1987

Kinetics Of Growth And Toxin Production By Zymocidal Saccharomyces Cerevisiae

John Blair Wallace

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KINETICS OF GROWTH AND TOXIN PRODUCTION BY ZYMOCIDAL *SACCHAROMYCES CEREVISIAE*

by

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Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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November, 1986

ISBN 0-315-36090-9

ABSTRACT

Zymocidal strains of Saccharomyces cerevisiae excrete protein toxins which attack and kill other sensitive contaminant yeast strains that might be present during fermentation. A systematic perusal of the literature revealed that very little or no research has been done on the kinetics of cell growth and zymocidal toxin production by S. cerevisiae, and its stability characteristics. This thesis represents the first attempt to study systematically, and under controlled conditions, the kinetic parameters of the zymocidal system.

In this study, batch and continuous steady-state culture techniques were used to study the affects of pH, temperature and initial glucose concentration on the production of zymocidal toxin and biomass. In addition; gel electrophoresis methods were used to identify the plasmids present in the zymocidal S. cerevisiae strain associated with the excretion of the zymocidal toxin.

The zymocidal toxin concentration was quantitatively using the Well-test method. Correlations of the zymocidal toxin concentration versus the clearing diameter were derived from the experimental data.

The batch and continuous culture results were analyzed and correlated to develop new kinetic models for cell growth, glucose uptake, ethanol and extracellular toxin production. A new kinetic model was proposed to explain the observed reduced rate of glucose uptake by zymocidal S. cerevisiae in the presence of toxins in the fermentation medium. The new model corrects the Monod equation to account for the

effects of the zymocidal toxin and immunity molecules on the cell wall. It was hypothesized that the toxin or the immunity molecule interfers with the uptake of glucose by the cell by causing an increase in the mass transfer resistance at the cell membrane. Fitting of continuous culture data to the new model suggested that a specific zymocidal toxin concentration of 11.69-Units/g of cells would be sufficient to stop all glucose uptake by the cell.

Cell-free broth was used to study the stability of the toxin at different conditions of pH and temperature. An Arrhenius temperature deactivation model was developed for the zymocidal toxin and the appropriate kinetic parameters were evaluated and correlated with respect to temperature and pH. The optimum pH range for toxin activity was found to be 4.2 to 4.6, and the thermal deactivation was found to be first order with a deactivation rate of 14.5-kcal/mole at pH=4.5.

Acknowledgements

I wish to express my thanks to my thesis supervisor, Dr. A. Margaritis, for suggesting this research topic and for his support and guidance throughout the course of this research. The author greatly acknowledges the help with the gel electrophoresis analyses provided by Dr. C. Panchal and his staff at the Labatt Brewing Company Ltd., London, Ontario. I wish to also thank Ms. Joanne Knight and Mr. Bruce Wallace for assisting me in sampling during the various experiments completed for this thesis.

Lastly, I would like to thank my wife, Virginia, for typing and correcting this manuscript. Her patience and understanding was unbounded and greatly appreciated.

Financial support of this research project came from the Natural Sciences and Engineering Research Council of Canada (N.S.E.R.C.) Operating Grant No. A4388 awarded to Dr. A. Margaritis.

TABLE OF CONTENTS

LIST OF PHOTOGRAPHIC PLATES

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

Symbol	Description	Units
A	Proportionality Constant in Arrhenius Relationship	h^{-1}
C	Concentration of Active Enzyme	$g\ L^{-1}$
C_0	Initial Concentration of Active Enzyme	$g\ L^{-1}$
D	Dilution Rate	h^{-1}
E	Deactivation Energy	$kcal\ kgmole^{-1}$
F	Feed Rate	$L\ h^{-1}$
k	Reaction Rate Constant	h^{-1}
K_m	Monod or Saturation Constant	$g\ L^{-1}$
m	Specific Rate of Substrate Uptake for Cellular Maintenance	h^{-1}
P	Product Concentration	$g\ L^{-1}$
P_m	Maximum Ethanol Concentration - Growth	$g\ L^{-1}$
P'_m	Maximum Ethanol Concentration - Product	$g\ L^{-1}$
q_s	Specific Substrate Uptake Rate	h^{-1}
R	Gas Constant (4.1872 kcal $kgmole^{-1}$ K^{-1})	
R_m	Optimization Residual	
r	Rate Constant of Enzyme Deactivation	$g\ L^{-1}\ h^{-1}$
$\bar{r}_c$	Average Reaction Rate in Time Interval Δt	$g\ L^{-1}\ h^{-1}$
S	Limiting Substrate Concentration	$g\ L^{-1}$
$\bar{S}$	Average Substrate Concentration in Interval Δt	$g\ L^{-1}$
t	Time	h
t_{lag}	Length of Lag Phase	h
T	Temperature	K or $^{\circ}C$
V	Reactor Volume	L
v	Specific Rate of Product Formation	h^{-1}
v_t	Specific Rate of Ethanol Production in the Presence of Ethanol	h^{-1}
v_o	Specific Rate of Ethanol Production at Zero Ethanol Concentration	h^{-1}
X	Biomass Concentration	$g\ L^{-1}$
X_o	Initial Biomass Concentration	$g\ L^{-1}$
$\bar{X}$	Average Biomass Concentration in Time Interval Δt	$g\ L^{-1}$
Y_g	Yield Factor for Cell Growth	
Y_p	Yield Factor for Biomass Formation	
$Y_{p/s}$	Overall Product to Substrate Yield	
$Y_{p/x}$	Overall Product to Biomass Yield	
$Y_{x/s}$	Overall Biomass to Substrate Yield	
$Y_{z/s}$	Overall Toxin to Substrate Yield	$U\ g^{-1}$
$Y_{z/x}$	Overall Toxin to Biomass Yield	$U\ g^{-1}$
Z	Zymocidal Toxin Concentration	$g\ L^{-1}$
Z_m	Maximum Specific Toxin Concentration	$U\ g^{-1}$
Z_o	Initial Zymocidal Toxin Concentration	$g\ L^{-1}$
Z_x	Specific Toxin Concentration	$U\ g^{-1}$
z_p	Clearing Zone Diameter	mm
z	Zymocidal Toxin Equivalent Concentration	$U\ L^{-1}$

Greek Symbols

α	Stoichometric Constant, Eqn 4.12	
β	Proportionality Constant, Eqn 4.13	h^{-1}
δ	Exponent, Eqn 4.24	
γ	Exponent, Eqn 4.23	
λ	Exponent, Eqn 6.8	
μ	Specific Growth Rate	h^{-1}
μ_1	Specific Growth Rate in the Presence of Inhibitor	h^{-1}
μ_o	Specific Growth Rate at Zero Inhibitor Concentration	h^{-1}
μ_{max}	Maximum Specific Growth Rate	h^{-1}

CHAPTER 1

INTRODUCTION

Microbes influenced man's development long before man was aware of their existance mostly through their ability to create or destroy some of the sources of his pleasure and livelihood. Through all of these many years microorganisms have been responsible for many processes which are taken for granted or as a fact of life. Examples of such processes include: enriching soil, destroying crops, fermenting drinks, spoiling foods, destroying waste and affecting health and general well-being. For many thousands of years these processes have continued undisturbed and undiscerned.

It is evident there has been an increasing interest in the potential of commercial exploitation of metabolic processes. The array of products available from conventional cellular systems has been augmented by modified cellular systems created using genetic engineering, hybridization, cell fusion or rare mating techniques (Stewart, 1985). Nowhere have these advances had more influence than in the pharmaceutical industry where quantities of high valued antibiotics or other types of drugs can be effectively and efficiently be produced. In most cases these specialized molecules cannot be efficiently synthesized in any other way.

Some recent advances in the production of specialized products included the production of hepatitis vaccine (Dawes, 1984), and the production of human interferons (Hitzeman et al., 1981 and 1983) and the production of the acid protease chymosin used in the milk-clotting step of cheese production (in: Stewart et al., 1984) by genetically modified strains of Saccharomyces cerevisiae. Cells of the bacterium Escherichia coli have been genetically modified to produce human growth hormone and insulin (in: Panchal et al., 1984).

As yeasts have been the microorganism most intimately involved with the progress and development of man, it seems most appropriate to study yeast cell systems when trying to assess biochemical principles and processes. The main contribution of yeast to man's development has been based on the ability of certain strains to rapidly and efficiently convert sugars to ethanol and carbon dioxide, and as a result effect fermentation. Examples of sugar solutions used in fermentations of this type are grain extracts, and grape juices (Stewart et al., 1984). It has been suggested that the future importance of yeasts in industrial applications will diminish due to the limited number of metabolites produced.

Seeming to contradict these ideas of the limited application of yeasts, it is an obvious fact that many species of yeast (particularly of the genus S. cerevisiae) produce the important primary metabolite ethanol for use as a beverage preservative or as a fuel. Also, as mentioned earlier, various strains of S. cerevisiae have been genetically modified to produce important and specialized metabolites.

In the fermentation industry a total of 1014 strains of the Saccharomyces species have been isolated over the past 50 years (Rosini et al., 1982).

S. cerevisiae and other related species are favoured for use as experimental organisms for research into the basic biochemical, metabolic and genetic processes of their life cycle (Stewart et al., 1984). Stable haploids, diploids and polyploids have been easily cultured from this eukcaryote and have been subjected to extensive analysis.

There are many uses of S. cerevisiae as an industrial organism, and as a tool in basic biochemical analysis. The scientific literature is full of studies which serve to elucidate the products and the biochemistry of this well researched (yet still surprising) organism. A complete knowledge of the life cycle, genetics, and ultimate capabilities of S. cerevisiae have still to be fully uncovered.

From this extensive use of S. cerevisiae it is easy to see that study of this yeast and its peculiarities will assist in all phases of basic and applied biochemical knowledge. Search for cellular systems to act as the hosts for genetic manipulation should concentrate on the changes which will occur in the cell after the gene splice is inserted. Therefore, study of naturally occuring cellular systems that already show some of the characteristics required in the genetically modified organism is important.

Of importance in the analysis of microorganisms is the process of medium and large molecule secretion. Important compounds, such as insulin and the human growth hormone, are produced within a cell, and are secreted to the extracellular medium. The processing of these molecules and the effects these molecules have on the overall growth of the producing organism is an important consideration.

A naturally occuring property of some yeasts is their ability to excrete a medium weight, proteinaceous molecule which is lethal to other yeasts of similar genera. These cellular systems have provided insight into the genetics of the production and release of extracellular proteins and other molecules. Brewers and other producers of beverage and fuel alcohol may be interested in using these zymocidal cell systems to reduce contamination in large scale fermentation systems. Of course, some of the production characteristics, and not just the genetics, of these cell systems are needed before application to large scale systems is possible.

A systematic perusal of the research literature revealed that very little information is available on the kinetics of growth and toxin production by zymocidal yeasts. These types of yeasts may have important industrial applications both in beer and wine fermentations. The main objective of this thesis research was to carry out original fundamental studies on the factors which control the kinetics of cell growth and toxin production by zymocidal yeast strains. The kinetic models and kinetic parameters for cell growth and toxin production developed in this study might be used not only for both bioreactor

design but also for the control of fermentation processes employing zymocidal yeast strains.

CHAPTER 2

LITERATURE REVIEW

It has been reported that some strains of Saccharomyces cerevisiae produce an extracellular toxin protein that attaches to the cell wall of other sensitive strains and kills them (Makower and Bevan, 1968, and Berry and Bevan, 1972). These zymocidal or "killer" cell yeasts have recently received attention by some researchers who tried to determine the structure, mode of action and the method of production of this proteinaceous toxin. Overall, these zymocidal strains promise to reveal some interesting aspects of the life cycle of S. cerevisiae, the processes involved in protein excretion, and the effects of extracellular proteins on growth and product formation during fermentation. Almost all of these studies were done at the petri dish or small shake flask fermentation level.

Review papers have been written to describe aspects of the biochemistry and the microbiology of production and excretion of the toxin, as well as the genetics of the transferring of zymocidal genetic material during cell reproduction, Bussey,(1981), Tipper and Bostian,(1984), Wickner,(1978), Wickner,(1981), Wickner,(1983), Wickner and Leibowitz,(1975) and Young and Yagiu,(1978). Since this thesis deals with the kinetics of bench scale fermentations for cell growth

and production of zymocidal toxin, we present a summary overview of the aspects of zymocidal production related to this work.

2.1 Discovery of the Zymocidal Toxin

Makower and Bevan (1963) were the first to discover zymocidal strains of S. cerevisiae, classifing some of the S. cerevisiae strains into three phenotypes; namely 'killer', 'sensitive', and 'neutral'. They reported that when killer yeast cells were grown with sensitive yeast cells on the same agar medium (or in the same broth), a high proportion of the sensitive yeast cells were killed. When killer strains were grown with neutral strains no killing of any of the cells was observed. Makower and Bevan (1963) concluded that some strains of S. cerevisiae were able to produce an extracellular toxin capable of killing sensitive strains, while other strains were immune to the zymocidal toxin.

Since this initial discovery, considerable work has been devoted to finding other microorganisms which excrete toxin molecules. By using a reference sensitive strain of S. cerevisiae NCYC 1006 and a killer S.cerevisiae strain NCYC 738, Rosini, (1983), examined a total of 1,273 strains of various yeast genera for their killer, sensitive, or neutral phenotypes. The results of that analysis shown in Table 2.1, indicate that one-third of the S. cerevisiae strains had zymocidal properties. In addition, a total of 707 of the S. cerevisiae strains tested were found to be sensitive, and another 44 appeared to be neutral. Except for a large majority of killer strains found with Hansenula anomala (71.4%), no other killer strains were found.

Since Rosini's work, killer strains of Kluyveromyces lactis (Panchal et al., (1985), and Sugisaki et al., (1983)) and Pichia kluyveri (Middlebeck et al., (1980) have also been isolated.

Analysis of other strains for killer phenotypes, by Rogers and Bevan, (1977), produced results similar to those presented in Table 2.1. Imamura et al., (1974a), (1974b), were able to isolate both the killer and the sensitive strains from their sake fermentations. The characteristics of these sake killer strains were similar to the killer strains of S. cerevisiae.

As shown in Table 2.1, note that during the early analysis of the zymocidal strains, an additional phenotype called "killer-sensitive" was introduced. It was used to describe yeast strains which were able to kill the sensitive strains. But, these strains were also found to be sensitive to the toxins produced by other killer strains, suggesting that more than one killer toxin system might exist.

All killer-sensitive phenotype designations were assigned based on the results of a test similar to the Well-test method used in antibiotic analysis (Bussey, 1973). To perform a Well-test for zymocidal toxin analysis, two yeast strains are required. The first strain is mixed and poured in a molten agar medium to form a lawn containing 10^5 cells. A sample of the second strain, containing 10^2 cells, is then spotted on the surface of the lawn. The plate is then incubated for 24 - 48 hours at a suitable temperature. If the second strain is able to produce a zymocidal toxin, and this toxin is able to

TABLE 2.1

OCCURRENCE OF THE KILLER PHENOTYPE IN SELECTED STRAINS OF VARIOUS YEAST GENERA*

SPECIES	NO. OF STRAINS TESTED	K	S	N	K-S
Saccharomyces cerevisiae	621	8(1.3)*	561(90.3)	37(6.0)	15(2.4)
ex. *S. bayanus*	104	0 -	93(89.4)	5(4.8)	6(5.8)
ex. *S. chevalieri*	28	0 -	24(85.7)	2(7.1)	2(7.1)
ex. *S. italicus*	29	0 -	29(100)	0 -	0 -
Total	782	8(1.0)	707(90.4)	44(5.6)	23(2.9)
Saccharomyces rosei	59	0 -	0 -	59(100)	0 -
S. uvarum	42	0 -	27(64.3)	15(35.7)	0 -
Saccharomycodes ludwigii	30	0 -	0 -	30(100)	0 -
Schizosaccharomyces octosp.	9	0 -	0 -	9(100)	0 -
Sansenia spora valbiensis	49	0 -	0 -	49(100)	0 -
Hansenula anomala var. anomala	56	40(71.4)	0 -	16(28.6)	0 -
H. californica	18	0 -	0 -	18(100)	0 -
H. minuta	6	0	0 -	6(100)	0 -
H. saturnus	9	0 -	0 -	9(100)	0 -
H. subpelliculosa	5	0 -	0 -	5(100)	0 -
Kluyveromyces veronae	25	0 -	0 -	25(100)	0 -
Pichia fermentans	14	0 -	0 -	14(100)	0 -
P. membranaefaciens	24	0 -	0 -	24(100)	0 -
Kloeckera africana	3	0 -	0 -	3(100)	0 -
K. apiculata	103	0 -	0 -	103(100)	0 -
K. corticis	14	0 -	0 -	14(100)	0 -
Torulopsis stellata	25	0 -	0 -	25(100)	0 -

*Figures in parentheses refer to the percent distribution of the different phenotypes.

*Source: Rosini, 1983

Legend :

K = Killer (Produces a Zymocidal Toxin)
S = Sensitive (Killed by Toxin Produced by other Zymocidal Strains)
N = Neutral (Neither produces, nor is killed by the Zymocidal Toxin)
K-S = Killer - Sensitive (Produces a Zymocidal Toxin, but is killed by other Zymocidal Toxins)

kill the sensitive strain which formed the lawn, a circular clearing zone will appear in the lawn. The size of this clearing zone is dependent on the sensitivity of the cells in the lawn and the amount of toxin produced by the zymocidal strain. If no clearing zone appears in the agar lawn after a known killer strain is spotted onto the lawn, then the strain forming the lawn is given the 'resistant' phenotype. Cross testing of all yeast strains, using known killers and sensitive strains is then required to determine the killer-sensitive phenotype of each individual strain. The results of this type of analysis are qualitative and only allow determination of the phenotype designations. Refinement of the analysis system is necessary before a quantitative zymocidal classification is possible.

Careful scrutiny of results obtained from all of the analyses of S. cerevisiae and other yeast strains, have led to the conclusion that there are at least 11 different extracellular toxins (Wickner, 1979). The cross testing of these strains was used to explain the existence of the killer-sensitive phenotype strains. Young and Yagui,(1978) introduced a new classification system which was later refined by Wickner, (1979) to expand the original phenotype designations. In this system, yeast strains were denoted as K_1 , K_2 , K_3 , ..., K_{11} . The 'K' denotes the ability of the strain to produce a killer toxin, and the subscript number indicates which of the eleven toxins is produced. Special mention should be made of the K_1 toxin as having been the zymocidal toxin first discovered by Makower and Bevan (1963). Resistance of a yeast strain to a particular toxin is denoted by a similar system. The symbols of R_1 , R_2 , R_3 , ..., R_{11} are used to denote the resistance to the corresponding toxin 1, 2, 3, ...,

11. It should be noted that a particular strain is not killed by its own toxin. Therefore, a cellular system described by using the symbols $K_1 R_1$ is able to produce and is resistant to the number one toxin. Table 2.2 reviews the cross reactions of the various yeast strains able to produce toxins and show resistance to other various toxins.

To keep the extent of killer systems in perspective, it should be noted that the results shown in Tables 2.1 and 2.2 were generated from experiments on petri dish solid media. Many of the killer strains will produce a toxin that is not stable enough in liquid broth to kill sensitive cells (Young and Yagui, 1978). These results also show that the killer character is much more prevalent in laboratory yeast strains than in wild-type strain. This was first noticed by Fink and Styles (1972). These workers suggested that the killer character was a cytoplasmic element which could only be stabilized in a cell strain under laboratory conditions.

2.2 Determination of Toxin Size and Structure

Initially, analysis of cell free broth indicated that the factor responsible for toxin action was a proteinaceous molecule that had small amounts of bound polysaccharides. This led early researchers to indicate that the toxin was a glycoprotein (Bussey, 1972). Since the time of this early analysis, the K_1 killer toxin has been purified 79,000 fold to homogenity (Palfree and Bussey, 1978) using a complex procedure of ultrafiltration of the culture medium, polyethylene glycol precipitation of the toxin, dissociation of the toxin from mannaprotein by urea, and glyceryl-controlled pore glass gel filtration to separate

TABLE 2.2

KILLER YEASTS: RANGE OF KILLING AND IMMUNITY

		STRAINS TESTED FOR SENSITIVITY											
		KILLER STRAINS											
		RESISTANCE SPECIFICITY											
SPECIES	KILLER SPECIFICITY	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	
Saccharomyces	K1	R	S	S	S	R	R	R	R	R	R	R	S
Saccharomyces	K2	S	R	R	S	R	R	R	R	R	R	R	S
S. capensis	K3	R	R	R	R	R	R	R	R	R	R	R	
Torulopsis glabrata	K4	R	R	R	R	R	R	R	R	R	R	R	
Debaromyces vanrijii *Hansenula anomala* *Hansenula subpelliculosa*	K5	R	R	S	S	R	R	R	R	R	R	R	
Kluyveromyces fragilis	K6	R	S	S	S	R	R	R	R	R	R	R	
Candida valida *Pichia membranaefaciens*	K7	R	R	S	S	R	R	S	R	R	R	R	
Hansenula anomala	K8	R	S	S	S	R	R	S	R	R	R	R	
Hansenula mrakii	K9	S	S	S	S	S	R	R	R	S	R	R	
Kluyveromyces drosophilarum	K10	S	S	S	S	S	S	S	S	S	S	R	S
Torulopsis glabrata	K11		S									S	R

*Source. Wickner, 1979

Legend :

S = Sensitive (Killed by the Zymocidal Toxin K_a)

R = Resistant (Not killed by the Zymocidal Toxin K_a)

the toxin from the mannaproteins. Electrophoresis showed that the toxin migrated as a single band of 11,000 ± 1,000 MW on sodium dodecyl sulfate-polyacylamide gel. The identification of the single protein band as being the toxin protein was further confirmed by the absence of this protein in the medium from zymocidal strains cured of the killer plasmid. To further verify this protein as the toxin, the purified material was chromatographed on SP-Sephadex G25, and the toxin activity and the protein co-chromatographed almost exactly. Earlier difficulties in purification of the toxin were caused by its high affinity for mannaprotein which was lost from the cell wall into the medium during cell growth. The active toxin protein purified by Palfree and Bussey, (1979) has no detectable carbohydrate, less than two hexasyl residues per polypeptide chain. Purification and concentration of cell free broth from sensitive strains did not show a protein of this size upon gel electrophoresis.

Further analysis of the protein structure was determined and it was found to be a single polypeptide chain having a length of approximately 109 amino acids, a pI value of 4.5, as determined by isoelectric focusing (Bussey, 1981) and a calculated molecular weight of 11,470 (Palfree and Bussey, 1979). Proteolytic digestion of the toxin by the V8 protease of Staphylococcus aureus has shown that the toxin lacks proline, and arginine, but is very rich in acidic amino acids (ie 21% of the total). It has also been determined that the toxin is hydrophobic because there are insufficient hydrophilic amino acid residues to expose to the solvent, even if a spherical shape is assumed (Palfree and Bussey, 1979).

The size of the toxin molecule derived from the sake fermentations has been reported as being as large as 50,000 MW by ultrafiltration and gel electrophoresis (Imammura et al., 1974a). Since no urea treatment was used to dissociate the mannan or mannaproteins from the toxin molecules, there may be some error in determining the molecular weight of the toxin.

2.3 Detection of Toxin in Cell Free Broth Samples

Determination of zymocidal toxin concentrations in cell free broth samples from fermentations using the method of Palfree and Bussey (1979), outlined above was difficult due to the large sample volumes required for each analysis. In addition, any quantitative analysis of the results from this analysis would be difficult due to the complexity of the overall process. It is therefore necessary to find a more direct and easier method to quantatatively determine the toxin concentration in broth samples.

Attempts to correlate the extracellular protein concentration with zymocidal activity have met with limited success (Panchal, personal communication 1986). Colorimetric protein analysis procedures such as the Lowry test (Lowry et al., 1951) require more concentrated protein solution than is available in extracellular samples from yeast fermentations. To add to the inaccuracy, it has been estimated that the zymocidal toxin can comprise as little as 5 - 15% of the total extracellular proteins (Bussey, 1981).

Additional complication in the determination of the zymocidal toxin concentration is due to the toxin's affinity for mannan while in liquid solution. As mentioned previously, the zymocidal toxin is most likely hydrophobic, and therefore, it attaches itself very tightly to mannan or mannaprotein present in the liquid broth. As indicated, this association led early researchers to believe that the protein was a glycoprotein (Bussey, 1972, and Woods and Bevan, 1968). To release the toxin from the mannan, urea treatment is required.

These problems were encountered by Woods and Bevan (1968) as they attempted to use the Folin - Ciocaltea test (1927) for protein combined with various cell broth pre-treatments. These authors, and others, concluded that the modified Well-test method represented the easiest and most reliable test for zymocidal protein concentration determination.

To determine the zymocidal toxin concentration in a cell free broth sample, the broth can be spotted onto a pre-poured lawn of sensitive cells and incubated for 24-48 hours. The size of the clearing zone formed by the diffusion of the broth sample through the agar lawn is directly proportional to the concentration of the toxin in the sample. As long as the sample size, the number of sensitive cells and the agar remains consistant, this test has been shown to be accurate (Ouchi et al., 1978).

2.4 Toxin Stability

The effectiveness of zymocidal toxin in killing sensitive cells is very much a function of the environmental conditions. The zymocidal toxin is much more stable in solid media than in liquid broth. Zymocidal toxins produced by genetically stabilized yeast strains have been found to be more stable than toxins from 'wild-type' yeasts. Therefore, it is very important to determine the optimum environmental conditions for toxin activity and stability.

2.4.1 pH Stability of Zymocidal Toxin

The optimum pH range for zymocidal activity has been found to be 4.2 - 4.6 for most of the toxins, although some toxins have been found to have a slightly wider optimum pH range in both solid and liquid media. Outside of this optimum pH range, the toxin does not immediately lose its ability to kill. It does, however, rapidly lose its activity (Rogers and Bevan, 1978) and inactivation at high pH levels is irreversible, due to conformational changes in its structure (Bussey, 1981). It appears as though the pH deactivated toxin has the same molecular weight as the activated toxin species. Clearly, care to insure that the pH remains within the optimum for proper activity is an important consideration when employing zymocidal toxins in fermentation systems.

It was found that glycerol has the ability to broaden the pH optimum "window" but, significant concentrations of the glycerol (20 to

30%) would be required before significant effects could be obtained (Ouchi et al., 1979).

2.4.2 Heat Stability of Zymocidal Toxin

As the toxin is a protein of natural origin, the temperature stability is an important property, especially if zymocidal systems are to be employed in beverage production. On agar, the toxin has been found to be stable at temperatures up to 42° C, and in liquid broth the protein is rapidly deactivated at lower temperatures (Woods and Bevan, 1968). The heat stability of the toxin in liquid broth can be increased substantially by the addition of 20 to 30% glycerol (Ouchi et al., 1978), and concentrations of glycerol less than 20% did not enhance thermal stability.

The concentration of glycerol produced during fermentations by S. cerevisiae, or by sulphite induction (Bispling and Rehm, 1982) are too low to produce stabilization of the toxin. It would therefore be necessary to add glycerol to fermentations when conditions adverse to the toxin stability are employed, which of course is not desirable in any large scale application of zymocidal strains. Clearly, maintenance of the optimal environmental conditions to maximize toxin activity is a desired beneficial objective.

2.4.3 Other Factors Affecting Toxin Stability

The zymocidal toxin has also been found to be unstable in high ionic strength solutions, especially in the presence of chaotropic

agents. The toxin is stable in antichaotropic agents such as sulphate (Bussey, 1981).

Vigorous mixing, such as, for 15 minutes in a vortex genie, can also rapidly deactivate the toxin (Wickner and Leibowitz, 1976). Very little or no deactivation is reported at mixing rates that are characteristic in fermentation systems. These observations suggest the effect of shear on the stability and activity of the zymocidal toxin.

2.4.4 Synergistic Affects

It was found by Bussey (1981) that the zymocidal toxin is stable indefinitely in liquid solutions containing 15% glycerol at a pH of 4.6 and a temperature of 4 $^{\circ}$ C (Bussey, 1981). Clearly this set of conditions cannot be adequately (or conveniently) maintained in a fermentation process. Therefore, analysis of the kinetics of toxin degradation with respect to the pH and temperature effects is very important, and is subsequently one of the main objectives of this thesis research.

Imamura and his co-workers have reported that zymocidal strains isolated from sake fermentation systems show the same characteristics (with respect to heat, pH, and vigorous shaking) as the toxins derived from *S. cerevisiae* strains (Imamura et al., 1974a).

2.5 Toxin Production Characteristics

Early analysis of zymocidal toxin production by cells of S. cerevisiae resulted in the determination that toxin production required complex nitrogen and carbon sources such as bacto-peptone and yeast extract to produce high titres of the toxin (Palfree and Bussey, 1979). It was also discovered that growth of zymocidal producing S. cerevisiae at elevated temperatures, "cured" (or removed) the ability of the strain to produce the toxin (Fink and Styles, 1972, and Wickner, 1974). These results led these workers to conclude that elevated temperatures (37 - 40 ^{o}C) caused an irreversible loss of the ability to produce the toxin, most likely by mutation. Cycloheximide, which inhibits protein synthesis in the cytoplasmic ribosomes, has been shown to stop killer activity (Fink and Styles, 1972). These results showed the early workers that killer activity is dependant on a protein (or proteins) coded on cytoplasmic ribosomes.

The only batch growth data available in the scientific literature suggest that toxin production by zymocidal S. cerevisiae takes place during the exponential growth phase, with no accumulation during the stationary growth phase (Imamura et al., 1974a, and Palfree and Bussey, 1979). Also noted by Palfree and Bussey (1979) was that the rate of production of active toxin did not appear to be directly related to the rate of cell growth.

These early analyses only considered accumulation of active toxin and did not account for toxin deactivated by the environmental conditions in the batch fermentor. A complete kinetic analysis, which

includes both the active and inactive toxin is needed before production characteristics can be accurately assessed. To achieve this, the modified Well-test method must be used to measure the active toxin concentration. The overall kinetics of toxin deactivation can then be utilized to estimate the total active and inactive toxin concentration. Clearly, this early, preliminary work suggested the need for a more in-depth basic study of the kinetics of both cell growth and toxin production.

2.5.1 Genetics of Toxin Production

Initial findings by other research groups led to the suggestion that the killer characteristic is actually inherited between the yeast strains as a dominant, non-Mendelian, genetic element (Bevan et al., 1973). In addition, it was found that the specific cytoplasmic genetic element is a double-stranded (ds), $1.2 \times 10^6 - 1.5 \times 10^6$ MW dsRNA which has been named "M", and it is encapsulated in a large molecule protein coat (Bevan et al., 1973, Herring and Bevan, 1974).

An additional cytoplasmic component is required for a specific cell strain to maintain the M dsRNA. This dsRNA is larger than the M particle and has been given the name "L". This L particle has a $2.5 \times 10^6 - 3.0 \times 10^6$ MW and has been shown to produce a large molecular weight protein present in the coats of both the M and L particles (Tipper and Bostian, 1984). Some yeasts have been shown to possess the L particle but not the M. In these strains, the L dsRNA appears to act as a cytoplasmic parasite which apparently does not affect the growth cycle of the cell in any way (Wickner, 1979). All cells of S.

cerevisiae are able to maintain and reproduce the L dsRNA. This is true even when the L dsRNA is introduced to a strain that does not normally contain any "L".

These particles seem to resemble viruses as both the L and the M dsRNA strands are encapsulated in the protein coating (Berry and Bevan, 1972, Bevan et al., 1973). These, and other dsRNA containing particles found in fungi, are sometimes called mycoviruses by some authors. Since viruses are able to pass from cell to cell extracellularly, but these 'mycoviruses' cannot, they can not be called true viruses (Wickner, 1984). This has led researchers to describe the "L" and "M" dsRNA systems and similiar systems in fungi as "Virus-Like Plasmids or Particles" (VLP) (Herring and Bevan, 1977, Breunn, 1980). Since these particles can not be transmitted extracellularly, inheritance of the ability to produce the killer toxin must be possible only through forced or natural cytoplasmic mixing. This would in turn suggest that the maintenance and replication of both the L and M dsRNA particles are a function of the host cell and therefore must be controlled by the genetics of the host cell (Wickner, 1978).

Cytoplasmic mixing that is accomplished by rare mating, natural, or forced techniques has allowed researchers to generate diploid, haploid, and polyploid S. cerevisiae strains possessing the killer characteristic (Young, 1981 and Siki et al., 1985). These strains are generally more stable than wild - type killer strains and they are able to produce higher titres of toxin than the wild type strains. These laboratory stabilized strains enable researchers to study the cellular processes without the worry of loss of killer

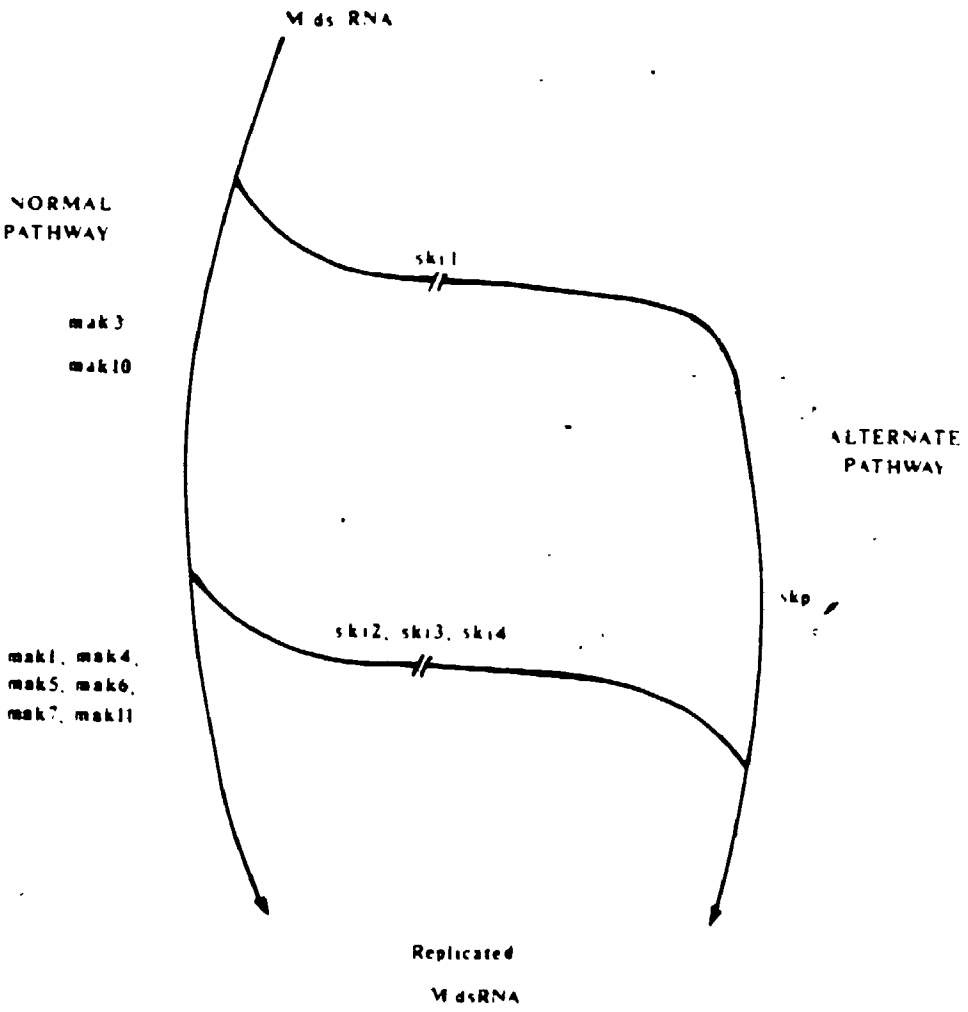
activity. Killer yeasts for sake fermentations have successfully been constructed by inducing cytoplasmic mixing between the killer and sensitive strains (Ouchi et al., 1979, Seki et al., 1985). The fermentation characteristics of these new killer strains were found to resemble the regular sake brewing strains, producing sake of a satisfactory quality.

2.5.1.1 The M dsRNA VLP

Replication and maintenance of the M dsRNA must be a function of the host cell system. It was determined that a minimum of 28 chromosomal genes (known as maintenance of the killer, or mak genes) are required to maintain and replicate the M dsRNA (Wickner, 1984). While the individual role of each gene (or gene product) in the maintenance of the killer plasmid is unknown, the overall process confirms the dependence of the M dsRNA on the host's genetic system. Mutants in each of the 28 mak genes cause the host cell to lose its M dsRNA while still being capable of retaining its L dsRNA. This ability shows how the maintenance and the replication of L and M plasmids are carried out by separate enzymes or genetic systems (Bevan et al., 1973, Ridley and Wickner, 1983).

Four additional genes have been found which result in an increased production of killer toxin (Tipper and Bostian, 1984). Recessive mutations in any of these four chromosomal genes, when combined with the mak mutations, produce strains that are still able to replicate the M dsRNA and produce high titres of the killer toxins. This suggests that two separate enzymatic pathways exist for the

Figure 2.1 - Heuristic Model of M dsRNA Replication showing the interaction of the mak and ski genes. The alternate pathway is normally inaccessible to M ds RNA because of the ski proteins. This model also assumes the existance of skp genes which are required for the alternate pathway, but not for the normal pathway. (Wickner, 1979)


M ds RNA
NORMAL PATHWAY
mak3
mak10
ski1
ALTERNATE PATHWAY
skp
ski2, ski3, ski4
mak1, mak4, mak5, mak6, mak7, mak11
Replicated M dsRNA

maintenance and replication of the M dsRNA (Wickner, 1979). Wickner further suggests that the wild - type killer strains utilize the "normal" pathway (as controlled by the mak genes), and that this pathway is blocked in most wild - type strains by mak mutants (Wickner, 1983) resulting in loss of the killer character.

Access to the "alternate" pathway is negatively controlled at various points by the products of these super killer or "ski" genes. It has been demonstrated that mutants deficient of the "ski" genes are able to produce large amount of killer toxin (Palfree and Bussey, 1979). This research further showed how the killer toxin produced from laboratory ski mutant strains is more stable to heat and other treatments than wild - type strains. In most cases, laboratory killer strains are actually stable ski mutants. Since mutation in any of the 28+ mak genes will cause the loss of the ablility of the cell to maintain and reproduce its M dsRNA plasmids, it appears obvious why the killer characteristic is seldom found in the wild - type strains.

It has also been postulated by various researchers that the M dsRNA codes for both the immunity to the toxin as well as the toxin itself. This is based on the observation that loss of the ability of a strain to produce the toxin is accompanied by a loss of immunity to the affects of the toxin. Therefore, the intercellular processing of the M dsRNA protein for the toxin/immuntity structure is essential to the understanding of the mode of action of the toxin and the ability of the yeast cell to be immune to the toxins effects.

2.5.1.2 The L dsRNA VLP

From the earliest discovery of the L and M dsRNA particles, the interdependency of M upon L has been well documented (Bevan et al., 1973). Cells that are deficient in L cannot maintain the M dsRNA particle. More recent research showed that both the M and the L containing particles are coated with a major 75,000 MW polypeptide (Herring and Bevan, 1977). This protein is the major product coded by the L dsRNA. Since the M dsRNA is only able to produce the toxin/immunity molecule, M plasmids cannot be maintained without the L dsRNA particles and its product. No other use has been determined for the existence of the L dsRNA system in any S. cerevisiae strain analyzed (Tipper and Bostian, 1984).

2.5.2 Zymocidal Activity and Immunity

2.5.2.1 Mode of Action of the Toxin

Treatment of sensitive cells with killer toxin for 40-50 minutes at 22 - 24 $^{\circ}$ C, will produce a leakage of intracellular ATP, potassium ions, and a stoppage of all macro molecular synthesis (Bussey et al., 1973). While evidence clearly indicates that this is an effect of the killer toxin, the time delay suggests that the killing is a complex process. Nevertheless, leakage of ATP and intracellular potassium has been a useful marker of toxin actions. Zymocidal strains from sake fermentations have been shown to be able to kill sensitive strains only during the logarithmic phase of growth of the sensitive cells. Stationary cells of sensitive sake strains are unaffected by the sake killer toxins. Toxins derived from glucose grown cells of S.

cerevisiae have been reported to be able to kill sensitive strains throughout the entire growth cycle. Stationary cells or ethanol grown sensitive cells require a longer treatment time before they are killed (Wickner, 1981).

Kotani et al. (1977) studied a K_1 killer strain from sake yeast, and they showed that the death of toxin - treated cells was aided by ADP and alternately prevented by calcium. Furthermore, these researchers found that ATP leakage by toxin treated cells was promptly halted by the addition of calcium to the medium. Sensitive cells, when treated with toxin for one hour, were reduced to 2 to 4% viability if plated on a yeast extract - peptone - dextrose medium. However, 94 to 100% of the cells were viable if plated on the same medium with an additional amount of (0.1 M) $CaCl_2$. Kotani and his co - workers then proposed a two stage model for the killing action. The first stage being reversible and the second stage irreversible, but blocked by calcium and promoted by ADP. The second stage of this killing action, as proposed by this model, would be accompanied by ATP and potassium leakage from the cell. Similar results were obtained by Middlebeck et al., (1980) for the killer toxin produced by Pichia kluyveri on sensitive cells of S. cerevisiae. In this work, potassium was shown to prevent death in the sensitive strain, while sodium did not demonstrate any affect. Complete recovery of toxin treated cell suspension was possible after an incubation for three hours in a suitable medium.

