Testing Monod: growth rate as a function of glucose concentration in *Saccharomyces cerevisiae*

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Declaration:

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and I have not previously in its entirety or in part submitted it at any university for a degree.

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Signature Date

Abstract.

The complexity of microbial systems has presented serious obstacles to the quantification of fermentation processes. Using computer modelling techniques progress has been made in monitoring, controlling and optimising microbial systems using material balancing techniques and empirical process models. The Monod equation is among the most commonly used models and is based on empirical findings with no mechanistic basis. Monod presents a simple model to describe the growth of a cell in a defined nutrient environment. The Monod equation is mathematically analogous to the formula that was proposed by Michaelis and Menten to describe enzyme kinetics. Both equations describe a hyperbolic function with a half-saturation constant (K_s in the monod equation and K_m in the Michaelis Menten equation) but the meaning of the two saturation constants K_s and K_m is different. In number of studies K_s and K_m are used as if they are equivalent. In contrast to Michaelis-Menten kinetics, which describes a process catalysed by a single enzyme, Monod kinetics describes an overall process involving thousands of enzymes.

The Monod equation describes the specific growth rate of a microbial cell as the function of a limiting substrate concentration. The aim of this study was to test this principle, for *Saccharomyces cerevisiae* VIN13 under glucose limited aerobic chemostat conditions. The VIN13 was observed to follow the Monod description and when compared with other growth kinetic models gave one of the best fits to the data. A functional relationship between the half-saturation constant, K_s, and Michaelis Menten constant, K_m, was there after derived. This was achieved by using metabolic control analysis (MCA) to explain when K_m of the transporter becomes equal to the K_s. Using the deductions obtained from MCA a core kinetic model was then formulated to demonstrate that the K_s can either be smaller, equal or higher than the K_m of the transporter, depending on the flux control distribution in the model.

Opsomming.

Die kwantifisering van fermentasieprosesse word ernstig belemmer deur die kompleksiteit van mikrobiale sisteme. Deur gebruik te maak van rekenaar-ondersteunde modelleringstechnieke vir die opstelling van massa balans vergelykings en empiriese prosesmodelle is vordering gemaak in die waarneming, beheer en optimalisering van mikrobiale sisteme. Die Monod vergelyking is een van die mees gebruikte groeimodelle en is gebaseer op empiriese bevindings - die model het nie ‘n meganistiese grondslag nie. Die Monod vergelyking is wiskundig ekwivalent aan die vergelyking wat opgestel is deur Michaelis en Menten vir die beskrywing van ensiemkinetika. Beide vergelykings beskryf ‘n hyperboliese kurwe met ‘n konstante wat die halfversadigingswaarde aangee vir substraat (K_s in die Monod vergelyking en K_m in die Michaelis-Menten vergelyking), maar die betekenis van die twee versadigingskonstantes is verskillend. In ‘n aantal studies word die Ks en Km waardes gebruik asof hulle gelyk is aan mekaar. In teenstelling met die Michaelis-Menten kinetika wat ‘n enkel ensiem-gekataliseerde reaksie beskryf, beskryf die Monod vergelyking ‘n proses wat duisende ensieme behels.

Die Monod vergelyking beskryf die spesifieke groeitempo van ‘n bakteriële sel as ‘n funksie van die beperkende substraatkonsentrasie. Die doel van hierdie studie was om hierdie beginsel te toets vir *Saccharomyces cerevisiae* VIN13 wat onder glukose beperkte, aerobiese kondisies in ‘n chemostat gekweek word. Die VIN13 groei kon goed beskryf word met die Monod model, wat in vergelyking met ander groeimodelle een van die beste passings vir die meetpunte het gegee. Vervolgens is ‘n funksionele verwantskap afgelei tussen K_s en K_m; deur gebruik te maak van metabole kontrole analise (MCA) kon verduidelik word wanneer die K_s gelyk is aan die K_m van die transporter vir die beperkende substraat. Deur gebruik te maak van die MCA analise is ‘n eenvoudige kinetiese model opgestel om aan te toon dat die K_s kleiner, gelyk aan of groter kan wees as die K_m van die transporter, afhanklik van die fluksie-kontrole verdeling in die model.

I dedicate this thesis to my Grandfather, Vuyisile Mrwebi…

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SYMBOLS AND ABBREVIATIONS

Symbol	Definition	Dimensions
Y_x	Biomass yield	g Dry weight/ g glucose
K_s	Half-saturation constant	mM
K_m	Michaelis Menten constant	mM
K_{eq}	Equilibrium constant	Dependent on reaction
α_{Vi}	Internal cell volume	L/ Wet weight
X	Biomass concentration	Optical density at 600nm
S	Growth-limiting substrate concentration	mM
F	Flow rate	$ml.h^{-1}$
V	Volume culture	ml
S_r	Medium substrate concentration	mM
C_x	Cell mass synthesised	g.-dry weight. $liter^{-1}$
C_s	Substrate for cell mass synthesis	mM
C_p	Formed product	mM
C_o	Dissolved oxygen	%
C_c	Dissolved carbon dioxide	%
$F_{L,in}$	Inflow	$ml.h^{-1}$
$F_{L,out}$	Outflow	$ml.h^{-1}$
V_L	Liquid phase volume	ml
C_{in}	Vector of concentrations in the inflow medium	mM
Q	Vector of reaction rates due to biotic phase	$mmol.g^{-1}.h^{-1}$
q_s	Glucose uptake rates	mmol/ g Dry weight/h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}
μ	Specific growth rate	h^{-1}
K_b	Blackman affinity constant	mM
K_i	Inhibition constant	mM

Symbol	Definition	Dimensions
$V_{\_t}$	Total culture volume	L
$V_{\_max}$	Maximum capacity	$mmol.h^{-1}$
$K_{\_T}$	Tiessier affinity constant	mM
n	Moser parameter	-
$Glc_{\_ext}$	Extracellular glucose concentration	mM
$Glc_{\_in}$	Intracellular glucose concentration	mM
$Glc_{\_feed}$	Medium glucose concentration	mM
p	Pump rate	$ml.h^{-1}$
$C^{J}_{v_{\_i}}$	Flux-control coefficient	
R^{J}_{s}	Response coefficient	-
$C^{X}_{v_{\_i}}$	Metabolite concentration-coefficient	-
$\varepsilon^{v_i}_{X}$	Elasticity coefficient	-

"…The study of the growth of bacterial cultures does not constitute a specialized subject or a branch of research: it is a basic method of microbiology."

