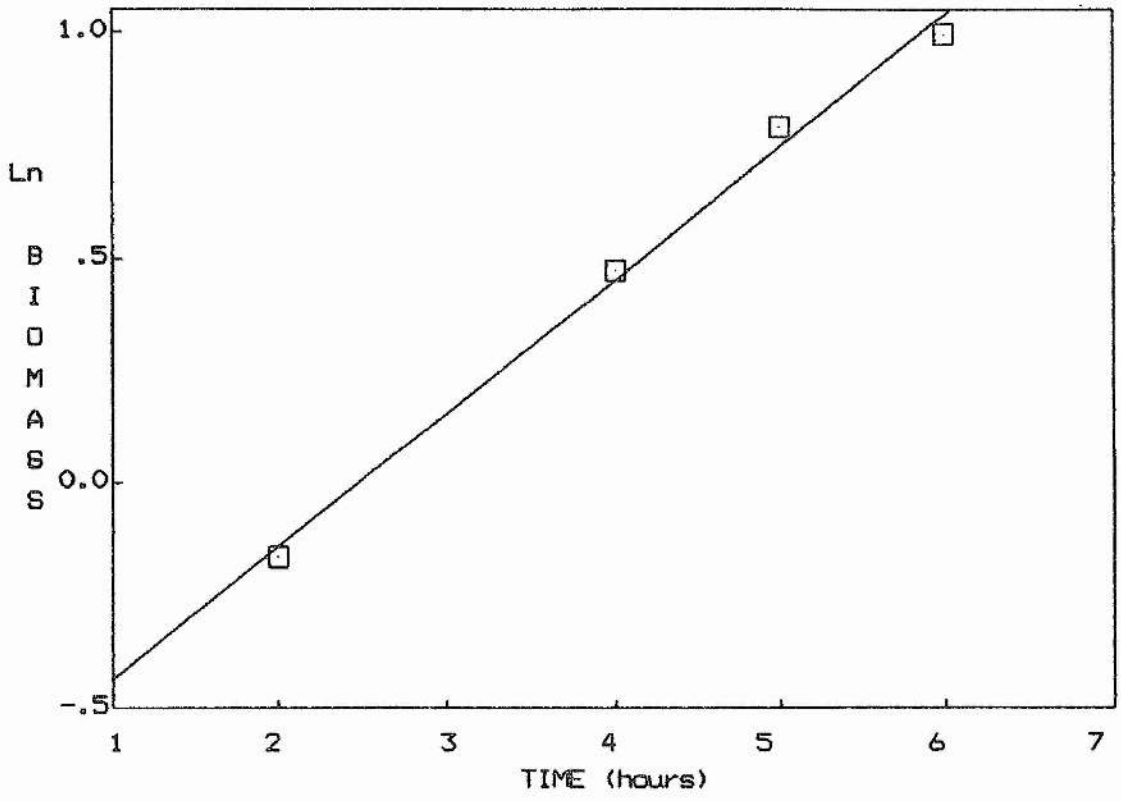
C. lipolytica 1055 UNDER DIFFERENT BATCH CONDITIONS

CARBON & NITROGEN SOURCES	MAXIMUM SPECIFIC GROWTH RATE (h^{-1})
TWEEN-80 & YEAST EXTRACT	0.27
TWEEN-80 & AMMONIUM SULPHATE	0.14
POLYETHYLENE GLYCOL & YEAST EXTRACT	0.13
GLYCEROL & YEAST EXTRACT	0.12
YEAST EXTRACT	0.12

N.B. The linear regressions were obtained by a Technical Graph Plotting and Curve Fitting Software (Techni-Curve).
The correlation coefficients were higher than 0.99.