Using drugs to affect ATP synthesis and utilization, Skipper and Bussey (1977), and Bussey et al., (1979) studied the role of the cell's energy state on its own sensitivity to toxin. An ethanol - grown

sensitive strain was made partially resistant to the yeast killer toxin by antimycin, cyanide, or carbonyl cyanide m-chlorophenyl hydrazone (CCCP). None of the drugs tested prevented the killing of the more sensitive glucose - grown cells but, 2,4-dinitrophenol (DNP) and CCCP both prevented potassium release. These results have led these authors to propose a two - stage model similar to that of Kotani et al. (1977). The first stage being the binding of the toxin to a receptor site on the sensitive cell and the second stage, the DNP- or CCCP- sensitive potassium leakage.

Since the toxin must bind to the cell wall before the cell is killed, analysis of the binding site of the toxin on the cell wall may lead to some indication of how the immunity to the toxin works. Tipper and Bostian (1984) reported that the toxin action involves an initial, rapid, energy independent binding of the toxin to a cell wall receptor that has been identified as 1,6 β - D-glucan (Wickner, 1981, and Bussey et al., 1979). Normal sensitive strains of yeast can contain as many as 1.1×10^7 of these sites per cell. As few as 2.8×10^4 toxin molecules actually need to be attached to these sites in order to kill the cell (Bussey et al., 1979). The binding of the toxin to the cell - wall receptor of the sensitive cells is very similar to the tight association of toxin to mannan in concentrated toxin preparations. Although the binding is reversible, the attachment is nonetheless very strong (Bussey, 1981).

The second stage of the toxin action is an energy dependent process which transfers the toxin to its action site on the cytoplasmic membrane (Tipper and Bostian, 1984). The action of the killer toxin

from this point on seems to involve the selective functional damage to the plasma membrane (Kagan, 1983). The affects of this damage includes the leakage of potassium, the stoppage of active transportation of amino acids, and the acidification of the cell interior (de la Pena, 1981, Tipper and Bostian, 1984, and Wickner, 1981). This series of events is reported to be similar to that of the effects of bacterial colicins which have been shown to produce channels in membranes (Wickner, 1981). This second, energy dependent step in the killing process suggests a reason for the differences noted between the sensitivity of logarithmic phase and stationary phase yeast cells. As reported earlier, stationary phase sensitive sake yeast strains and ethanol grown sensitive S. cerevisiae are both less sensitive to the toxin action. This suggests that there is a difference in the energy state of these cells as compared to glucose grown cells (Skipper and Bussey, 1977).

2.5.2.2 Maturation and Expression of the Toxin

Until fairly recently, the process of maturation and expression of the zymocidal toxin was unknown. Initial attempts to map the M dsRNA for the K_1 toxin were reported by Wickner (1983). This report outlined a model for the M_1 structure, where the M_1 dsRNA codes for the K_1 toxin. The model proposes that an A-U rich bubble separates a 32,000 MW toxin precursor molecule from a 19,000 MW protein of an unknown function.

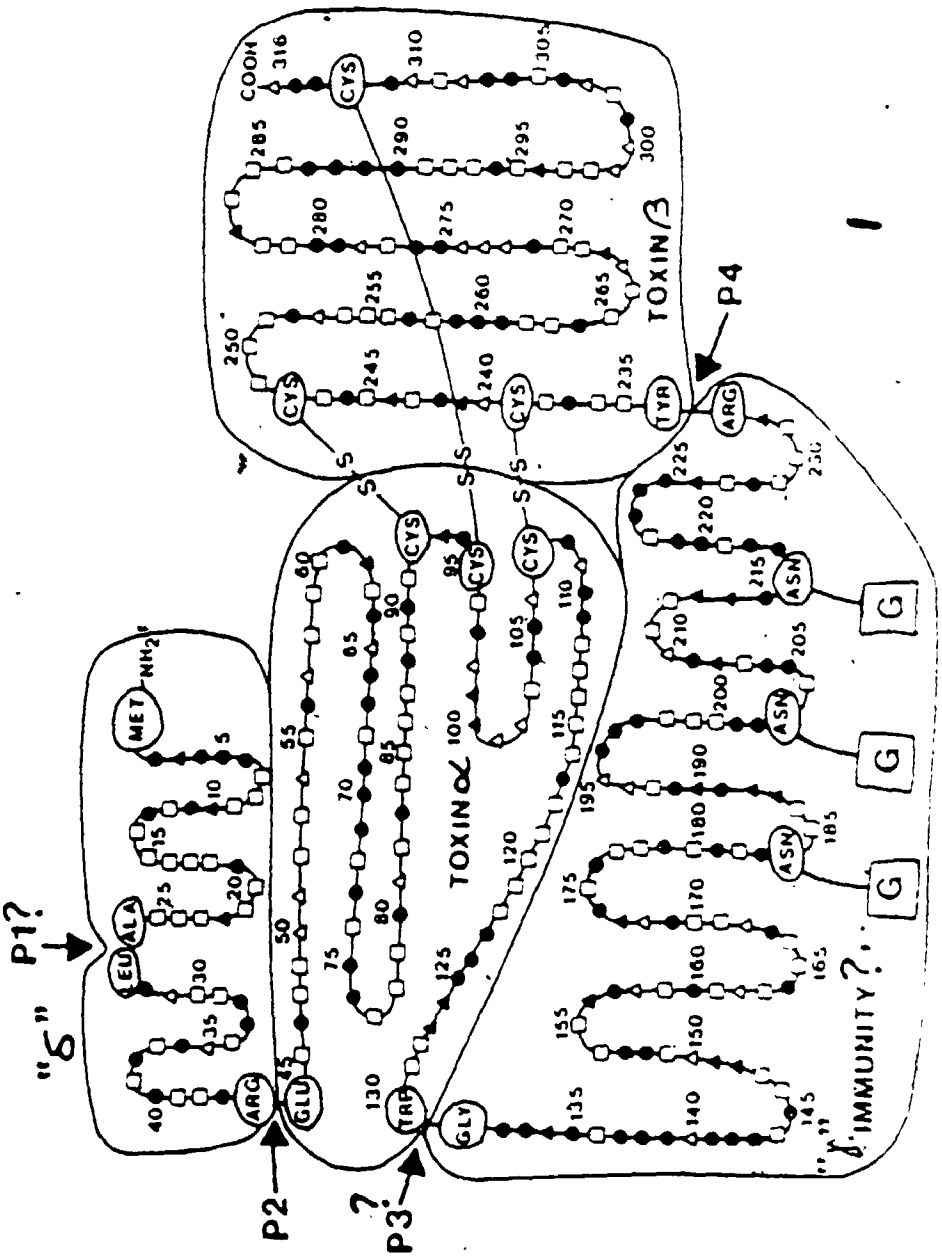
A complete map of the M_1 dsRNA gene was elucidated very recently (Bostian et al., 1984). Based on this work, these researchers

suggest that the toxin exists as a two component system similiar to human insulin. This led these workers to further propose a toxin maturation process similiar to that of insulin. The schematic diagram of the proposed toxin precursor molecule is shown in Figure 2.2.

Essentially, the toxin precursor, as coded by the M dsRNA, is processed in the same way as other secreted proteins (Tipper and Bostian, 1984). In this particular case, the 32,000 MW toxin precursor molecule is composed of four distinct regions. The first area (δ) is 1,600 MW in size, and it is considered to be a leader protein as no other purpose has been discovered for its ultimate use. Analysis of culture broths to find a protein of this size would confirm the above hypothesis. The remaining toxin precursor molecule is composed of an α and a β portion (each having a 9,500 MW) and an additional γ portion of 13,000 MW. The α and the β portions are the two components of the toxin system that are attached together by disulfide bonds in a manner similar to those of insulin. The large portion (γ) of the toxin precursor molecule is assumed to be the molecule which enables the zymocidal cell to be immune to the affects of the toxin. It is proposed that this γ portion is able to bind to the outside surface of the toxin producing cell, and so it effectively blocks the cell wall receptors from accepting the toxin molecules. It is also assumed that the γ portion will position itself such that its glycosylated portions are exposed. This therefore would allow the toxin to bind to the outside of the immunity layer (Bostian et al., 1984).

Since the structure of the γ subunit as proposed by Bostian et al., (1984) cannot span the cell membrane, nor, attach itself to the

Figure 2.2 Schematic Domain Structure of the Toxin Precursor or Preprotoxin. Disulfide bonds drawn between the subunits are arbitrarily chosen. Glycosylation sites (G) are indicated. Symbols: - hydrophobic amino acids; - acidic amino acids; - basic amino acids; - other. (Tipper and Bostian, 1984)


"δ"
P1?
P2
?
P3
P4
TOXIN α
TOXIN β
"γ" IMMUNITY?
MET
NH2
LEU
ALA
ARG
GLU
TRP
GLY
CYS
TYR
ASN
G
COOH

1,6 β - D-glucan receptor sites, these authors proposed that the subunit must attach itself to cell membrane proteins.

Three recessive chromosomal genes, known as kre 1 through to kre 3 for killer resistant, have been shown to play some role in the immunity of the host cell to the effects of the toxin (Wickner, 1979). Kre 1 and kre 2 as mutant - sensitive strains did show a decrease in the cell wall binding of the toxin, however the kre 3 mutants showed no apparent difference. The Kre 1 and kre 3 mutant strains were shown to be resistant to all toxins tested. Alternately, the kre 2 strains were only resistant to S. cerevisiae K_1 toxin. The α and β subunits of the toxin molecule have been found to contain a high content of charged and hydrophobic amino acids (Bussey et al., 1979). This excess charge, which resides mostly in the β portion, is responsible for the toxin having a pI of 4.5. In addition, the α and β subunits are joined by disulfide linkages which will separate only after the β portion has attached itself to a cell wall receptor site.

A parallel between the maturation of the toxin and that of insulin was drawn by Tipper and Bostian (1984). Both the insulin and the toxin are processed by the removal of a leader and internal peptides. Thus, what remains are two disulphide - linked peptides. The insulin 'C' peptide is smaller that the γ peptide removed from the toxin precursor but, the insulin 'C' peptide is assumed to only ensure that the insulin precursor exceeds a minimal size for the mammalian secretion pathway. A much more important role for the γ subunit is suggested by its size. As outlined above, this γ subunit is thought to be the immunity determinant and in addition, this γ subunit may

mask the active site of the toxin until it is released. Thus, it will protect the producing cell from the effects of the toxin.

Continuing the parallel between insulin and the killer toxin, the model of Tipper and Bostian suggests that three cleavages are needed to produce the toxin from the precursor molecule. For both the toxin and insulin, the trypsin - like linkages must be separated and probably involve serine proteases. Processing of the toxin would then most likely occur in the endoplasmic reticulum and result in the release of the separated γ subunit, the δ leader and the disulphide linked α - β subunits.

2.5.2.3 Factors Affecting Toxin Action

Of major concern in the applications of zymocidal strains is the effects of temperature and pH on the killing ability of the toxin. As outlined previously, the pH range to maximize activity is a narrow band of 4.2 to 4.6. Outside this optimum range or as temperatures increase, the toxin is rapidly deactivated. The killer strains have been shown to lose their ability to produce the toxin at temperatures above 37 ^{o} C.

Long et al., (1982) studied the effects of temperature on the in vitro transcription of the L and M plasmids of a S. cerevisiae killer strain. It was found that transcription of the M- dsRNA showed a marked inhibition at 37 ^{o} C, and became completely inactive at temperatures higher than 42 ^{o} C. Alternately, the L-dsRNA was unaffected at the same 37 ^{o} C temperature, but exhibited a slight increase in

activity at 42 °C. These results indicate that the 'heat curing' of killer S. cerevisiae strains in vitro may be due to a direct influence on the M-dsRNA particle, and thus is not completely due to the suggested inactivation of some of the mak genes (Wickner and Leibowitz, 1976).

The pH influence on the binding of toxin to sensitive yeast cells was a topic investigated by Imamura et al., (1975). These workers discovered that in addition to its inactivation at non-optimum pH levels, the killer toxin was able to bind to cells in the pH range of 3 to 8. It was found that at pH values greater than 9, the toxin did not bind to the cell wall receptors. It was also noted that the bound killer toxin could be subsequently removed from the killer (or sensitive) cell wall receptors by adjusting the pH of the suspension to values between 9 and 11, for a period of 20 minutes while at a temperature of 3 °C. This phenomenon occurs well outside of the normal pH range for yeast growth, and likewise outside of the pH optima for zymocidal toxin activity. This should not create any undo complications during most yeast fermentations.

2.6 A Comment on the Name: Zymocidal or 'Killer'

Before the (1963) discovery of killer toxins in yeast, it was established that there existed cytoplasmic virus-like particles which contained strands of dsRNA in filamenteous fungi. None of these 'mycovirus' systems, including the yeast killer system, has been shown to be capable of extracellular transmission (Tipper and Bostian, 1984, Bussey, 1981). It has been suggested that the zymocidal toxins are more

accurately mycocins, due to their similarity to bacteriocin (Woods and Bevan, 1968). The VLP system would then be more accurately called a mycovirus. Although possibly more accurate and proper than the names 'zymocidal' or 'killer', the added older names are more descriptive. Further work is necessary at the microbiological and biochemical levels to fully assess the appplicability of the mycocin and mycovirus terminologies (Wickner, 1983).

The killer characteristic of yeast strains, especially in the S. cerevisiae genera, can very effectively kill sensitive cells of similar genera. It is interesting though, that there are so few wild strains of yeast with the killer characteristic. Two different proposals have been put forward to explain this phenomenon. The complexity of the wild-type replication and maintenance mak gene system must be considered. Since mutation in any of these 28+ mak genes will cause the loss of the M plasmid, care to insure proper environmental conditions is important. Wild-type strains, which are subjected to all ranges in environmental conditions, would most-likely lose their ability to produce the toxin. Firstly, the wild killer strains have been shown to be very inefficient assassins. Only by laboratory isolation and stabilization can the killer character become truly potent (Tipper and Bostian, 1984). Secondly, expression of the killer character requires two dsRNA virus-like particles: the L and M. Both of these dsRNA particles are required, and transmission only can occur by cytoplasmic mixing. Since cytoplasmic mixing transfers the genetic material between cells and chromosomal genes control the overall replication and excretion process, a mutation which would result in a strain acquiring the killer character is impossible. Only under a

particular set of conditions can a previously sensitive strain be made killer. This set of conditions is easily developed in the laboratory, but is seldom attainable in nature. These two theories suggest that the killer character is easily lost by wild-type strains. Once lost, the killer character is not easily regained. Therefore it is not surprising that so few wild-type killer strains exist.

2.7 Industrial Experience with Zymocidal Systems

Killer yeast strains have not been applied in industrial fermentations as of yet, but they have been reported as being contaminants in both batch and continuous beer production processes. The first report of killer yeast strains as contamination in beer production was reported by Mauli and Thomas (1972). These workers isolated a killer strain of yeast from production-scale, two-stage, continuous fermentations. They reported that a concentration of only 2% killer cells would produce sufficient toxin to give the killer yeast strain an advantage in the continuous fermentation over the desired brewery strain which was rapidly killed. The beer produced by the killer strains acquired a fruity "off"-flavour which has been described as as herbal or phenolic, thus demonstrating the adverse effects of the killer strains on the quality of beer.

The discovery of a killer strain as a contaminant in batch beer production systems was not reported until 1979 (Taylor and Kirsop, 1979). During these fermentations, the wort fermented much more slowly and the green beer had the same phenolic aroma as that reported in the continuous systems. The strain isolated in the batch fermentation

system was morphologically different from the killer strain isolated by Maule and Thomas (NCYC 738), and it also differed slightly in its pH optima for toxin activity, when compared to other killer strains such as NCYC 1001. All subsequent reports of the fermentation properties of these wild type killer yeast in laboratory beer fermentations indicate that they ferment the wort slower than normal brewery strains (Young, 1983). In most cases, these wild strains also produced the phenolic off-flavours reported in the industrial batch and continuous systems.

Much work has been completed to show the overall influence of the killer character on the production of beer (Young, 1983, Hammond and Eckersley, 1984, Young and Philiskirk, 1975). All of these workers crossed a brewery strain of S. cerevisiae with a killer strain, by using forced mating, to successfully transfer the killer character. The beer produced with this altered strain differed very little from regular fermentations which use the non-killer strains. Their efforts demonstrated how it is possible to transfer the advantageous killer trait to regular brewing strains without compromising the quality of the final product. This development suggests that two advantages in industrial applications can be offered by the usage of the new killer strains (Young, 1981). Firstly, these new systems can be immune to the toxins produced by other wild killer contaminants, which can allow the brewery strain to grow better and to compete better than the contaminant strain. Secondly, the toxin produced by the killer brewery strain will prevent growth of any other sensitive wild-type yeast strains. Any sensitive wild-type strain that may invade the fermentor as a contaminant will be killed quite effectively by the zymocidal toxin. The small amounts of toxin present in the finished beer product,

can be easily denatured by pasteurization, which is part of the beer making process.

So far, the genetically modified new killer brewery strains have only been applied to qualitative batch fermentations of beer wort.

2.8 Future Applications for the Zymocidal and Related System

There is general consensus among researchers that the genetically modified zymocidal yeast strains have important industrial applications in the following three areas:

The first application is in basic biochemical research. The killer system may be a useful model for several of the phenomena which are characteristic of some of the other eukaryotes. Control of the replication of viruses and plasmids within yeast systems is important but, the research into the role of the host in this process is limited due to the complexity of the genetics.

The second possible application is in the analysis of the mechanism of protein production and its secretion. This killer system and its mechanism for protein secretion is analogous to such processes as hormone production and to such other products which are available from genetically modified microorganisms. This application of combined genetic and biochemical approaches does promise to make the killer system very useful: For example, Tipper and Bostian (1984) have already drawn a parallel between the zymocidal toxin maturation and expression

to that of insulin. Further research will lead to more extensive applications.

A third possible application of genetically modified zymocidal strains is in the batch and continuous ethanol fermentations. The killer systems will protect the fermentation from contamination by another killer strain and any sensitive wild-type strains. The kinetics of cell growth, toxin and ethanol production of zymocidal strains in both batch and continuous systems have not been studied. The major research objective in this thesis was to study the kinetic characteristics of cell growth, and the production of ethanol and toxin, and these data may be used for further scale-up at the pilot plant and industrial scale levels.

The commercial application of zymocidal systems is only possible if truly stable, zymocidal yeast strains can be isolated. Currently, considerable work is being done in this area by Bussey and Meaden (1985) and Hjortso et al. (1985), to quantify plasmid losses and to propose methods to improve the stability of all genetically modified zymocidal strains.

CHAPTER 3

SCOPE AND RESEARCH OBJECTIVES OF THIS WORK

In the view of the many advantages of genetically modified zymocidal yeast strains and their potential commercial use in batch and continuous fermentations, the following research objectives will be pursued:

1. Isolation and purification of the toxin protein by gel electrophoresis.

2. Isolation and identification of the plasmids responsible for toxin secretion by gel electrophoresis.

3. Study of the stability of the toxin at different condrtions of pH and temperature. This will lead to the determination of deactivation rate kinetic expressions and temperature and pH optima.

4. Assess the affects of temperature on the kinetics of the batch culture of zymocidal Saccharomyces cerevisiae.

5. Study of the pH effects on the kinetics of the batch culture of zymocidal S. cerevisiae.

6. Determine the effects of the initial glucose concentration on the kinetics of the batch culture of zymocidal S. cerevisiae.

7. Study of the temperature effects on the kinetics of the continuous culture of zymocidal S. cerevisiae.

8. Correlate these kinetic studies to determine the influence of the toxin on the growth and product formation of zymocidal S. cerevisiae.

Study of these parameters will allow a greater understanding of the applicability of zymocidal yeast strains to industrial fermentation processes and in laboratory analyses.

CHAPTER 4

THEORETICAL CONSIDERATIONS ON THE GROWTH KINETICS OF MICROORGANISMS

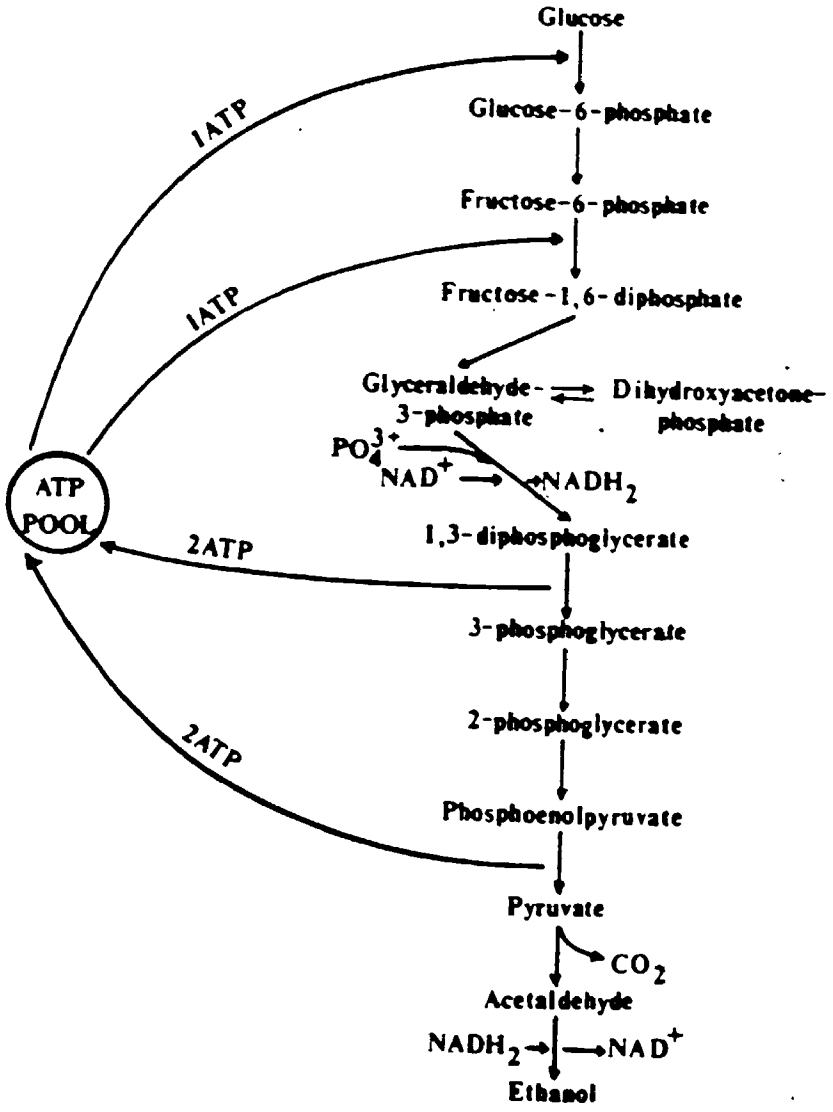
4.0 Biochemical Pathway for Anaerobic Growth of Saccharomyces cerevisiae

Product formation and growth, by any microorganism, are complex processes that involve many steps. The conversions and processes are not easily quantifiable in mathematical expressions. The overall chemical equation used to describe how yeast will convert glucose to both ethanol and carbon dioxide under anaerobic conditions can be written as follows:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_6O + 2CO_2 \qquad 4.1$$

But this process requires 10 successive, enzyme - catalyzed reactions which occur under anaerobic conditions. The conversion of glucose to pyruvate, and then to ethanol has been thoroughly researched and the overall pathway is shown in Figure 4.1 (Aiba et al., 1973). As depicted, glycolysis is the process of converting the six carbon glucose into two molecules of three carbon pyruvate and is called the Embden-Meyerhof-Parnas (EMP) pathway. From pyruvate, the final products

Figure 4.1 - Embden - Meyerhof - Painas Metabolic Pathway showing the steps to convert glucose to ethanol and carbon dioxide. This figure is simplified for illustrative purposes. It should ne noted that each molecule of glucose produces two molecules of ethanol. (based on Aiba et al, 1973)


Glucose
Glucose-6-phosphate
Fructose-6-phosphate
Fructose-1,6-diphosphate
Glyceraldehyde-3-phosphate
Dihydroxyacetone-phosphate
PO_4^{3+}
NAD^+
$NADH_2$
1,3-diphosphoglycerate
3-phosphoglycerate
2-phosphoglycerate
Phosphoenolpyruvate
Pyruvate
CO_2
Acetaldehyde
$NADH_2$
NAD^+
Ethanol
ATP POOL
1ATP
1ATP
2ATP
2ATP

formed are dependent on the microorganism in use and upon the conditions under which the growth occurs. For example, under aerobic conditions, yeasts will convert the pyruvate to both carbon dioxide and water via the T.C.A. cycle (Lehninger, 1982).

Fermentation is a general term used to denote the anaerobic bioconversion of glucose, or other carbohydrates, into various products. Of interest in this study is the alcoholic fermentation of glucose to ethanol, under anaerobic conditions, using a zymocidal producing strain of Saccharomyces cerevisiae.

Glycolysis is a complex process utilized by cellular systems in order to derive energy contained in the glucose molecule. This conversion stores the energy as ATP (adenosine triphosphate). As shown in Figure 4.1, the overall conversion of glucose to ethanol does produce a net total of two moles of ATP per mole of glucose consumed. This ATP is used as the energy source for the complex enzymatic reactions, and conversions, that occur throughout a growing cell. Another compound which plays an important role in fermentation is the NADH (nicotinamide adenine dinucleotide) which is a coenzyme used as a transient carrier, or intermediate, for the hydride ions, between enzymatic reactions.

Each conversion in the glycolysis pathway is catalyzed by an enzyme (or enzymes) which have been created by other metabolic processes within the cell. Considering the three different chemical transformations outlined above, the overall anaerobic conversion of glucose to ethanol can be written very simply as:

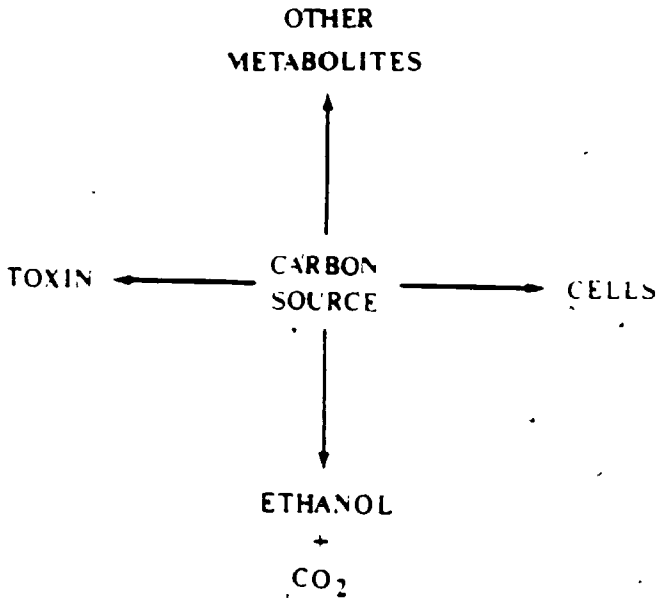
$$C_6H_{12}O_6 + 2ATP \longrightarrow 2C_2H_6O + 2CO_2 + 4ATP \qquad 4.2$$

All of the carbon that is utilized by the living cell is not converted to ethanol. Some is needed to maintain the structure of the cell, to maintain the enzymatic machinery within the cell, and to allow growth and reproduction of the cell itself. The carbon and energy utilized in this way, is generally referred to as 'maintenance energy' (Aiba et al., 1973). This maintenance energy can represent a measurable portion in the overall carbon balance.

In the case of the zymocidal toxin producing strain of S. cerevisiae, and other yeasts, a portion of the glucose is converted to both the toxin and the immunity proteins, outlined in chapter 2 of this thesis. Generation of proteins, such as the toxin, other enzymes, and other components of the cell, also require sources of nitrogen, phosphate, sulphate and other compounds and trace materials such as metals.

A schematic representation of the uses of carbon within an anaerobically growing cell of zymocidal yeast is shown in Figure 4.2. In any model or kinetic analysis, the glucose mass-balance, and hence the uses for carbon within the system, must be considered.

Figure 4.2 - Schematic of Carbon Use in an Anaerobic Yeast System


OTHER
METABOLITES
TOXIN
CARBON
SOURCE
CELLS
ETHANOL
+
CO_2

4.1 Mathematical Formulation of the Overall Kinetics

The process of the conversion of glucose to ethanol is, like all other biochemical processes, an enzyme catalyzed sequence of simple reactions. In most fermentations, one substrate is completely utilized or reaches a concentration that limits growth of the culture. Assuming that one of the enzymatic conversions in the overall process is rate limiting, then the kinetics of the utilization of substrate has been shown to be related to the cell growth rate according to the Monod equation (Monod, 1949).

$$\mu = \frac{\mu_{max} S}{K_M + S} \qquad 4.3$$

Equation 4.3 is an empirical analogy to the Michaelis-Menten equation which was derived theoretically from single enzyme kinetics. The constant 'K_M' in the Monod equation is called the 'saturation constant'. This constant represents the substrate concentration at which the specific growth rate, (μ), is half of its maximum. In the Michaelis-Menten equation, the constant 'K_S' is a ratio of the actual reaction rates involved in a single enzymatic conversion (Lehninger, 1982). The Monod equation is an empirical model of the relationship between the limiting substrate concentration, and the specific growth rate. This equation does not include any terms to account for product inhibition, substrate inhibition, or any of the other common problems encountered in fermentation systems. Therefore, this simple model may require some modification before it can be applied to the complex interactions which occur in cellular systems.

Other kinetic equations have been proposed and used to fit experimental data (Bailey and Ollis, 1986) and are given by equations 4.4, 4.5 and 4.6.

Tessier Model $$\mu = \mu_{max}(1 - e^{-S/K_S}) \quad 4.4$$

Moser Model $$\mu = \mu_{max}(1 - K_S S^{-\lambda})^{-1} \quad 4.5$$

Contois Model $$\mu = \mu_{max}\left(\frac{S}{BX + S}\right) \quad 4.6$$

4.2 Definition of Kinetic Parameters

4.2.1 The Batch Growth Curve

Four basic growth regimes or phases may be identified in the batch growth curve of a typical microorganism (Bailey and Ollis, 1986). Initially, after inoculation of the batch bioreactor, a short lag phase occurs, and during this time period, the cells are adjusting their enzymatic systems to meet the new environmental conditions in the bioreactor. After the lag phase there is a rapid growth period during which the number of cells increases exponentially. This stage in the batch growth is referred to as the exponential (or logarithmic) growth phase. When the substrate is utilized to such a level that it limits the cell growth, the cell culture enters a stationary phase, and at this point the cell population achieves its maximum size. Eventually, the number of cells declines and this phase is referred to as the death or decline phase.

The transition between any of the phases can usually be distinguished more clearly if the growth curve is reploted in semi-logarithmic. The equation relating the specific growth rate to the slope of the growth curve at any time, t, is given by equation 4.7.

$$\mu = \frac{1}{X} \frac{dX}{dt} \qquad 4.7$$

Integrating equation 4.7 between the initial conditions of

$$X = X_0 \quad \text{at} \quad t = t_{lag} \qquad 4.8$$

gives the cell concentrations as a function of time, as shown in equation 4.9.

$$X = X_0 e^{\mu(t - t_{lag})} \qquad 4.9$$

4.2.2 Kinetics of Substrate Utilization

In most cases, the utilization of a limiting substrate by a growing cell culture exhibits much the same trends (or phases) as the biomass. Initially, as the cells adjust to their new environment little or no substrate is consumed. As the cell population begins to grow and enters the exponential growth phase, the substrate is consumed more rapidly. It then decreases exponentially in the same way as the cells increase exponentially. As the substrate concentration reaches its limiting concentration, the cell population enters the stationary phase, and substrate utilization slows dramatically. The substrate

consumed during the stationary phase is generally used for endogenous metabolism.

Considering the exponential phase of substrate utilization, the decrease in the specific substrate concentration (when plotted with respect to time) should be a straight line on semi-logarithmic axis. Therefore, a relationship similar to equation 4.7 can be utilized. This equation is shown by equation 4.10.

$$q_s = \frac{-1}{\chi} \frac{dS}{dt} \qquad 4.10$$

Similarly, a specific product formation rate can be defined according to equation 4.11.

$$q_p = \frac{1}{\chi} \frac{dP}{dt} \qquad 4.11$$

At high substrate concentrations, the substrate may inhibit the cell growth and, in most cases, this inhibition may prevent the exponential growth of the biomass. Instead, only linear growth and substrate uptake is observed (Shukla et al., 1984). Cell strains which are able to utilize high substrate concentrations and produce high product concentrations are promisong from an economic point of view (Converti et al., 1984, Panchal et al., 1982b).

4.2.3 Product Formation Kinetics

The formation and accumulation of the products during a fermentation depends to a great extent on the substrate and product under consideration. In this research, the conversion of glucose to ethanol, and the production of the zymocidal toxin, was of interest.

When a substrate is stoichiometrically converted to biomass and a single product, the rate of formation of the product is said to be growth-associated and is shown in equation 4.12 (Aiba et al., 1973).

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} \qquad 4.12$$

In cases where the rate of product formation is dependent on cell concentration in the fermentor, the product formation rate is then said to be non-growth associated and is instead, controlled by a constitutive enzyme system (Bailey and Ollis, 1986). This relationship is given by equation 4.13.

$$\frac{dP}{dt} = \beta X \qquad 4.13$$

In many cases, a combined growth and non-growth associated model is a better representation of the kinetics of product formation. In this relationship, equations 4.12 and 4.13 are combined to yield equation 4.14.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad 4.14$$

By dividing equation 4.14 by the biomass concentration, and substituting equation 4.7 we obtain equation 4.15.

$$r = \frac{1}{X}\frac{dP}{dt} = \alpha\mu - \beta \qquad 4.15$$

The combined model given by equation 4.15 is also known as the Luedeking-Piret Model and proved to be extremely useful in fitting the product formation data from many batch and continuous fermentations (Bailey and Ollis, 1986). This kinetic expression is an expected form, when the products are a result of energy-yielding substrate utilization. Bailey and Ollis (1986) suggest that the constants α and β may be related to the energy used for growth and maintenance, respectively.

4.2.4 Cell and Product Yields

The overall biomass to substrate yield is defined by equation 4.16.

$$Y_{X/S} = \frac{X - X_0}{S_0 - S} = \frac{\Delta X}{-\Delta S} \qquad 4.16$$

The overall product yield is defined in a similar way, and is given by equation 4.17.

$$Y_{P/S} = \frac{P - P_0}{S_0 - S} = \frac{\Delta P}{-\Delta S} \qquad 4.17$$

An additional yield relationship used in many applications is the product produced per cell of biomass. This relationship is useful

in showing the overall product to biomass yiels and is shown in equation 4.18.

$$Y_{P/X} = \frac{P - P_0}{X - X_0} = \frac{\Delta P}{\Delta X} \qquad 4.18$$

The usefulness of these yield relationships is limited as these yield factors include all the substrate used for cell maintenance, product formation, and cell growth in single variables. These yield factors do, though, allow a rough estimation and act as comparison parameters between cell strains.

One relationship commonly used to relate the substrate utilization to cell growth and maintenance requirements of a cell is shown in equation 4.19 (Aiba et al., 1973). This equation separates the substrate utilized for growth from the substrate utilized for maintenance, but it does not account for the substrate converted to products.

$$\frac{dS}{dt} = \frac{1}{Y_{G}} \frac{dX}{dt} + mX \qquad 4.19$$

Another cell yield growth factor is defined by equation 4.20.

$$Y_{G} = \frac{\Delta X}{(-\Delta S)_{G}} \qquad 4.20$$

If significant amounts of substrate are utilized for product formation, then the model proposed in equations 4.19 is only applicable if the product is growth associated. In these cases, the substrate

utilized by the cell to produce the product will be included in α for the growth associated products or, included in β for the non-growth associated products. When significant amounts of product are formed, the model should be expanded to include the substrate utilized in the creation of these products (Damiano et al., 1985 and Shukla et al., 1984). A substrate balance can then be expressed according to equation 4.21.

$$\frac{dS}{dt} = \frac{1}{Y_G}\frac{dX}{dt} + \frac{1}{Y_P}\frac{dP}{dt} + mX \qquad 4.21$$

4.2.4.1 Importance of Maintenance Energy Requirements

Confusion in regard to the importance of the magnitude of the specific rate of substrate uptake for maintenance has been considered by many authors (Goma et al., 1979, Esmer et al., 1981, Karsch et al., 1983). Each group of researchers has come to different conclusions in regard to the optimum value for the maintenance requirement based on experimental results as defined by equation 4.20.

Assuming that ethanol is the only significant product (other than biomass), and the rate of the production of ethanol can be represented by the combined growth and non-growth associated model, then equation 4.21 can be combined with equation 4.14 to give equation 4.22.

$$\frac{dS}{dt} = \left(\frac{1}{Y_G} + \frac{\alpha}{Y_P}\right)\frac{dX}{dt} + \left(\frac{\beta}{Y_P} + m\right)X \qquad 4.22$$

This equation shows the effect that the products have on the overall substrate utilization rate. Analysis of experimental data using equations similar to 4.22 will result in the removal of much of the confusion regarding maintenance requirements.

4.3 Inhibitory Effects in Ethanol Production

4.3.1 Ethanol Effects

It is well known that fermentation of glucose, or other sugars, into ethanol will be limited by the concentration of ethanol accumulating in the fermentor. Considerable work has been devoted to the prediction of the inhibiting effects of ethanol on the cell growth rate and on the product formation rate. This has been especially so for S. cerevisiae fermentations (Levenspiel, 1980, Righelato et al., 1981, Hoppe and Hansford, 1982, Luong, 1985). Luong (1985) reviewed all of the proposed models which were used to describe these ethanol inhibition effects. He proposed that the effects of ethanol on the growth and product formation by S. cerevisiae can best be described by two similar kinetic models. This work showed that the effect of ethanol on the growth of S. cerevisiae has been successfully modelled by a kinetic expression and it relates the inhibited growth rate with the uninhibited growth rate, as given by equation 4.23.

$$\frac{\mu_i}{\mu_o} = 1 - \left(\frac{P}{P_m}\right)^{\delta} \qquad 4.23$$

The effect of ethanol concentration on its own production rate by S. cerevisiae can be modelled by a similar kinetic expression given by equation 4.24.

$$\frac{v_i}{v_0} = 1 - \left(\frac{P}{P_m'}\right)^{\gamma} \qquad 4.24$$

Luong (1985) used a typical strain of S. cerevisiae and the published results of other workers in order to derive the values for various constants in equations 4.23 and 4.24. He found that the maximum allowable ethanol concentration affecting growth was 112 g/L, and the ethanol producing capability of S. cerevisiae was inhibited at an ethanol concentration of 115 g/L. The constants of δ and γ were estimated to be 1.41 and 1.69 respectively.

The fermentation temperature also plays a role in the inhibition caused by ethanol in a growing yeast culture (Brown and Oliver, 1982, Leao and van Uden, 1985). These effects have been shown to become most significant at temperatures greater than 40 °C with the ethanol concentration more than 80 g/L.

4.3.2 Other Inhibitory Effects

As previously discussed, the inhibitory effects of ethanol on the cell growth rate and subsequent ethanol production rate have been known for some time. Since high biomass concentrations are important for the rapid production of alcohol, the cell recycle systems, and immobilized cells, have become of great interest, especially in the production of fuel alcohol (Margaritis and Merchant, 1984, Margaritis et al., 1981).

Recently, Cysewski and Wilke (1977) used a cell recycle system in order to increase the biomass concentrations and vacuum to reduce the ethanol concentration. These authors reported that under low bleed (high recycle) conditions, toxic materials present in the system inhibited yeast growth (or the product formation). They suggested that non-volatile toxic substances managed to accumulate in the fermentor. These toxins reached such a level as to severely limit the yeast metabolism. Other workers (Margaritis and Wilke (1972, 1978a, 1978b), Maiorella et al., 1983, Damiano et al., 1985, Shin et al., 1985) have reported similar findings even when using many different methods to remove the primary product (the ethanol) as outlined below.

Margaritis and Wilke (1972, 1978a, 1978b) used a rotating microporous membrane bioreactor system (Rotofermentor) to concentrate yeast cells and simultaneously remove ethanol and other non-volatile inhibitory products during the course of fermentation. Damiano et al., (1985) used a continuous yeast fermentation with cell recycle by ultrafiltration membrane. These workers reported that some type of inhibitory compound caused a decrease in both the specific growth rate and specific ethanol productivity as compared to studies with no cell recycle. Maiorella et al. (1983) further showed that the fermentation by-products (such as acetic acid, formic acid, 1-propanol, etc.) were not major factors in this secondary inhibition of yeast growth and productivity.