"It would be a foolish enterprise, and doomed to failure, to attempt to review briefly a subject which covers our whole discipline. Unless, of course, we considered the formal laws of growth as a method for their own sake, an approach which has repeatedly proved sterile"

---J. Monod, 1949

CHAPTER 1

1 General introduction

Microbial growth kinetics has been the subject of many scientific studies and has many implications for our society. Biotechnology, traditionally heavily based on microorganisms is an important discipline for research institutes, agriculture, pharmaceuticals and food industry.

Progress in biotechnology has created a need to quantify metabolic processes of microorganisms so that they can be most thoroughly and efficiently exploited. The potential rewards of an improved quantification, enabling better understanding and control of microbial processes are most certainly great: (1) increased yield of microbial products, (2) increased rate of product formation, (3) maintenance of microbial product quality and uniformity and (4) attainment of process uniformity. The development of new experimental techniques has been much more rapid than the tools for analysis of these experimental data. Thus, while it is possible to measure a multitude of components in cells growing under well-controlled conditions, our understanding of the cell physiology is still very limited. It is becoming increasingly clear that to come to a better understanding of the regulation and control of cellular processes we will have to use quantitative techniques. Due to the multitude of cellular processes and their non-linear interactions we are often limited to computer models for interpretation of the experimental data.

The growth of microbial cells can be viewed from various perspectives and with varying degree of complexity, depending on whether we distinguish between individual cells in a reactor and whether we examine individual metabolic reactions occurring within the cell.

While a detailed model of growth could consider all the reactions occurring within each cell with variations from cell to cell in a population, such a model would be very unwieldy [2].

The need for methods and tools for simpler data analysis is clear, not only in academic research, but also in industry where a minimum of time is spent on data analysis and a lot of potentially profitable information is inevitably lost [2]. By combining experimental work and mathematical modeling, it is possible to provide meaningful and quantitative interpretation of experimental results while also revealing new aspects of microbial physiology [2].

The reason to specifically focus on unstructured, non-segregated models is that, in order to improve a system, it is beneficial to first explore avenues that provide a simple approach. The Monod equation, which is the main focus of this study, presents a simple model to describe the growth of a cell (whereas a cell itself is a complex system) in a defined nutrient environment. It is the most commonly applied model to estimate cell growth and substrate biodegradation. The model equation describes the specific growth rate of a microbial cell as the function of a limiting substrate concentration. The objective of this study is to test this principle for *Saccharomyces cerevisiae* under glucose limited aerobic chemostat conditions. The Monod description is based on empirical findings and has no mechanistic basis. The second objective of this study is to derive a functional relationship between the Monod constant K_s and Michaelis Menten constant K_m of the microorganism's transporter for growth limiting substrate.

Chapter 2 will introduce very briefly the importance of biotechnology in general, and use *Saccharomyces cerevisiae* as a model of biotechnology applications. Subsequently several external factors are listed and responses of yeast to changes in the factors are described. Some of the different cultivation techniques that are used for yeast are discussed and compared. The chapter finishes with a description of growth models with a specific focus on simple models such as Monod.

Chapter 3 is a description of the methods that were used in the experiments described in this thesis. *Saccharomyces cerevisiae* VIN13 was cultivated under glucose limited aerobic chemostat conditions. The steady state residual glucose concentration was measured at various dilution rates. The objective was to experimentally verify whether the specific growth rate can be described as a function of the residual substrate concentration.

In chapter 4, the experimental results are presented. A relationship between the specific growth rate of *S. cerevisiae* and the substrate concentration was determined in chemostat cultures. Furthermore the effect of dilution rate on biomass concentration is also presented. A shift from an oxidative metabolism to a respiro-fermentative metabolism is proposed on the basis of glucose yields and ATP yields. The Monod equation along with other unstructured models was fitted to the experimental data.

In chapter 5, using metabolic control analysis (MCA), it was shown that it is possible to relate the K_s of the cell to the K_m of the transporter, under certain conditions.

In chapter 6, a core kinetic model is presented that illustrates the principles formulated in chapter five. The assumptions that were applied in chapter five were used to design a core model. It is demonstrated that the K_s can either be smaller, equal to, or higher than K_m of the transporter, depending on the flux control distribution in the model.

Chapter 7 is a general discussion. This chapter summarizes the findings and places them in a broader context.

The aim of this study was to test the empirical findings of Monod on yeast, and to further develop a mechanistic understanding of K_s, the half-saturation constant of Monod.

CHAPTER 2

2 Literature Review

2.1 Introduction

Cells are universal units of life that are made up of molecules and in which more than a thousand reactions can take place simultaneously. Acquiring better understanding of cell physiology and biochemical processes provides insight into a range of issues that directly impact our everyday life (such as pharmaceuticals and food industry). The molecular cell composition and physiology form the vital machinery of an organism. An organism is either unicellular or multi cellular, and cells are categorized as either prokaryotic or eukaryotic. Prokaryotes are single celled organisms such as bacteria, and are usually preferred for biological analysis and industrial production of certain compounds. This is because they are relatively smaller; grow faster and are less complex than eukaryotes [34].

However, yeast and other eukaryotic cells are suitable hosts for cloning eukaryotic genes. This is because prokaryotic cells are sometimes unable to produce functional proteins from eukaryotic genes even when all signals necessary for gene expression are present, since most eukaryotic proteins must undergo posttranslational modification [34].

Saccharomyces cerevisiae is a widely used industrial microorganism for reasons of its well-studied biological activities and its ability to utilize cheap materials for growth and production. For instance, *S. cerevisiae* has been used since the very early days of microbial fermentation history for wine and beer production and the leavening of bread [22, 40]. The former production

stages are usually performed in batch conditions and in non-aerated vessels. In contrast, the production stages for biomass formation for the latter processes are highly aerobic processes occurring in a fed-batch manner in order to maintain the sugar concentration at a low level aiming at maximizing respiratory metabolism [1]. The *S. cerevisiae* biomass, mainly in the form of baker's yeast, represents the largest bulk production of any single-cell microorganism throughout the world [52]. Several million tons of fresh baker's yeast cells are produced yearly for human food use [31].