APPENDIX 2

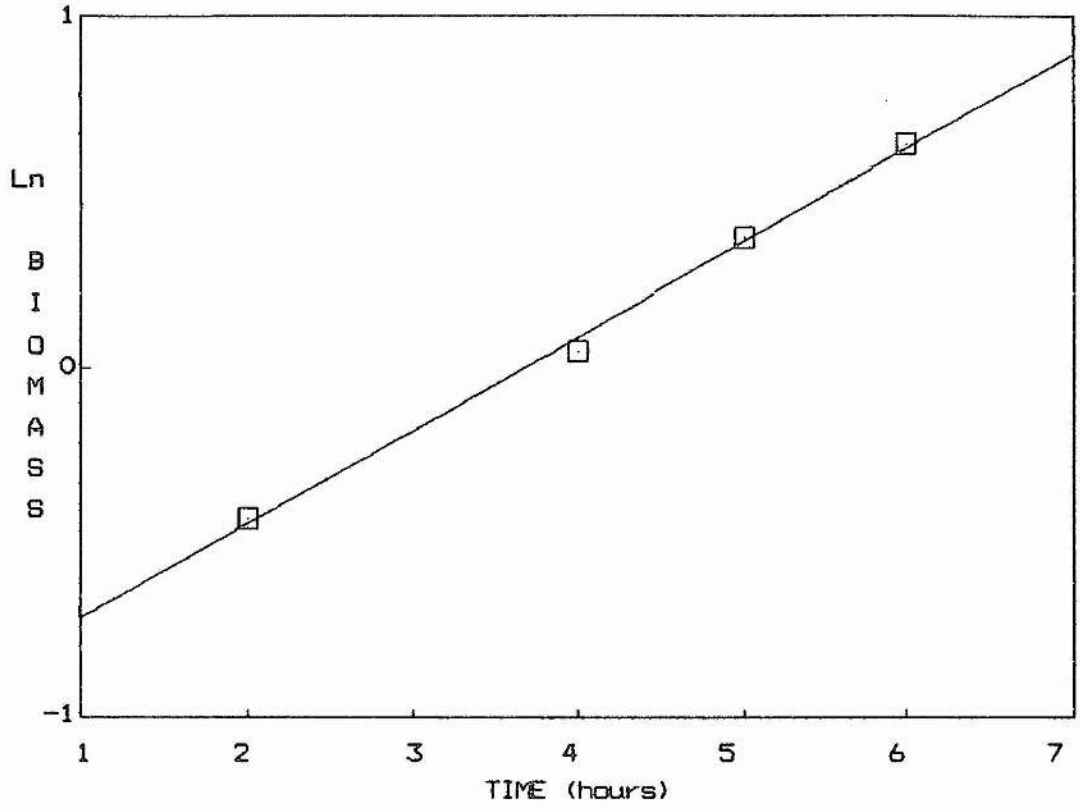
LINEAR REGRESSION OF NATURAL LOGARITHM OF BIOMASS AGAINST TIME FROM EXPONENTIAL GROWTH PHASE OF *C. lipolytica 1055* GROWN IN 20 g/l GLUCOSE AND 5 g/l YEAST EXTRACT AT 30°C



Slope	.2945944
Y intercept	-.7297275
Correlation coefficient	0.9967
Standard error	4.985642E-02

APPENDIX 3

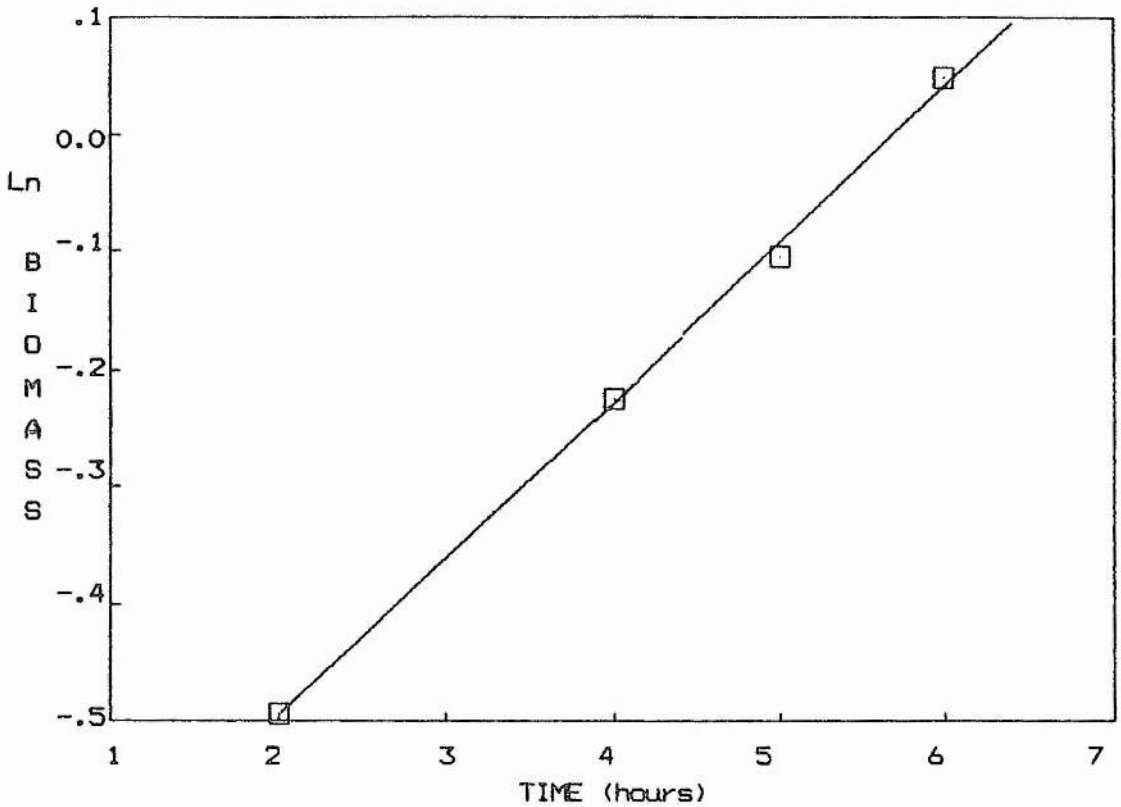
LINEAR REGRESSION OF NATURAL LOGARITHM OF BIOMASS AGAINST TIME FROM EXPONENTIAL GROWTH PHASE OF *C. lipolytica 1055* GROWN IN 2 g/l GLUCOSE AND 0.5 g/l YEAST EXTRACT AT 30°C