Shin et al. (1985) studied the effects of non-volatile substances on both the yeast growth rate and the ethanol productivity of S. cerevisiae. The non-volatile substances, such as proteins and

salts, were shown to reduce the yeast growth rate and ethanol productivity, but not the maximum cell concentration. Thus it was concluded that these compounds appear to inhibit the cell growth in a competitive mode. Since Maiorella et al. (1983) showed that the primary fermentation by-products were not the cause of the inhibition, Shin and his co-workers used both sterilized and non-sterilized waste media in their batch fermentations. This was done in order to study the effects of fermentation by-products upon the growth and the productivity. The results of these batch experiments showed that sterilized waste medium did bring about better growth and product formation than the non-sterilized waste medium. Therefore, this suggests that proteins present in the non-sterilized waste medium could indeed inhibit cell growth. These proteins were not present in the sterilized medium because they would be denatured by the sterilization process.

This inhibitory effect is of great concern in the application of zymocidal yeast strains to both batch and continuous systems. The zymocidal toxin and immunity molecules are both extracellular proteins which will accumulate in the fermentation broth. These proteins will bind to the cell wall, of the host cell, and may cause inhibitory effects, especially in continuous experimentation.

4.4 Environmental Effects in Ethanol Fermentations

4.4.1 Effects of temperature on Cell Growth

The series of enzyme catalyzed reactions, which make up the glycolysis pathway, are affected by temperature. Enzyme - catalyzed reactions have been shown to follow the Arrhenius type of relationship

within the boundaries of the thermal stability of the enzyme and its production mechanism. In a cellular system, it is difficult to conveniently isolate the enzymatic step which is most influenced by temperature. During balanced cell growth, a single parameter, the specific growth rate, is used to characterize the population growth kinetics (Bailey and Ollis, 1986).

At low to medium temperatures, the data can be easily analyzed in terms of the specific growth rate, μ , and temperature relationship shown in equation 4.25. A plot of the logarithm of the growth rate versus the inverse of the absolute temperature should produce a straight line with slope -E/R and intercept ln(A).

$$k = Ae^{-E/RT} \qquad 4.25$$

This relationship applies to the typical range of fermentation temperatures and at higher temperatures, thermal deactivation takes place resulting in the decrease of the specific growth rate of the microorganism.

4.4.2 Effects of pH on Cell Growth

It is well established that the activities of enzymes involved in different biochemical reactions are pH dependent. This dependency effects the specific growth rate of the whole cell. The specific growth rate of a microorganism can be greatly influenced by pH. The range of pH in which microorganisms are able to grow and/or produce products at the most optimum rate is generally limited. Yeasts have been shown to

be able to grow at pH values from 3.0 to 8.5, with the optimum at pH values between 4 and 5. Clearly, the optimum pH value for growth and/or product formation is an important consideration when analyzing a particular microbial strain for possible large scale applications.

4.4.3 Temperature and pH Deactivation of Proteins

In section 2.4.2 it was noted that the toxin molecule produced by the zymocidal yeast strains is easily and rapidly deactivated by temperature. Since this toxin acts by attaching itself to the wall of a sensitive cell, and then attacking it, it is felt that the deactivation kinetics will be similar to the deactivation kinetics of enzymes.

Denaturation due to temperature increases for both proteins and enzymes, occurs mainly as a result of changes in the structure of the molecule. The simplest model used to express the deactivation kinetics assumes that the protein molecules undergo an irreversible structural change. This deactivation would then occur at a rate which is proportional to the active enzyme concentration (Hill, 1977).

$$\text{[illegible]} = -kC \qquad 4.26$$

Assuming that the system is closed, well-mixed and free of other factors that would influence the deactivation system, the active protein concentration will decrease with time according to equation 4.27.

$$\frac{dC}{dt} = -kC \qquad 4.27$$

If the initial concentration of toxin in the system at zero time is assumed to be C_o, then equation 4.27 can be integrated to give equation 4.28.

$$\ln\left(\frac{C}{C_o}\right) = -kt \qquad 4.28$$

Thus, a plot of the concentration of active protein versus time using a semi-logarithmic scale should produce a straight line having the slope of -k, provided that this model is indeed valid. Correlation of the rate constants at the particular environmental conditions used in each deactivation experiment can be used to produce an overall kinetic expression.

Indications from previous workers (Bussey, 1981) suggest that the deactivation of the zymocidal toxin involves a conformational change in the structure as the active and inactive forms of the toxin have been shown to be of equivalent molecular weight.

4.5 Application of Kinetics to Batch and Continuous Experiments Data

4.5.1 Batch Growth Kinetics

Levenspiel (1980) derived many equations (for different reactor configurations) based upon the Monod, substrate - limited kinetic expression and included product inhibition effects where appropriate.

This derivation was based on the assumption that the Monod rate equation 4.29 can be used to mathematically express the kinetics of all growth and product formation.

$$r_c = \frac{\mu_{max} SX}{k_m + S} \qquad 4.29$$

Substituting the product inhibition effects from equation 4.23 into equation 4.29, a generalized kinetic expression can be derived for the growth and product formation of a particular system. This expression is given by equation 4.30. This result assumes that the maximum specific growth rate shown in equation 4.29 is maximum specific growth rate in the presence of the inhibitor. The maximum specific growth rate shown in equation 4.30 is then the actual maximum specific growth rate if inhibition was not present.

$$r_c = \mu_{max}\left(1 - \left(\frac{P}{P_m}\right)^{\delta}\right) \frac{SX}{K_m + S} \qquad 4.30$$

At low initial substrate concentrations (or when the limiting inhibitory product concentration is large), the inhibition does not slow the reaction, therefore:

$$\mu_{max}\left(1 - \left(\frac{P}{P_m}\right)^{\delta}\right) \longrightarrow \mu_{max} \qquad 4.31$$

At high substrate concentrations (such that $S >> K_m$), the uptake of substrate does not slow down, but the overall reaction rate reduces to a linear form, according to equation 4.32.

$$r_c = \mu_{max}\left(1 - \left(\frac{P}{P_m}\right)^{\delta}\right) X \qquad 4.32$$

Integration of equation 4.30 to give an overall change in concentration with respect to time profile for a batch process, has been proposed in the scientific literature.

Converti and his coworkers (1984) applied an integrated form of this equation to the batch experimental data. They were studying the effects of high substrate concentrations on the kinetics of S. cerevisiae strains. They utilized the Halwach graphical method which assumes a constant biomass concentration in the system. Since a constant biomass concentration only occurs in the stationary phase of a batch fermentaion, the Halwach method is of little value in studying the growth and product formation kinetics during the exponential growth phase.

Other reports of applying integrated forms of the Monod equation (or equation 4.30) have led to similar restrictions to those outlined above. It therefore seems more logical to utilize equation 4.30 in a differential form. Thus, by assuming a small time interval Δt, equations 4.29 or 4.30 can be rearranged, and utilized to evaluate the kinetic constants from any batch experimental data.

For batch kinetics (with no product inhibition effects), equation 4.30 can be used to evaluate the Monod constants by using a plot of $\bar{X}/\bar{r}_c$ versus $1/\bar{S}$ (Levenspiel, 1980), where the variables are the time averaged biomass concentration, rate of increase in biomass and substrate concentration respectively.

$$\frac{\bar{X}}{\bar{r}_c} = \left(\frac{1}{\mu_{max}}\right) + \left(\frac{K_m}{\mu_{max}}\right)\left(\frac{1}{\bar{S}}\right) \qquad 4.33$$

Analysis in this way should lead to a straight line with slope K_m / μ_{max} and intercept $1/\mu_{max}$, assuming no product inhibition effects. Deviation from linearity or improbable results would indicate product inhibition effects. Other kinetic parameters such as the specific growth rate can be derived directly from a semi-logarithmic plot of the experimental data.

Further kinetic parameters, such as whether the products are growth or non-growth associated, and their maintenance requirements, will be evaluated where necessary from the data derived from batch culture experiments. Utilization of equations such as equation 4.21 to batch data require more sophisticated curve fitting techniques than the derivation of the Monod constants. In these cases, mathematical expressions for the substrate uptake, biomass formation and product formation will be evaluated.

4.5,2 Continuous Growth Kinetics

4.5.2.1 Overall Mathematical Expressions

Calculation of kinetic parameters from continuous experimental data is generally much easier than calculations from batch data because steady-state can be achieved. Mass balances of the limiting substrate, the products, and the biomass can be written for a single, well-mixed, continuously stirred bioreactor (CSTB) to yield some very useful relationships.

In a typical bioreactor shown in Figure 4.3, mass balances for the biomass, products, and substrate are shown in Equations 4.34 through 4.36 (Aiba et al., 1973).

$$\frac{dX}{dt} = D(X_0 - X) + \mu X \qquad 4.34$$

$$\frac{dP}{dt} = D(P_0 - P) + Y_{P/X}\,\mu X \qquad 4.35$$

$$\frac{dS}{dt} = D(S_0 - S) - Y_{S/X}\,\mu X \qquad 4.36$$

Where the dilution rate, D, is given by equation 4.37.

$$D = \frac{F}{V} \qquad 4.37$$

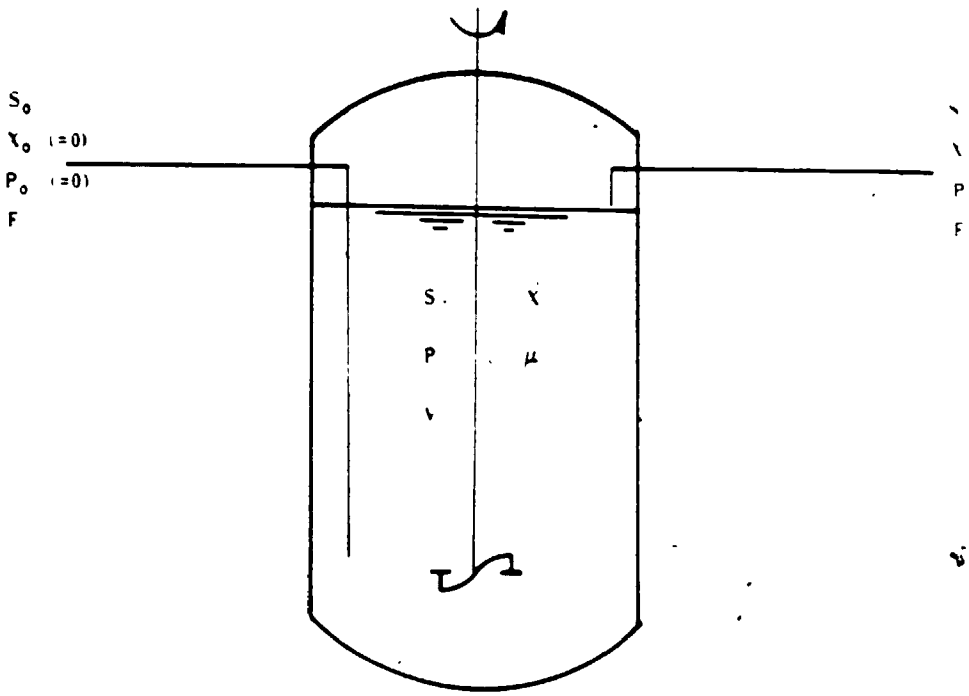
Assuming the feed is free of biomass and products, and the steady state is reached in the CSTB, then equation 4.34 can be reduced to equation 4.38.

$$D = \mu \qquad 4.38$$

Equation 4.38 therefore indicates that the growth rate of the microorganism in a continuous bioreactor system is equal to the dilution rate. This of course, is only true as long as the dilution rate is not larger than the maximum specific growth rate ($D < \mu_{max}$), beyond which cell washout will occur.

These results can be applied to the Monod kinetic expression to model the expected kinetics of an actively growing continuous fermentation. Substituting equation 4.38 into equation 4.3 leads to equation 4.39.

Figure 4.3 - Schematic of a Continuous Bioreactor (ideal mixing)
S - Substrate concentration.
X - Biomass concentration.
P - Product concentration.
F - Feedrate.
V - Reactor volume.
μ - Specific growth rate.
Subscript 'o' - inlet conditions.


S_0
X_0 (=0)
P_0 (=0)
F
S
X
P
μ
V
V
P
F

$$D = \frac{\mu_{max} S}{K_m + S} \qquad 4.39$$

Or, upon rearranging, this leads to equation 4.40.

$$S = \frac{K_m}{\frac{\mu_{max}}{D} - 1} \qquad 4.40$$

From this equation, it can be seen that at low dilution rates the substrate concentration will approach zero. As the dilution rate is increased, the substrate concentration in the bioreactor will increase as long as the dilution rate remains less than the maximum specific growth rate. At dilution rates greater than the maximum specific growth rate, growth of the culture cannot be maintained within the fermentor, and washout occurs.

If we continue to ignore product inhibition effects, equation 4.39 can be rearranged to enable determination of the kinetic parameters directly from the continuous experimental data. Rearranging equation 4.39, a Lineweaver - Burk relationship is easily derived according to equation 4.41 (Ngian et al., 1977, Aiba et al., 1973).

$$\frac{1}{D} = \frac{K_m}{\mu_{max}} \frac{1}{S} + \frac{1}{\mu_{max}} \qquad 4.41$$

Therefore, a plot of the inverse of the dilution rate versus the inverse of the substrate concentration should produce a straight line with slope K_m/μ_{max} and intercept $1/\mu_{max}$.

Since this thesis research deals with ethanol fermentation, and the production of a proteinateous toxin molecule, the inhibition of

these two products cannot be ignored. The effects of ethanol on the growth rate of yeast was reviewed in section 4.3.1, but the effects of the toxin are unknown. Derivations of the kinetics from those defined in equation 4.39 will be as a result of these products effects.

Maintenance requirements for the continuous systems can be determined by using equation 4.21 (Dimiano et al., 1985), which can be rewritten as a complete mass balance for the substrate as shown in equation 4.42 (assuming that the feed is free of biomass and product).

$$\frac{dS}{dt} = D(S_0 - S) - mX - \frac{DP}{Y_P} - \frac{DX}{Y_G} \qquad 4.42$$

At steady state, the left hand side of this equation is zero and therefore, this equation can be rearranged to yield equation 4.43.

$$\frac{S_0 - S - \frac{P}{Y_P}}{X} = \frac{m}{D} + \frac{1}{Y_G} \qquad 4.43$$

Thus, a plot of the left hand side of this equation versus the inverse of the dilution rate should yield a straight line with slope 'm' and intercept $1/Y_G$.

As indicated in the literature review, the toxin is only produced by actively growing cells in the exponential phase. Therefore, the toxin is most likely a growth associated product, and the substrate utilized to produce the toxin would be included in $1 Y_G$.

Kinetic parameters derived under batch growth conditions may be quite different than those calculated from continuous growth

conditions. This arises mostly from differences in the behaviour of the strain during the batch process where the environmental conditions change with time, as compared to the steady-state continuous process where the conditions remain constant. Tabera and his co-workers (1985) found that kinetic data derived from yeast in batch experiments may be different from those obtained under steady-state, continuous culture conditions.

The possibility of some oscillation in continuous culture of S. cerevisiae may exist and therefore, the fermentation system must be controlled to reduce the chances of oscillations. Parulekar et al. (1986) and Jobses et al. (1986) both reported that oscillations in continuous fermentations of S. cerevisiae could be eliminated by maintaining the dissolved oxygen concentration above 78% or below 20% saturation. Since in our research we deal only with anaerobic fermentations, oscillations should not be observed.

CHAPTER 5

EXPERIMENTAL MATERIALS AND METHODS

5.1 Microorganisms

The two strains of Saccharomyces cerevisiae used in this experimentation were kindly provided by Dr. C. Panchal of the Production Research Department of the Labatt Brewing Company, London, Ontario. The first strain used was Saccharomyces cerevisiae - Labatt collection #1465, and it was originally obtained from H. Bussey. This haploid killer strain (K_1 R_1) was used as the model zymocidal toxin producing strain in this research, and it is specifically referred to by Palfree and Bussey (1979) as A8207B, (α, his4C - 864). The second yeast strain used in this analysis was an S6 sensitive strain of Saccharomyces cerevisiae - Labatt collection #1438. This strain is a wild-type having no zymocidal activity and was used to detect the active toxin concentration produced by the killer zymocidal yeast strain.

Both of the strains were maintained in 10-mL portions of buffered liquid media (c.f. 5.2.2). They were transferred weekly in order to insure that a high level of cell viability was maintained. Both of the strains were replenished, on a monthly basis, from cell

maintenance agar medium (c.f. 5.2.4) to keep a constant and consistent liquid inocula supply.

Cells in liquid broth were kept at 24 °C in an incubator (Precision Scientific - Model 805). Yeast cells on the maintenance agar were initially incubated for 72 - 96 hours at 24 °C, and then were stored at 5 °C until needed. The lower temperature used in these cultures slowed down the cell metabolism in order to reduce the chances of cell death and lysis.

The inocula prepared and used in the experiments were 10-ml portions of bufferred medium (c.f. 5.5.2). They were inoculated from the liquid maintenance stock and incubated at 24 °C for a period of 24 hours prior to use. The carbon source in the inocula was glucose.

5.2 Media Composition

All chemicals used in the media and the analyses, with the exception of those listed below, were obtained from CanLab and they were Baker Analyzed Reagent quality. The bacto-peptone used in all of the media was obtained from the Difco Laboratories. The yeast extract was obtained from Gist-Brocades NV lot 1850/1 and the agar used for the cell maintenance slants and for the toxin concentration assay was obtained from the Sigma Chemical Company. The glucose was obtained from the Corn Products Company of Engelwood, N.J., and it was U.S.P grade Dextrose.

5.2.1 Yeast Growth and Production Medium

The medium used for cell growth and toxin production had the following composition in grams per litre of distilled water: Bacto-peptone, 3.5; yeast extract, 3.0; potassium phosphate, (monobasic), 2.0; ammonium phosphate, 1.0; and magnesium sulphate, (heptahydrate), 1.0. The carbon source used in this medium was glucose at a concentration between 20 and 200-g/L depending on the experimental conditons used.

5.2.2 Cell Maintenance and Inoculation Medium

Both of the cell strains were maintained in 10-mL portions of sterile liquid media which contained the basic media components listed in section 5.2.1 having a carbon source of 20-g/L glucose. This medium was buffered to a pH of 4.5 by the addition of a 0.1-M (total ionic strength) citric acid - phosphate buffer. The buffer required the addition of 6.472-g of citric acid 7.33-g of sodium phosphate-dibasic to each litre of medium prepared. The amount of sodium phosphate added to the media was reduced in order to attain the correct phosphate concentration in the final media, because some phosphate is added as potassium phosphate in the basic media composition.

Inocula were composed to include the above basic components along with a carbon source of 20-g/L glucose. The sensitive strain, Saccharomyces cerevisiae -1438, was grown in 125-mL volumes of the cell maintenance media contained in 500-mL shake flasks that had 20-g/L glucose. These cultures were inoculated with 10-mL portions from the

sensitive strain maintenance culture. They were then incubated at 25 °C for 24 hours in a rotary shaker (New Brunswick Scientific, Model G - 25) at 200 rpm. These cultures were used in the toxin detection and concentration assay (c.f. 5.3.5).

5.2.3 Cell Maintenance Agar Medium

Both of the yeast strains used in this analysis were maintained on solid media which had the following composition: glucose, 20-g/L; yeast extract, 2.0-g/L; and agar, 20-g/L. Slants of this medium were prepared in 20-mL screw-top tubes, and they were loop inoculated with the yeast strains. These cultures were incubated for 72 - 96 hours at 24 °C, and then they were stored at 5 °C until used. Each month, one tube of each of the yeast strains was used to renew the liquid maintenance cell lines and also to inoculate other maintenance agar tubes.

5.2.4 Toxin Assay Medium

The medium used in the toxin assay consisted of the basic components listed in section 5.2.1 with the addition of 20-g/L of glucose. It was buffered with 6.472-g/L of citric acid and 7.33-g/L of sodium phosphate dibasic. This medium also contained 20-g/L of agar and 20-mg/L of methylene blue. For each toxin concentration assay it was necessary to use this medium in two forms, namely, 25-ml test-tubes containing 10-ml of toxin assay medium, and petri dishes containing 10-ml of this same medium.

Large volumes of 4 - 5 litres of this medium were prepared at one time. This was sterilized as either 15-mL portions in 25-mL test tubes, or in 500-mL flasks. The media was poured as a 10-mL layer into 100 x 15-mm petri dishes. An equal number of tubes and plates were produced from each batch of medium. After this preparation, the tubes and plates were sealed in plastic bags. They were then stored at 5°C to keep a constant supply of consistent plates for the analyses.

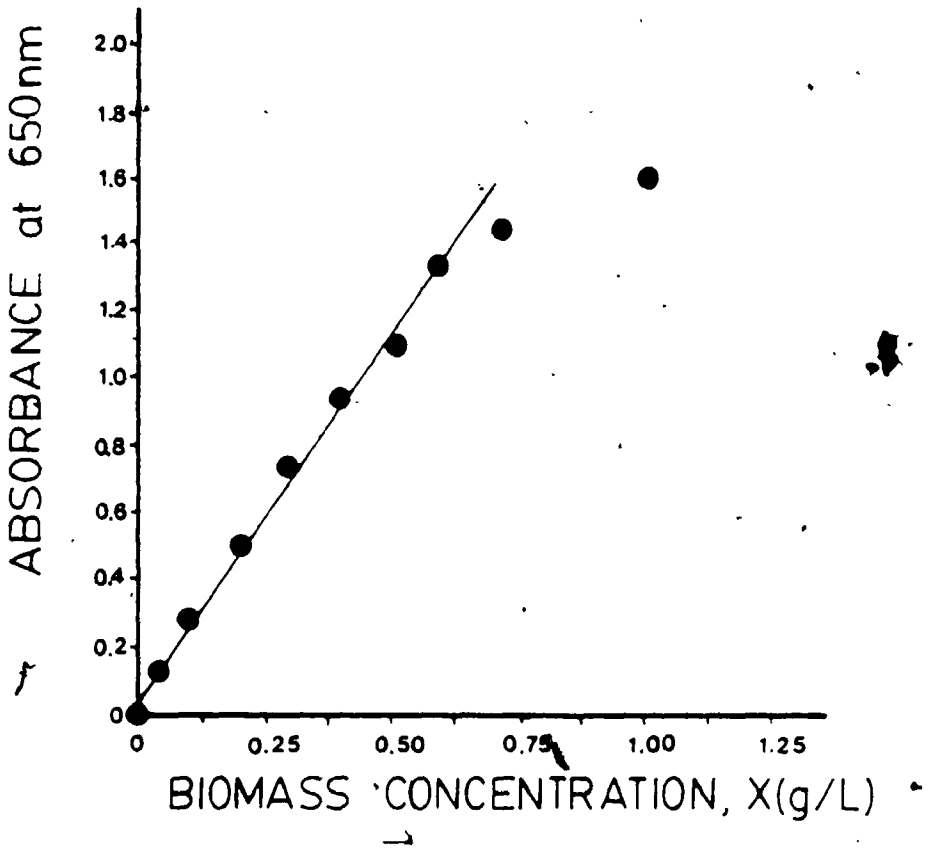
5.3 Analytical Methods

5.3.1 Biomass Concentration of Zymocidal Strain

Cell concentrations for the zymocidal yeast Saccharomyces cerevisiae -1465 were determined by measuring its absorbance at 650-nm on a Philips -Pye Unicam UV/VIS Spectrophotometer, (Model PU 8610, cuvette - 1-cm path length). Samples were prepared by centrifuging a 5-mL portion of cell broth at 1,000-rpm for 10 - 15 minutes (International Clinical Centrifuge - Model CL). The cell pellet was resuspended in an appropriate amount of sterile, distilled water to give a total cell concentration between 0.0 and 0.6-g/L. The absorbance was then measured using sterile distilled water as the blank. The supernatant from the centrifugation was transferred to a sterile test tube and was used in the toxin assay and for testing to determine the concentrations of ethanol and reducing sugars.

A typical biomass calibration curve for the Saccharomyces cerevisiae strain -1465 is shown in Figure 5.1. The calibration data was derived as follows: two 20-mL portions of stationary phase broth were filtered through each of two pre-weighed filter papers (1.2- μm

Figure 5.1 - Biomass Calibration Curve - Zymocidal Strain. Calibration Curve X = 0.45996 Y - 0.02441. Correlation Coefficient 0.99.


ABSORBANCE at 650nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.25
0.50
0.75
1.00
1.25
BIOMASS CONCENTRATION, X(g/L)

and 47-mm). One additional filter paper was pre-weighed and wetted. These were dried to become a constant weight at 105 °C, and were subsequently weighed (Mettler H15 balance). After centrifugation and resuspension, the absorbance of distilled water dilutions of some cell broth were measured. For each dilution, the cell concentration (g/L) was calculated from the dry weights, and then correlated with the appropriate absorbance value. Linear regression was used to determine the correlation curve for the points shown in Figure 5.1.

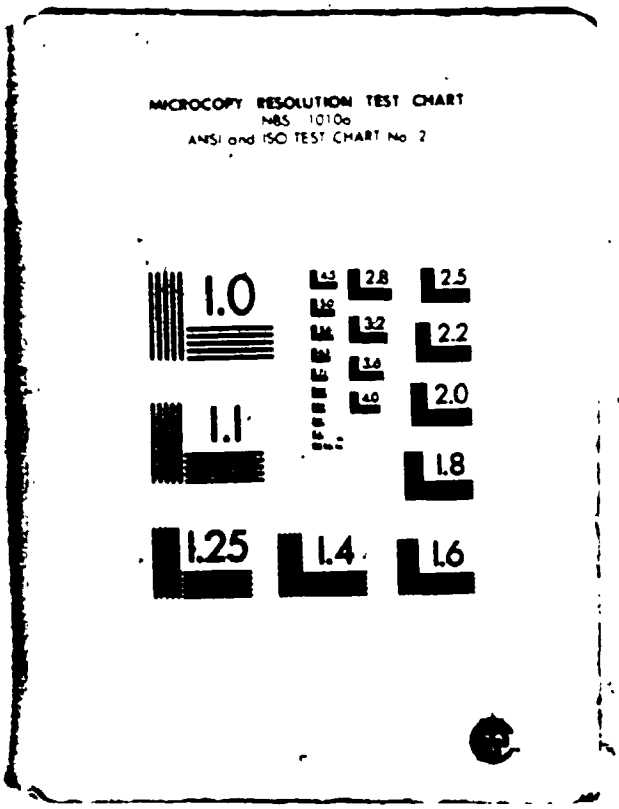
5.3.2 Cell Numbers - Sensitive Strain

The numbers of cells in a sample of the sensitive strain Saccharomyces cerevisiae -1438 was determined using a hemocytometer. A drop of the cell broth (diluted to contain approximately 25 x 10^6 cells/mL) was aseptically transferred to the hemocytometer and was observed under a light microscope at a magnification of 400 times (American Optical, Model - Phase Star - one-ten). The cells were counted within the double-scribed area on the hemocytometer. From this count, and from knowing the dimensions of the scribed counting area, the cell number concentration can be found by using Equation 5.1:

$$\text{Cells/ml} = \text{Count in screen area} \times 2.5 \times 10^5 \qquad 5.1$$

5.3.3 Total Reducing Sugars

The concentration of reducing sugar in the medium was measured by using the dinitrosalicylic acid method (DNS) of Miller et al. (1960). One litre of reagent was prepared in the following manner: 11.3-g of sodium hydroxide were dissolved in 500-mLs of distilled

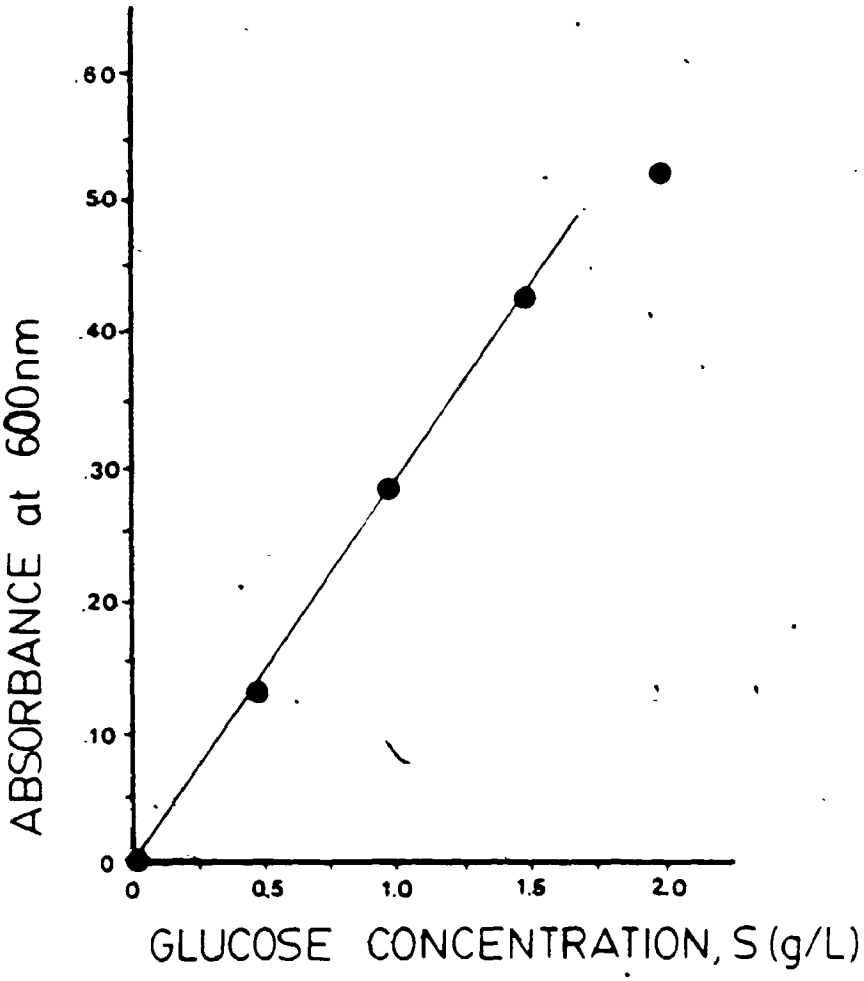

MICROCOPY RESOLUTION TEST CHART
NBS 1010a
ANSI and ISO TEST CHART No 2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8

water. 0.565-g of sodium sulphite, 11.3-g of 3,5-dinitrosalicylic acid, 226.0-g of sodium potassium tartrate (Rochelle Salt), and 2.0-g of phenol were all added with stirring. The solution was then made up to a 1-L total volume, and it was stored in a light protected repipettor (Oxford Laboratories Pipettor).

The analytical procedure used to determine the glucose concentration in the cell free broth samples was adapted from Miller et al (1960). One millilitre samples of centrifuged cell broth were diluted to contain between 0.2 and 2.0-g/L of reducing sugars. Then, in a 50-mL test tube, to these samples was added 3-mLs of the DNS reagent. After being vortexed, the tubes were placed in boiling water for exactly 5 minutes (±1 second). They were then placed into a cold water bath. To each was added 20-mLs of distilled water (Repipet™ - Labindustries Inc.). After being vortexed, the solutions were poured into cuvettes and the absorbance was measured at 600-nm against a reagent/distilled water blank (Philips - Pye Unicam UV/VIS Spectrophotometer Model PU 8610). The blank was prepared by using 1.0-mL distilled water and 3.0-mLs of DNS reagent. Next it was heated, and then diluted with 20-mLs of distilled water by the same method as when handling the broth samples.

The sugar concentration was then determined from a calibration curve that uses glucose as the standard sugar for all fermentations. A calibration curve was generated for each sugar analysis in this manner: an appropriate mass of glucose was weighed into a 500-mL volumetric flask, it was dissolved into the distilled water to yield a final concentration of 2.0-g/L. In other volumetric flasks, this solution was

Figure 5.2 - Glucose Calibration Curve - DNS Method


ABSORBANCE at 600nm
.60
.50
.40
.30
.20
.10
0
0
0.5
1.0
1.5
2.0
GLUCOSE CONCENTRATION, S (g/L)

then used to prepare the sugar solutions of concentrations between 0.5 and 2.0-g/L. The DNS test described above was then performed on each of these standards. A typical calibration curve for glucose is depicted in Figure 5.2.

5.3.4 Ethanol

Gas - liquid chromotography was used to measure ethanol concentrations using appropriate calibration standards. A Varian Model 3700 gas chromatograph was used in this analysis having the characteristics and the set points as listd in Table 5.1.

A 1.0-μL sample of centrifuged cell broth was diluted in order to contain less than 1.0-g/L ethanol. This was injected into the gas chromatograph (10-μL Hamilton Microlitre Syringe), and the peak height (as measured from the base line) was then compared to the peak height of a 1.0-μL ethanol standard. One standard injection was made after each sample injection.

Periodically, the column temperature of the gas chromatograph was increased up to 200 °C for 90 - 120 minutes in order to clean the column. This was required as the sensitivity (which is measured by the standard peak height) would be reduced after a series of 10 to 20 sets of sample/standard injections.

TABLE 5.1

SET POINTS FOR GAS CHROMATOGRAPH

(Varian - Model 3700)

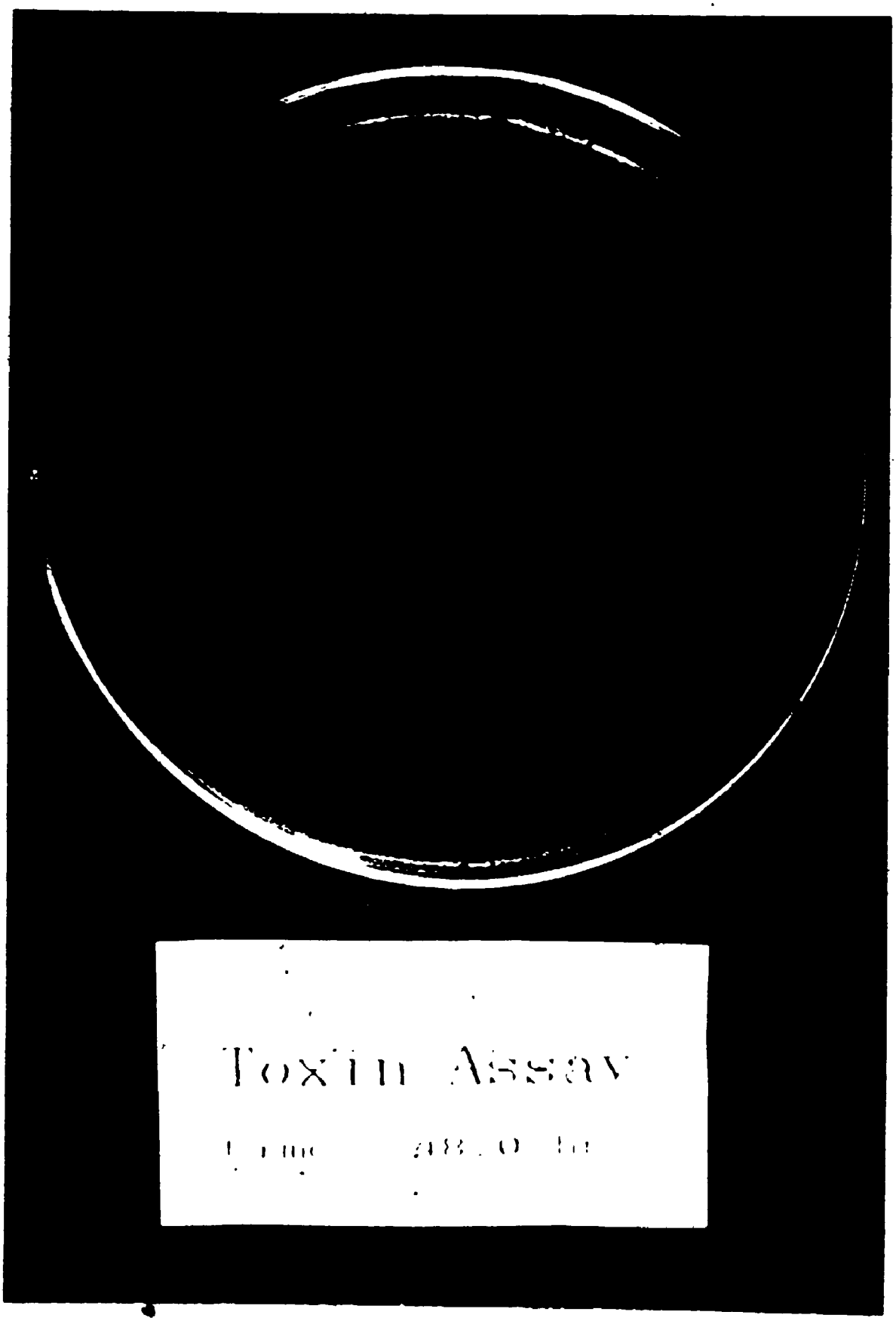
Column - 6-m, 1/16" Stainless Steel, packed with Chromosorb 102		
Sample Size:		1 or 2 µL
Sample Concentration:		0 - 0.5 g/L
Temperatures	- Injector	160°C
	- Ion Detector	170°C
	- Column	150°C
Attenuator		16
Range		10-11
Gas Flow Rates	- Nitrogen	30 ml/min
	- Hydrogen	30 ml/min
	- Air	100 ml/min

5.3.5 Toxin Concentration

Active toxin concentrations in the liquid broth were measured using the methylene - blue spotting assay of Panchal et al (1985). Approximately 10^5 cells of the sensitive wild-type strain (Saccharomyces cerevisiae -1438) were added to a test tube that contained 10-mL of molten toxin assay medium (c.f. 5.2.4) and was held at 40 °C in a water bath. The toxin medium - cell suspension was next vortexed for a period of 5 to 6 seconds. Then it was poured onto the surface of a petri-dish that contained 15-mLs of cooled toxin medium. These overlays were allowed to harden (by cooling) for approximately 20 minutes. Four, 10- μL portions of centrifuged cell broth (Oxford Adjustable Sampler - model 8885 - 300205) were then placed into four separate spots that dotted the surface of the sensitive strain agar lawn. The plates were incubated at 22 °C for 24 to 48 hours in order to allow the growth of the sensitive strain, and therefore, achieve development of the clear zone. The plates were then examined under light so that the clearing zones could be measured accurately. A photograph of a developed clearing zone toxin assay plate is shown in Plate 5.1.

The clearing zone that develops around the 'killer - broth' spot is a function of two variables: the volume of the original sample placed upon the sensitive lawn and, the concentration of the toxin in the sample. Therefore, in order to calibrate this test two separate experiments were performed. The first experiment was used to determine the optimum size of the sample to be placed upon the lawn of wild-type yeast cells. This was accomplished by growing cells of the killer

Plate 5.1 - Developed Toxin Assay Plate. Ten microlitre spots of toxin containing broth on 10^5 sensitive lawn. Forty-eight hour incubation time.