Products of yeast fermentation processes are of great importance for humankind, as they are widely used in everyday life, directly as drugs or food, or indirectly as materials in the pharmaceutical, food and chemical industries. The main products are; single cell (yeast), primary metabolites (citric acid, ethanol, glutamic acid etc.), secondary metabolites (antibiotics), enzymes (amylase, protease, lipase), therapeutic proteins (insulin, interferon, human growth hormones) and vaccines (hepatitis A, B) [1].

Yeast is publicly accepted as non-pathogenic i.e. a safe producer (GRAS) [50]. Since the whole genome of *S. cerevisiae* has been sequenced, several kinds of analyses have been applied in order to assign functions to orphan genes [5,18]. In many of these analyses, the aim has been to determine how different genes (those with known function and unknown function) interact with each other [3] to enable the cell to respond to different stimuli, release the products to the environment, regulate their metabolism, divide and to regulate up-take of nutrients and grow [67]. In addition to its GRAS status, *S. cerevisiae* can be relatively easily manipulated genetically, and also be grown on simple and cheap media under well-defined conditions.

In this chapter I will give a literature review of different cultivation techniques used in microbiology, with specific forms on (modeling) the functional relationship between the residual substrate concentration and growth rate in the chemostat cultures.

2.2 Cultivation techniques

Yeast can be cultivated either in batch, fed-batch or continuous fermentation depending on the objective of the experiment. For optimization of gene expression, producing a specific compound (e.g. drug or enzyme) or for biomass production it is usually essential to optimize the growth conditions. When optimizing yeast growth conditions, several factors should be taken into consideration such as: medium components, temperature, pH, and dissolved oxygen concentration as well as agitation speed [44].

2.2.1 Batch cultivation

Batch fermentations are commonly used for optimization of growth conditions. Microbial growth can be quantified via measurements of cell number in a culture, but for practical reasons usually optical density or dry weight is determined to follow changes in biomass concentration. In classic batch type of cultures bacterial growth follows three phases (lag phase, exponential phase and stationary phase). This is a disadvantage of batch culture, i.e. during the growth period the environmental conditions change due to changes in environmental conditions (pH, substrate depletion and product accumulation). Any growth phase could be of interest to the experimenter depending on the objective of cultivation [14].

Studies on exponential growing cells are often focused at optimizing growth conditions for either improvement of specific growth rate or product formation rate. Stress is sometimes evoked on cells to optimize expression of a certain gene, and this can occur either during exponential phase or stationary phase.

Batch cultivations are also crucial for growing cells to be harvested for resting cell metabolism experiments. Resting cells are non-growing cells that are re-suspended on buffer with substrate that has to be converted to a product.

Batch cultivations can be performed either under uncontrolled conditions, such as when pH and

gas mixing is allowed to change, or controlled conditions such when pH and dissolved oxygen are kept constant.

2.2.2 Fed batch cultivation

Usually smaller bioreactors are used for optimization stages and operated under batch conditions. Often larger bioreactors are commonly used for the up-scaling stages using the fed-batch technique, i.e. the nutrients are fed at a variable rate to the culture broth to avoid substrate inhibition, as well as to provide an increasing measure of control. The problem is then the determination of the best feed-rate of substrate as a function of time, where the meaning of *best* varies from problem to problem. Fed-batch bioreactors may be operated in a variety of ways by regulating the feed rate in a predetermined manner (feed-forward control) or by using a feedback control. The most commonly used are constantly fed, exponentially fed, and extended fed-batch. In extended fed-batch cultivation, the feed rate is regulated to maintain the substrate concentration constant until the bioreactor is full. However, the application of extended fed-batch is hindered by the lack of online sensors for substrate [38].

2.2.3 Continuous cultivation

There are two different types of continuous cultures, auxostat type with medium addition coupled to bacterial activity and the chemostat with constant medium addition. The fundamental importance of chemostat culture became more apparent only after the formulation of the basic theory by Monod [35] and Novick & Szilard [39]. The theory states that it should be possible to fix the specific growth rate of an organism if its extra-cellular environment is maintained constant. This is achieved by a continuous inflow of fresh medium and removal of culture effluent at equal and constant rate. The medium is made up in such a way that a single chemical species is growth rate limiting (a change in the concentration of the species alters the growth rate of the organism)

while changes in all other nutrient concentrations have no effect. When maintained sufficiently long a steady state is achieved at which the specific growth rate of the organism is equal to the dilution rate (flow rate/volume of the culture) of the chemostat. Thus, the experimenter, simply via controlling the medium supply rate, sets the specific growth rate.

The chemostat has played an important role in microbiology and population biology to increase our understanding of both environmental and industrial biotechnological processes [29]. The chemostat is useful for ecological studies, giving insight into the effect of extracellular environment to metabolic activities. There has been a debate concerning the role of the nutrient transporter that links the intracellular environment with the extracellular environment through transportation of nutrients. Considerable research effort has focused on glucose transporters of *Saccharomyces cerevisiae* due to their large number [7, 16, 42].