Slope	.2668882
Y intercept	-.9807943
Correlation coefficient	0.9984
Standard error	3.115587E-02

APPENDIX 4

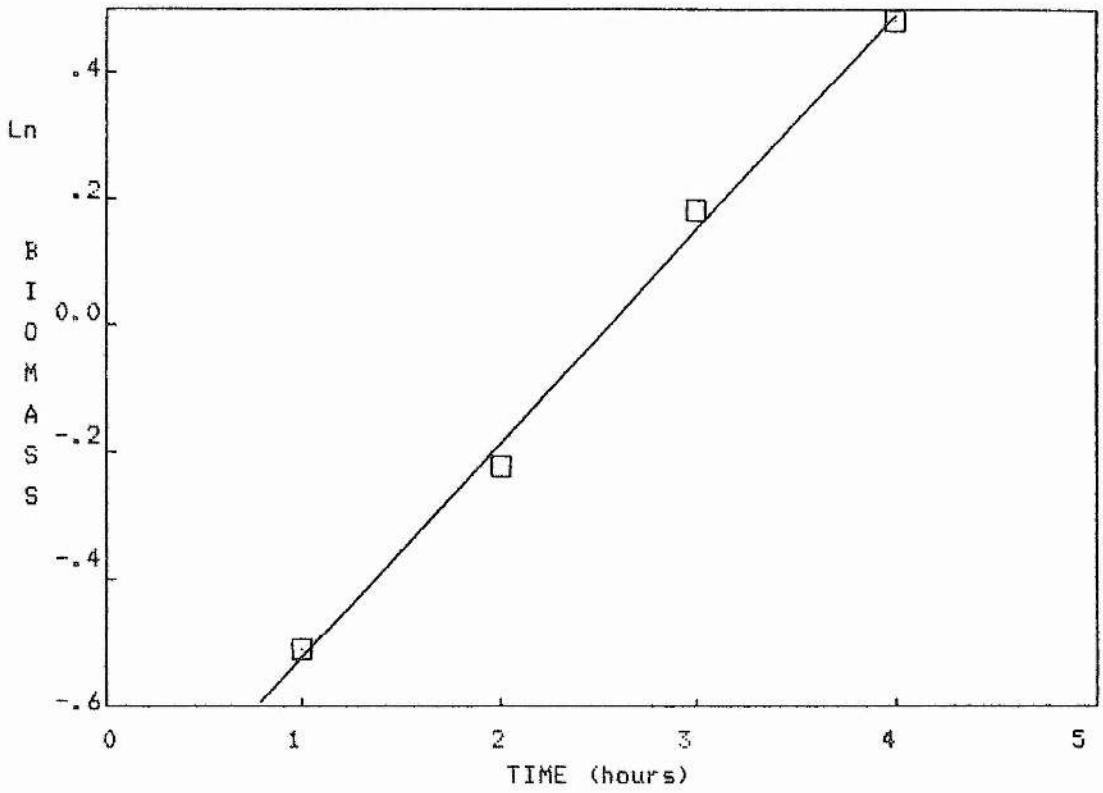
LINEAR REGRESSION OF NATURAL LOGARITHM OF BIOMASS AGAINST TIME FROM EXPONENTIAL GROWTH PHASE OF *C. lipolytica 1055* GROWN IN 4 g/l TWEEN-80 AND 2 g/l AMMONIUM SULPHATE AT 30°C



Slope	.1361032
Y intercept	-.7695714
Correlation coefficient	0.9988
Standard error	1.423457E-02

APPENDIX 5

LINEAR REGRESSION OF NATURAL LOGARITHM OF BIOMASS AGAINST TIME FROM EXPONENTIAL GROWTH PHASE OF *C. lipolytica 1055* GROWN IN 3.2 g/l OLIVE OIL AND 0.5 g/l YEAST EXTRACT AT 30°C



Slope	.3385221
Y intercept	-.8636105
Correlation coefficient	0.9978
Standard error	3.555442E-02