Toxin Assay

strain Saccharomyces cerevisiae -1465 in 125-mL of buffered inoculation medium contained in a 500-mL shake flask kept in a rotary shaker, at 175-rpm and 18 °C for 24 hours. The cells were then removed by centrifugation (Sorval - RCB-2, 100-rpm, 5 °C, 20-min.), and the supernatent was decanted into the sterile test tubes. Samples of 5, 10, 15, and 20-μL were then spotted onto the four separate plates, which contained the sensitive yeast strain lawn. The clearing zones were measured after the incubation period was over and the mean values for each sample volume and their standard deviations were calculated and plotted in Figure 5.3. As can be seen from this figure, the optimum sample volume was 10-μ L because this had the smallest standard deviation. A second experiment was used to establish a direct quantitative relationship between the diameter of the clearing zone and the toxin concentration. To do this, the killer cells were grown in 125-mL of inoculation medium, which contained 50-mL of glucose, held in a 500-mL shake flask under the same conditions as for the previous experiment. The supernatant containing the killer toxin was again recovered by centrifugation and was diluted in a pH of 4.5, citric acid - phosphate buffer to the concentrations of 1/10, 2/10, 3/10, ..., 10/10 of the original broth concentration. These dilutions were then spotted onto toxin assay plates using 10-μL spots, and they were incubated for 24 to 48 hours. The clearing zones were then measured, and correlated against the original toxin concentration in the broth. The results of this are plotted in Figure 5.4. Note that the concentration of zymocidal toxin shown in Figure 5.4 was shown as percent concentration equivalents. This was done to indicate that the total toxin concentration in defined units is unknown. In the remainder of this thesis toxin concentration will be reported in UNITS/L where

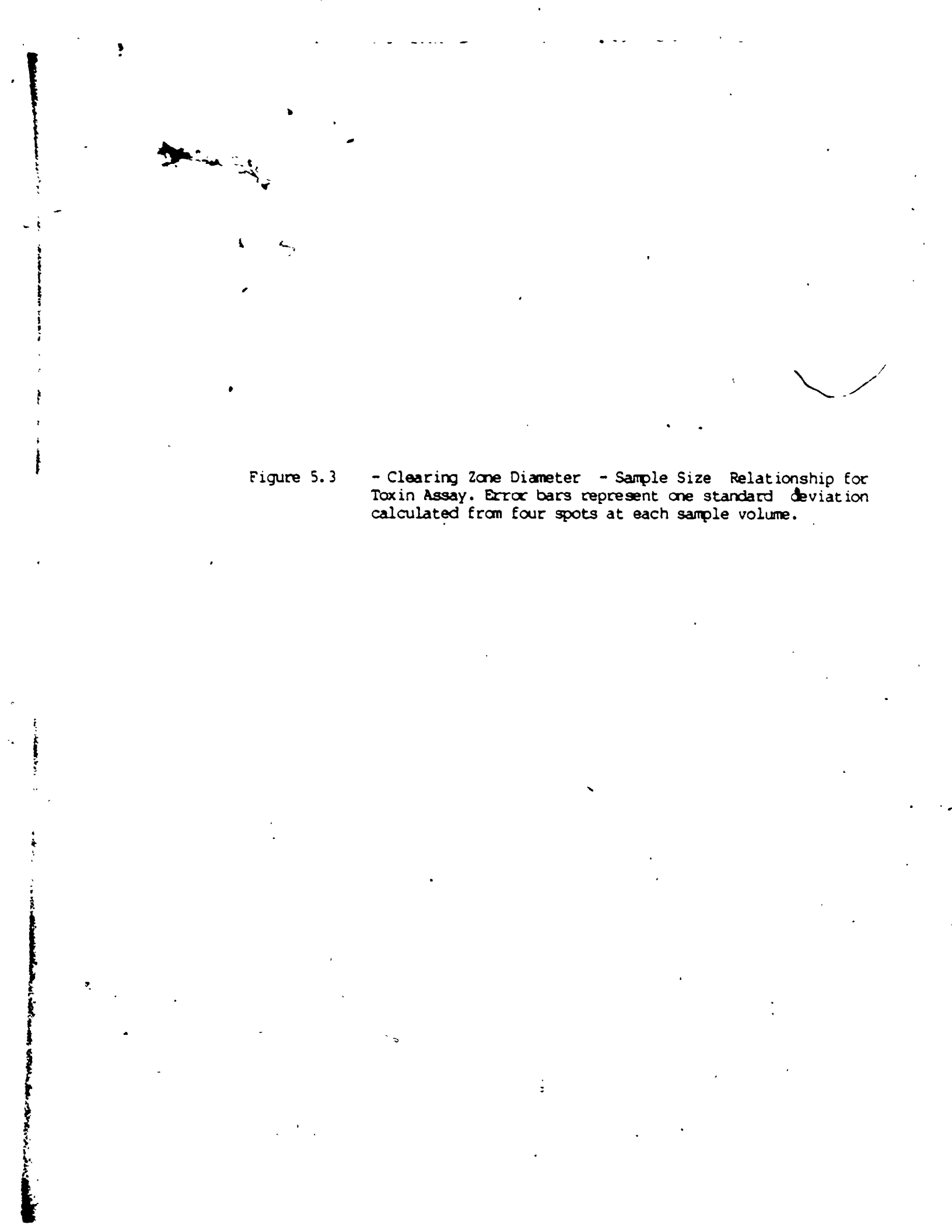


Figure 5.3 - Clearing Zone Diameter - Sample Size Relationship for Toxin Assay. Error bars represent one standard deviation calculated from four spots at each sample volume.

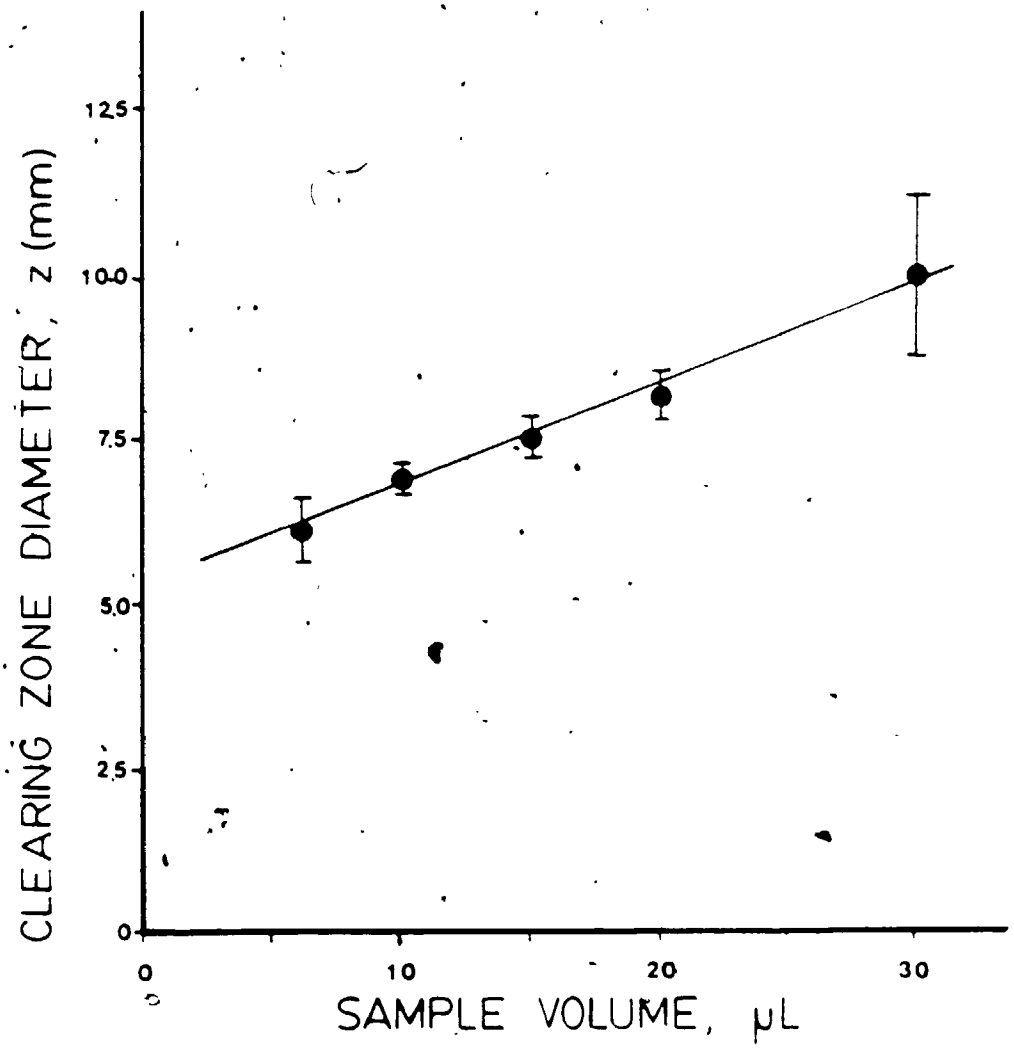
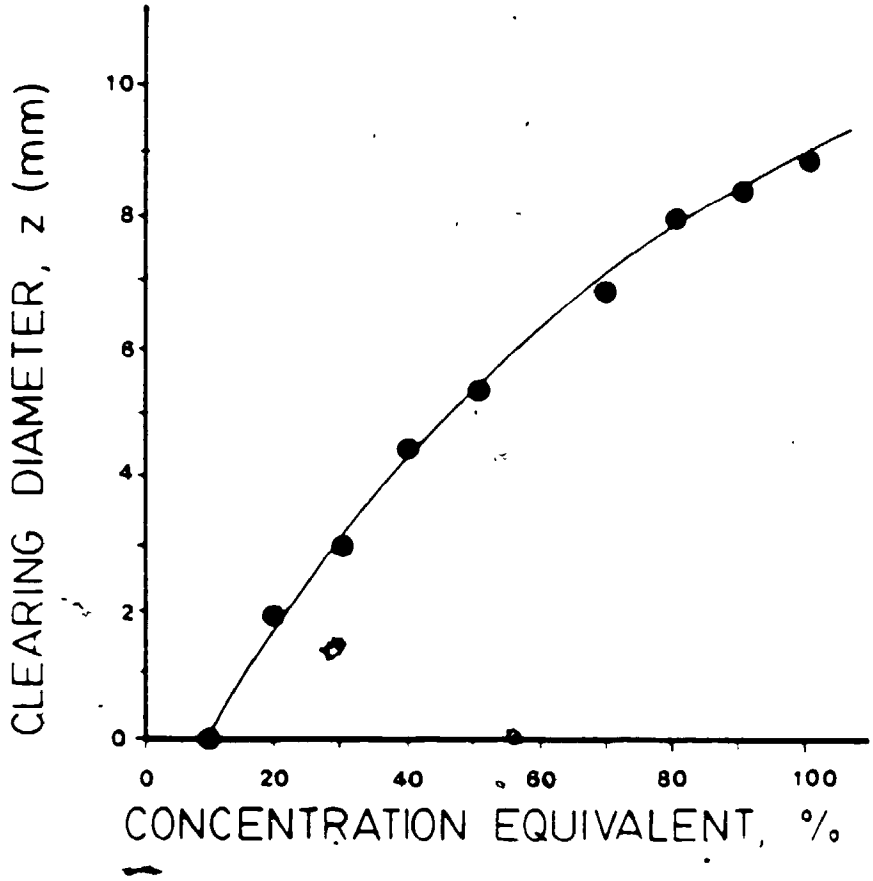

CLEARING ZONE DIAMETER, z (mm)
12.5
10.0
7.5
5.0
2.5
0
0
10
20
30
SAMPLE VOLUME, µL

Figure 5.4 - Toxin Calibration Curve. Equivalent toxin concentrations were made from a series of dilutions of cell free late log phase broth.


CLEARING DIAMETER, z (mm)
0
2
4
6
8
10
0
20
40
60
80
100
CONCENTRATION EQUIVALENT, %

one UNIT/L is equivalent to the amount of toxin which generates a 9-mm clearing zone. As can be seen from this figure, the sensitivity of the test to high toxin concentrations is limited by the diffusional characteristics of the agar medium. Therefore, samples containing high toxin concentrations would have to be diluted in the buffer solution in order to obtain spot diameters in the range of 0 to 9-mm which allows for accurate active toxin concentration determination.

5.3.6 Total Toxin Concentration and Purification

5.3.6.1 Toxin Production

To measure the purity and concentration of the extracellular toxin, a 12-L batch of killer strain culture was harvested during its early stationary phase. The cells were grown in a 14-L Microferm Fermentor (New Brunswick Scientific, model MF - 114) at a temperature of 20°C with the pH being controlled to a level of 4.5 by the automatic addition of either hydrochloric acid or sodium hydroxide. The pH controllers used in all experimentations were model 5997 pH controllers (Horizon Ecology Inc.). The pH probes employed were autoclavable Ingold probes (Type 465).

A 250-mL inoculum was prepared in a rotary shaker at 20°C for 24 hours at 175-rpm and used in the 14-L batch experiment. After 36 hours of batch growth, the cells in the 10-L broth solution were removed by ultrafiltration using a Millipore HVLP membrane (0.5-μm) in a Pellicon Cassette System (Millipore Corp.). The filtration rate was set to 500-mL/min with a recirculation rate of 1,000-mL/min at an inlet pressure of 10-psig.

5.3.6.2 Concentration and Purification Procedures

The extracellular macromolecules remaining in the clarified cell broth were subsequently concentrated by ultrafiltration across a Millipore PTGC ultrafiltration membrane (10,000-MW) in the Pellicon Cassette System using a back pressure of 10-psig. The filtration rate was adjusted to 150-mL/min, and the recirculation rate was 600-mL/min. The remaining steps in the purification and electrophoresis procedures were completed in the labs of the Production Research Department of the Labatt Brewing Company with the assistance of Mr. Peter Zygora. The proteins thus present in the clarified and concentrated cell broth were then precipitated by the addition of ammonium sulphate. The amount of ammonium sulfate added was such that the total concentration was 80% of saturation. The resulting precipitate was collected by centrifugation, and it was resuspended into 10.5-mL of buffer.

5.3.6.3 Toxin Gel Electrophoresis and Total Concentration

Samples of both the unprecipitated and the resuspended protein solutions were applied to separate 20% SDS Polyacrylamide Gels. These were compared to samples of a known protein solution. The known solutions had been applied onto the same gel as the resuspended protein solution to act as standards. The gel was then submerged horizontally in trisglycine buffer in the electrophoresis apparatus. After two hours at 70-V, and a current of 30-mA, the gel was removed from the apparatus and subjected to silver staining. As electrophoresis will separate the different proteins by the charge on the proteins in the mixture, both

the samples and the marker solutions produced a distinct band pattern, depending on the presence of various molecular weights. The width of each band on the gel is proportional to the amount of that protein in the original preparation. Since a known solution of protein was applied as a marker, the overall size of each protein band could be evaluated.

To evaluate the band sizes, the gels were photographed and the negatives were scanned in a Densitometer to determine the relative sizes of the different protein bands (Kipp and Zonen - model DD2). The results from the densitometer were automatically traced onto a chart recorder (Micrograph BD5). The area under the chart recorder trace (for each band) is directly related to the size of the individual bands on the gel. Therefore, the overall concentration of each band can then be estimated from the area under the standard bands.

5.3.7 Plasmid Analysis

To determine the presence of both L and M dsRNA plasmids in the killer yeast strain, a 25-mL sample of late logarithmic phase cells were centrifuged for 20-min at 2,000-rpm. The supernatent was removed, and the cell pellet was chilled in an ice-water bath to be subsequently transported to the Production Research Department of the Labatt Brewing Company, London, Ontario. With the assistance of Lynda Bast of the Labatt Brewing Company, the chromosomal DNA and RNA was isolated, and then detected by gel electrophoresis by using the following method:

The nucleic acids were isolated following the lysis of the cell pellet in buffer that contained 1% sodium dodecyl sulfate. The

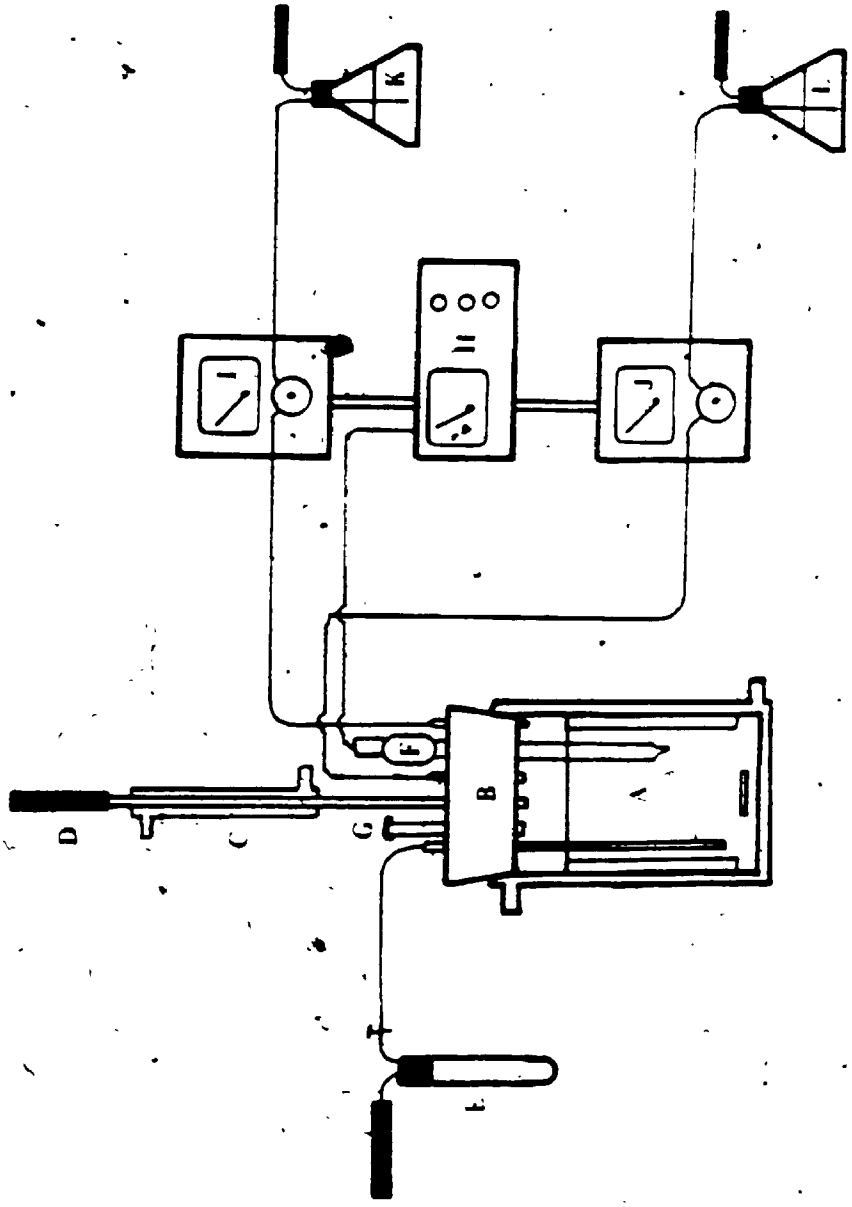
chromosomal DNA and RNA was precipitated with salt (1-M NaCl), and the supernatent was subjected to one phenol extraction (saturated with 10-mM Tris, pH = 8.0) followed by two chloroform extractions. After extensive dialysis, the nucleic acids were precipitated with cold 95% ethanol at -20° C. After centrifugation, the pellets were air dried and resuspended into buffer. The electrophoresis was performed using a 0.7% agarose gel which was submerged horizontally in Tris acetate buffer (40-mM Tris, 5-mM sodium acetate, 1-mM EDTA, pH = 7.4) at 20-V, 50-mA for 21 hours. The buffer was replaced with fresh buffer after 8 hours of electrophoresis. DNA and RNA bands became visualized after staining the gel with ethidium bromide (0.5- μg/mL) and observing them under an ultra-violet transilluminator. This gel was photographed and the photographic negative was scanned by the densitometer (see 5.3.6.3) in order to determine the relative sizes of each DNA and RNA band. A marker track of known molecular weight nucleic acids was used to estimate the position of the L and M dsRNA plasmid bands.

5.4 Experimental Apparatus for Batch Experiments

All batch fermentations used to study the environmental factors were completed in a 2-L (total volume) bioreactor system as depicted in Figure 5.5. The vessel itself was a 2-L tempering beaker (Cole Parmer, model number R-3773-30) fitted with a set of stainless steel baffles. The top of the bioreactor unit was a number 16 rubber stopper, fitted with a sample port, a gas outlet port, and acid and base addition ports made of 1/4" glass tube. Two additional ports in the stopper were 1/2" in diameter, they were used for inoculations and for the pH probe (Ingold, Type 465). The acid and base ports were attached through Sigma

Figure 5.5 - Schematic of Batch Fermentation Apparatus.

A - 2-L Total volume fermentor vessel.
B - Rubber stopper - fitted with access ports.
C - Exit gas condenser.
D - Exit gas filter.
E - Sample port and tube.
F - pH probe.
G - Inoculation port.
H - pH meter and controller.
I - Base solution pump.
J - Acid solution pump.
K - Base solution reservoir.
L - Acid solution reservoir.


K
A
B
D
C

pumps (Sigma - motor Comp., AL.4.E) by separate 1/4" latex tubing to the two 1,000-mL buffer solution flasks. The pumps and the pH probe were connected to a pH controller (Horizon Ecology Comp., model 5997). The pH in the fermentor was controlled to a desired level through the automatic addition of 0.1-M hydrochloric acid or 0.2-M sodium hydroxide. The temperature of the fermentor was controlled with an immersion heater and pump (Haake model E1) in a 20-L water bath. Tubing routed the water from the pump through to the jacket of the tempering beaker, and then back to the water bath. A cold water cooling loop was also included in the temperature bath to allow for accurate control at temperatures lower than ambient. Outlet gases from the fermentor were cooled in a cold water condensor and routed through a glass wool packed air filter.

At the beginning of a particular experiment, 1.5-L of liquid medium was prepared and transferred to the bioreactor. The pH - buffer flasks were filled, and the fermentor vessel with all of its attachments was sterilized in an autoclave, at 121° C for 30 minutes.

After the fermentor reached the desired fermentation temperature but prior to inoculation, a sample of the medium was drawn from the fermentor. The pH of this sample was measured (Beckman Zeromatic, SS-3), and the pH controller was turned on and was adjusted accordingly. The pH meter was calibrated using reference buffer solutions of pH 4.01 and 7.00 (Canlab H 7590-4 and H 7590-7). The fermentor was then inoculated aseptically with a 10 -mL killer strain inoculum. A sample was drawn from the fermentor immediately after inoculation, and also at regular intervals through the course of the

fermentation and they were analyzed for biomass, glucose, ethanol and toxin concentrations.

5.5 Experimental Apparatus for Continuous Growth

The fermentation vessel used in continuous culture experiments was similar to the batch vessel and is shown in Figure 5.6. An additional port was added to the vessel to allow medium feeding while the sample port was removed and replaced with an exit port from the system. These entry and exit ports were fitted with 1/4" latex tubing through pumps to the 20-L medium and the product storage vessels. The feedpump was a Masterflex 1 to 100-rpm pump (R-7520-30) fitted with a Masterflex R-7013-13 head which uses 0.030 inch (R-6424-13) tubing.

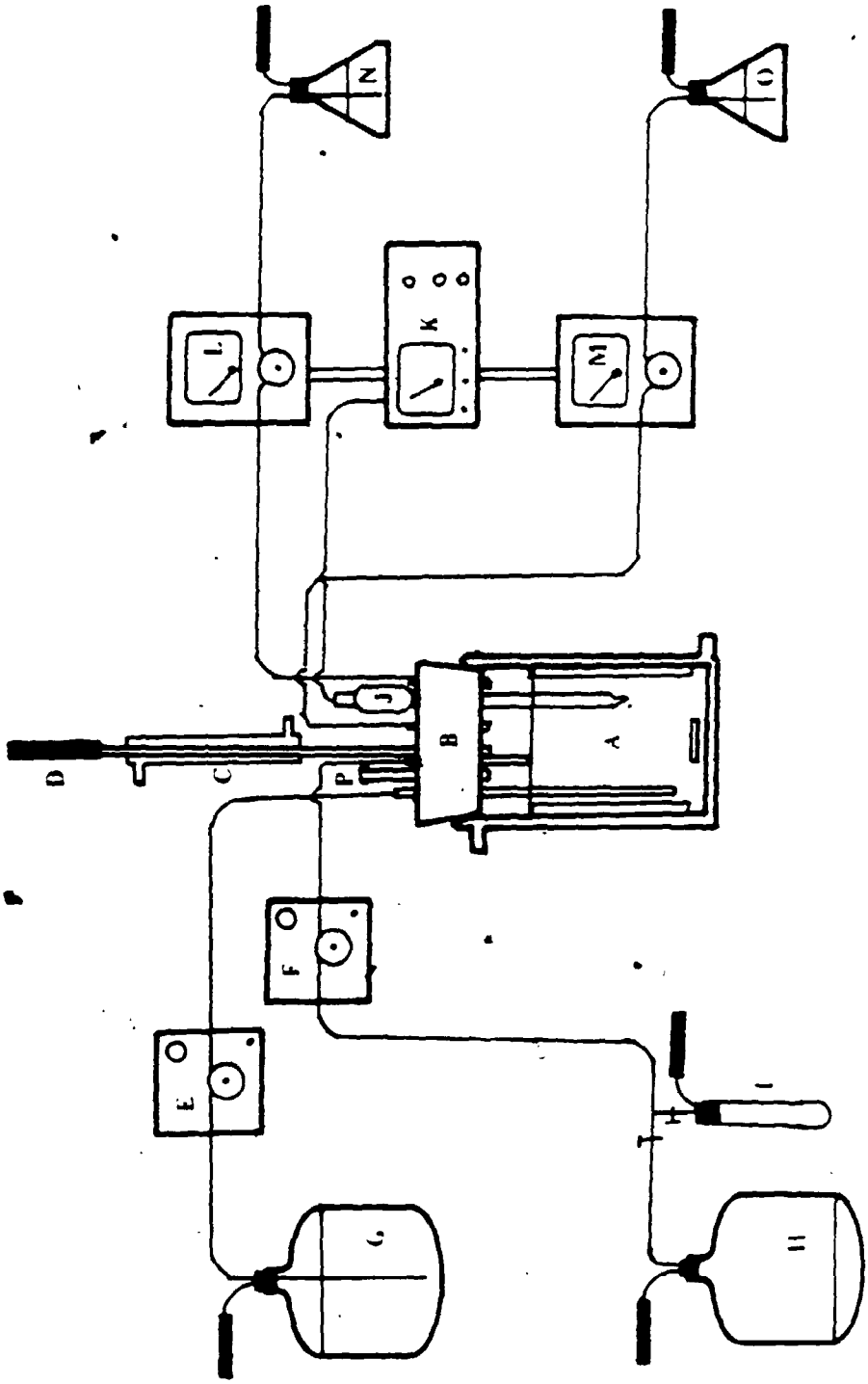
The product pump was a Masterflex 6 to 600-rpm pump (R-7520-05) fitted with a Masterflex R-7014-20 Head which uses 0.064 inch (R-6424-14) tubing. Between this pump and the product storage vessel, a sample port was added to allow the aseptic product to be extracted.

At the beginning of each experimental run, the exit and entry ports were sealed. The fermentor vessel was filled with 1.5-L of cell growth and production medium. The fermentor vessel was then sterilized, and was set up in the same manner as for the batch experiments. A large volume (20-L) of medium was prepared, subsequently sterilized in the feed tank, and then connected to the fermentor vessel.

After inoculation, the batch growth was allowed to proceed near the end of the exponential growth phase and at this point, the feed and

Figure 5.6 - Schematic of Continous Fermentation Apparatus

A - 2-L Total volume fermentor vessel.
B - Rubber stopper - fitted with access ports.
C - Exit gas condenser.
D - Exit gas filter.
E - Feed pump.
F - Product pump.
G - Feed reservoir.
H - Product reservoir.
I - Sample Port.
J - pH Probe.
K - pH meter and controller.
L - Base solution pump.
M - Acid solution pump.
N - Base solution reservoir.
O - Acid solution reservoir.


N
O
K
L
M
A
B
C
D
E
F
G
H
I

product pumps were set to the appropriate feed rates, and appropriate level control of the fermentor.

5.6 Sampling Techniques

Biomass, ethanol, reducing sugars, and toxin concentration were determined for each sample withdrawn from the batch (or continuous) runs. An 8 to 10-mL sample was aseptically withdrawn from the fermentor and transported to the laminar flow hood (Canlab, VBM 600). A 5 -mL portion of the sample as transferred by pipette to a sterile centrifuge tube. After centrifugation, the supernatent was transferred to a sterile test-tube, and the cell pellet was saved for biomass concentration determination.

A 10-μL portion of sensitive strain was mixed with a 15-mL portion of molten toxin concentration medium, and it was poured aseptically into a petri dish. This lawn was allowed to solidify, and then four 10-μL samples of the sample supernatent were spotted on the surface. The petri-dish was left in the laminar flow hood for 4 to 6 hours before it was transferred to the incubator. This time period allowed the spotted samples to absorb into the agar. The remainder of the supernatent was stored at 5°C for reducing sugar analysis. Ethanol concentration was determined by using a dilution of the remaining whole broth sample. Details of the specific tests utilized are outlined in sections 5.3.1 through 5.3.5.

CHAPTER 6

EXPERIMENTAL RESULTS AND DISCUSSION

6.0 Initial Experimental Runs

Four, initial batch runs were completed at different temperatures and pH values in order to analyze the limitations of both the fermentor and the control equipment. These initial experiments served to set-up the boundaries for the ranges of temperature and pH.

Each of the initial runs was completed as a batch experiment using the 1.5-L fermentor system outlined in section 5.4. The temperature range which could be accurately attained in the fermentor for the course of a typical fermentation was assessed to be between 15 and 45° C. It was also shown that the contents of the fermenter vessel were at the same temperature as the contents of the temperature control system and 35-L water bath. This enabled ease in temperature control and monitoring throughout the course of the fermentation studies. The pH could be accurately controlled to be between pH 3 and pH 8.

These boundaries should allowed a wide enough window in order to accurately assess the production and growth kinetics of the zymocidal yeast strain.

6.1 Toxin Presence and Concentration

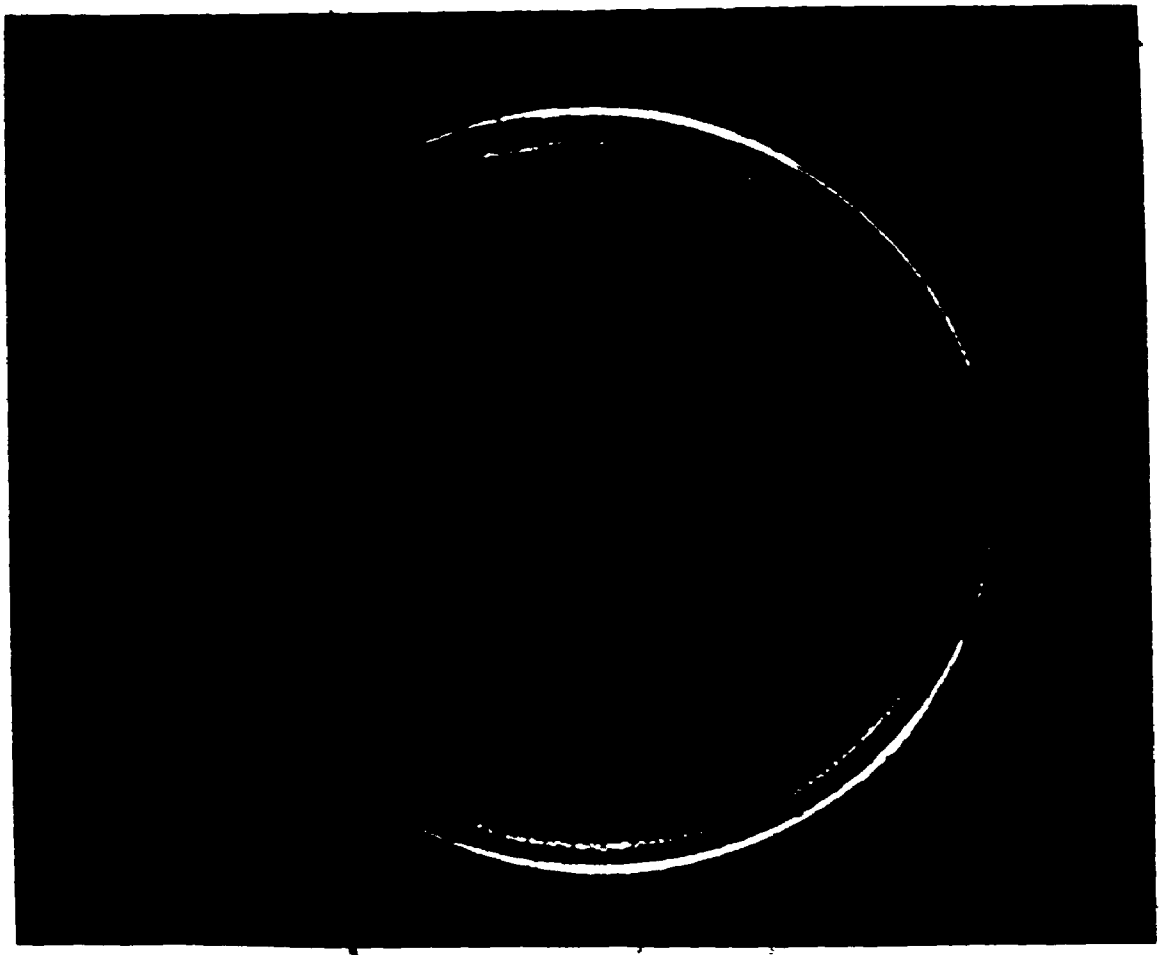
Toxin production by the zymocidal strain was detected by the well - test method. To show that the strain used in this analysis was capable of producing the zymocidal toxin, a 10- μL portion of cell broth containing 10^2 cells was spotted onto a sensitive lawn. A photograph of this test is shown in Plate 6.1.

The overall concentration of toxin in the liquid broth was evaluated by the concentration/electrophoresis method (section 5.3.6). A 10-L batch of growth and production medium was prepared and sterilized in the 14-L New Brunswick fermentor. After the temperature and pH were adjusted to be 20 $^{\circ}$ C and 4.5 respecitively, the fermentor was inoculated with a 1-L zymocidal inoculum. This inoculum was grown for a period of 24 hours in a rotary shaker at 25° C.

At the end of the exponential phase of growth, the fermentor contents were subjected to ultrafiltration to remove the cells. Further ultrafiltration was used to concentrate the extracellular proteins from the original 11-L to 1-L. (See section 5.3.6 for details of this process.)

The proteins from a 500-mL portion of this concentrate were then precipitated by the addition of ammonium sulphate to 80% saturation. The precipitate was removed by centrifugation and weighed to be 3.5-mg. The precipitate was then resuspended into 10.5-mL of buffer for use on the electrophoresis gel.

Plate 6.1 - Zymocidal Strain on a Sensitive Lawn of Saccharomyces cerevisiae on a 10 lawn. Initial zymocidal cell concentration - 100 cells.



Zymocidal S.c. - Strain 1465

on a 10⁵ lawn of sensitive

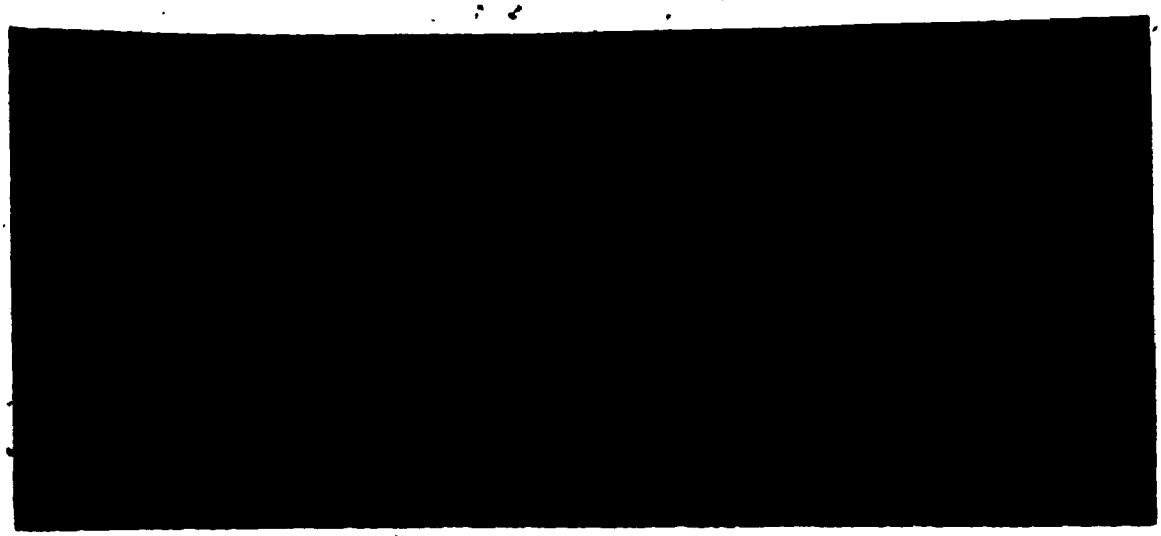


Plate 6.2 shows the gel which resulted from unprecipitated toxin concentrate. As can be seen on this plate, many other proteins are present in the concentrate broth. Separation of these proteins by precipitation is essential before analysis is possible.

An electrophoresis gel was run using the precipitated proteins as shown in Plate 6.3. Four different size samples were used, and the resulting lanes were scanned by a densitometer. As can be seen in this plate, the toxin band is distinguishable at a molecular weight less than the 14,000 MW marker band in all of the lanes. Scans of the photographic negative showed that a 5- μL sample of the precipitated proteins produced a 0.3- μg toxin band. Based on the densitometer scans, the total amount of toxin in the precipitated sample was 630-g. Before precipitation, the total amount of toxin present in the 1-L sample concentrated broth was then 1.26-mg. Therefore, the total concentration of toxin in the original 11-L of fermentation broth was 0.115-mg/L.

Assuming that only 50% of the toxin and other proteins are precipitated by the ammonium sulphate treatment (Panchal, personal communication, 1986), the overall toxin concentration is estimated to be 230- μg/L.

The average clearing zone diameter created by application of the cell free broth from the original fermentation was calculated to be 7.0-mm. This allows for approximate conversion of the zone diameters to

Plate 6.2 - Electrophoresis Gel of Unprecipitated Protein Solution.
Band A - extracellular protein bands.
Band B - 11,800 MW toxin.
See text for purification and electrophoresis procedure.

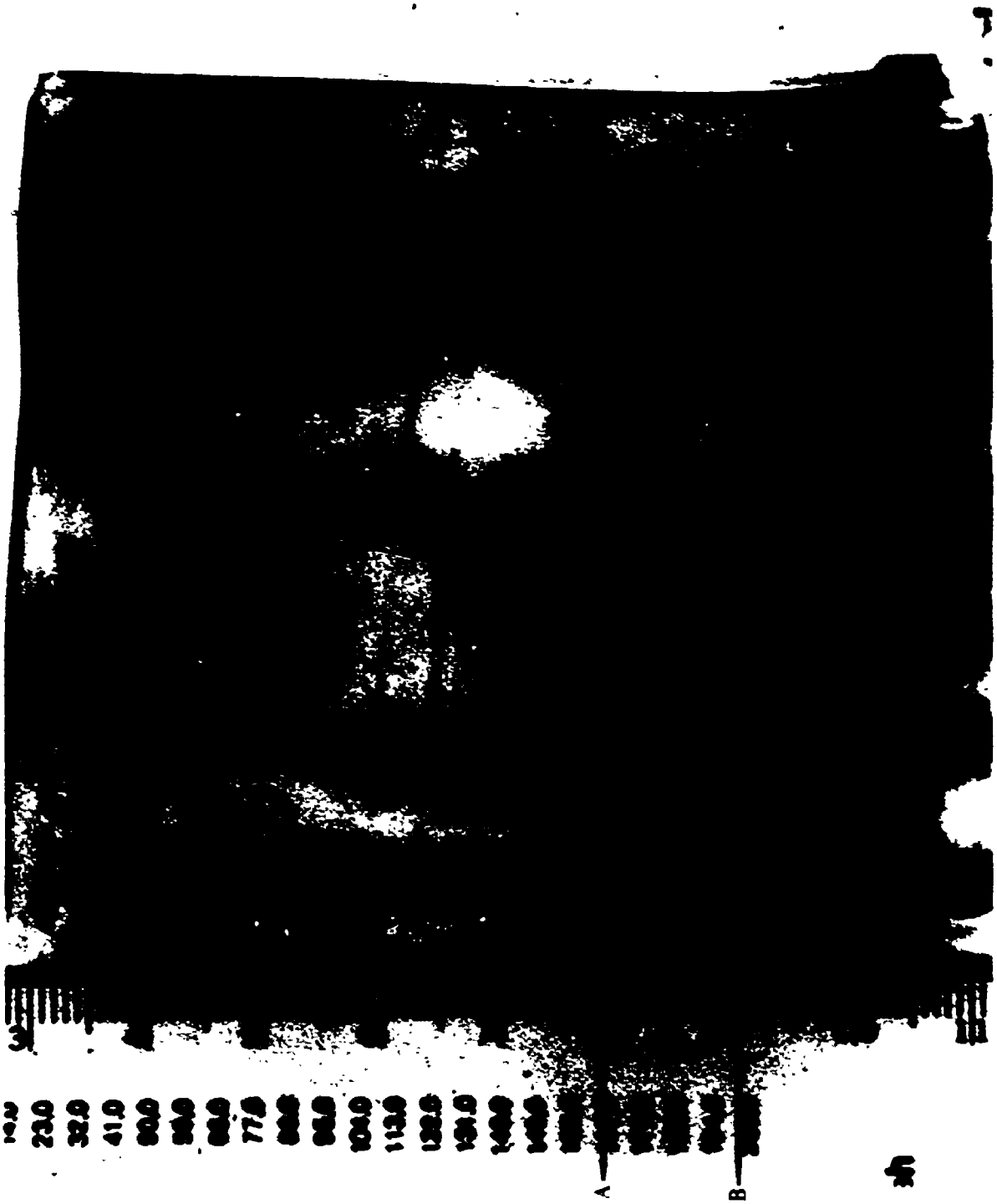

A
B

Plate 6.3 - Electrophoresis Gel of Precipitated Protein Solution at indicated sample sizes.
Band A - 11,800 MW toxin.
Band B - 14,000 MW extracellular protein.
Band C - other cellular proteins.