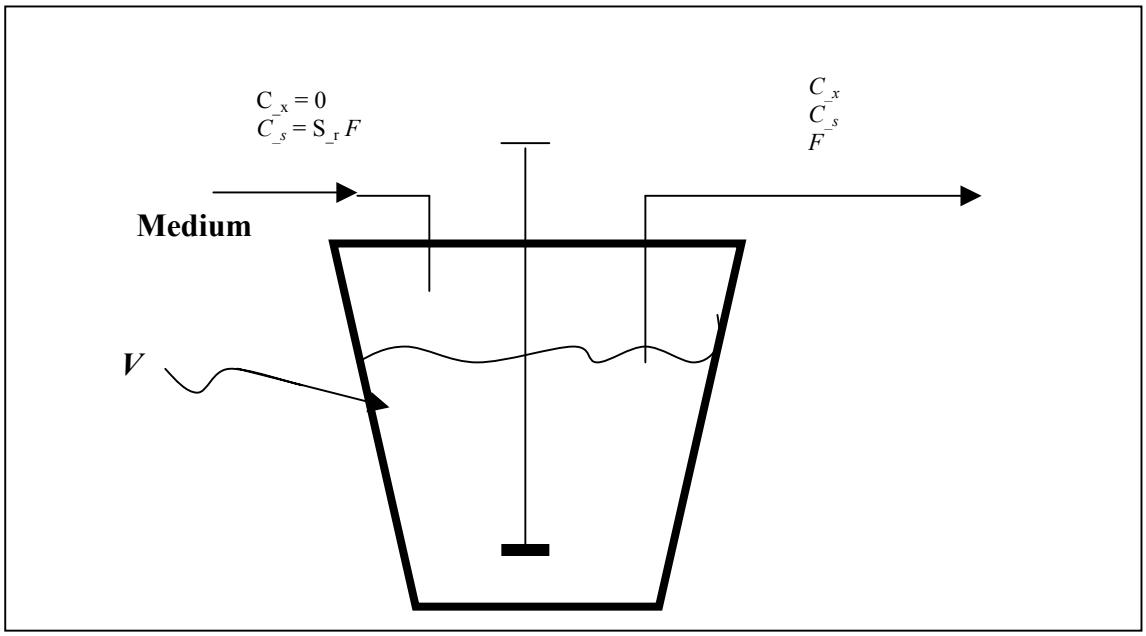

Figure: 2.1: The chemostat (diagrammatic). The biomass and growth-limiting substrate concentrations at different points are represented by C_x and C_s respectively; F = flow rate, V = volume culture. Medium generally contains no biomass and has a substrate concentration S_r, effluent has biomass (C_x), substrate concentration (C_s), and is renewed at constant flow rate (F) as the medium is fed into the culture, V= constant.

2.3 General cultivation model structure

Generally the following phases can be distinguished in biotechnological processes: the liquid phase, gas phase, and the biotic phase, the latter consisting of the cells or enzymes. Oxygen mass transfer from the gas phase to the liquid phase can have a key role in these bioreactor processes [38]. Below a threshold oxygen concentration the metabolism of *S. cerevisiae* changes from oxidative to fermentative. Thus in addition to the external glucose concentration also the oxygen availability influences the metabolism of *S. cerevisiae*. In laboratory bioreactors operative conditions are normally chosen to avoid mass transfer limitations [13]. For example, this is achieved by continuous feed of air for aerobic conditions or nitrogen for anaerobic conditions at a specific flow rate by the ability to monitor the saturation of dissolved oxygen and the ability to set agitation speed in such a way that it influences the oxygen mass transfer.

The reactions that are catalyzed by microorganisms take place in the liquid phase, with the following relevant variables: Cell mass or biomass (C_x), synthesized from the available substrates. Substrates, C_s, act as free-energy source and substrate for synthesis of biomass and products, C_p. Dissolved gases, mainly oxygen, C_o, and carbon dioxide, C_c, which are connected to the gas phase by mass exchange, are also cell substrates. In Figure 2.2 the corresponding structure of a model of a biotechnological process is shown, which is independent of type of reactor and mode of operation [38]. It consists of the liquid phase, a gas phase, and cell phase:

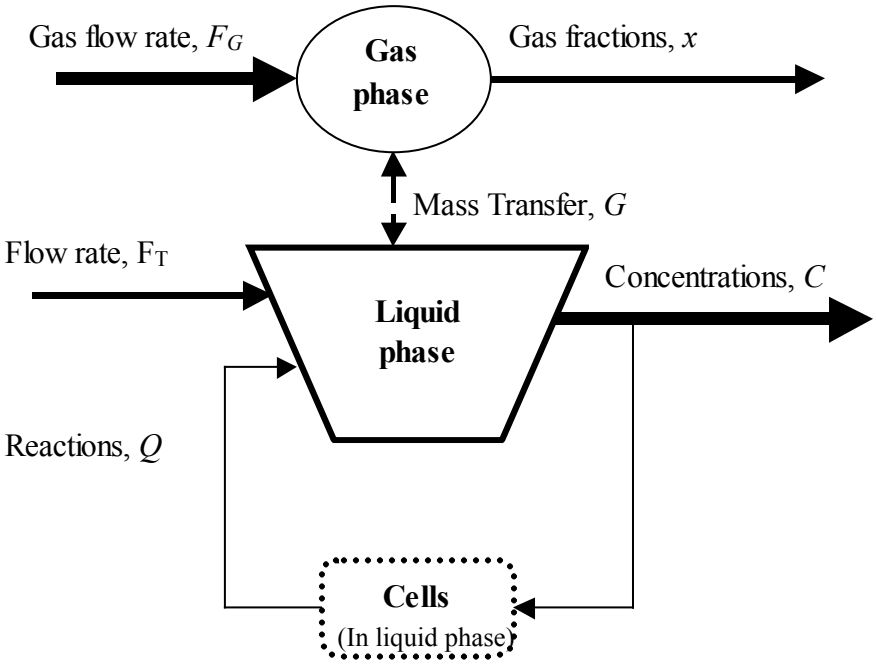

Figure 2.2: Structure of models for biotechnological processes.

2.3.1 Modeling the liquid phase of a well-stirred bioreactor

The liquid phase of a well-stirred tank bioreactor can be modeled using input, output and consumption mass balances:

$$\frac{dC}{dt} = \frac{F_{\_L,in}}{V_{\_L}} \cdot (C_{\_in} - C) + Q + G \qquad (2.1)$$

$$\frac{dV_{\_L}}{dt} = F_{\_L,in} - F_{\_L,out} \qquad (2.2)$$

Where C is the vector of concentrations in the liquid phase of reactants (C_x, C_s, C_p, C_o, C_c), F_L,in and F_L,out are the respective inflow and outflow rate of the medium (and gases). V_L is the liquid phase volume, C_in is the vector of concentrations in the inflow medium, Q is the vector of reaction rates mostly due to biotic phase in the liquid phase (this includes substrate consumption rate, product formation rate and specific growth rate), and G is the vector of gas phase exchange with the liquid phase and biotic phase [38]. The above model equations can be used to describe different kinds of processes as listed in Table 2.1:

Table 2.1: Unique properties of different cultivation techniques [38].

Cultivation Technique	Dilution and Washout Rates	Liquid phase volume
Batch cultivation	$F_{t,in} = F_{t,out} = 0$	V_L = constant
Fed batch cultivation	$F_{t,in} \neq F_{t,out} = 0$	$V_L \neq$ constant
Semi-continuous cultivation	$F_{t,in} \neq F_{t,out} \neq 0$	$V_L \neq$ constant
Continuous cultivation	$F_{t,in} = F_{t,out} \neq 0$	V_L = constant

To describe the overall process, models that deal with physical properties of fermentation processes, and kinetic models that describe physiological interactions of intracellular activities with the extracellular conditions have to be established [38]. The biological rates (Q) can be expressed as a specific rate multiplied by the biomass concentration:

$$Q = q \cdot C_x \qquad (2.3)$$

Growth is dependent on catabolic and anabolic pathways, where substrate is utilized to derive free energy, building blocks and reducing power via the first pathway to derive the latter pathways. The specific growth rate of an organism has been described as a function of a single limiting substrate using two parameters, K_s and μ_{max}. Where K_s is half saturation constant and μ_{max} is maximum specific growth rate. The K_s parameter is an empirical constant with no mechanistic meaning, and is normally referred to as the affinity of a cell for its substrate. In view of the complexity of the microorganism, it is remarkable that growth can be adequately described using just two parameters. The clear dependence of μ on S indicates that the interaction of the microorganism and its substrate must be important. We have chosen to investigate the interaction of yeast with glucose.

2.4 Effect of growth conditions on glucose transporters of *Saccharomyces cerevisiae*

Being a uni-cellular organism yeast is subject to large changes in its growth environment, either due to its own activity, i.e. substrate consumption and product formation, or due to other factors, e.g. temperature changes in night day regimes. Yeast is well adapted to these varying conditions [26].

Central to this adaptation is a variable gene expression leading to the expression of enzymes appropriate to the prevailing nutrient regime; both gene expression and protein turnover are influenced by the environmental conditions [26]. *S. cerevisiae* is a Crabtree positive yeast, i.e. it produces ethanol even under aerobic conditions as long as high glucose concentrations are present [65, 67]. It has been shown that with high concentrations of sugars, the glycolytic flux of *S. cerevisiae* can attain very high levels leading to considerable alcohol production [26].

Common industrial carbon sources like molasses and wort are composed of a mixture of sugars, but glucose is the preferred carbon and free-energy source of *S. cerevisiae* (this phenomenon is called glucose repression)[22, 41, 43]. This results to sequential utilization of other available sources such as fructose, mannose, galactose, maltose, or ethanol, even though simultaneous utilization would make the process shorter and beneficial for commercial production [9, 26, 40].

Transport across the plasma membrane is the first, obligatory step of hexose utilization. A large family of related proteins in yeast cells facilitates the hexose sugar uptake [12, 27, 43]. Research on glucose uptake in the yeast *S. cerevisiae* has focused on the number of hexose transport (HXT) genes, on the characteristics of distinct protein-mediated systems, and on the role of putative transport proteins such as SNF3 [11]. *S. cerevisiae* can deal with an extremely broad range of sugar concentrations. It can effectively metabolise glucose at concentrations as high as 2M in drying fruits down to micro-molar concentrations (Kruckeberg reported a range of 1M – 100μM) [27, 41]. This suggests the presence of highly regulated glucose uptake system to enable yeast to function optimally at a wide range of glucose concentrations [8, 32]. It has been suggested that the yeast transport step of sugar exerts a high level of control on the glycolytic flux [62, 72]. Moreover it has also been postulated that the transport system is a component of a glucose-sensing complex, and maybe directly involved in the initial sensing of glucose by yeast [28].

Genetic, physiological, and biochemical evaluation of glucose uptake in *S. cerevisiae* has shown that the process is complex involving many gene products. There are 18 putative hexose transporter genes HXT1-17 and GAL2, and two hexose sensor genes SNF3 and *RGT2*. Several lines of evidence implicate the HXT family of proteins to be part of a sugar transport super family [11].

It has furthermore been shown that other yeast genes from HXT8-17 (except HXT12) and HXT5 are able to mediate uptake of hexose if overexpressed. This suggests that most of the yeast Hxt proteins are able to transport hexose but some of them are only used under specific conditions and for specific purposes, which are not clear [8].

Genes *HXT*1-4 and *HXT*6-7 encode the major glucose transporters of *S. cerevisiae*[32, 51]. They have been shown to be regulated by glucose concentration [8, 11, 12, 27, 32, 38, 43, 43, 47, 68]. Glucose transport in yeast exhibits dual kinetics, with a high- and a low-affinity kinetic component, the relative concentrations of which depend on the culture conditions. The kinetics observed are the result of the differential expression of the HXT genes, whose products have different affinities for glucose. HXT1 and HXT3 encode low-affinity transporters (K_m=50mM to 100mM), HXT2 and HXT4 encode intermediate-affinity transporters (K_m=10 mM), and HXT6 and HXT7 encode high-affinity transporters (K_m 1mM to 2mM) [11, 12, 27, 32, 38, 43, 43, 47, 68].

Saccharomyces cerevisiae strains expressing different sugar kinases levels show different glucose transport kinetics and transcribe different glucose transporters as determined from mRNA levels [40]. The presence of glucose leads to a variety of responses that ensure its preferential use, via modulation of enzyme activity to repression or induction of genes. A great number of proteins participate in the process of glucose repression, including hexokinase II, which is encoded by the gene *hxt*2. During exponential growth on low glucose concentrations, an *hxk2* null strain exhibited high-affinity hexose transport associated with an elevated transcription of the genes *htx2* and *hxt7*, encoding high-affinity transporters, and a diminished expression of the *hxt1* and *hxt3* genes, encoding low-affinity transporters [47].

Deletion of the *hxt7* gene in the *hxk2* mutant eliminated a substantial proportion of the high-affinity component of glucose uptake during exponential growth on glucose. The double mutant showed a component with very high affinity for glucose (K_m = 0.19 mM) but had a low activity. The activity is thought to be ascribed to *hxt8* to *hxt17*. Petit et al concluded that the very high-affinity component is due to the high level of HXT2 expression observed in the *hxk2* strain grown at high glucose

concentrations. But it is not yet clear if that is the real affinity of the transporter or is just appearing as a result of *hxk2* deletion [47].

2.5 Modeling of microbial growth

Combination of experimental works with mathematical modeling makes it is possible to provide meaningful and quantitative interpretation of the experimental results and also revealing new aspects of microbial physiology. Kinetic models can be constructed at varying degrees of detail, from non-structured (black box) approaches to segregated structural models were each individual is treated separately with detailed intracellular reactions. The question being addressed determines which model is best for a particular study. The growth of microbial cells can be viewed from various perspectives and with varying degree of complexity, depending whether we distinguish between individual cells in a reactor and whether we examine individual metabolic reactions occurring within the cell. While detailed models of growth could consider all the reactions occurring within each cell with variations from cell to cell in a population, such model would be very unwieldy [38]. In the following section we give a brief overview of the different kinds of kinetic models that have been developed for the description of microbial growth with a specific focus on non-structured, non-segregated models.