A
B
C

absolute toxin concentration. This relationship is shown in equation 6.1.

$$Z = \frac{230}{-} z_D \qquad 6.1$$

This relationship must be corrected to reflect the non-linear relationship between the clearing area and the equivalent concentration shown in Figure 5.5. A better relationship between the overall concentration and the zone diameter would then be shown in equation 6.2.

$$Z = \frac{230}{0.71} z \qquad 6.2$$

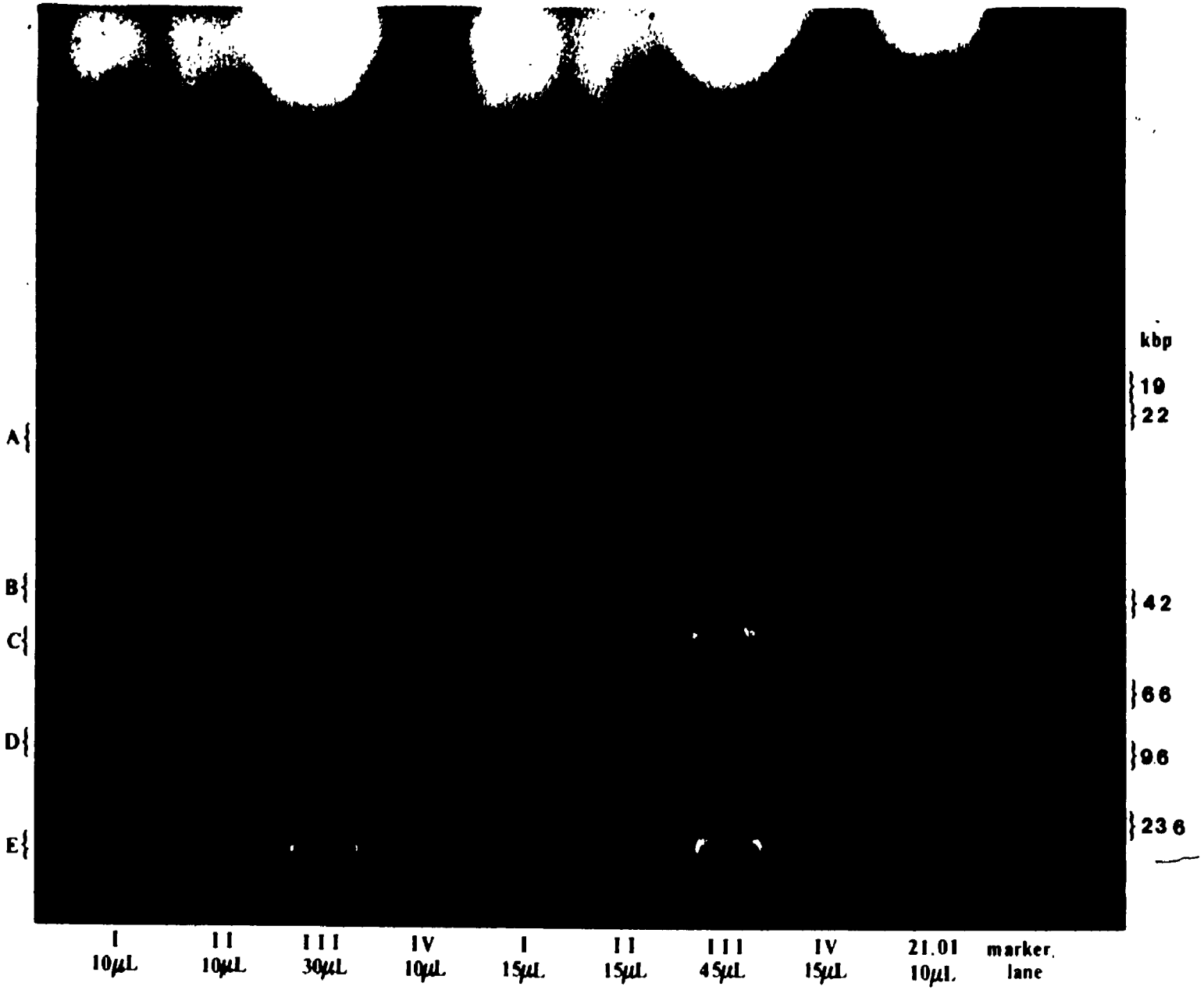
6.2 Plasmid Analysis

6.2.1 Plasmid Presence in Killer Strain

Initial tests were completed to determine the presence and approximate number of L and M plasmids in the killer yeast strain. Four, 25-mL samples were withdrawn from a 1.5-L batch fermentation during the logarithmic growth phase. The batch fermentor was controlled at a temperature of 20° C and a pH of 4.5. The whole broth sample was centrifuged and the resulting cell pellet was subjected to the plasmid analysis technique outlined in section 5.3.7. The resulting gel was photographed and is shown in Plate 6.4. The first lane is a marker band of proteins of known molecular weight. This lane will allow estimation of the molecular weight of the L and M plasmids. The second lane is a

Plate 6.4 - Electrophoresis Gel of Cellular Nucleic Acids.

Band A - M ds RNA.
Band B - Superhelical DNA.
Band C - L ds RNA.
Band D - Open loop DNA.
Band E - Large molecular weight DNA/RNA and other nucleic acids.


kbp
19
22
42
66
96
236
A
B
C
D
E
I 10µL
II 10µL
III 30µL
IV 10µL
I 15µL
II 15µL
III 45µL
IV 15µL
21.01 10µL
marker lane

11.

nucleic acid extract of a different superkiller strain. This lane was included to allow some comparison of the plasmids from different strains. The remaining eight lanes shown in Plate 6.4 are different sized samples of the four nucleic acid extracts from the original four samples. The size of each sample is shown on the photograph.

As shown in Plate 6.4, each nucleic acid extract from the killer strains produced five distinct bands on the gel. The band which migrated the furthest was estimated to have a molecular weight of 2.0 kilobase pairs (kbp), and was identified as the M dsRNA plasmid by comparison to work done by other researchers (Wickner, 1979 and Palfree and Bussey, 1979).

The second distinct band at approximately 3.8-kbp was identified as 2-μm superhelical DNA (Panchal, personal communication, 1986). Between 50 and 80 copies of this small DNA strand exist in the cytoplasm of yeast cells. This band was used to estimate the number of copies of the L and M plasmids by densitometer analysis. By referring to section 2.4.2 of this thesis, one can see that the overall size of the L plasmid is approximately twice that of the M plasmid. The third and largest distinct band at approximately 4.5-kbp was identified as the L plasmid band because of its size when compared to the M band. Comparison of these results to other published results also suggest that this large band is created by the L plasmids. The fourth faint band at 6.6-kbp was identified as the 2-μm open-circular DNA (Panchal, pers. comm., 1986). Saccharomyces cerevisiae cells possess these short strands in a similar quantity as the superhelical DNA. The fifth band, at approximately 24-kbp, is the nuclear DNA along with other large

nuclear and cytoplasmic nucleic acid components of the cell. No separation of these large fractions appears as they are at the limit of this analysis. Separation of these large nucleic acids is possible, but it is of little significance in this analysis.

6.2.2 Plasmid Numbers

A densitometer comparison of each band on the photographic negative of the gel was used to estimate the number of copies of the L and M plasmids. As reported earlier, each cell of S. cerevisiae contains between 50 and 80 copies of the superhelical DNA. Comparison of the density of this band to the L and M bands allowed estimation of the numbers of these plasmids. The densitometer results indicated that between 100 and 160 copies of the M plasmid, and between 250 and 400 copies of the L plasmid exist in each cell of the killer strain used throughout this analysis. Analysis of the lane from the different strain of S. cerevisiae resulted in similar estimated numbers of the plasmids.

6.3 Kinetics of Toxin Inactivation

6.3.1 Temperature and pH Effects

The effects of temperature and pH on the stability of the zymocidal toxin in liquid broth is of prime concern in this analysis. Studies were undertaken to determine the deactivation of the toxin at different temperatures and pH values. A single 1.5-L batch of the killer strain sustained at 20° C and a pH of 4.5 was grown until the end of the logarithmic phase was detectable by a biomass analysis. The

fermentor broth was centrifuged at 5,000-rpm for 10-min. at a temperature of 5 °C to remove the cells. The cell free broth was then divided into five separate portions and stored at 5 °C in a refridgerator.

Each portion of the centrifuged broth was then further subdivided into five 10-mL samples and added to separate, sterile 50-mL test tubes that already contained 10-mL portions of a sodium phosphate - citric acid buffer. The five tubes had pH values of 3, 4, 4.5, 5 and 6 respectively. A total of 25 tubes were created in this way. Five at each pH. Each set of five buffered tubes created from the five portions of the original broth were then placed in refridgerators, incubators, or environmental rooms in order to adjust the temperature to the desired value. Temperatures of 7, 21.8, 24.5, 30 and 40 °C were used in this analysis.

Therefore, in each incubator, five test tubes at different pH values were available for use to study the inactivation of the toxin. Samples from each tube were taken periodically and assayed for toxin concentration, over an eight hour period.

After a 48 hour incubation, the clearing zones caused by the active toxin were measured and then converted to equivalent concentration units by using Figure 5.5. From this data, the percentage of original toxin activity was evaluated. The results of this analysis were plotted in accordance with equation 4.24, and a linear relationship was noted. A typical set of results for temperature

Figure 6.1 - Temperature Deactivation of the Zymocidal Toxin pH = 4.5.

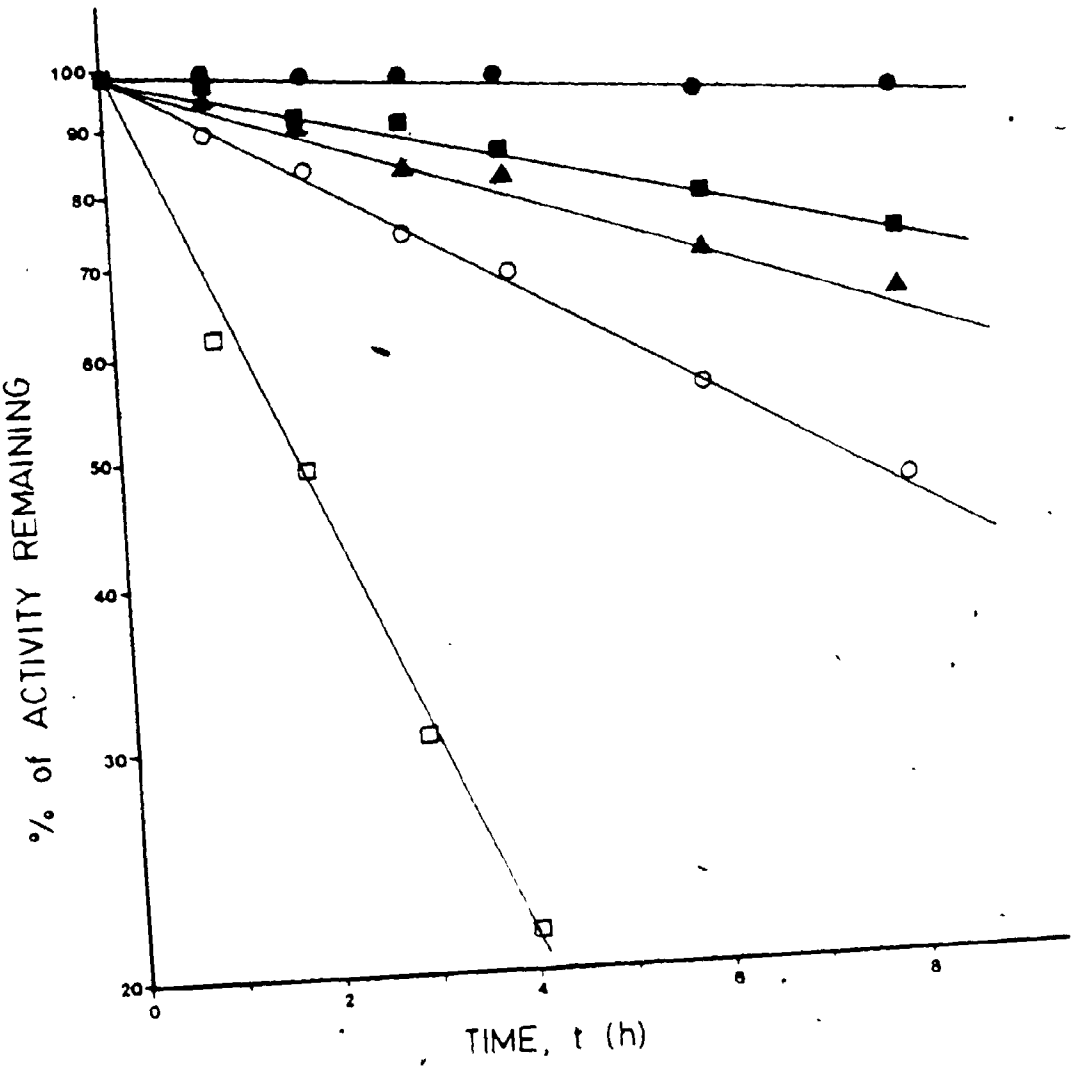
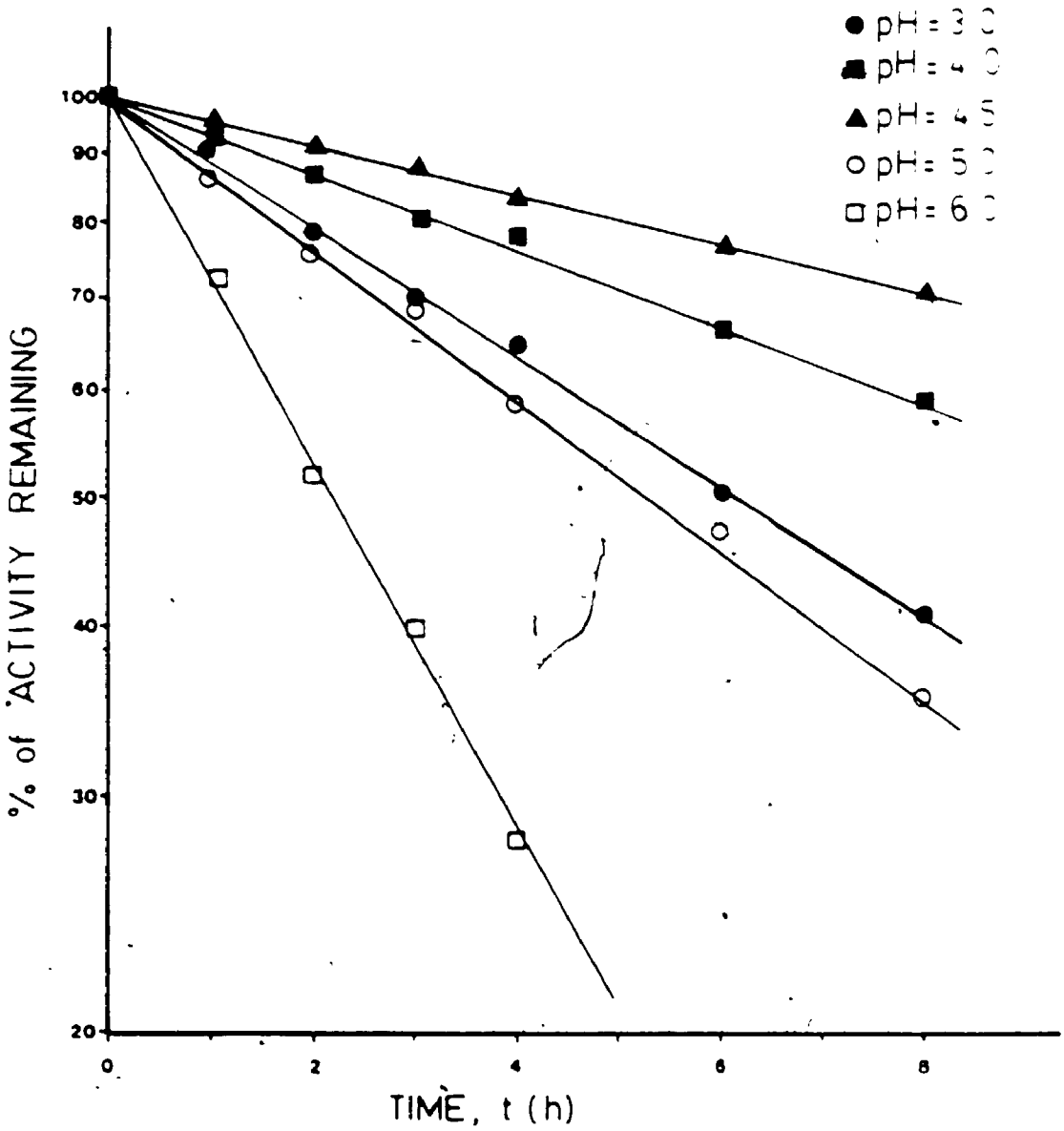

● T = 7°C
■ T = 21.8°C
▲ T = 24.5°C
○ T = 32°C
□ T = 41°C
% of ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
0
2
4
6
8
TIME, t (h)

Figure 6.2 - pH Deactivation of the Zymocidal Toxin
Temperature = 21.8° C.


pH = 3.0
pH = 4.0
pH = 4.5
pH = 5.0
pH = 6.0
% of ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
0
2
4
6
8
TIME, t (h)

inactivation is shown in Figure 6.1, and for pH inactivation in Figure 6.2.

As shown in these figures, the toxin is rapidly deactivated at reasonably low temperatures and at pH values outside of the 4.0 to 4.6 range. The slopes of the lines on Figure 6.1 were used to the determine the Arrhenius rate constant in accordance with equation 4.21. Figure 6.3 is an Arrhenius plot of the data shown in Figure 6.1. As shown in Figure 6.3, a linear relationship was found between the logarithm of the deactivation rate constant k and the inverse of the absolute temperature. This allows the calculation of the deactivation energy (E), and the proportionality constant (A) in the Arrhenius equation 4.21.

Based on the data shown in Figure 6.3, the deactivation energy and the proportionality constant were evaluated to be 14.5-kcal/mole and 2.53×10^9 h^{-1} respectively. The value of the deactivation energy calculated for this S. cerevisiae based toxin is lower than the 55-kcal/mole reported by Kotani et al. (1977). The toxin used by Kotani and his co-workers was produced by sake yeast. The sake toxins have been shown to be more stable than the S. cerevisiae derived toxins as is confirmed by these results.

The rate of deactivation of the toxin as a function of pH is shown in Figure 6.4, which includes the kinetic data from Figure 6.2. As shown in Figure 6.4, the rate of deactivation of the toxin is very sensitive to pH. At pH values outside the optimum range of 4.0 to 4.6,

Figure 6.3 - Arrhenius plot of the Thermal Deactivation Rate of the Toxin

pH = 4.5.

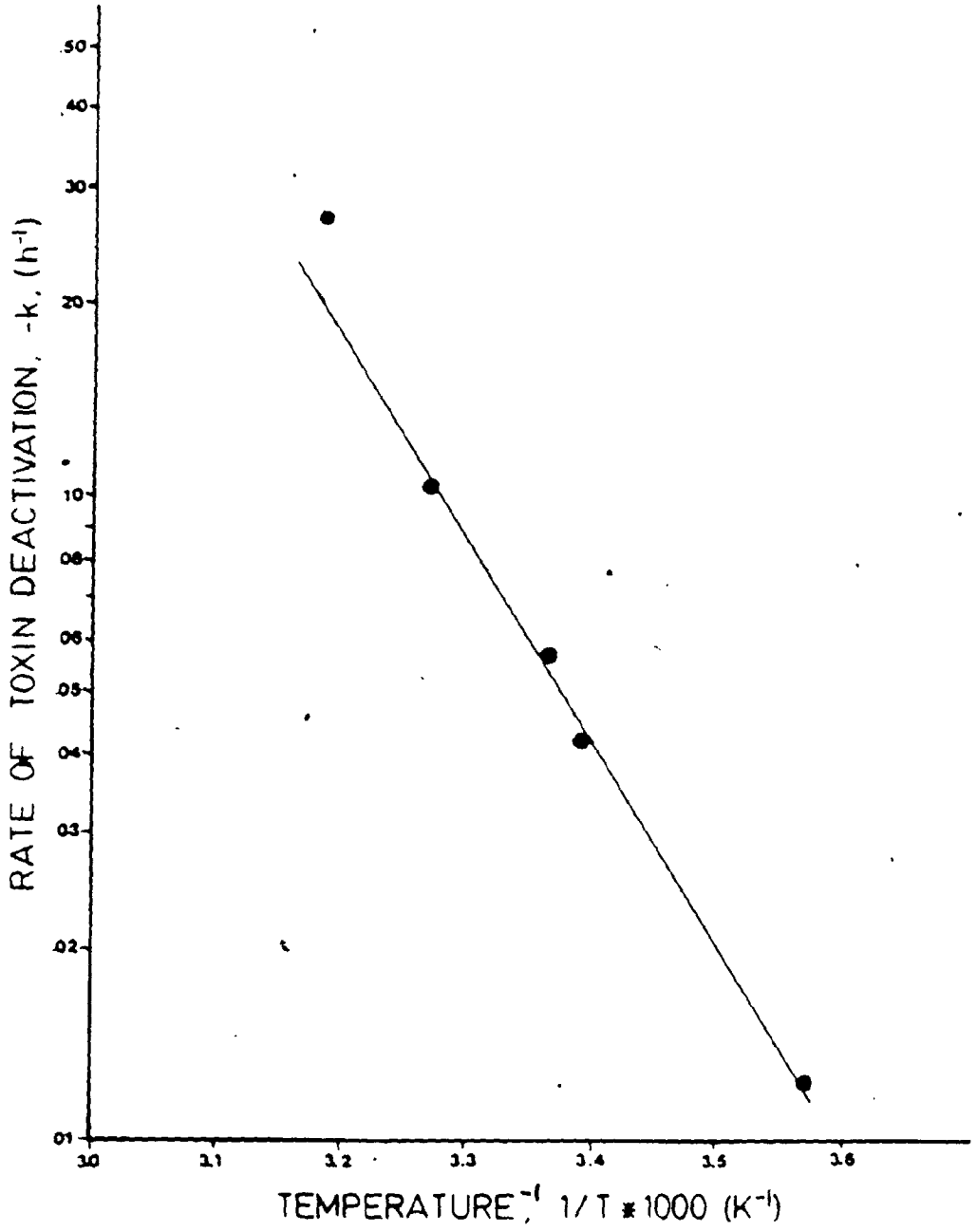
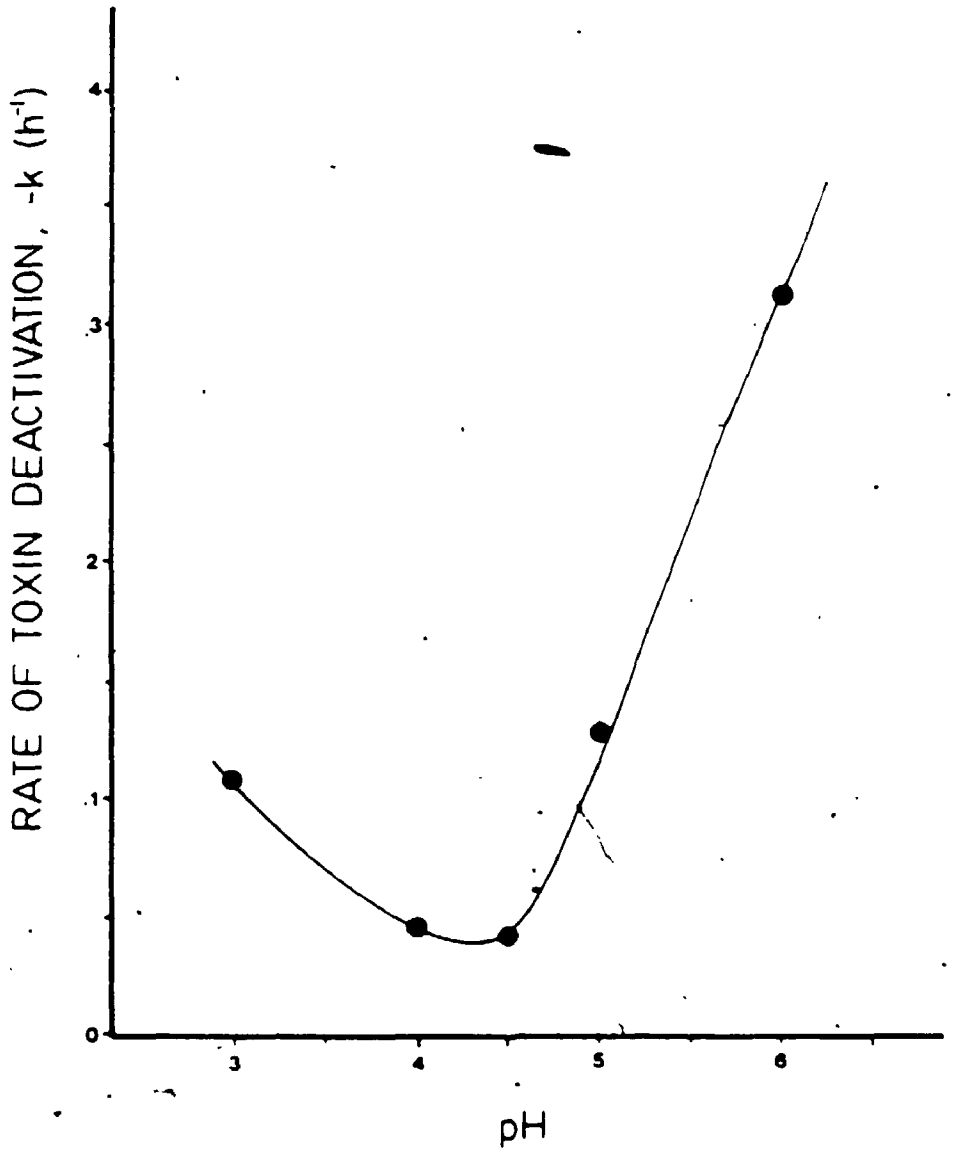

RATE OF TOXIN DEACTIVATION, -k, (h⁻¹)
.50
.40
.30
.20
.10
.08
.06
.05
.04
.03
.02
.01
3.0
3.1
3.2
3.3
3.4
3.5
3.6
TEMPERATURE⁻¹, 1/T * 1000 (K⁻¹)

Figure 6.4 - pH Effects on the Thermal Deactivation Rate of the Toxin
Temperature = 21.8° C.


RATE OF TOXIN DEACTIVATION, -k (h⁻¹)
0
1
2
3
4
3
4
5
6
pH

the rate of deactivation is very high. This is especially true at pH values higher than 4.6.

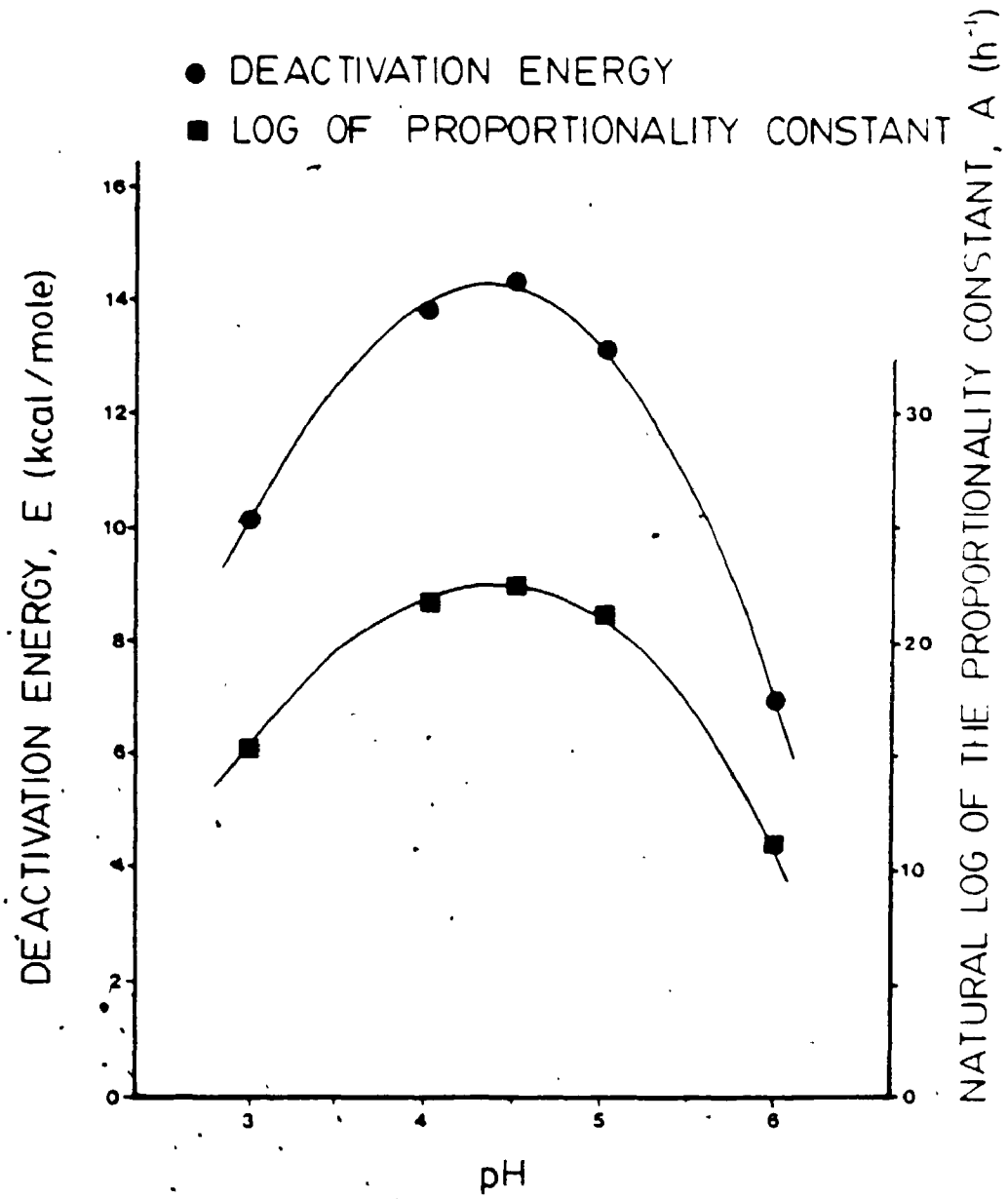
It is interesting to note that the optimum pH range for toxin activity is very similar to the optimum pH range for yeast growth. Yeasts have been shown to grow best at pH values between 4 and 5 (Bailey and Ollis, 1986). This suggests that the zymocidal toxin was originally produced to protect the cell when it reproduced most rapidly.

Continuing with calculations from figures similar to those outlined for Figures 4.1 through to 4.4, the overall effect of pH and temperature on toxin deactivation may be calculated. Figure 6.5 shows the deactivation energies and the proportionality constants as functions of pH for each set of samples analyzed. Since the rate constants are a function of temperature, the overall effect of pH and temperature is shown in this figure.

The results evaluated above will allow the determination of the total active and inactive toxin concentrations during a fermentation. These results show that at a constant pH, the deactivation of the active toxin is consistent with first - order kinetics of decay. The rate of deactivation of the toxin follows a typical Arrhenius relationship with respect to temperature.

The overall deactivation rate equation for the toxin may be, therefore, maybe given by equation 6.3.

Figure 6.5 - Overall Temperature and pH Effects on Toxin Deactivation. Deactivation energies and proportionality constants where evaluated from the temperature deactivation data according to the Arrhenius relationship.


● DEACTIVATION ENERGY
■ LOG OF PROPORTIONALITY CONSTANT
DEACTIVATION ENERGY, E (kcal/mole)
NATURAL LOG OF THE PROPORTIONALITY CONSTANT, A (h⁻¹)
pH
0
2
4
6
8
10
12
14
16
0
10
20
30
3
4
5
6

$$\frac{Z_A}{Z_T} = e^{-kt} \qquad 6.3$$

The rate constant of toxin deactivation is given by Equation 6.4.

$$k = A\, e^{-\frac{E}{RT}} \qquad 6.4$$

In this Arrhenius equation, both A and E are functions of the broth pH. These values can be estimated by the use of Figure 6.5, or calculated using the correlations shown in Equations 6.5 and 6.6 which were derived from the experimental data.

$$\ln (A) = -.602(pH)^3 + 4.01(pH)^2 - .567(pH) - 3.09 \qquad 6.5$$

$$\frac{E}{R} = -96.9(pH)^3 - 16.4(pH)^2 + 5690(pH) - 8990 \qquad 6.6$$

These correlations are plotted with the data points in Figures 6.6 and 6.7.

The differential form of equation 6.3 will be of more use in this analysis and is shown in equation 6.7.

$$\frac{dZ_A}{dt} = -kZ_A \qquad 6.7$$

6.3.2 Ethanol Effects

The series of experiments outlined above were repeated using a new batch of cell-free fermentation broth. This set of analyses not only included the pH and temperature effects on the toxin but also included ethanol effects.

Figure 6.6 - Proportionality Factor Correlation. The correlation curve fit to the data using optimization techniques. See the text for details.

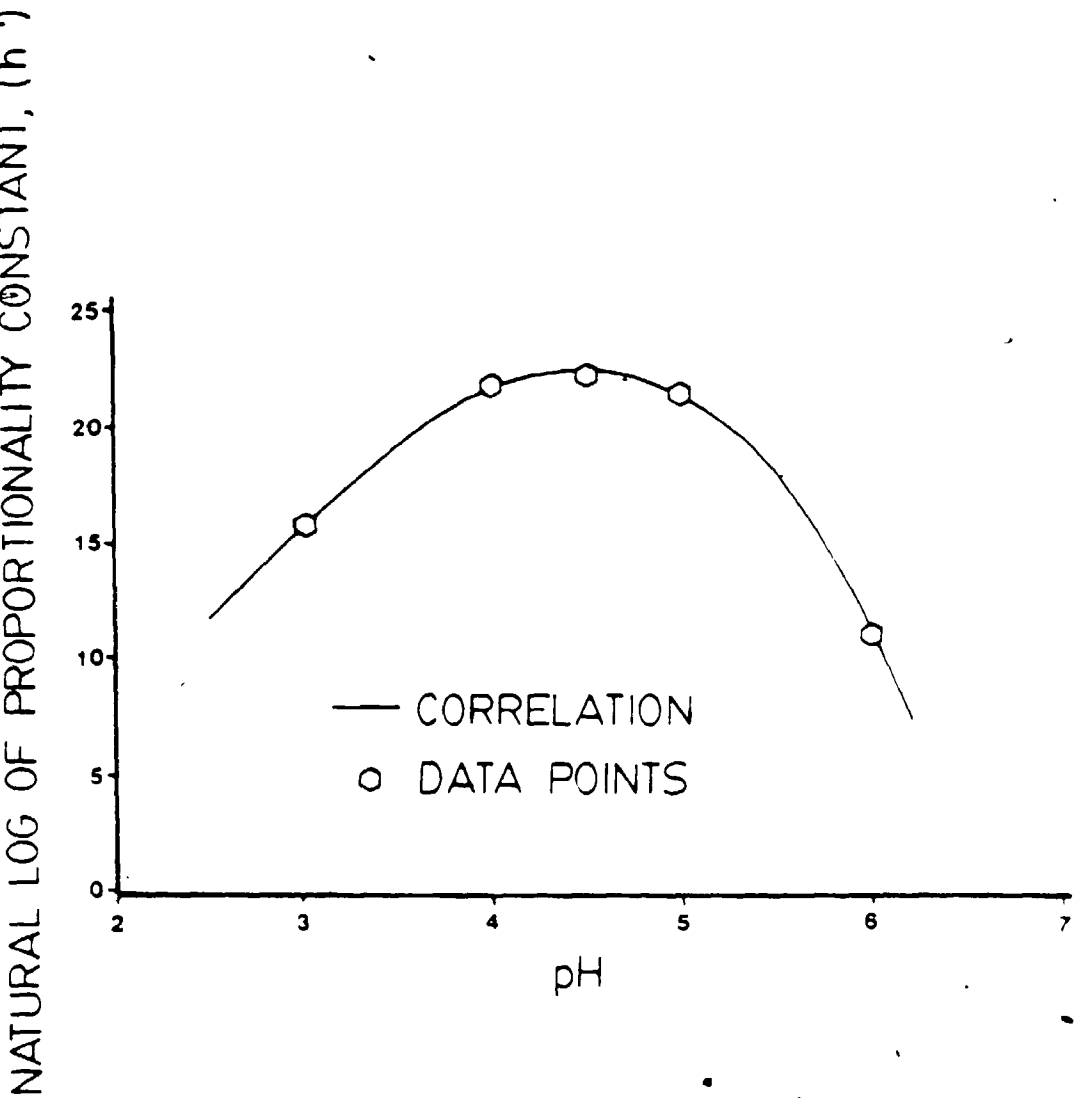
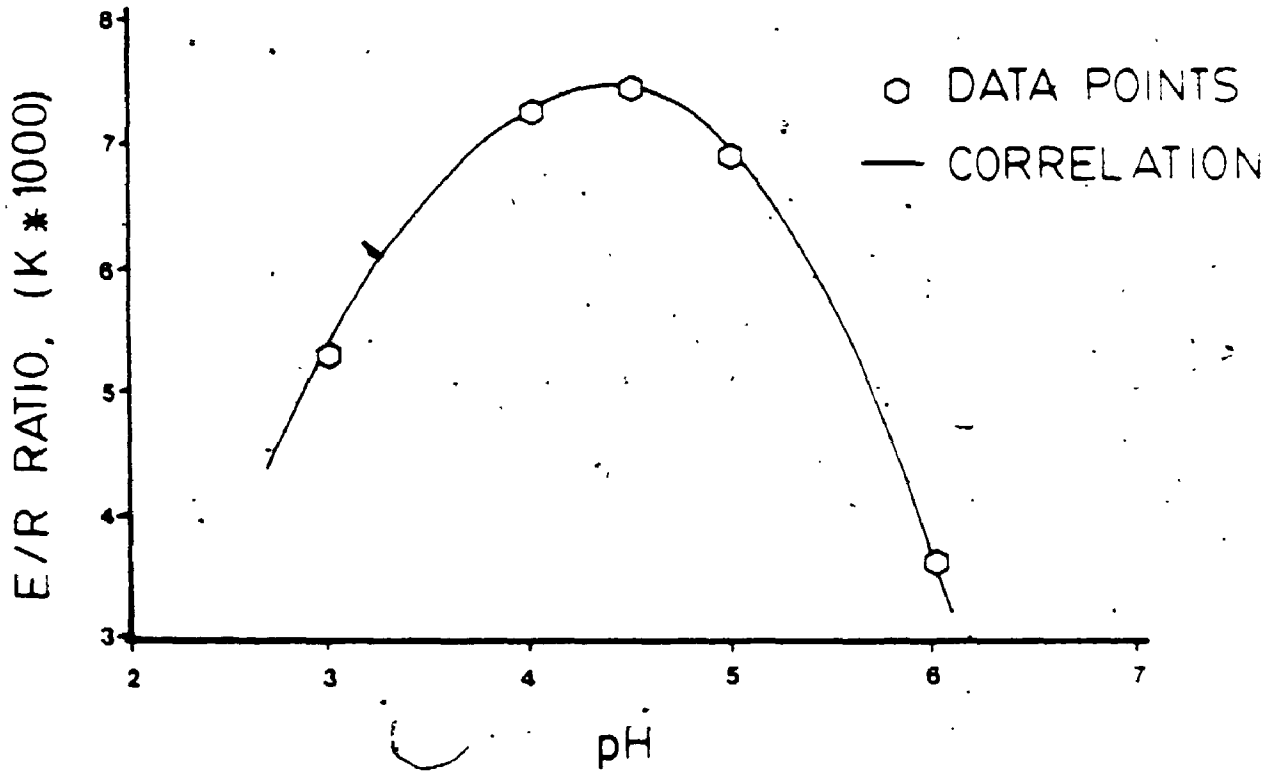

NATURAL LOG OF PROPORTIONALITY CONSTANT, (h^{-1})
25
20
15
10
5
0
2
3
4
5
6
7
pH
CORRELATION
DATA POINTS

Figure 6.7 - Activation Energy Correlation. The correlation curve was fit to the data using optimization techniques. See the text for details.


E/R RATIO, (K*1000)
8
7
6
5
4
3
2
3
4
5
6
7
pH
DATA POINTS
CORRELATION

The samples were again measured into test tubes, containing buffer, in order to adjust the pH to the desired level. Five test tubes at each pH level were set-up. Subsequently, these five tubes (at each pH level) were adjusted to 0, 10, 20, 30, and 50-g/L ethanol by the addition of small amounts of 95% ethanol. Final ethanol concentrations were determined by gas-chromatography. Each set of 25 pH/ethanol samples were then placed into an environmental room pre-set to the desired temperature (Conviron Systems, model C918). After the samples reached the temperature of the environmental room, the initial samples were then taken. Additional samples were taken from all tubes over the course of an eight hour period. Due to the number of samples at each temperature (25), this analysis was completed over a period of 5 days. The cell-free broth not being used were stored at 4 ^{o} C to reduce deactivation of the toxin.

Analysis of the results from the experiments showed no significant difference from the results outlined in the previous section. Ethanol did not increase (or decrease) the rate of deactivation of the toxin. As the ethanol concentrations utilized in this analysis were typical for fermentation systems, it was concluded that at the ethanol concentration levels tested, no measurable deactivation effects of ethanol on the toxin was detected.

Simple experiments were conducted to show the significance of inactivation by centrifugation (10-min at 1000-rpm) or by gentle agitation. For the centrifugation inactivation experiments, the activity of samples of cell broth that were subjected to centrifugation

were compared to samples of cell broth which were filtered to remove the cells. Similarly, the activity of agitated cell free broth samples (24-hr, 24° C, 75-rpm) were compared to similar samples stored for the same time period and temperature but unagitated. The results of both sets of these experiments showed that there was no significant inactivation of the toxin caused by the centifugation or the gentle mixing.

6.4 Batch Production of Zymocidal Toxins

6.4.1 Temperature Effects

The first set of experiments were carried out in order to determine the effect of temperature on cell growth and toxin production. The bioreactor was set-up as previously outlined, then was adjusted to a pH of 4.5 and the temperatures were 15, 20, 25, 30, 35, 37, 40 and 45° C.

Figure 6.8 shows typical growth results at 20 ° C and a pH of 4.5. After a lag of approximately 15 hours, the exponential cell growth phase began. This phase lasted for approximately 35 hours and the glucose concentration was reduced to almost zero. The cell culture then entered the stationary phase.

The ethanol concentration increased during the logarithmic growth phase, having little or no accumulation during the stationary phase. A similar pattern is apparent with the active toxin concentration. The toxin concentration began to decrease during the stationary phase, most likely due to temperature and pH deactivation.

Figure 6.9 shows the batch growth curves for the zymocidal strain at 35 ° C and pH = 4.5. Accumulation of active toxin during the exponential growth phase was greatly reduced at 35 C. As indicated in the deactivation kinetics section of this report (sec. 6.3), the deactivation of the toxin is greatly influenced by temperature. This explains why such low toxin levels were attained at this temperature.

Figure 6.10 shows the batch growth kinetics at 37° C. At this temperature, much less biomass was produced with very little or no accumulation of toxin. The ethanol production was also lower. At temperatures greater than 37 ° C, no growth was detectable after 70 hours.

Using the biomass growth data for each batch experiment completed in the temperature study, the specific growth rate of the zymocidal strain was evaluated from the exponential growth phase. Figure 6.11 shows the Arrhenius plot of μ versus 1/T. As shown, the growth rate reaches a maximum at a temperature of 35 ° C, and then rapidly declines as the temperature is further increased.

An overall activation energy for the growth of the zymocidal strain was estimated for the temperatures of 15, 20, 25 and 30° C. A straight line was fitted through these points in accordance with the Arrhenius relationship and the activation energy was then calculated to be 11.2-kcal/mole.

Figure 6.8 - Typical Growth Curves for Batch Growth.
Temperature = 20° C, pH controlled to 4.5.

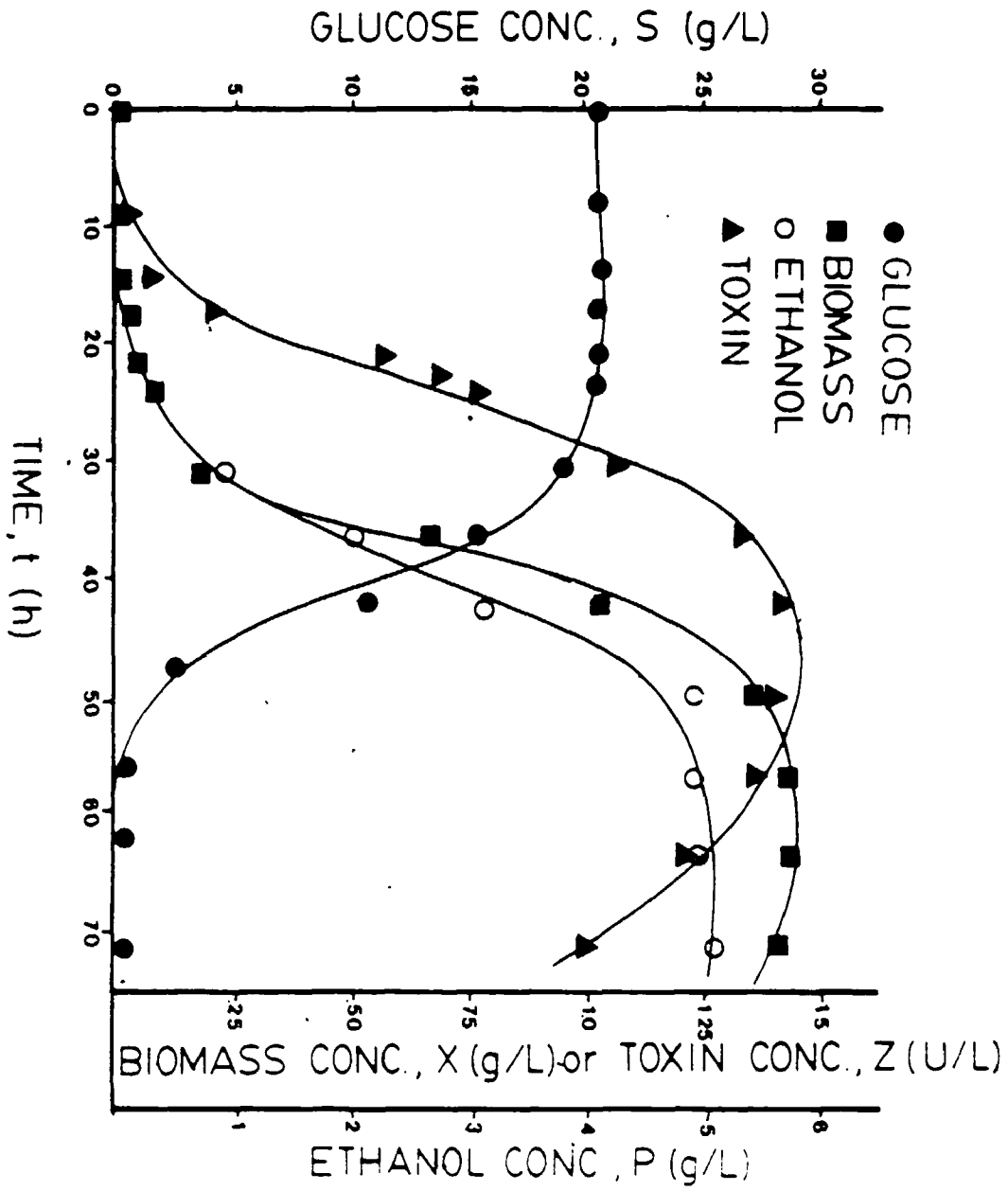

GLUCOSE CONC., S (g/L)
0
5
10
15
20
25
30
TIME, t (h)
0
10
20
30
40
50
60
70
● GLUCOSE
■ BIOMASS
○ ETHANOL
▲ TOXIN
BIOMASS CONC., X (g/L)-or TOXIN CONC., Z (U/L)
ETHANOL CONC., P (g/L)

Figure 6.9 - Typical Growth Curves for Batch Growth.
Temperature = 35° C, pH controlled to 4.5.

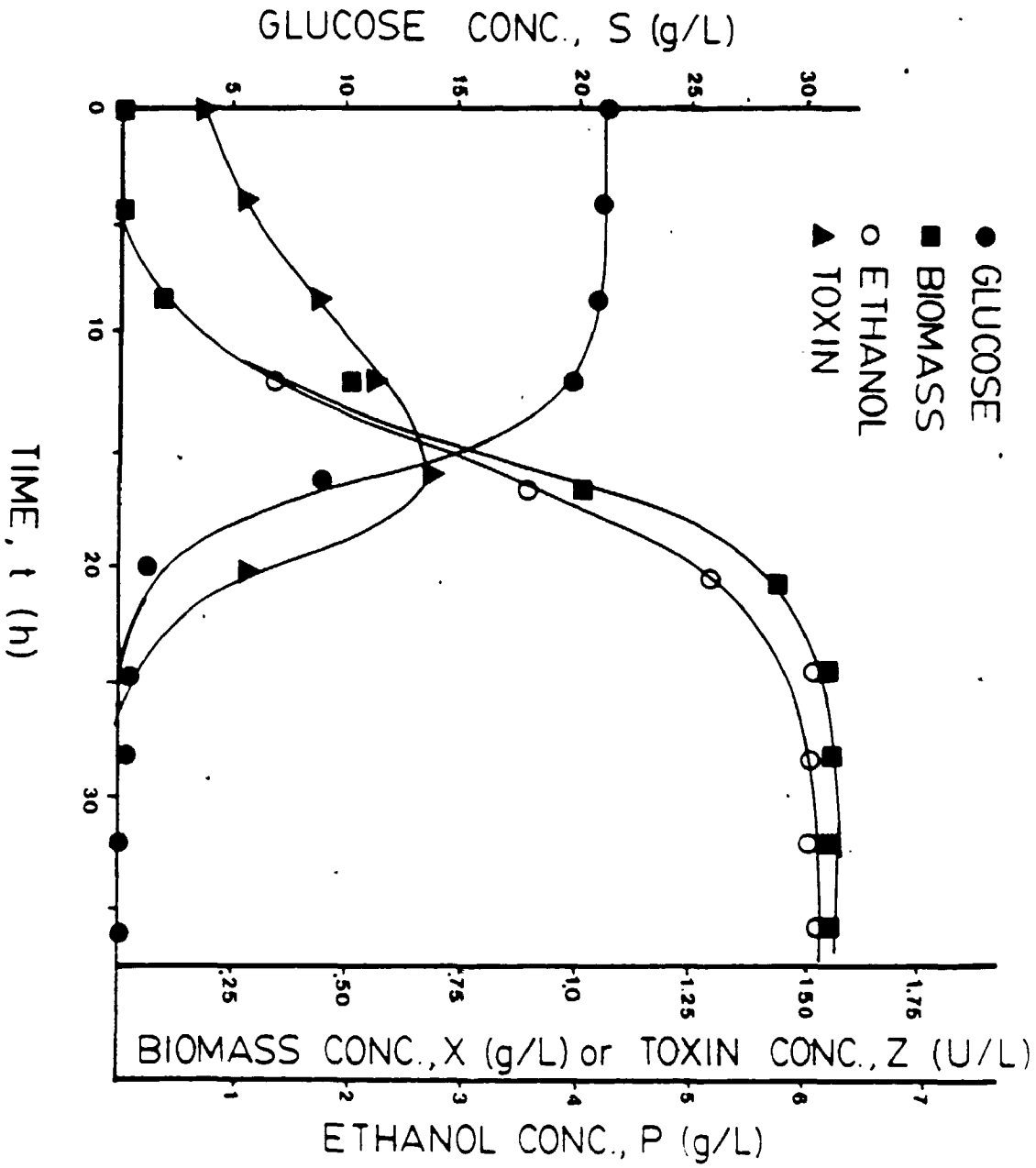
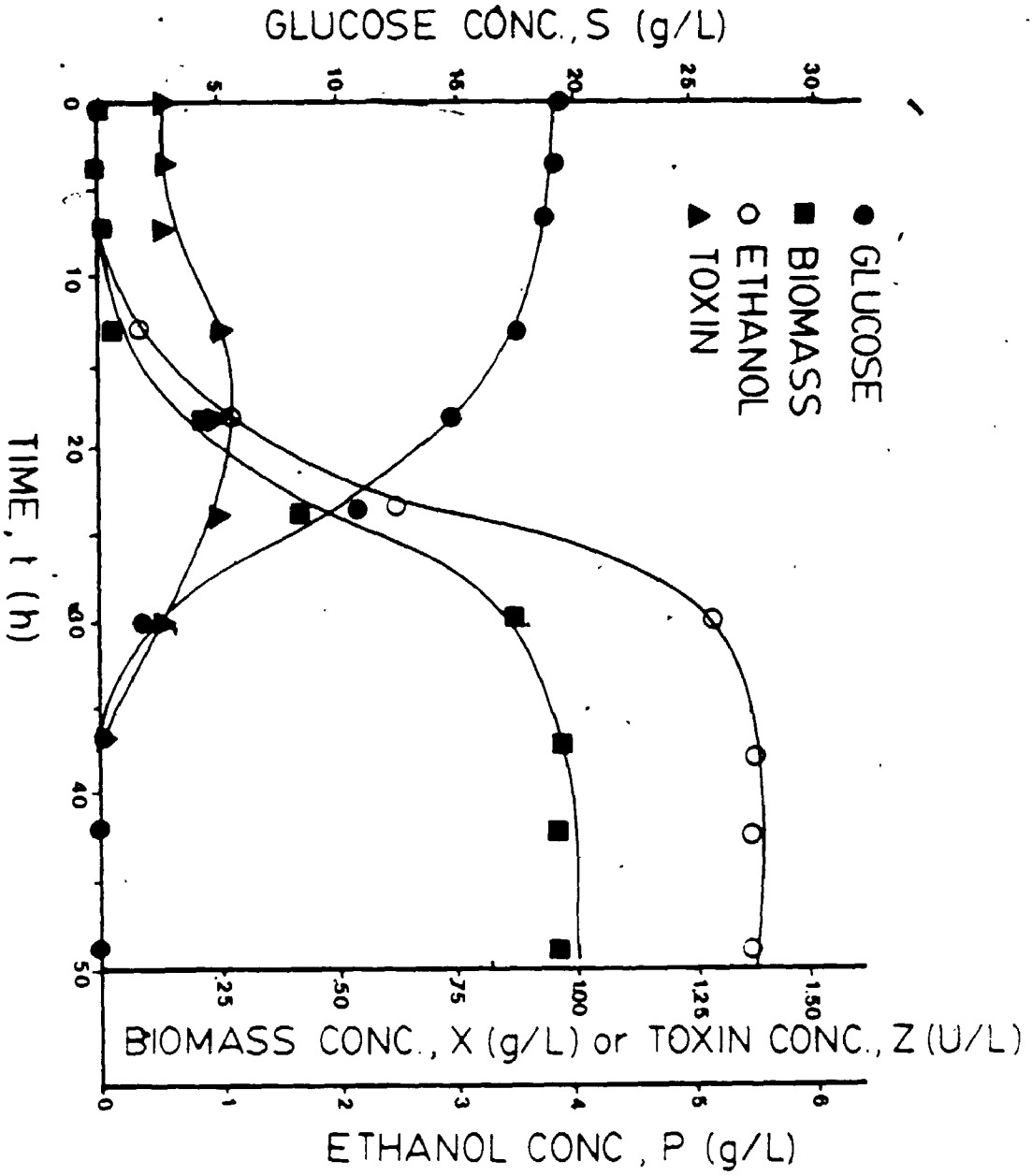

GLUCOSE CONC., S (g/L)
5
10
15
20
25
30
0
10
20
30
TIME, t (h)
● GLUCOSE
■ BIOMASS
○ ETHANOL
▲ TOXIN
.25
.50
.75
1.0
1.25
1.50
1.75
BIOMASS CONC., X (g/L) or TOXIN CONC., Z (U/L)
1
2
3
4
5
6
7
ETHANOL CONC., P (g/L)

Figure 6.10 - Typical Growth Curves for Batch Growth.
Temperature = 37° C, pH controlled to 4.5.


GLUCOSE CONC., S (g/L)
0
5
10
15
20
25
30
TIME, t (h)
0
10
20
30
40
50
● GLUCOSE
■ BIOMASS
○ ETHANOL
▲ TOXIN
25
50
75
100
125
150
BIOMASS CONC., X (g/L) or TOXIN CONC., Z (U/L)
0
1
2
3
4
5
6
ETHANOL CONC , P (g/L)

Figures 6.12 through 6.14 show the various yield factors at different temperatures. Figure 6.12 shows that the optimum temperature for conversion of substrate to biomass occurs at 30° C. Conversion of substrate to ethanol reaches a maximum at temperatures between 30 and 35 ° C, as shown in Figure 6.13. This figure also shows that the cellular yield of product is nearly constant with respect to temperature. The ethanol yield only reaches 55% of the theoretical at 30 °C.

Figure 6.14 on the other hand, shows that the highest yield of active toxin occurs at a much lower temperature than optimum growth. The substrate to active toxin, and the biomass to active toxin yields both reach their maxima at a temperature of 20 ° C. This would then suggest that in order to optimize zymocidal activity, low culture temperatures are necessary. These lower temperatures increase the time needed to affect a complete fermentation, and also reduce the overall ethanol yield. It would therefore be very important to balance the zymocidal activity with respect to other process and economic parameters.

6.4.2 pH Effects

The effects of pH on the growth of the zymocidal strain were studied. After sterilization of the medium and fermentor, the pH controller was adjusted to the desired pH prior to inoculation. The temperature was set at 20 °C and the initial sugar concentration was 20-g/L in all of these experiments.

Figure 6.11 - Arrhenius relationship for Batch Growth Rates. Activation energy for growth evaluated for 15, 20 and 25 ^{o}C growth rates.

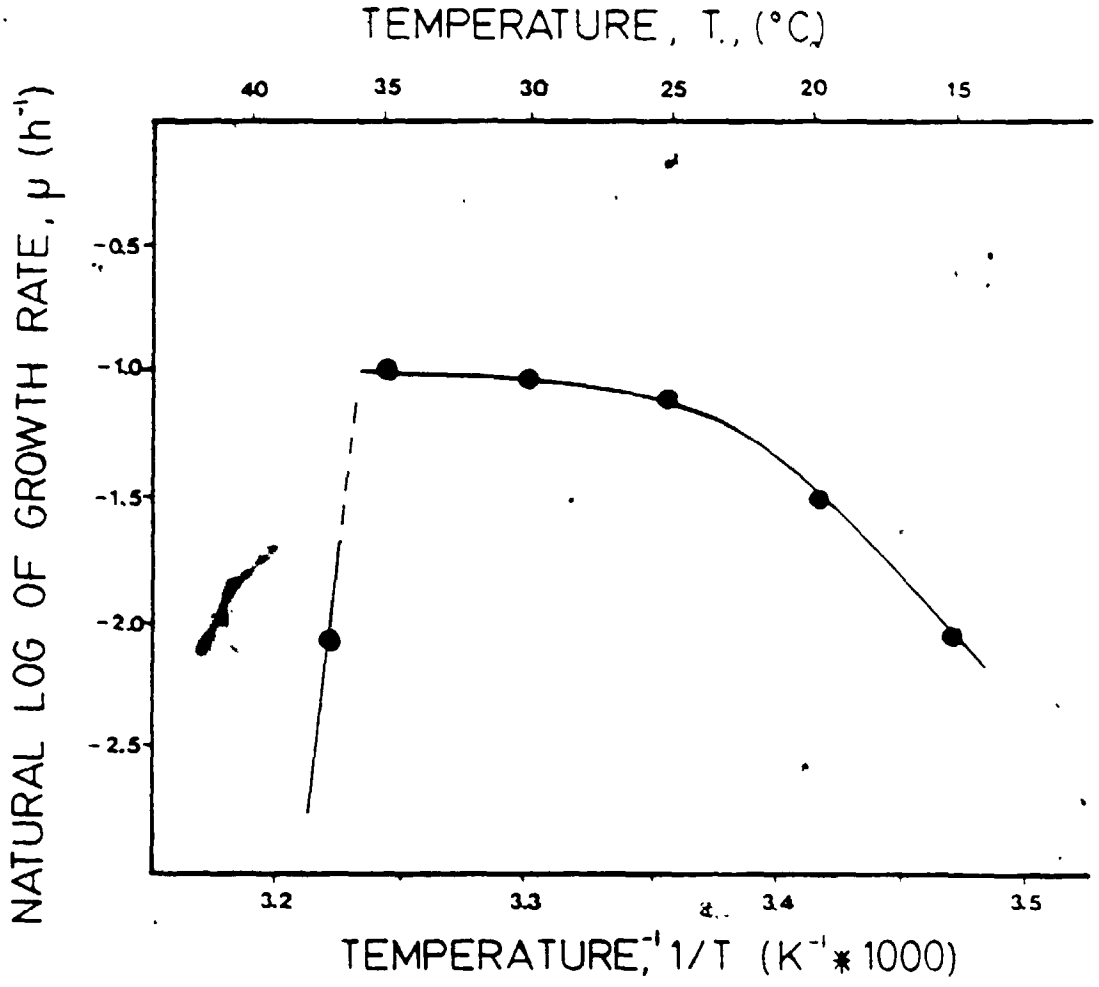

TEMPERATURE , T., (°C)
40
35
30
25
20
15
NATURAL LOG OF GROWTH RATE, μ (h⁻¹)
-0.5
-1.0
-1.5
-2.0
-2.5
3.2
3.3
3.4
3.5
TEMPERATURE, 1/T (K⁻¹ * 1000)

Figure 6.12 - Temperature Effects on Biomass to Substrate Yield. Controlled pH = 4.5.

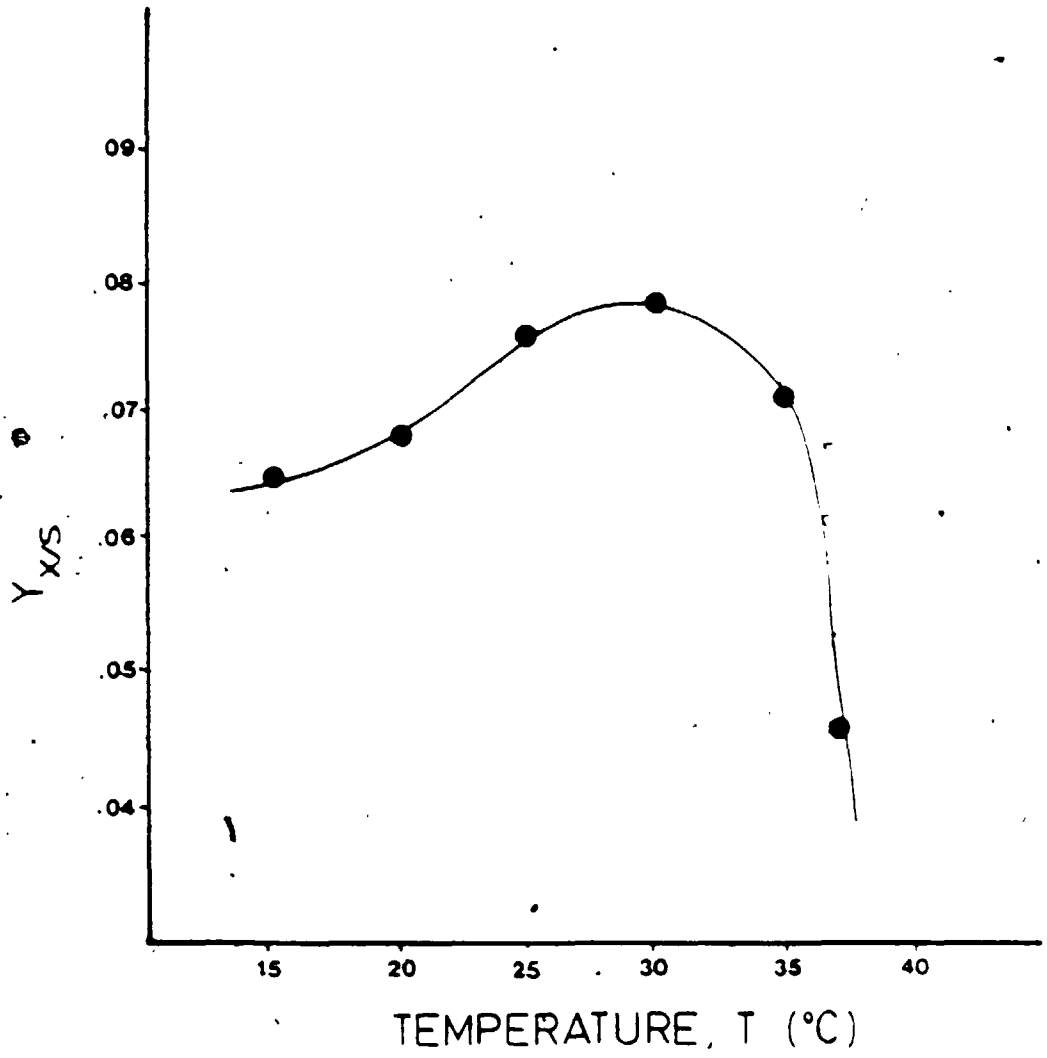

09
.08
.07
.06
.05
.04
$Y_{X/S}$
15
20
25
30
35
40
TEMPERATURE, T (°C)

Figure 6.13 - Temperature Effects on Ethanol to Substrate Yield and Ethanol to Biomass Yield.
Controlled pH = 4.5.

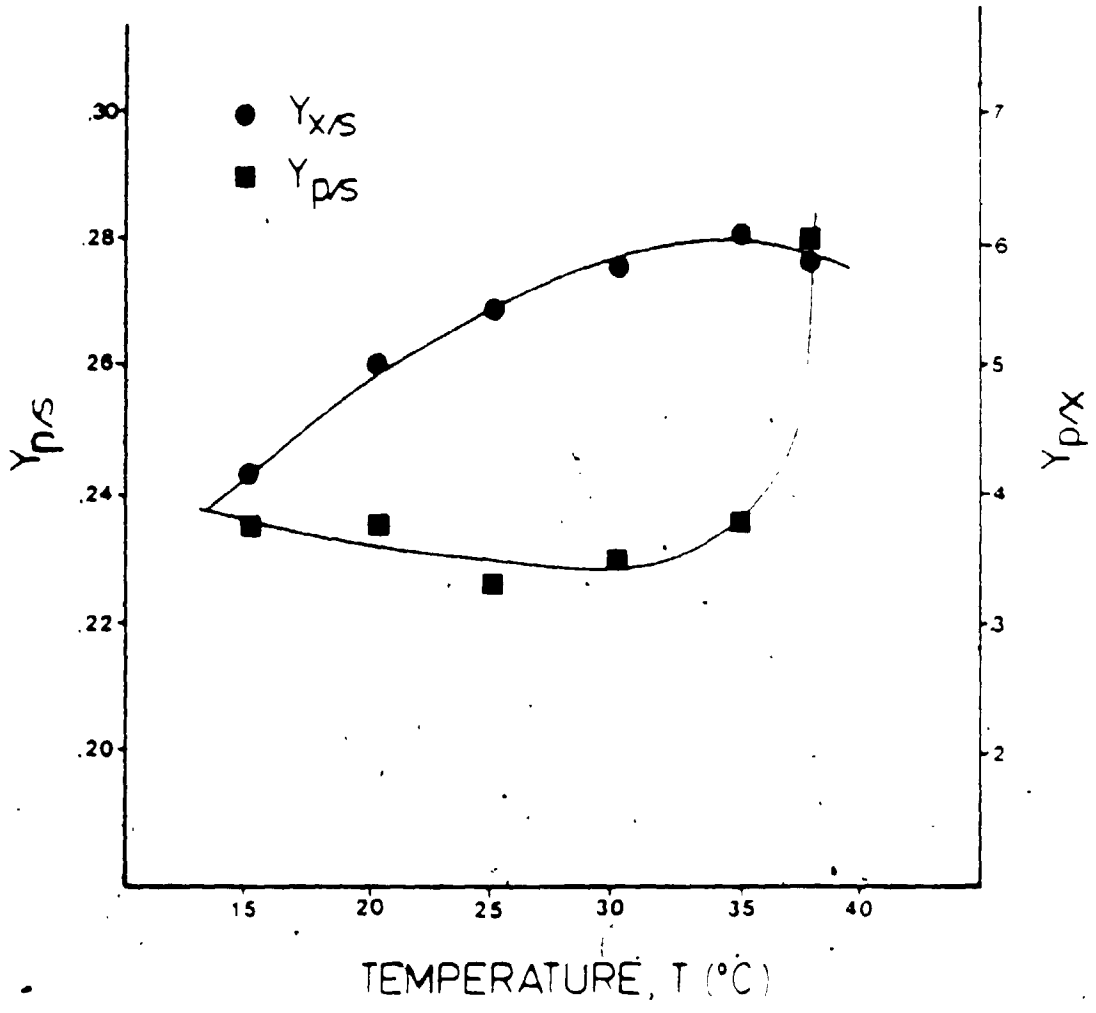
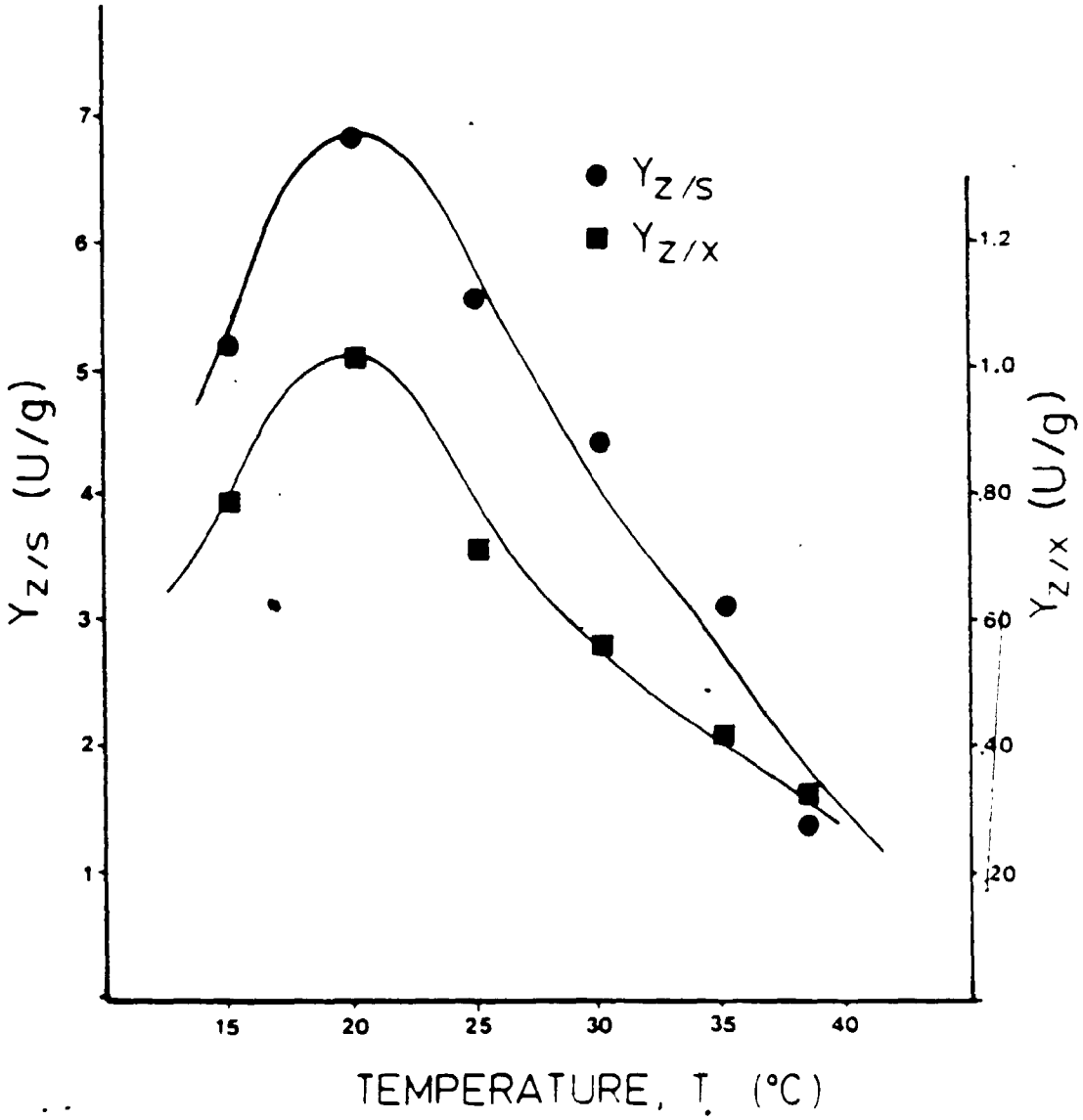

$Y_{X/S}$
$Y_{P/S}$
$Y_{P/X}$
.30
.28
.26
.24
.22
.20
7
6
5
4
3
2
15
20
25
30
35
40
TEMPERATURE, T (°C)

Figure 6.14 - Temperature Effects on Active Toxin to Substrate Yield and Active Toxin to Biomass Yield.
Controlled pH = 4.5.


$Y_{Z/S}$
$Y_{Z/X}$
$Y_{Z/S}$ (U/g)
$Y_{Z/X}$ (U/g)
7
6
5
4
3
2
1
1.2
1.0
.80
.60
.40
.20
15
20
25
30
35
40
TEMPERATURE, T (°C)

The pH values assessed in this analysis were: 3.0, 4.0, 4.5, 5.0, 6.0 and 7.0. The accuracy of the pH controller was verified periodically by measuring the pH of fermentor samples.

The growth, substrate utilization and product formation curves were similar to those shown in Figure 6.8. The specific growth rates and biomass yields are plotted in Figure 6.15. As can be seen in Figure 6.15, the growth rate of the zymocidal strain was at a maximum in the pH range of 3.75 to 4.75. The specific growth rate falls dramatically outside of this range. The overall biomass yield calculated from the batch data is nearly constant over the whole pH range.

The ethanol concentrations accumulating in the fermentor were typical of yeast fermentations. The concentration reached a maximum of 6.75-g/L and a pH of 4.0. The effects of the culture pH on the ethanol yield are shown in Figure 6.16. The maximum yield obtained was 62% of theoretical, and it was attained at a pH of 4.0.

At optimum pH levels for toxin activity, the concentration reached its maximum. At these pH levels, the toxin slowly deactivated during the stationary phase of growth. At pH levels outside of the optimum range, the accumulation of toxin during the exponential growth phase was greatly reduced. The further that the pH level was from the optimum range, the lower the maximum concentration of toxins. Active toxin present in the liquid phase was rapidly deactivated during the stationary phase. The relationship between the pH and the maximum active toxin concentration is shown is Figure 6.17.

Figure 6.15 - pH Effects on Specific Growth Rate and Biomass Yield.
Temperature = 21.8° C

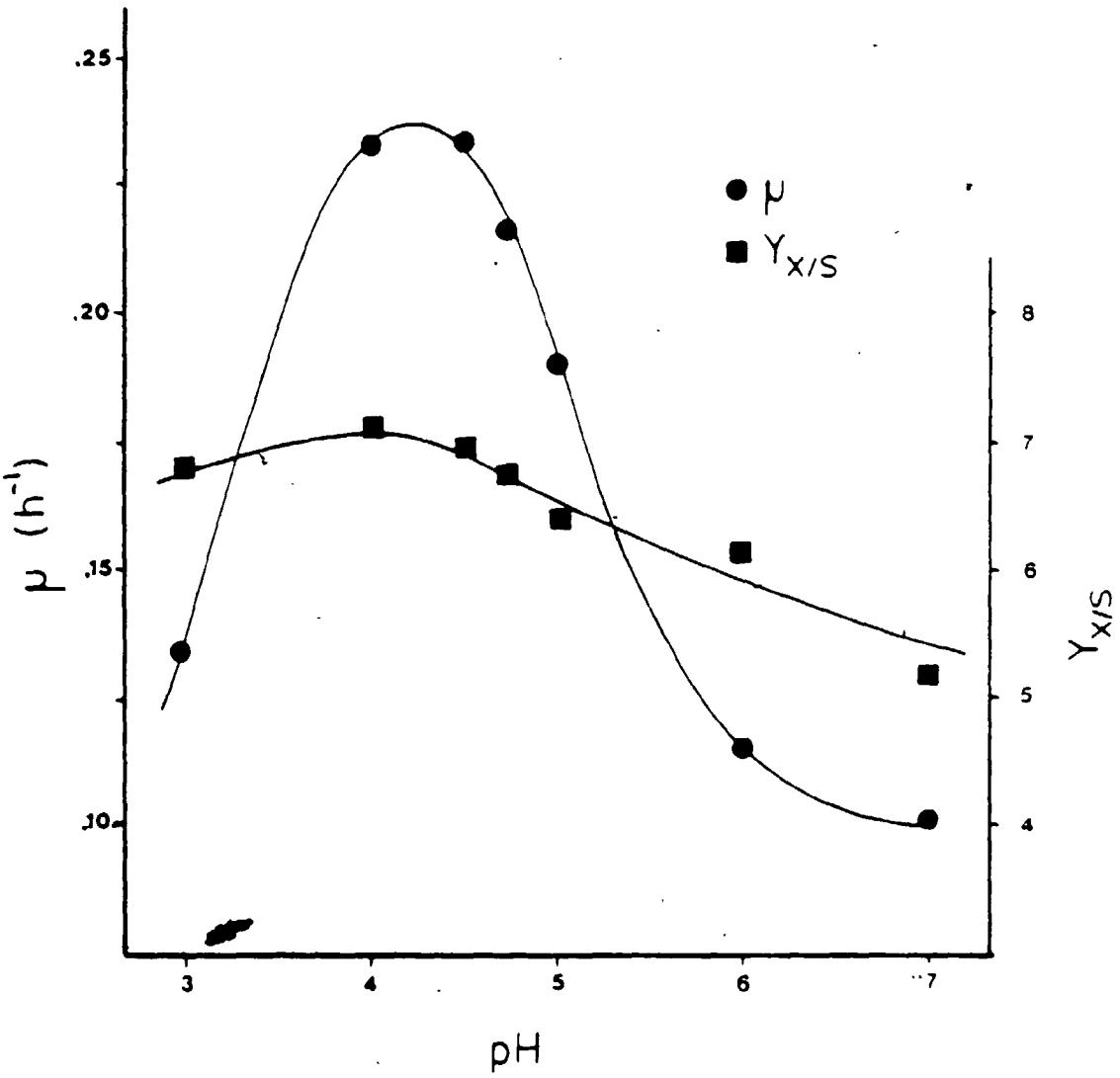
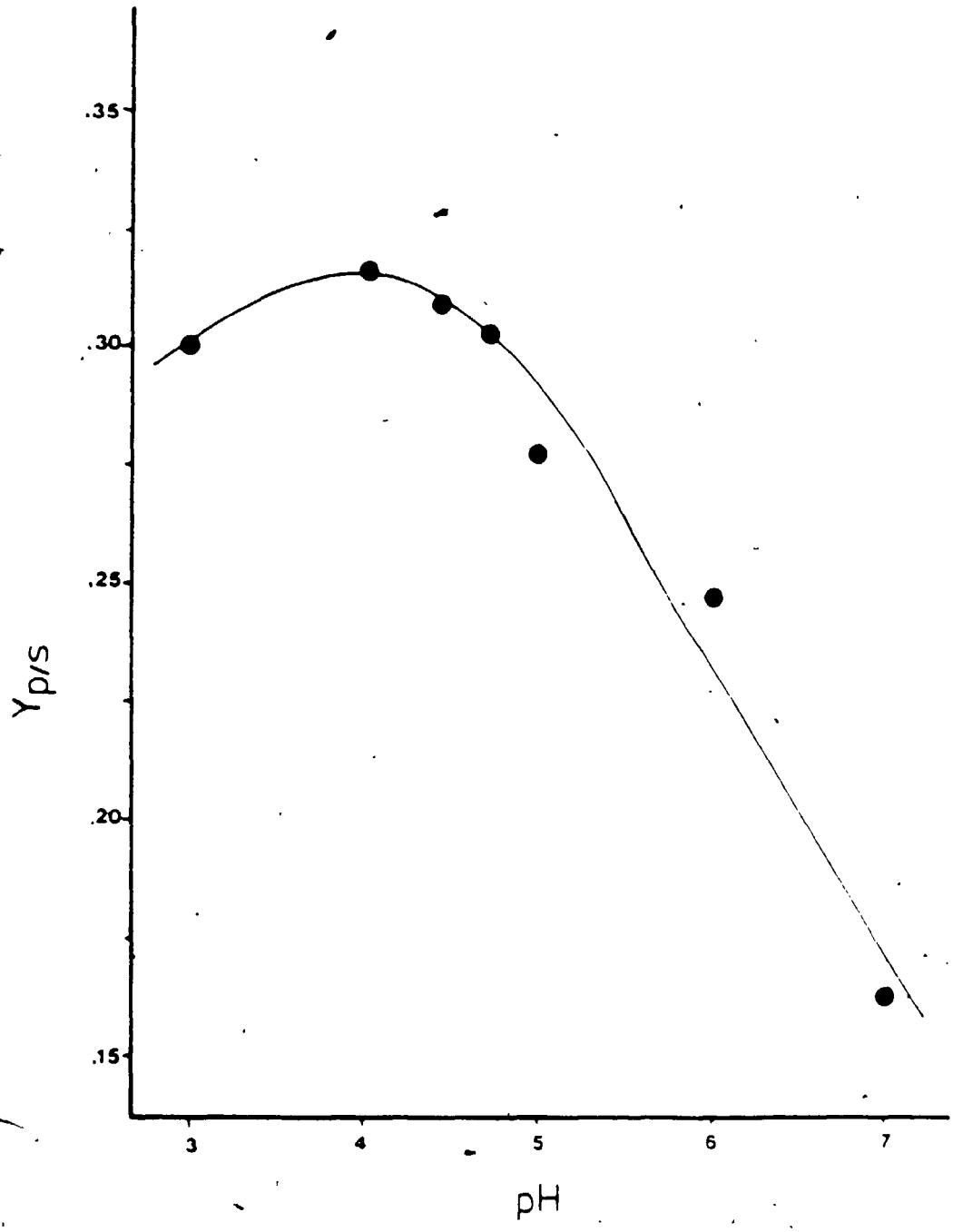

.25
.20
.15
.10
μ (h⁻¹)
● μ
■ Yx/s
8
7
6
5
4
Yx/s
3
4
5
6
7
pH

Figure 6.16 - pH Effects on Ethanol to Substrate Yield.
Temperature = 21.8° C


.35
.30
.25
.20
.15
$Y_{P/S}$
3
4
5
6
7
pH

All of the results derived from these pH analyses suggest that the optimum pH for the production of active toxin in batch culture is 4.5. These results also show that the influence of pH on the growth of the zymocidal strain is similar to other yeast strains.