2.5.1 Classification of models

2.5.1.1 Segregated versus non segregated models

Segregated models treat each cell independently, and a population as a collection of such distinct cells. They describe different morphological types of cells or cell ageing and sometimes describe the interactions between different cells. So when the population is split up into individual cells that are different from one another in terms of some distinguishable characteristic, the model is segregated. The usefulness of segregated models depends on our experimental ability to distinguish between cells in a population, which is often difficult [1, 38], and our understanding of the mechanism leading to these differences, which is often limited.

Non-segregated models treat the culture as a collection of average cells (identical cells), all with the same characteristics at any given time. They interact with the external environment, and can be viewed as one species in solution. The cell concentration can be described by one variable. Non-

segregated models have the advantage that they are mathematically simpler than the segregated models. [1, 17, 38].

2.5.1.2 Structured versus unstructured model

Structured models consider the internal states of the cells. They are called structured-models because they incorporate genetic, morphological, or biochemical attributes that collectively determine the physiological state of the biomass. This class of model has a great potential to describe growth phenomena since trends and responses can be recognized and the changes in the biomass composition measured [24, 31]. Such models are structured on the basis of biomass components such as concentration of metabolites, enzymes, DNA and/or RNA. With these models it becomes possible, for instance, to describe a lag or transient phase [14, 23, 28, 44].

Unstructured models take the cell mass as a uniform quantity without internal dynamics, i.e. the black box approach. Unstructured models view cells as an entity in a solution, which interacts with the environment (i.e. cell reaction rates are only related to biomass concentration and to the environment). The reaction rates depend only upon the microscopic conditions in the liquid phase of the bioreactor. These models view the cell as a single species in a solution and attempt to describe the kinetics of cell growth based on cell and nutrient profiles. Therefore the models only contain kinetics of growth, substrate uptake and product formation. The simplest models describing the relationship between exponential growth, nutrient profile and product formation are unstructured models [33, 11, 13].

2.5.2 Unstructured growth models

The modeling of biotechnological processes began with the equation of Blackman in 1905 [6] and Monod in 1942 [35], which related the concentration of the limiting substrate to the growth rate of the microorganism using simple empirical equations. These equations provide a simplified theoretical basis that accounts for the more specialized cases represented by the commonly used empirical models of unstructured nature. Moser [36] and other many Monod based equations along with empirical parameters appearing in these equations can be estimated via data fitting yet miss mechanistic understanding [38].

2.5.2.1 Blackman model (1905):

Blackman gave one of the earliest overall growth descriptions in 1905 [6]. In the Blackman model it is assumed that at low substrate concentration the rate of uptake is proportional to substrate concentration and at high substrate concentration both uptake and growth rate are independent of the substrate concentration because some other nutrient or intracellular factor is limiting under those conditions [25].

The Blackman model makes a sharp transition from first order to zero-order when substrate concentration exceeds the half-saturation coefficient. It does not allow for a gradual transition from zero-order to first-order kinetics, i.e. the function is not smooth [38].

$$\begin{aligned} \mu &= \frac{\mu_{-\max} \cdot s}{K_{-b}} && if\ s < K_{-b} \\ \mu &= \mu_{-\max} && if\ s \geq K_{-b} \end{aligned} \qquad (2.4)$$

2.5.2.2 Monod model (1942):

The classical approach to modeling microbial growth is derived from the seminal work of Monod, about 50 years ago. The systematic description of bacterial growth and the ideas surrounding chemostat theory [35], (see also [44]) led to the notion that under certain conditions a limited number of growth constants define the behavior of bacterial cultures [15]. Given that few areas of biology were satisfactorily described by mathematical equations, these influential ideas put microbiology on a more satisfying scientific footing.

$$\mu = \frac{\mu_{-\max} . s}{K_{-s} + s} \qquad (2.5)$$

2.5.2.3 Haldane model (1930):

In addition to substrate limitation, inhibition of specific growth rate by substrates, products or biomass is quite often observed in biotechnological processes. Han and Levenspiel [17] give an extensive review of inhibition kinetics. Observations have shown that at high concentrations the substrate can also act as a toxic growth inhibitor. This is taken into account in the Haldane equation. On the bases of the additional inhibition term compared to the Monod equation, it is commonly viewed as an extension of the Monod equation. The Haldane model is the most cited model for inhibition kinetics [38].

$$\mu = \frac{\mu_{-\max} \cdot s}{K_{-s} + s + s^2 / K_{-i}} \qquad (2.6)$$

Growth of *Escherichia coli* on acetate can serve as an example where the Haldane equation for growth can be useful. Under aerobic conditions *E. coli* can use acetate as an carbon and free-energy source but at high concentrations, especially at low pH culture, acetate will become inhibitory due to its uncoupling activity. Since acetate is often produced as a side product in production processes with *E. coli* it is important to be able to incorporate the inhibitory effects in the overall growth rate description [19-20, 33].

2.5.2.4 Tiessier model (1942):

The exponential model (also know as Tiessier model) describes the specific growth rate as a continuous function of the substrate concentration like the Monod equation but the transition to saturating concentration is sharper than in the Monod equation. Bader [2] concluded that most of the published kinetics data fall between the curves for the Blackman model and the exponential model (Tiessier), which in turn lies in between the Blackman and Monod model description. [23, 61].

$$\mu = \mu_{-\max}(1 - e^{K_{-T} \cdot s}) \qquad (2.7)$$

2.5.2.5 Moser model (1958) and Contois model (1959):

The Moser and Contois models are derivations from the Monod equation developed in the 1950s, these models were never very popular but are occasionally used in experimental studies [36,10, 50,56]. The inclusion of a third parameter in the Monod equation as shown in equations 2.8, and 2.8, such as the parameter *n* in equation 2.8, or introduction of the *X* term as biomass in 2.9, did lead to a predictable improvement of fit of the equations to experimental data compared to a fit with the Monod equation [23].

$$\mu = \frac{\mu_{-\max} . s^n}{K_{-s} + s^n} \qquad (2.8)$$

[Moser, 1958]

$$\mu = \frac{\mu_{-\max} . s}{K_{-s} . X + s} \qquad (2.9)$$

[Contois, 1959]

2.5.2.6 Logarithmic model

The logarithmic model proposed by Westerhoff [69] also describes the specific growth rate, as a continuous function of the substrate concentration like the Monod equation but the transition to saturating concentration is less sharp than in the Monod equation. Senn H et al. [54] concluded that from the series of alternative models proposed in the literature the growth model proposed by Westerhoff is one of the very few that yields fits comparable in quality to the fits obtained for the Monod model. Their study showed that the logarithmic model fitted their data points slightly better than the Monod model [53]. The equation 2.10 model describes growth rate as a function of the logarithm of the substrate concentration, where b and a are parameters of the model. However, because the logarithmic model assumes an exponential dependence of cell growth on a single limiting substrate, it does not show any maximum specific growth rate for a cell, as a result one cannot define the *Ks* constant.

$$\mu = a + b \cdot \ln(s) \qquad (2.10)$$

At very low substrate concentrations this model would predict a negative growth rate, and the generally used kinetic descriptions always give a positive growth rate. However, Westerhoff et al. in his review concluded that there is no reason to consider logarithmic dependence of growth on

substrate concentration as an approximation to reality that is inferior to the more commonly used hyperbolic dependence [69].

The methods and models discussed in this chapter will be used in the subsequent chapters. They will be used to verify the hyperbolic dependence of *S. cerevisiae* growth rate on substrate. Subsequently we will formulate a mechanistic understanding for the affinity constant (K_s) of Monod, as it is the most commonly used kinetic description of growth.

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CHAPTER 3

3 Materials and Methods

3.1 Yeast culture methods

3.1.1 Yeast strain

Saccharomyces cerevisiciae wild type VIN13 strain was used in all cultivation experiments. It is a strain that is mainly used for wine making and was kindly donated by Department of Wine-biotechnology, Stellenbosch University.

The stock cultures were prepared by adding overnight shake-flask cultures to a sterile glycerol solution (final concentration 40 % (v/v)). One-milliliter vials of the stock were stored at –80^{o}C.

YPD agar plates (OXOID yeast extract, 1% (m/v); OXOID neutralized bacteriological peptone, 2% (m/v); D- (+)-glucose (dextrose), 2% (m/v) and Difco agar, 1.5% (m/v)) were prepared and used to inoculate from the frozen stock cells. The plates were incubated at 30^{o}C overnight. Subsequently they were stored at 4^{o}C for no longer than 6 weeks.

3.1.2 Culture medium

The carbon-limiting mineral medium contained the following (per liter): $(NH_4)_2$ SO_4, 5g; KH_2PO_4, 3g; $MgSO_4.7HO_2O$, 0.5g; EDTA, 15mg; $ZnSO_4.7H_2O$, 4.5mg; $CoCl_2.6H_2O$, 0.3mg; $MnCl_2.4H_2O$,1mg; $CuSO_4$.$5H_2O$, 0.3mg; $CaCl_2.2H_2O$, 4.5mg; $FeSO_4.7H2O$, 3mg; $NaMoO_4$.$2H_2O$, 0.4mg; H_3BO_3, 1mg; KI, 0.1mg. The final vitamin concentrations per liter were as follows: biotin, 0.05 mg; calcium pantothenate, 1mg; nicotinic acid, 1mg; inositol, 25mg; thiamine. HCl, 1mg; pyridoxine. HCl, 1mg; para-aminobenzoic acid, 0.2 mg.

The medium was sterilized by autoclaving at 121^{o}C for one hour together with vitamins. 20% stock glucose solution was prepared and sterilized separately by autoclaving at 121^{o}C for 15 minutes to avoid caramelization.

3.1.3 Pre-culture preparation

The above-described mineral medium with 2 % glucose concentration was used to incubate cells overnight in a shaking incubation (250 rpm) at 30^{o}C. The microscope was used to check for purity of the overnight-cultivated cells before inoculation to the chemostat.

3.1.4 Chemostat conditions

Chemostat cultures were run using New Brunswick Scientific, model Bioflo 110 fermenters (New Brunswick Scientific Co., Inc, New Jersey). The agitator speed was set to 250 rpm and temperature was controlled at 30^{o}C. The working volume was kept at 650ml and 700ml by a peristaltic pump. The pH was kept at 5.5+/-0.1 by a Bioflo 110 biocontroller (New Brunswick Scientific Co., Inc, New Jersey), via automatic addition of 1M NaOH.

The culture was maintained aerobic by constant flush with air at a flow rate of 20 l/h using a Brooks 5850E mass flow controller (Brooks Instrument BV, Veenendaal, The Netherlands). The dissolved O_2 concentration was monitored with a Metler Toled dO2 electrode Model InPro6110/160 (New Brunswick Scientific Co., Inc, New Jersey) and kept above 60% dO_2 saturation.

Carbon limiting medium was used with a glucose concentration of 1mM or 2mM. After inoculating the cells were first allowed to grow under batch conditions, subsequently when cells were glucose negative, the medium pump was switched on. A steady state was defined as the situation in which at least five volume changes had passed after the last change in growth conditions. This was verified by measuring the optical density of subsequent samples for a particular steady state dilution rate, and showed variation that was less than 5%. The chemostat cultivations were not allowed to run for more than a month to avoid working with mutated cells.

3.2 Sampling method

After establishment of a steady state, samples were taken and analyzed for biomass and residual glucose concentration.

A looping system was designed in a chemostat so as to remove the dead volume in the tube. For purity and biomass determinations cell were withdrawn from the chemostat into an empty vial. However, a sample for residual glucose was withdrawn from the chemostat into a vial with 10 ml of chilled 10% perchloric acid (PCA) in an approximate ratio of one part sample and one part PCA. The 50ml syringe connected to sterile filter was used to first create under-pressure before withdrawing to allow a rapid sampling. The exact amounts were determined by weighing the vial with the PCA before and after the sample.

The samples were then stored at 4^{o}C until further use. Before sugar determination the quenched samples were neutralized by slowly adding 5ml of 2 M K_2CO_3 and incubated on ice for 15 minutes. The neutralized sample was centrifuged in an Eppendorf Centrifuge 5804R at 20800 x *g* and 4^{o}C for 10 minutes to remove the precipitated salts and proteins.

Sampling required withdrawal of approximately 20 ml of liquid from the bioreactor, which corresponded to 3% of the culture volume. To account for the resulting disturbance in dilution rate individual samples were usually separated by 2 volume changes (3).