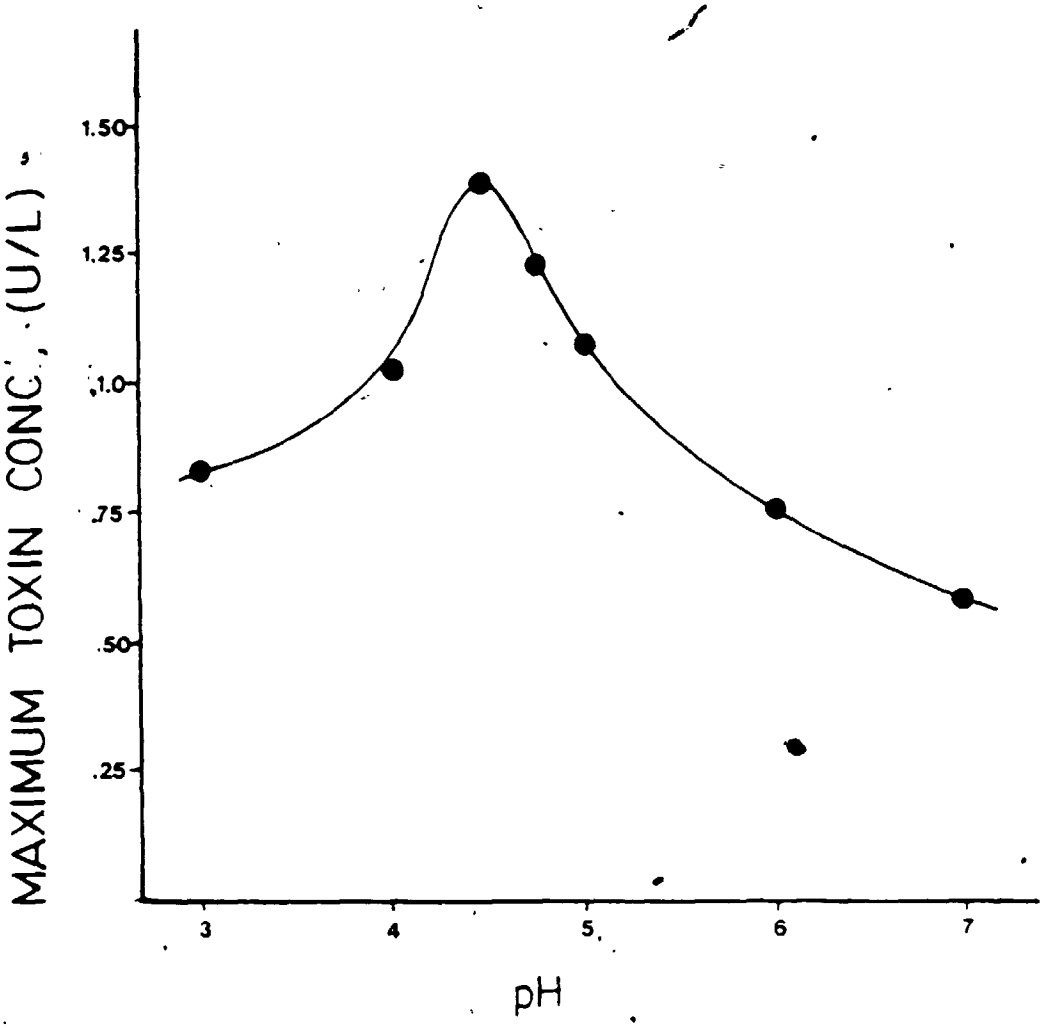
6.4.3 Initial Sugars Concentration

The effects of the initial sugars concentration on the growth of a yeast strain is an important consideration. Utilization of process streams containing high sugar concentrations is important in the fermentation industry. Yeasts which cannot utilize these streams produce lower ethanol concentrations and therefore increase distillation costs. Therefore, selection of a yeast (or bacteria) which will produce high ethanol concentration is an important economic decision (Karsch et al., 1983).

In these batch growth experiments, initial sugar concentrations of 20, 40, 80, 115 and 195-g/L were used. The batch culture temperature was set at 20° C and the pH was controlled to 4.5 prior to inoculation. The growth, substrate utilization and product formation curves for 20-g/L (Figure 6.8) and 40-g/L (Figure 6.18) were typical of non-inhibited growth. At initial glucose concentrations of 80-g/L and higher, substrate utilization and product formation was inhibited.

The results for an initial sugar concentration of 115-g/L are shown in Figure 6.19. Glucose uptake became almost linear after an initial period of approximately 25 hours. This figure is typical of the results obtained at 80 and 195-g/L.

Figure 6.17 - pH Effects on Maximum Toxin Concentration.
Temperature = 21.8° C


MAXIMUM TOXIN CONC., (U/L)
1.50
1.25
1.0
.75
.50
.25
3
4
5
6
7
pH

The specific growth rate and maximum biomass concentrations are plotted versus the initial sugars concentration in Figure 6.20. This figure shows that as the initial sugars concentration is increased, the specific growth rate of the system decreases. The maximum biomass concentration goes through a maximum at an initial glucose concentration of approximately 60-g/L.

These data suggest that the type of inhibition is not directly related to the initial sugars concentration. This is because the inhibition is present at low initial sugars concentrations. It would seem that some other effect, possibly the extracellular toxin, has an influence on the growth rate of the zymocidal strain.

Plotting the specific growth rate calculated from the initial sugars batch data versus the maximum specific active toxin concentration produces an interesting result. The maximum specific toxin concentration is calculated by dividing the maximum toxin concentration by the maximum biomass concentration. This plot is shown as Figure 6.21. As can be seen, the reduction in the specific growth rate is directly related to the specific toxin concentration. This figure then suggests that the inhibition is a result of the presence of the toxin protein.

Toxin inhibition is suggested for two reasons. Firstly, the specific growth rate decreases continuously with increasing initial sugars concentration. Normally, the specific growth rate goes through a maximum as the substrate concentration is increased. The results from

Figure 6.18 - Typical Growth Curves for Batch Growth.
Initial Sugars = 40-g/L
pH Controlled = 4.5
Temperature = 20° C.

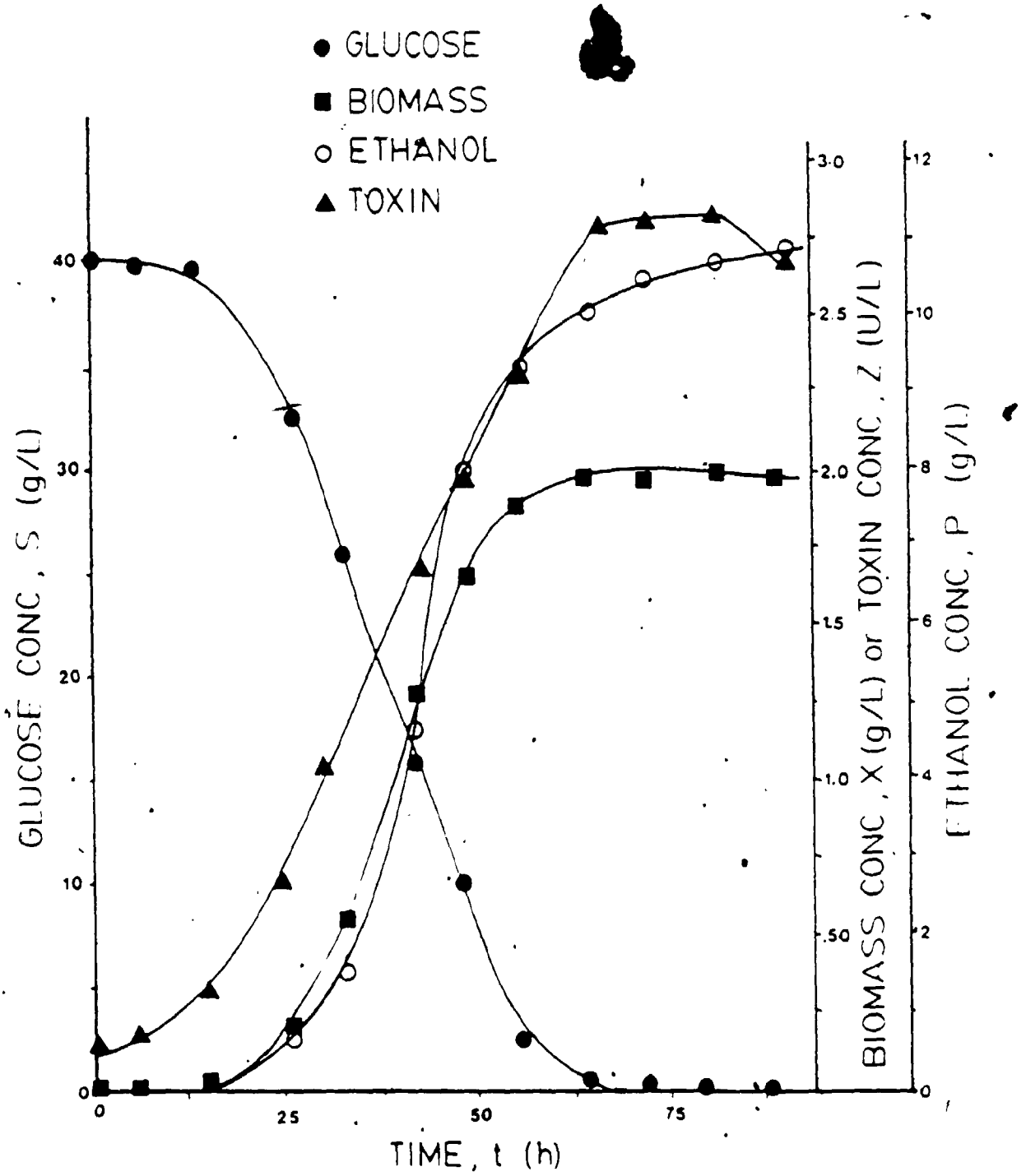

GLUCOSE
BIOMASS
ETHANOL
TOXIN
GLUCOSE CONC., S (g/L)
BIOMASS CONC., X (g/L) or TOXIN CONC., Z (U/L)
ETHANOL CONC., P (g/L)
TIME, t (h)
0
10
20
30
40
.50
1.0
1.5
2.0
2.5
3.0
2
4
6
8
10
12
25
50
75

Figure 6.19 - Typical Growth Curves for Batch Growth.
Initial Sugars = 115-g/L
pH Controlled = 4.5
Temperature = 20° C.

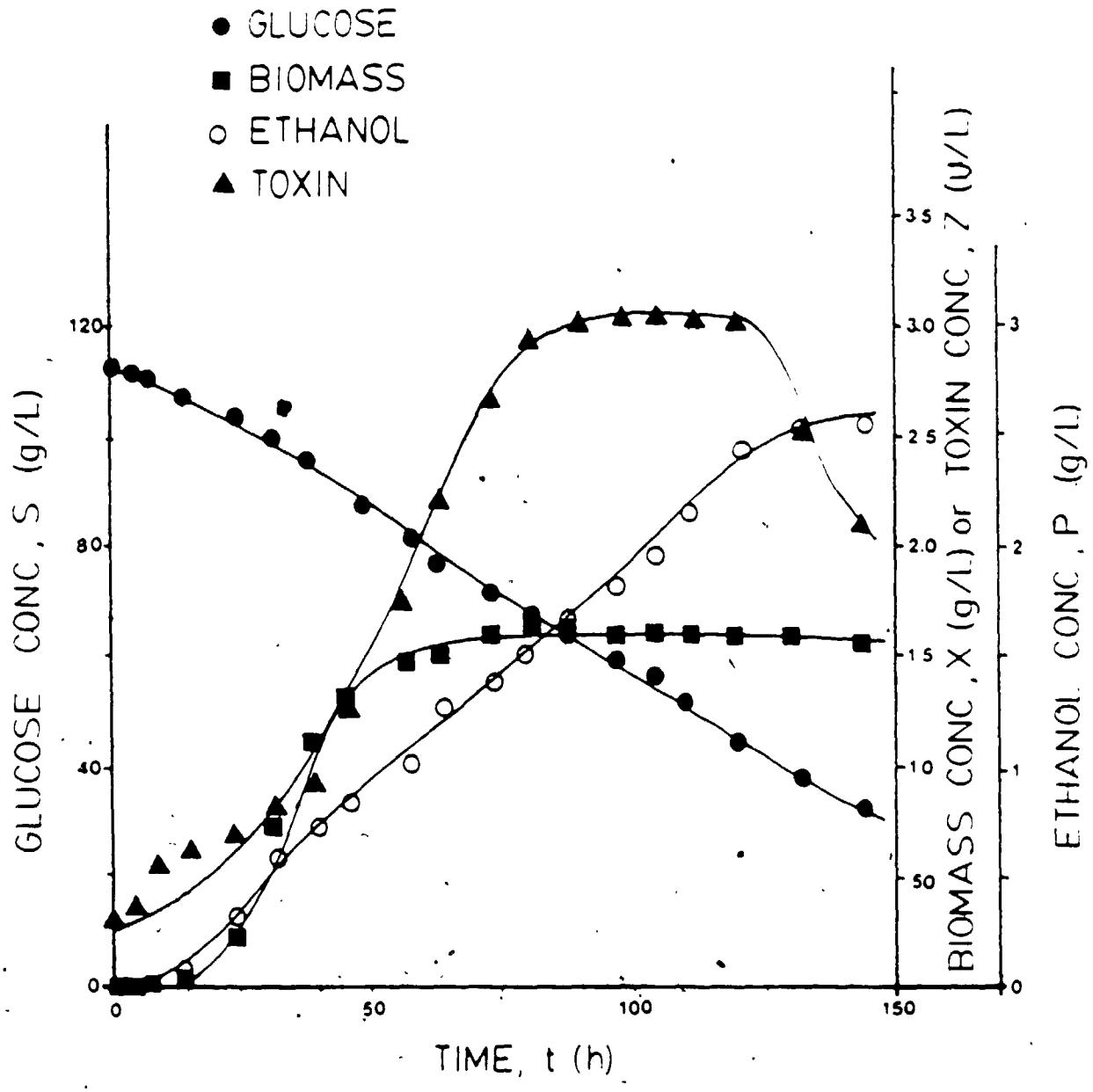

GLUCOSE
BIOMASS
ETHANOL
TOXIN
GLUCOSE CONC., S (g/L)
BIOMASS CONC., X (g/L) or TOXIN CONC., Z (U/L)
ETHANOL CONC., P (g/L)
TIME, t (h)
0
40
80
120
0
50
100
150
0
50
1.0
1.5
2.0
2.5
3.0
3.5
0
1
2
3

Figure 6.20 - Initial Sugars Concentration Effect on the Specific Growth Rate and Maximum Biomass Concentration
pH Controlled = 4.5
Temperature = 20° C.

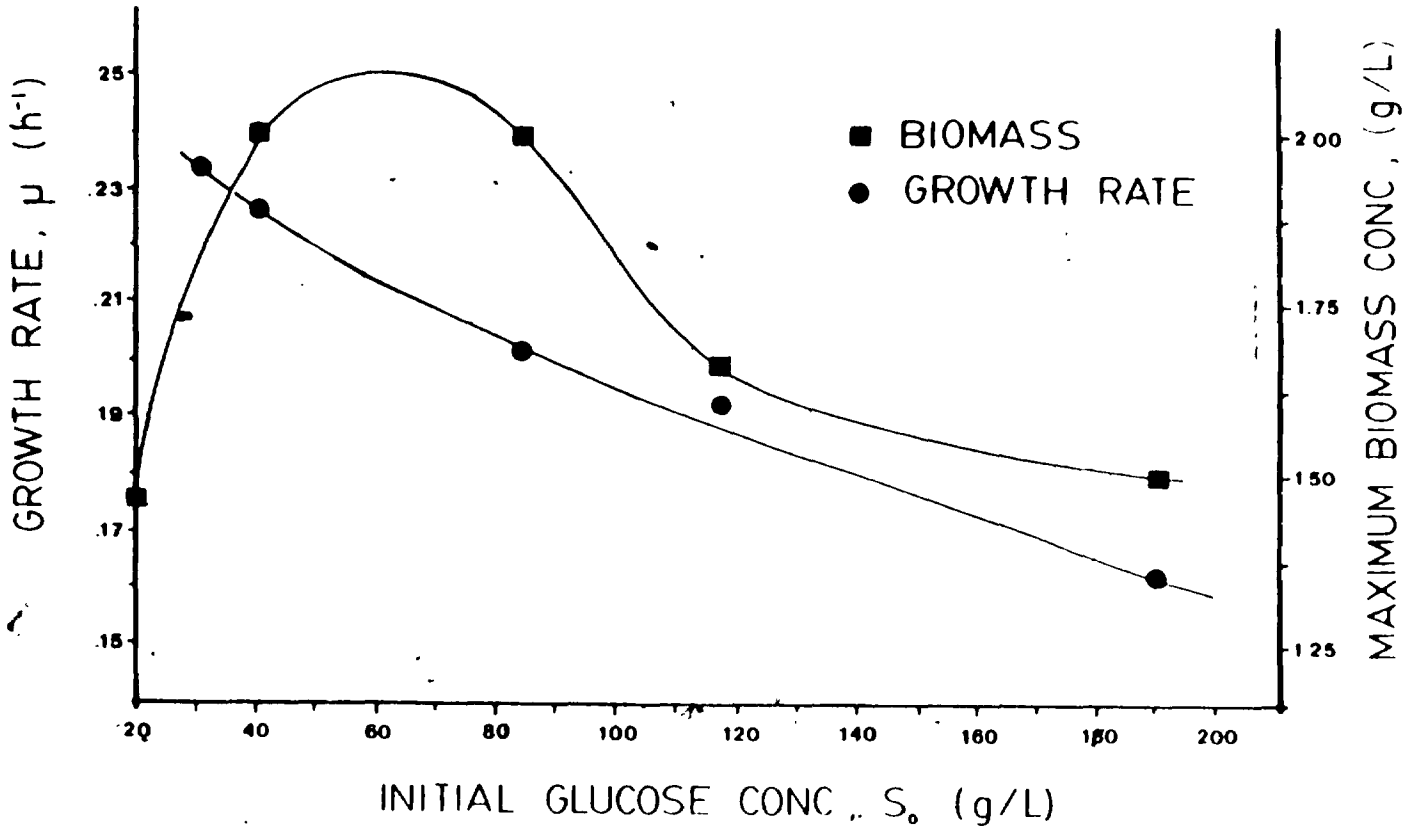

GROWTH RATE, μ (h⁻¹)
.25
.23
.21
.19
.17
.15
BIOMASS
GROWTH RATE
2.00
1.75
1.50
1.25
MAXIMUM BIOMASS CONC., (g/L)
20
40
60
80
100
120
140
160
180
200
INITIAL GLUCOSE CONC., S₀ (g/L)

Figure 6.21 - Inhibition Relationship Between Specific Growth Rate and Maximum Specific Toxin Concentration.
pH Controlled = 4.5
Temperature = 20° C.

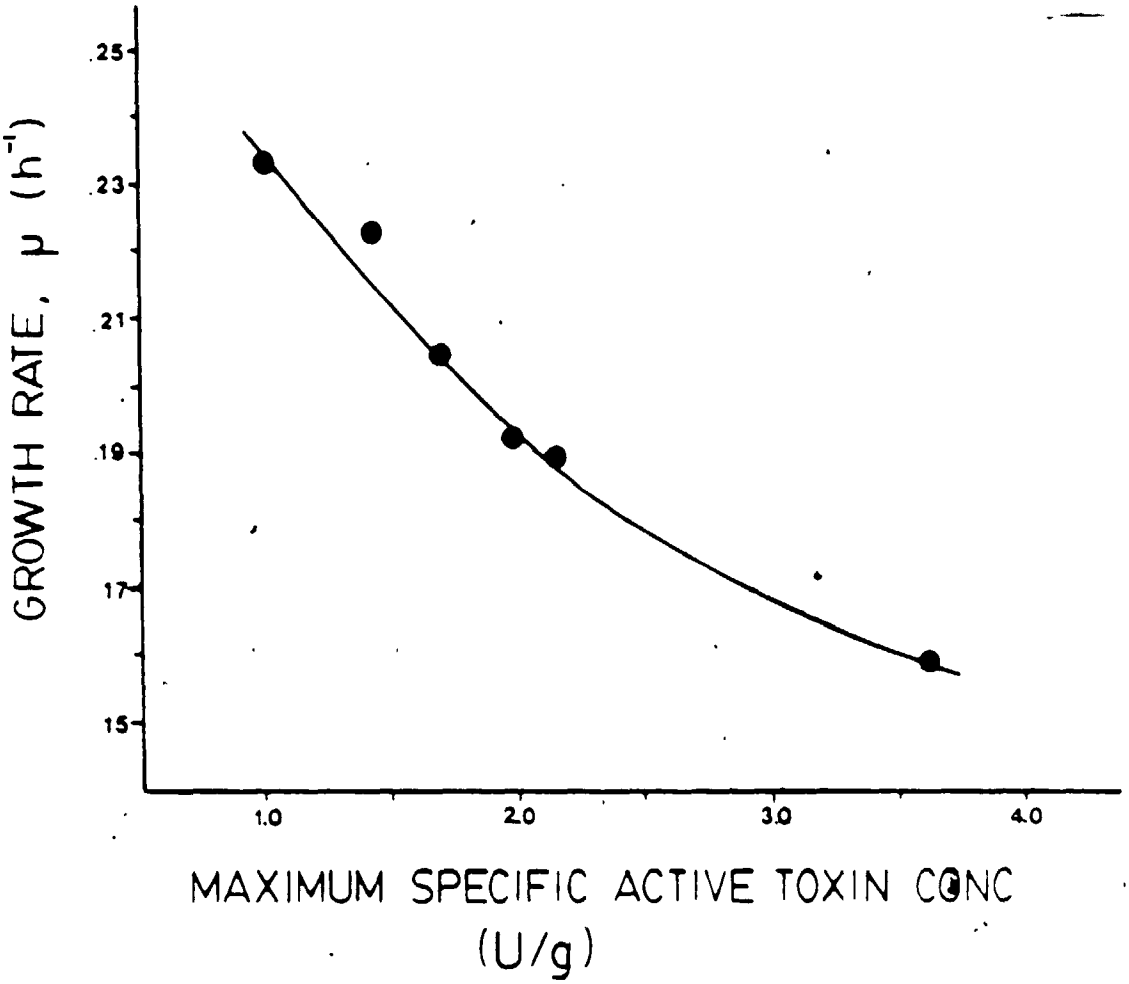
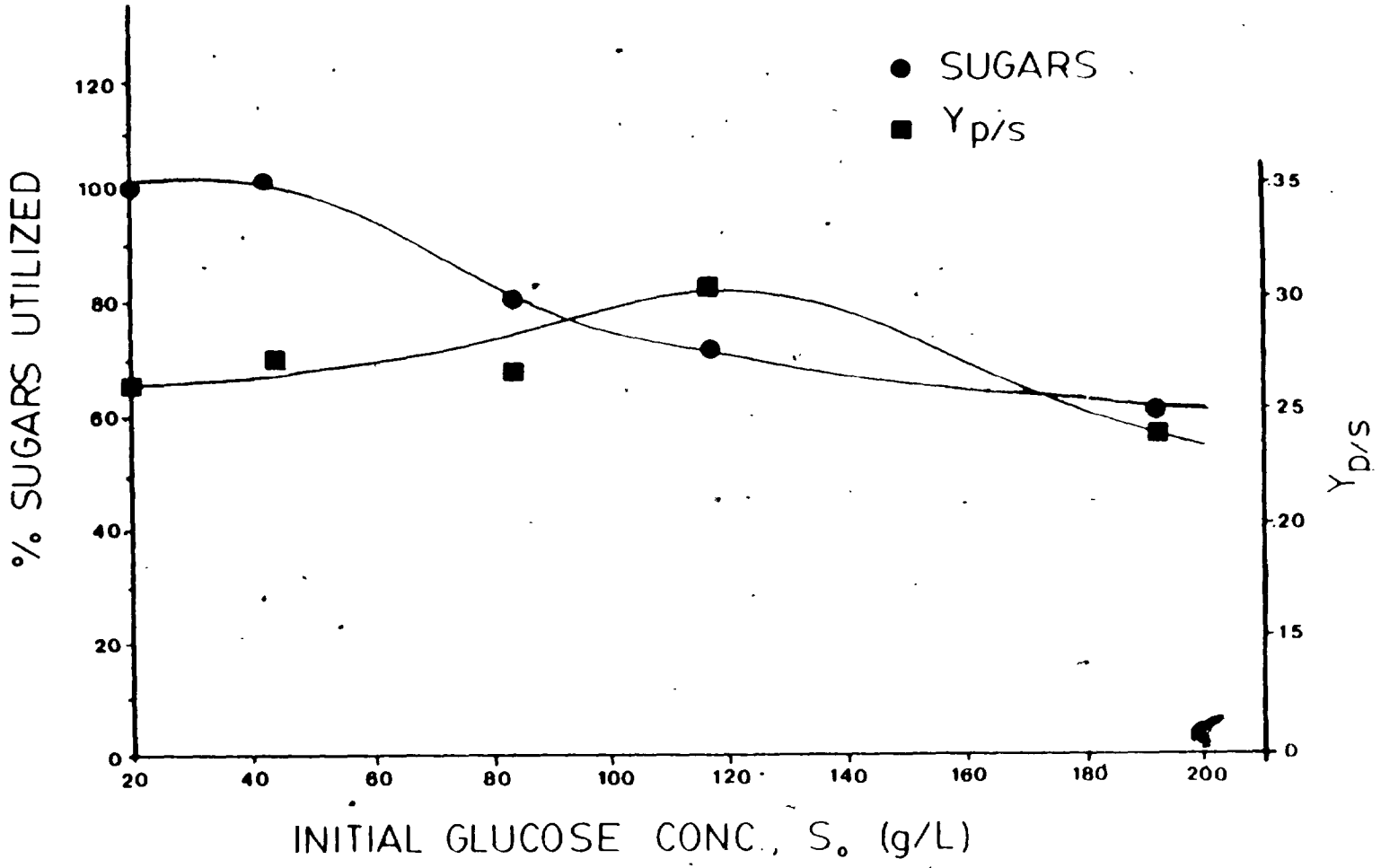

GROWTH RATE, μ (h⁻¹)
.25
.23
.21
.19
17
15
1.0
2.0
3.0
4.0
MAXIMUM SPECIFIC ACTIVE TOXIN CONC
(U/g)

Figure 6.22 - Initial Sugars Effect on Percent Sugars Utilized and Ethanol Yield.
pH Controlled = 4.5
Temperature = 20° C.


SUGARS
$Y_{p/s}$
% SUGARS UTILIZED
$Y_{p/s}$
120
100
80
60
40
20
0
35
30
25
20
15
0
20
40
60
80
100
120
140
160
180
200
INITIAL GLUCOSE CONC., S_o (g/L)

this analysis would suggest that the substrate inhibition begins at substrate concentrations below 20-g/L. Significant inhibition by substrate at concentrations lower than 20-g/L is impossible for yeast.

Secondly, the maximum biomass concentration attained in the system goes through a maximum at an initial substrate concentration of approximately 60-g/L. As shown in Figure 6.22, the per cent sugar utilized decreases with increased initial sugars concentration. Neither of these results appears to correlate with the decreases in the specific growth rate. Substrate effects can therefore probably be ruled out as causing the inhibition effects.

Figure 6.22 also shows that the yield for growth is nearly constant, regardless of the initial glucose concentration. The glucose to ethanol conversion was approximately 58% of theoretical. The maximum ethanol concentration attained was 27.5-g/L at an initial sugars concentration of 195-g/L glucose.

6.5 Continuous Experimentation

6.5.1 Experimental Results.

The batch experimental apparatus was reassembled to allow for continuous operation. The fermentor was filled with 1.5-L of production medium, and it was inoculated with 10-mL of a 24 hour-old zymocidal inoculum. At the end of the batch growth, the feed and product pumps were switched on, and continuous operation commenced.

A total of five continuous experiments were run at temperatures of 15, 20, 25, 30 and 35 $^{\circ}$ C using a feed concentration of 50-g/L glucose and the pH was controlled at 4.5.

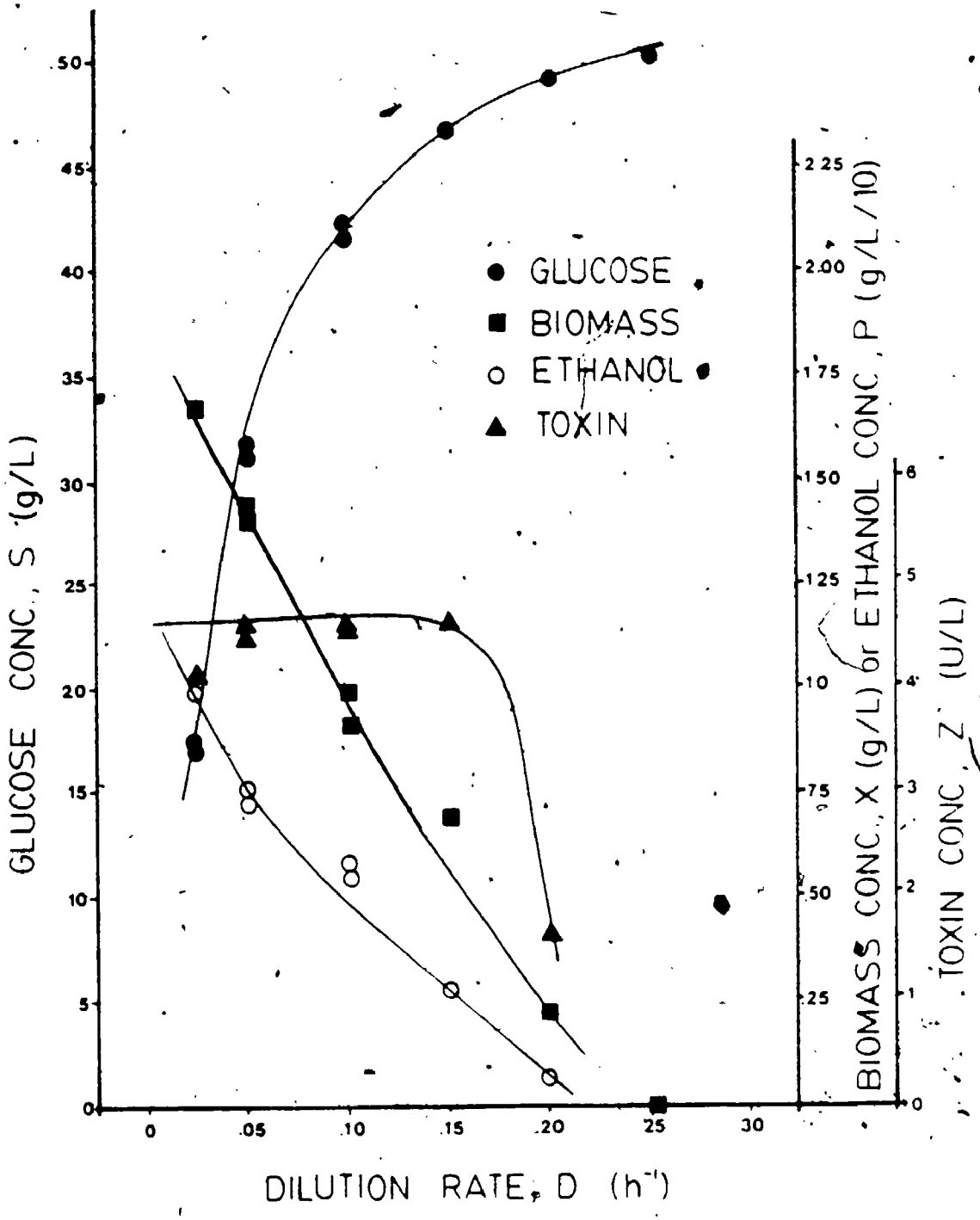
Various dilution rates were used in each continuous experimental run. Low dilution rates were used initially and the dilution rate was increased in steps, until wash-out occurred. At each dilution rate, samples were taken periodically to check for steady-state conditions.

The results of the continuous experiment at 20° C are shown in Figure 6.23. Similar results were obtained for the other temperatures used in this analysis and the results of analyses done at 30° C are shown in Figure 6.24.

All of the results of this analysis showed two interesting patterns. Initially, glucose uptake by the cells in the system was much lower that expected. High glucose concentrations in the product solution suggest than something has reduced the ability of the cells to use glucose. Secondly, and partially as a result of the reduced sugar uptake, ethanol productivity was greatly reduced. Very low ethanol concentrations were achieved in the continuous experimentation.

These results suggest that the specific growth rate of the organism in the presence of the extracellular protein, can be expressed by Equation 6.8. This expression is similar to those used in ethanol inhibition, as presented in Equation 4.23.

Figure 6.23 - Continous Experimental Results
Temperature = 20° C
pH Controlled = 4.5
Feed Substrate Concentration = 50-g/L.


GLUCOSE
BIOMASS
ETHANOL
TOXIN
GLUCOSE CONC., S (g/L)
BIOMASS CONC., X (g/L) or ETHANOL CONC., P (g/L/10)
TOXIN CONC., Z (U/L)
DILUTION RATE, D (h⁻¹)
0
5
10
15
20
25
30
35
40
45
50
0
.05
.10
.15
.20
25
30
.25
.50
.75
1.00
1.25
1.50
1.75
2.00
2.25
0
1
2
3
4
5
6

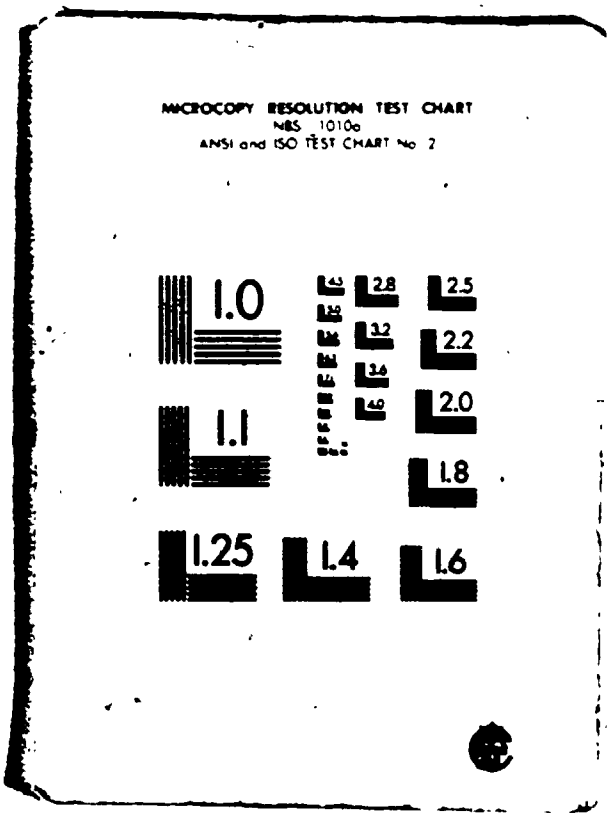
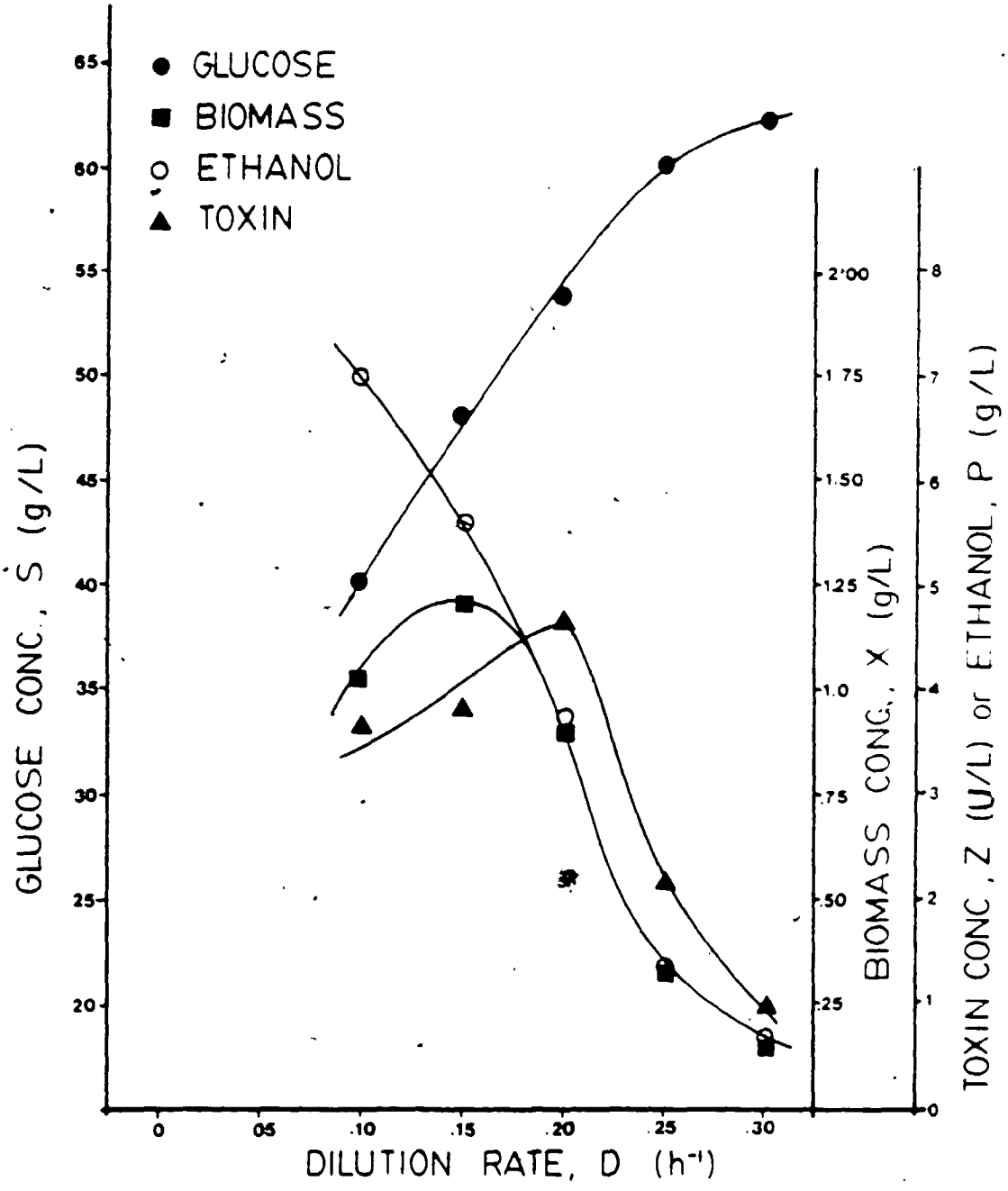

MICROCOPY RESOLUTION TEST CHART
NBS 1010a
ANSI and ISO TEST CHART No. 2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8

Figure 6.24 - Continous Experimental Results
Temperature = 30 °C
pH Controlled = 4.5
Feed Substrate Concentration = 62-g/L.


GLUCOSE
BIOMASS
ETHANOL
TOXIN
GLUCOSE CONC., S (g/L)
BIOMASS CONC., X (g/L)
TOXIN CONC., Z (U/L) or ETHANOL, P (g/L)
DILUTION RATE, D (h⁻¹)
65
60
55
50
45
40
35
30
25
20
2·00
1·75
1·50
1·25
1·0
·75
·50
·25
8
7
6
5
4
3
2
1
0
0
05
·10
·15
·20
·25
·30

$$\frac{\mu_i}{\mu_o} = \left\{ 1 - \left(\frac{Z_i}{Z_m} \right)^{\lambda} \right\}^{-1} \qquad 6.8$$

The proposed new model is designed to account for the mass transfer limitations caused by the layer of immunity and toxin molecules on the surface of the cell. It may be possible that as the specific toxin concentration increases, the amount of interference by the toxin on glucose uptake will also increase.

These criteria are met in the model proposed in equation 6.8. At low specific toxin concentrations, the right hand side of this model limits to one. This indicates no effects by the toxin on glucose uptake.

At high specific toxin concentrations, the right hand side of this model approaches infinity, this indicates a high level of inhibition. This would then suggest that an infinitely high driving force would be needed to effect mass transfer across the cell membrane and wall coatings.

Substitution of equation 6.8 into the kinetic expression for batch growth (equation 4.30) would result in an equation with four unknowns, namely: K_m, μ_{max}, Z_m, and λ. Use of the equation with the batch data would require estimation of at least two of the other variables. Since the of Levenspiel method (1980) used to solve this equation also relies on average values that were calculated from the batch data, use of this equation would be more difficult. The use of steady-state, continuous data would better allow the calculation of these kinetic parameters.

6.5.2 Toxin Presence on Cell Surface

It is proposed that the toxin is able to bind to the cell wall of sensitive cells (Wickner, 1981). It is also proposed that the toxin is able to attach itself to the immunity molecules which protect the cell wall of killer cells from the toxin. These immunity molecules are thought to attach to the cell wall of the killer cells. If this hypothesis is true, then it might be possible that the layer of immunity and toxin molecules on the cell surface may interfere with the transport of glucose into the cell. This would then lower the ethanol productivity and slow the growth rate of the cells. It is evident that more basic and fundamental work is needed to elucidate these basic phenomena of possible toxin interference with the uptake of glucose by the yeast cells.

To determine if the toxin molecules are bound to the cell walls of the zymocidal strain, a large (100-mL) sample of the continuous broth was collected. This sample was filtered (0.22-μm, 47-mm) and the cell free broth was then spotted onto toxin assay plates. The cell cake was then repeatedly washed with 10-mL volumes of pH 4.5 buffer solution. The results of this analysis are listed in Table 6.1 and shows that a significant amount of toxin can be liberated from the walls of zymocidal cells. This suggests that the binding of toxin to the cell wall receptor is reversible, as was reported in the literature. Since these cell-bound toxins do not kill the zymocidal cells, this may suggest that the immunity molecules are also present. A

TABLE 6.1

CELL CAKE WASH RESULTS

Original Sample:	100-ml
Wash Volume:	10-ml
	Equivalent Concentrations
Original Solution	1.24
Wash 1	0.98
Wash 2	0.93
Wash 3	0.92
Wash 4	0.91

diagram of the proposed cell wall, immunity molecules, and toxin molecules is shown in Figure 6.25.

6.5.3 Calculation of Kinetic Parameters.

Use of the continuous fermentation data to evaluate the kinetic parameters K_m and μ_{max} by using equation 4.38 is not possible and this equation must be modified to account for the toxin effects. By substituting equation 6.8 into the Monod kinetic expression (equation 4.3) and performing some rearranging, a kinetic expression can be derived which contains the terms to account for the inhibition caused by the toxin protein. The kinetic expression is shown as equation 6.9.

$$\frac{1}{\mu} = \left\{ 1 - \left(\frac{Z_x}{Z_m} \right)^{\lambda} \right\} \left(\frac{K_m}{\mu_{max} S} + \frac{1}{\mu_{max}} \right) \qquad 6.9$$

Substituting equation 4.37 into 6.9 leads to equation 6.10, the final form of this kinetic equation:

$$\frac{1}{D} = \left\{ 1 - \left(\frac{Z_x}{Z_m} \right)^{\lambda} \right\} \left(\frac{K_m}{\mu_{max} S} + \frac{1}{\mu_{max}} \right) \qquad 6.10$$

This equation contains four unknowns, namely: Z_m, λ, K_m, and μ_{max}. An estimate of at least one of these variables is needed before it can be fitted to the continuous fermentation experimental data.

Therefore, an estimate of the maximum specific growth rate should be possible be by extrapolating the biomass curves to zero in the data shown in Figures 6.23, or 6.24. By doing this, μ_{max} was estimated for each continuous experiment and these data are shown in

Figure 6.25 - Proposed Hypothetical Representation of the Immunity Receptor sites on the cell wall of the zymocidal yeast cell and their interaction with the toxin molecules. This figure shows the immunity or γ units bound to the cell wall receptor sites. This binding prevents the disulfide linked α and β toxin components from access to the cell wall. At high toxin/immunity concentrations the transport of substrate into the cell will be drastically reduced due to the presence of these layers on the cell wall. This is the basis of the proposed interference model.

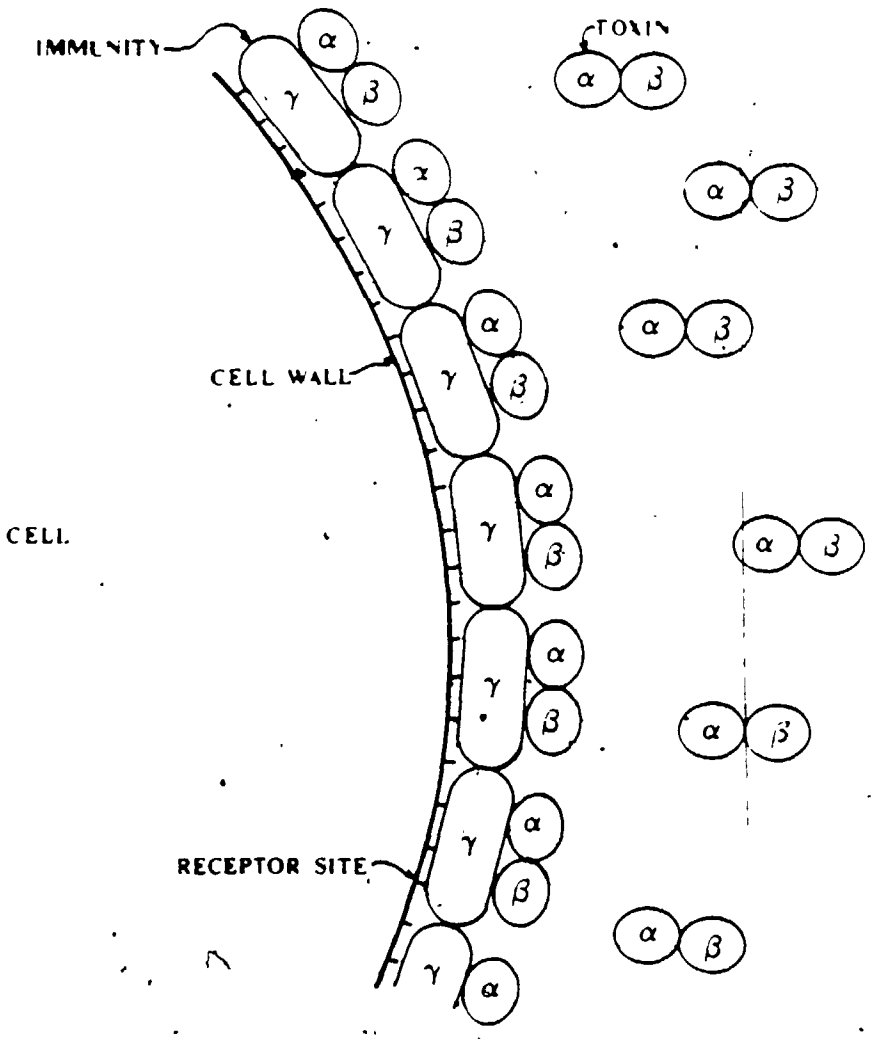

IMMUNITY
TOXIN
CELL WALL
CELL
RECEPTOR SITE
α
β
γ

TABLE 6.2

EXTRAPOLATION OF μ_{max} FROM CONTINUOUS DATA

TEMPERATURE °C	μ_{max} CONTINUOUS	μ BATCH
15	[illegible]	[illegible]
20	0.23	[illegible]
25	0.27	[illegible]
30	0.32	[illegible]
35	0.36	[illegible]

Table 6.2. As shown in this table, there is very good agreement between the maximum specific growth rate estimated in this way, and the maximum specific growth rates calculated from the batch fermentation data. The large error in the values at 15 °C were due to the low number of points in the extrapolation since only three flow rates were used before wash-out occurred.

With an estimate of the maximum specific growth rate, the continuous data can be fit to the kinetic model. Three variables remain, namely: K_m, Z_m, and λ. This requires the use of an optimization procedure such as OPDATA (TRIUMF/BCRC - VAX 750 computer).

The experimental data from the 20, 25, 30, and 35 °C were computer fitted to the kinetic model. A plot of the inverse dilution rate versus the inverse substrate concentration for a pH of 4.5 and a temperature of 20 °C is shown in Figure 6.26.

The optimization procedure used in this analysis minimized the residual error between the model and the data points. The optimization equation utilized is shown in equation 6.11.

$$R_E = \sum_{n=1}^{N} \left(M_{model} - M_{experimental} \right)^2 \qquad 6.11$$

The optimization was completed in two stages, initially the minimum residual was found with respect to the exponent, λ. These results are shown in Figure 6.27. The minimum residual was evaluated at an exponent of 0.26. The model and experimental data derived from the optimization are plotted in Figure 6.28. This figure shows that the

Figure 6.26 - Plot of the Continuous Culture Results according to the Inverse of the Monod Kinetic Model or the Lineweaver-Burk Relationship according to Equation 4.41.
Temperature = 20° C
pH Controlled = 4.5.

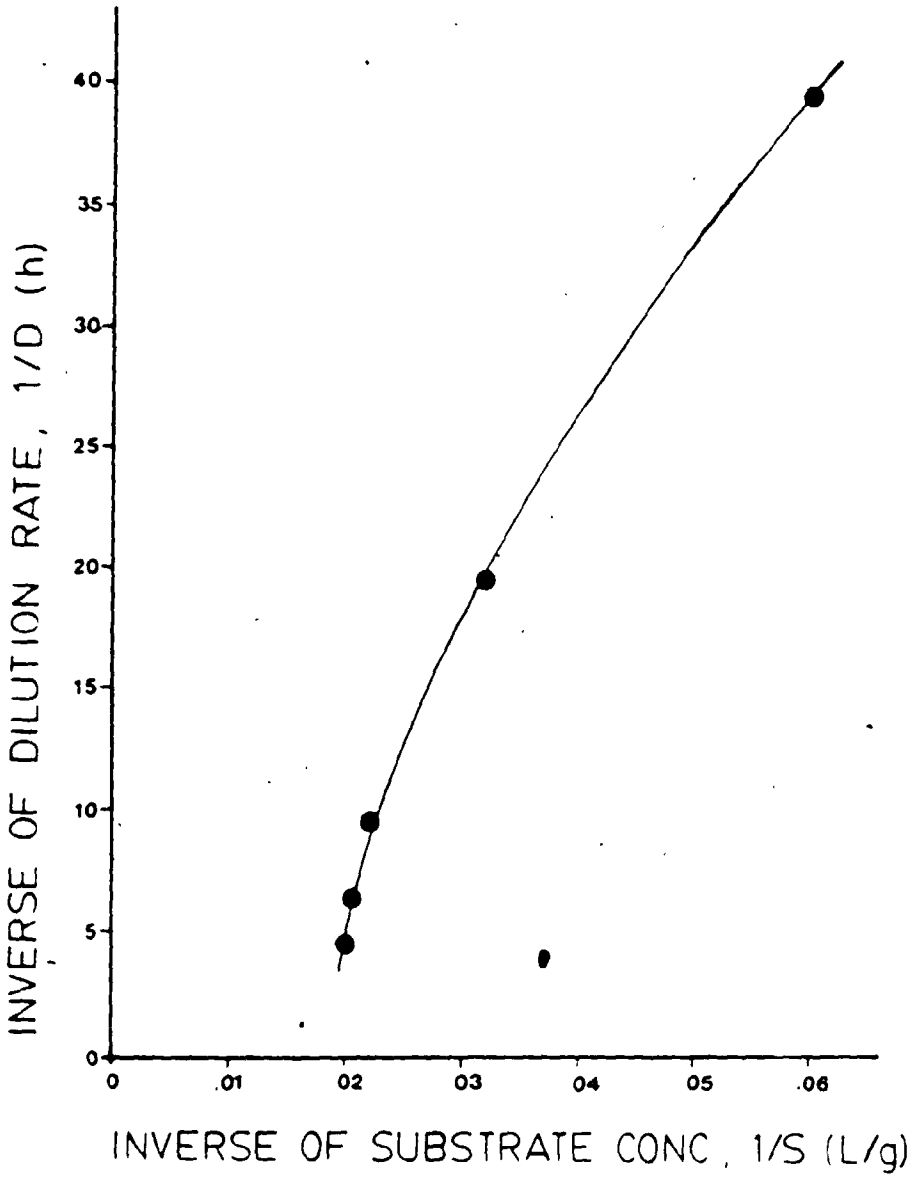

INVERSE OF DILUTION RATE, 1/D (h)
40
35
30
25
20
15
10
5
0
0
.01
02
03
04
05
.06
INVERSE OF SUBSTRATE CONC , 1/S (L/g)

Figure 6.27 - Optimization Results for Interference Model. Optimization of the Exponent Value, λ, for experimental conditions:
Temperature = 20° C
pH Controlled = 4.5.

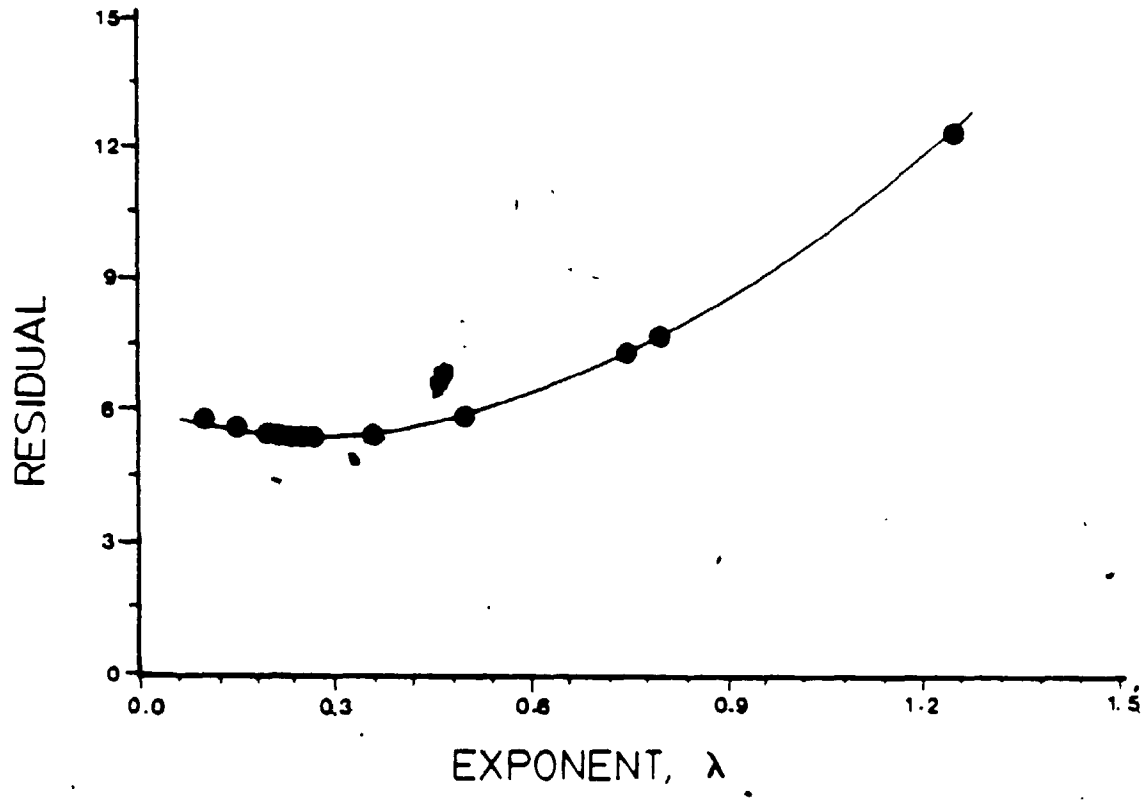
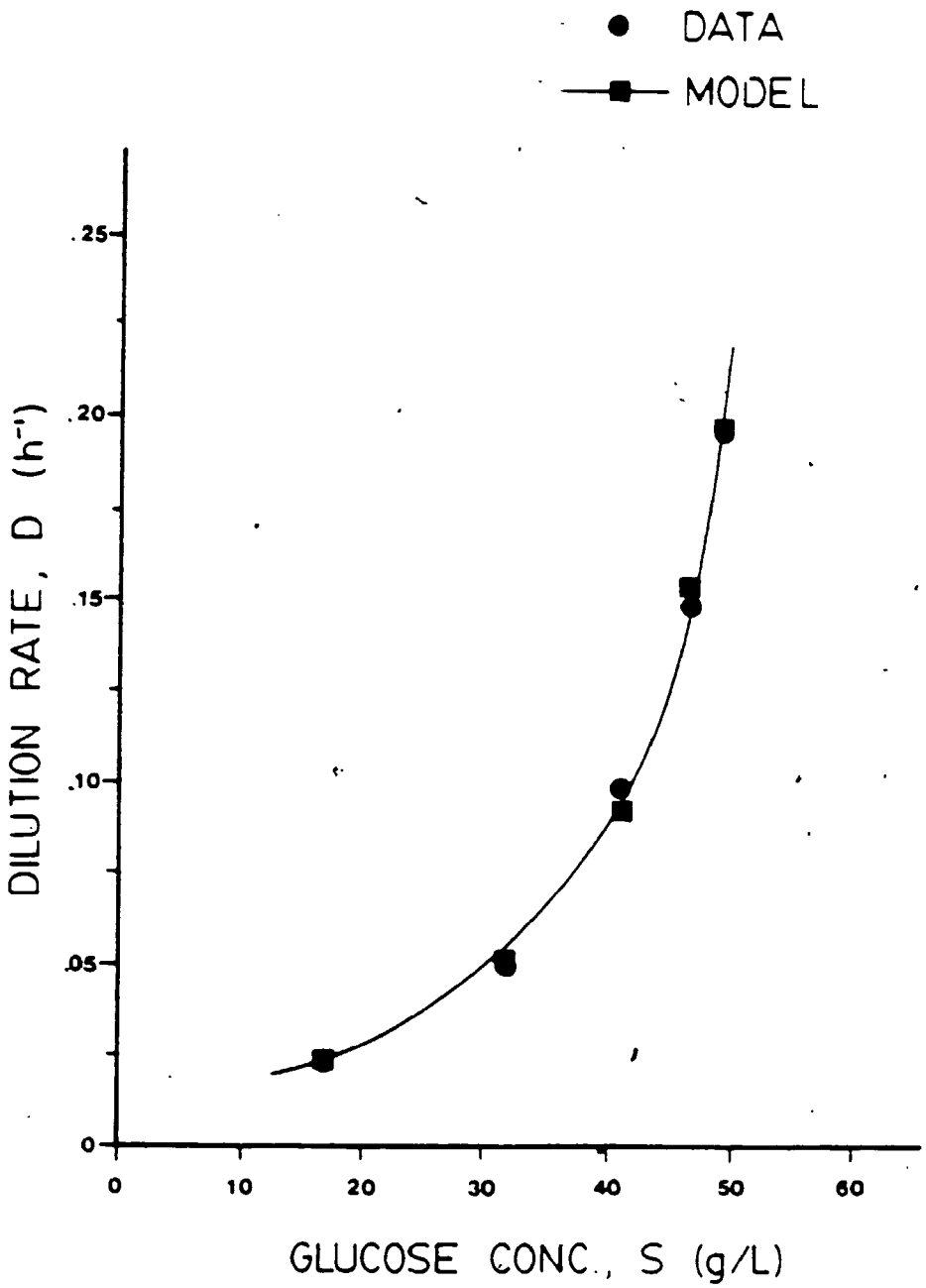

RESIDUAL
15
12
9
6
3
0
0.0
0.3
0.6
0.9
1.2
1.5
EXPONENT, λ

Figure 6.28 - Validity of Model Fit - Plot of experimental data and interference model estimates.
Temperature = 20° C
pH Controlled = 4.5.


DATA
MODEL
DILUTION RATE, D (h⁻¹)
.25
.20
.15
.10
.05
0
0
10
20
30
40
50
60
GLUCOSE CONC., S (g/L)

model does accurately represent the inhibition observed in this analysis.

Figures 6.29 and 6.30 show the optimized parameters at each continuous temperature. As shown in Figure 6.29, the maximum specific toxin concentration which totally inhibits growth was found to be 11.69-Units/g. As the temperature increases, the overall effects of the inhibition goes down, and at 35 ^{o} C the maximum specific toxin concentration was found to be 76.0-Units/g.

These results are consistent with the temperature stability characteristics of the toxin. At low temperatures, the toxin is most stable and would cause the most inhibition and at high temperatures, the toxin would be quickly denatured, and the toxin present on the cell surface would have to be constantly replaced. Therefore this might reduce the inhibition effects.

Figure 6.30 shows the temperature effects on the optimized values of the saturation constant K_m. As the temperature increases, there is a marked drop in the saturation constant. This result is normally masked by the effects of the extracellular toxin protein.

6.6 Proposed Model of Toxin Accumulation

The batch and continuous results suggest that the toxin production is growth associated, as its accumulation only occurs during growth. No accumulation of toxin occurred in the stationary phase.

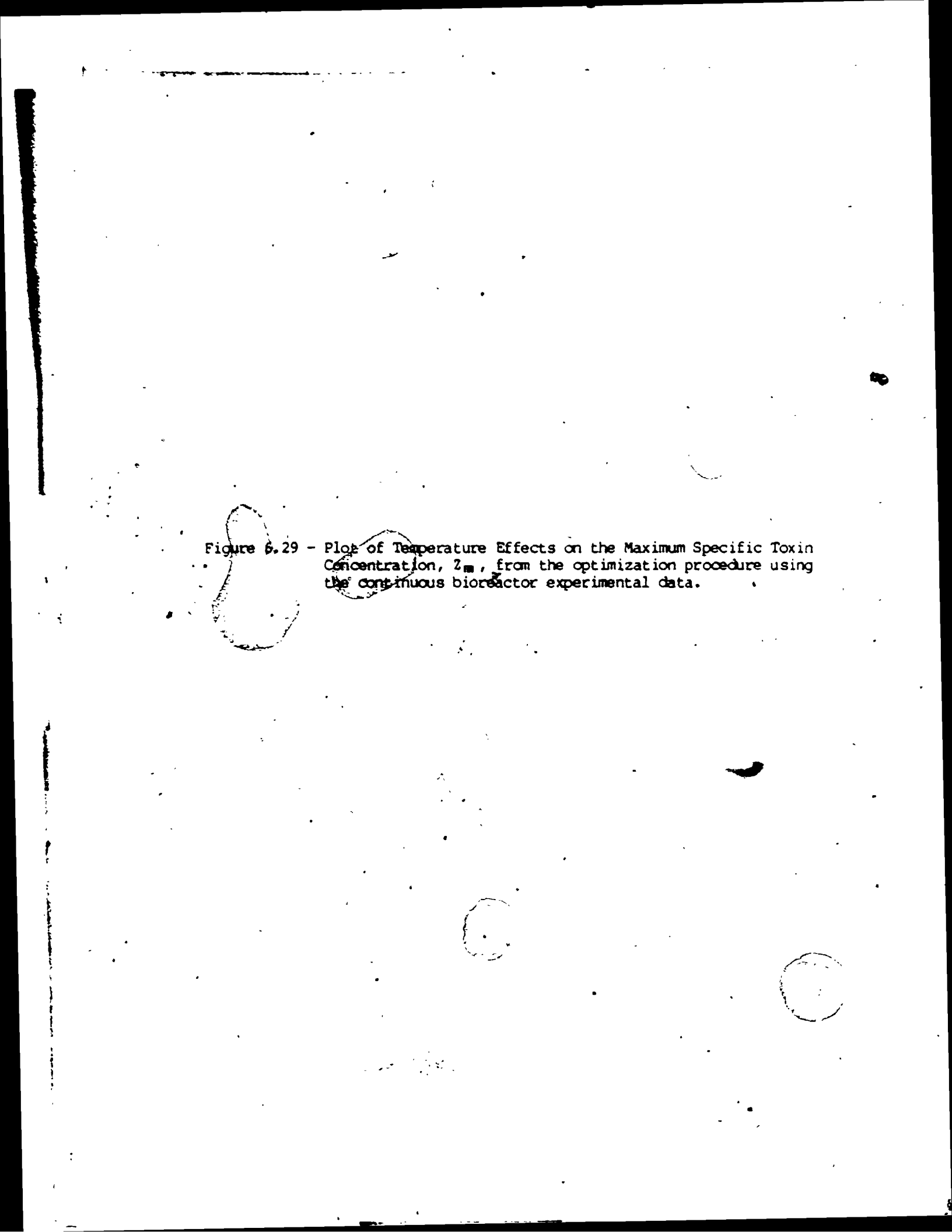


Figure 6.29 - Plot of Temperature Effects on the Maximum Specific Toxin Concentration, Z_m, from the optimization procedure using the continuous bioreactor experimental data.

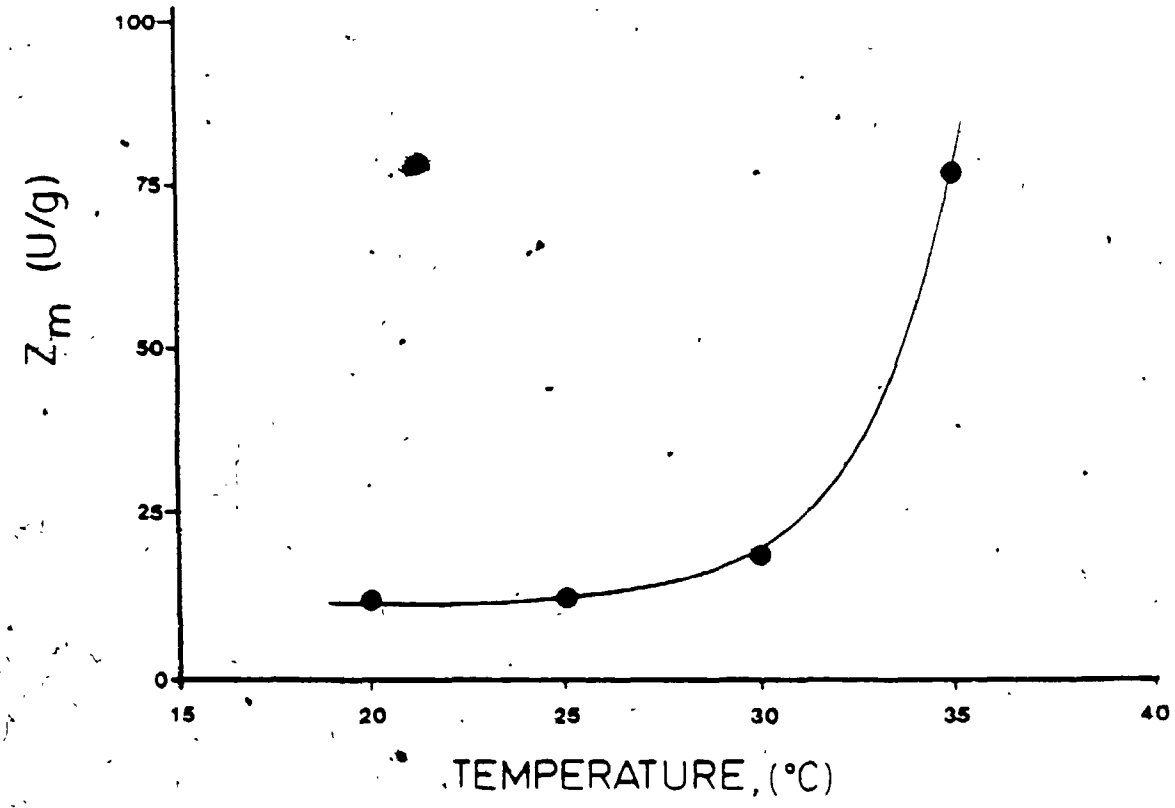
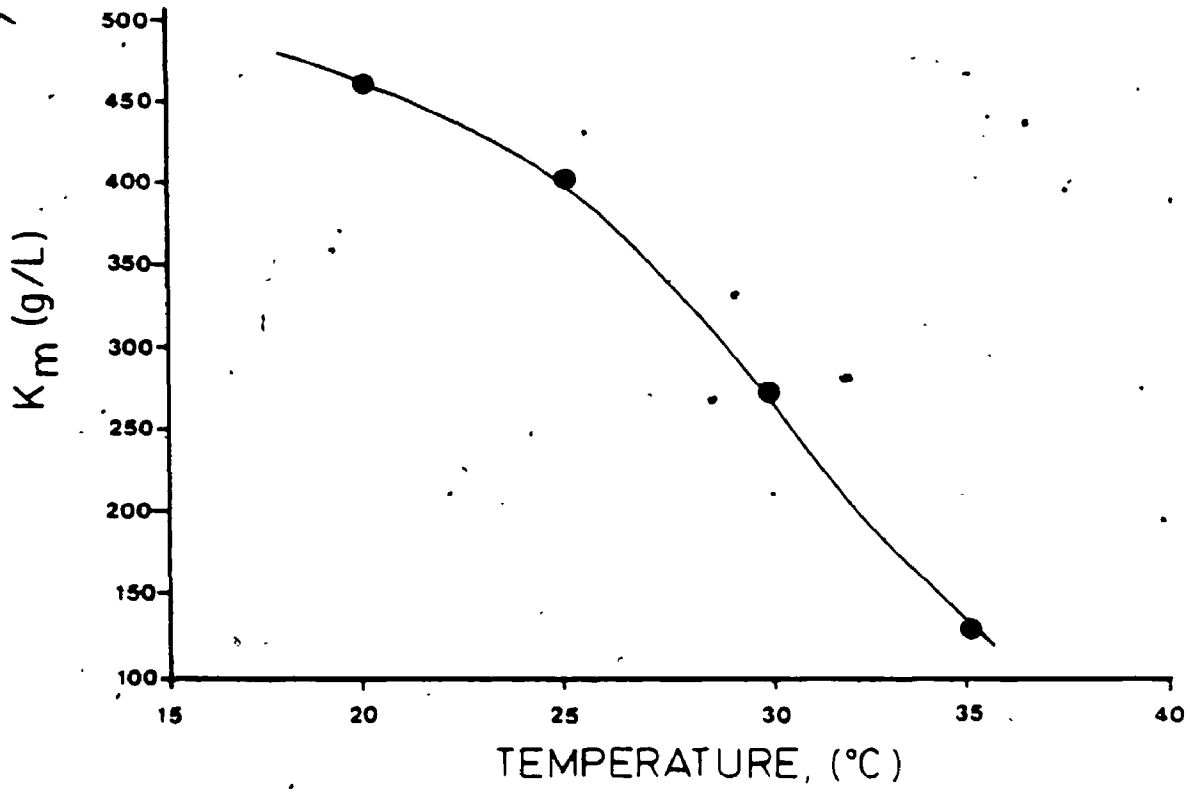

100
75
50
25
0
Z_m (U/g)
15
20
25
30
35
40
TEMPERATURE, (°C)

Figure 6.30 - Plot of Temperatues Effects on the Saturation Constant, K_m from the optimization procedure using the continuous bioreactor experimental data.


500
450
400
350
300
250
200
150
100
K_m (g/L)
15
20
25
30
35
40
TEMPERATURE, (°C)

With this assumption in mind, a differential mass balance on the toxin can be written in the form of equation 6.12. This equation states that the rate of increase in the active toxin concentration is equal to the rate of toxin production, less the rate of toxin deactivation.

$$\frac{dZ_A}{dt} = \frac{dZ_T}{dt} - \frac{dZ}{dt} \qquad 6.12$$

From the growth associated model, the rate of product formation is given by equation 4.12. The rate of toxin inactivation was derived earlier, and is given by equation 6.8. Substituting these equations into equation 6.12 leads us to equation 6.13.

$$\frac{dZ_A}{dt} = \beta X - kZ_A \qquad 6.13$$

In a batch culture, the rate of increase in biomass is related to time (according to equation 4.9). This equation can than be substituted into equation 6.13 and is given by equation 6.14.

$$\frac{dZ_A}{dt} = \beta X_0 e^{\mu(t-t_{lag})} - kZ_A \qquad 6.14$$

Integration of this equation leads us to an equation which may be used to predict the active toxin concentration in the liquid broth. Fitting of this data to experimental results is required before full functional use is possible. This equation is shown as equation 6.15.

$$Z_A = \frac{\beta X_0 e^{-\mu t_{lag}} e^{\mu t}}{\mu + k} - Ce^{-kt} \qquad 6.15$$

The use of equation 6.13 is much easier in the case of continuous data. At a steady-state, the left hand side of this equation is zero, and therefore it can be rearranged to give equation 6.16.

$$D\mathcal{L}_A = Y_{Z_a/X} \mu X - kZ_a \qquad 6.16$$

Much work to determine the applicability of these equations is required. Larger scale fermentations, where more samples can be taken during the exponential growth phase, would be required for accurate results to be obtained.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

The results of the analysis clearly show the characteristics of the zymocidal yeast strain Saccharomyces cerevisiae #1465. The toxin produced by the strain quite effectively killed sensitive cells, which was limited by the stability of the toxin in liquid broth. The toxin was shown to deactivate very rapidly at temperatures above 10° C and at pH values outside the optimum range 4.0 to 4.75.

The rate of deactivation of the toxin with respect to temperature at constant pH was found to be of first order, and temperature deactivation reaction rate constants followed the Arrhenius relationship. A kinetic model was derived, including correlations for the Arrhenius proportionality constant and activation energy. These correlations allow for calculation of the rate of toxin deactivation at any conditions of temperature and pH.

The growth rate of the cells in both the batch and continuous systems were shown to be adversely affected by increases in the active toxin concentration in the liquid phase. The toxin was shown to affect the glucose uptake of the cell. This interference resulted in low ethanol concentrations in both continuous and batch fermentations. The

toxin was found to be capable of binding to the cell wall of the producing cell. This binding of the toxin, and most likely the immunity molecule, is proposed to add a significant mass transfer resistance to the outside of the cell wall. This resistance may affect adversely the mass transfer (or active transport) of glucose into the cells. The overall effects of these phenomena are a slower growth rate, reduced substrate utilization, and low ethanol concentrations.

The proposed mass transfer effect was modelled by applying a correction factor to the Monod kinetic expression. This new model was applied to the continuous growth experimental data using computer optimization techniques. At temperatures where the toxin is most active, the optimization results showed that all growth would stop at a specific toxin concentration of 11.69-Units/g or higher. High toxin concentrations which developed during batch fermentations with high initial sugars, or during continuous fermentations, were detrimental to the ethanol productivity.

The results obtained suggest that there is limited application of the zymocidal strains in fuel alcohol and distilled spirit production. Zymocidal strains are only able to effectively ferment substrate streams at low temperatures (<20 $^{\circ}$C) and at low substrate concentrations (<60 -g/L). This would greatly increase time and distillation equipment needed to affect a complete fermentation and product purification.

The use of zymocidal yeast in beer production would require further study, and the lower fermentation temperatures of 10 - 15 $^{\circ}$ C

used in conventional brewing systems may provide possible applications. Study of the fermentation properties of the zymocidal strains on beer wort are needed before the advantages and/or disadvantages can fully be assessed.

Two models for the accumulation of the active toxin in the liquid broth were derived. These models were based on the observation that the production of the toxin appeared to be growth associated. These models, which used the deactivation kinetics, could be utilized to predict the active toxin concentration during batch or continuous fermentations. Evaluation of two constants in these models would be necessary before they could be applied. More research, with larger scale bioreactors, would be needed before accurate estimates of these constants can be made.

The similarity between the zymocidal toxin and the non-volatile toxin effects reported by some researchers is too striking to be ignored. As reviewed in Chapter 2 of this report, many researchers have reported inhibitory effects caused by non-volatile 'toxins' present in the liquid broth. The zymocidal toxin produces effects quite similar to these non-volatile 'toxins'. It is therefore concluded that these non-volatile toxins are proteinaceous by-products of naturally growing cells. These protein molecules may possibly effect the mass transfer of substrate and product near the cell surface in a similar fashion as the toxin-immunity system.

Use of different strains which are able to produce some of the different zymocidal toxins or other detectable proteins would be an

interesting application of the correlations derived in this thesis. These types of analyses may lead to the discovery of a zymocidal or other system which is not adverse to the effects of the extracellular proteins. Discovery of such a system could greatly benefit the fermentation industry

Immobilization of the zymocidal yeast strain in calcium alginate may lead to interesting results. The combined mass transfer effects of the substrate through the bead, of the substrate into the cell and of the toxin and products out, may lead to interesting modelling possibilities. More work is needed to study the possible effects of zymocidal cell immobilization and their application to ethanol production.

Lastly; study of the transient behavior of mixed sensitive and killer cultures may also allow for some modelling possibilities. Injection of a killer strain into a batch or continuous culture of sensitive cells may allow models to be derived to enable the prediction of contamination in large scale systems. Methods to detect the percent killer strain in diluted broth samples would have to be developed and perfected before this type of analysis would be possible.

At the present time, the use of zymocidal strains is limited to the laboratory scale. Application of these strains to either batch or continuous fuel or distilled spirit fermentations would be economically unsuitable until further developments are made. Study of zymocidal strains as model systems for protein secretion and in the study of strains interaction is a very promising way of obtaining important new

kinetic models which may be used in new industrial fermentations.

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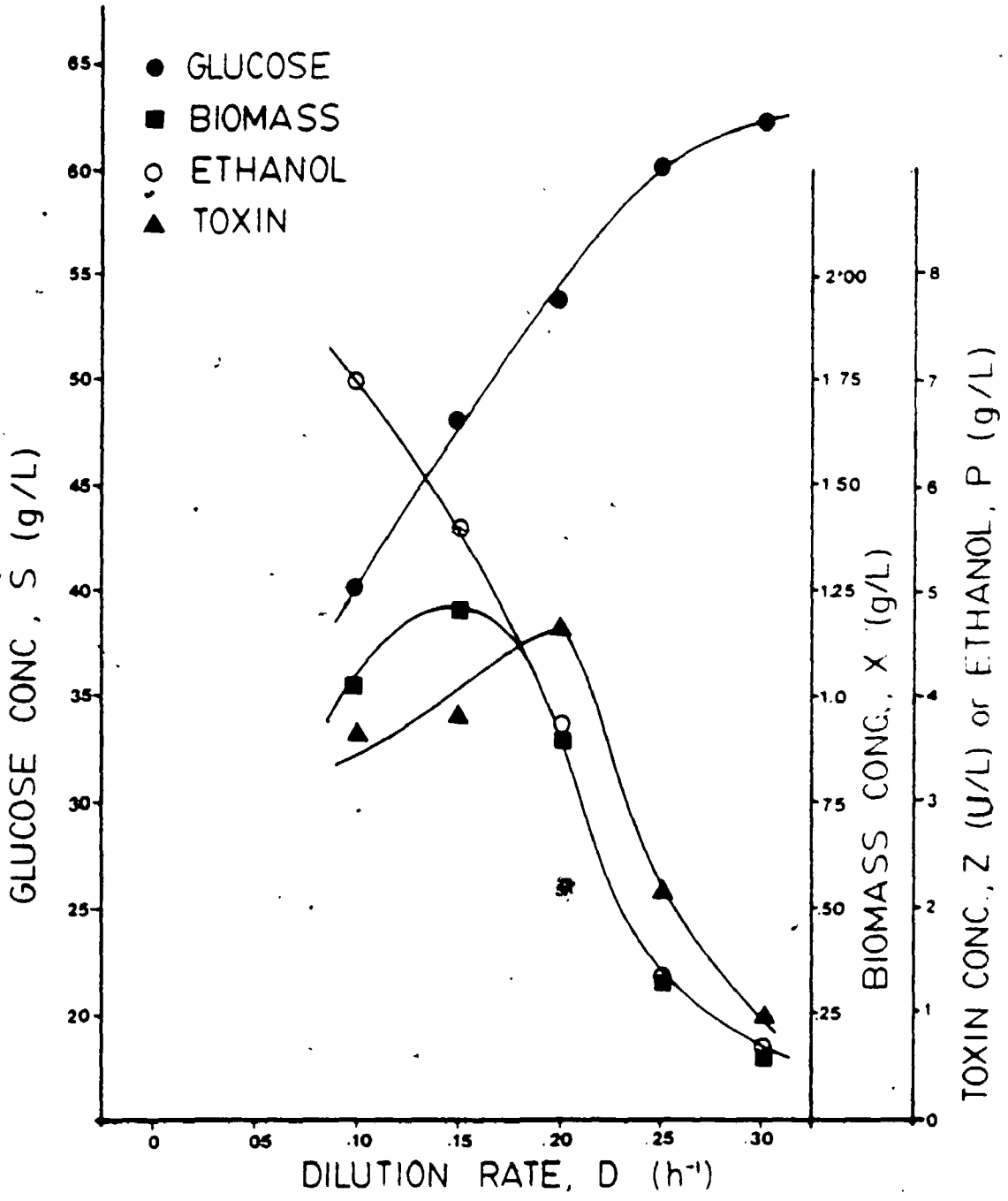
GLUCOSE
BIOMASS
ETHANOL
TOXIN
GLUCOSE CONC., S (g/L)
BIOMASS CONC., X (g/L)
TOXIN CONC., Z (U/L) or ETHANOL, P (g/L)
DILUTION RATE, D (h⁻¹)
65
60
55
50
45
40
35
30
25
20
2·00
1·75
1·50
1·25
1·0
·75
·50
·25
8
7
6
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2
1
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05
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