3.3 Biomass determination

Low biomass concentrations were obtained by cultivating cells at low medium glucose (energy-source) concentrations. Rather than investigating dry weights, which would require a large sample volume, optical density was measured at 600nm using a Jenway model 6110 spectrophotometer (South African scientific products, South Africa).

3.4 Residual glucose concentration determination

3.4.1 Preparation of buffer with coenzymes

A solution of triethanolamine hydrochloride was prepared with following concentrations: (MERCK), $MgSO_4.7H_2O$ (Saarchem (Pty) Ltd), NADP disodium salt (Boehringer Mannheim Gmbh –Germany), and ATP (Boehringer Mannheim Gmbh –Germany) to prepare a solution with the following final concentrations: 0.89M Tris-HCl; 0.01 M $MgSO_4.7H_2O$; 1.27 mM NADP and 8.26 mM ATP with the pH of 7.6. The solution was stored 4^{o}C [3].

3.4.2 Standard curve preparation

A range of dilutions was prepared from a fresh prepared 0.11mM glucose solution (5X; 2.5X; 1.67X 1.25X and 1X to a final volume of 333.3µl in a 1.3 ml cuvet). Then 333.3µl of milli-Q water and 333.3µl buffer with coenzymes (NADP and ATP) were added. A blank cuvet was prepared by mixing 666.6µl of milli-Q water with 333.3µl buffer in a cuvet.

3.4.3 Measuring for standard curve

Helios epsilon spectrophotometer (Spectronic Unicam, USA.) was used to measure the absorbance at 340nm before and after adding 7µl of a hexokinase/glucose-6phosphate dehydrogenase enzyme mix with a concentration of 340U hexokinase/ml at 25^{o}C with glucose and ATP as substrates and 170U glucose-6phosphate at 25^{o}C with glucose-6phosphate and $NADP^{+}$ as sustrates. The enzyme mix was added and reactions were allowed to run to completion by taking the 340nm absorbance reading after 20 minutes.

3.4.4 Samples

A volume of 333.33µl of the sample with 333.3µl milli-Q water and 333.3µl buffer with coenzymes was mixed in a 1.2 ml cuvet. The absorbance reading was recorded before and after the enzyme was added and allowed to stabilize.

3.5 Parameter estimation

The obtained residual glucose concentrations at varying dilution rates were analysed in a Lineweaver-Burk plot, Hanes-Wolff plot, Eadie-Hofstee plot, and direct-linear plot and by non-linear estimation using Mathematica. The data was transformed to estimate growth parameters i.e. K_s and μ_{max}. Westerhoff model, Blackman model and Teissier model parameters were estimated by non-linear regressions using Mathematica 5.0 software.

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CHAPTER 4

4 Experimental Results

4.1 Residual glucose concentration and biomass as a function of dilution rate

S. cerevisiae VIN13 was grown in glucose limited chemostat cultures. Glucose served as the carbon and free-energy source with a medium concentration of 1mM or 2mM. Low glucose concentrations were selected in order to obtain low biomass concentrations. It was crucial that the sampling method for the residual glucose concentrations was reliable, because measuring residual glucose concentrations was the core of this study. High biomass concentrations were avoided as they could easily result to significant changes of the residual glucose concentration during sampling. The 1mM resulted to cell washout for cells that were set to grow at high dilution rates (from $0.35h^{-1}$), and for that reason the glucose concentration in the medium was increased to 2mM. The steady state residual glucose concentration was measured at various dilution rates. As shown in figure 4.1 the measured residual glucose increased with increasing dilution rate. Doubling the glucose medium concentration did not affect residual glucose values. The medium glucose concentration was chosen such that a low biomass was obtained. Optical density was measured to estimate the biomass concentration. Biomass decreased when dilution rate was increased. At high dilution rates, a doubling of glucose medium concentration was expected to result to a biomass concentration change by more than a factor of two. This results because doubling the glucose medium concentration does not change the residual glucose concentration but increase the internalised glucose concentration. When the glucose medium concentration was doubled the internalised glucose concentration was increased of two. However, a doubled biomass concentration was observed as shown in figure 4.2.

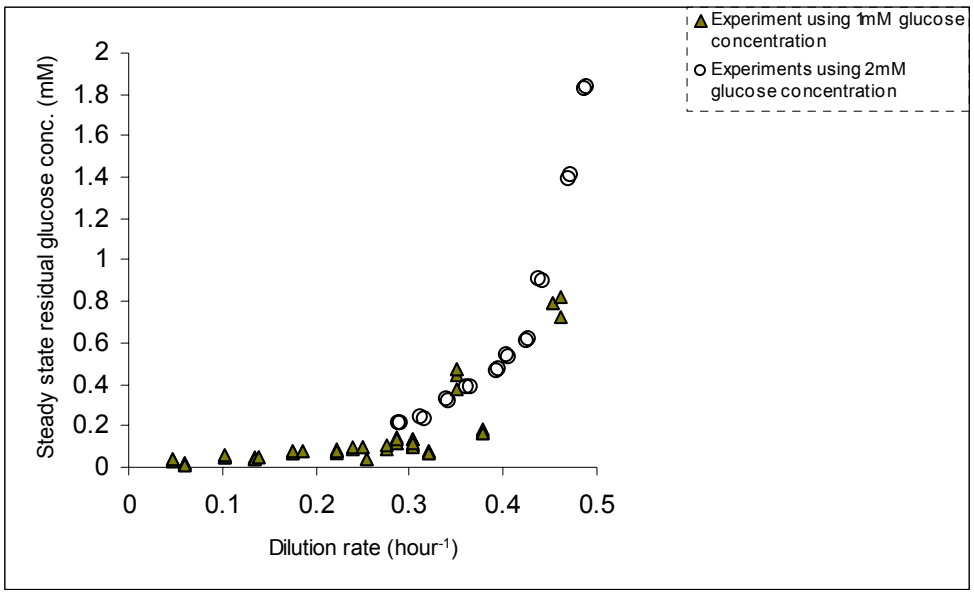

Figure 4.1: The effect of dilution rate on the residual glucose concentration. At steady state the specific growth rate is numerically equal to the dilution rate. This graph shows an observed increase of residual glucose with increasing dilution rates. A doubling of reservoir glucose concentration did not change residual glucose concentrations but was necessary to achieve steady states at high dilution rates. Data collected over 13 independent chemostat sets are shown. Points are single measurements.

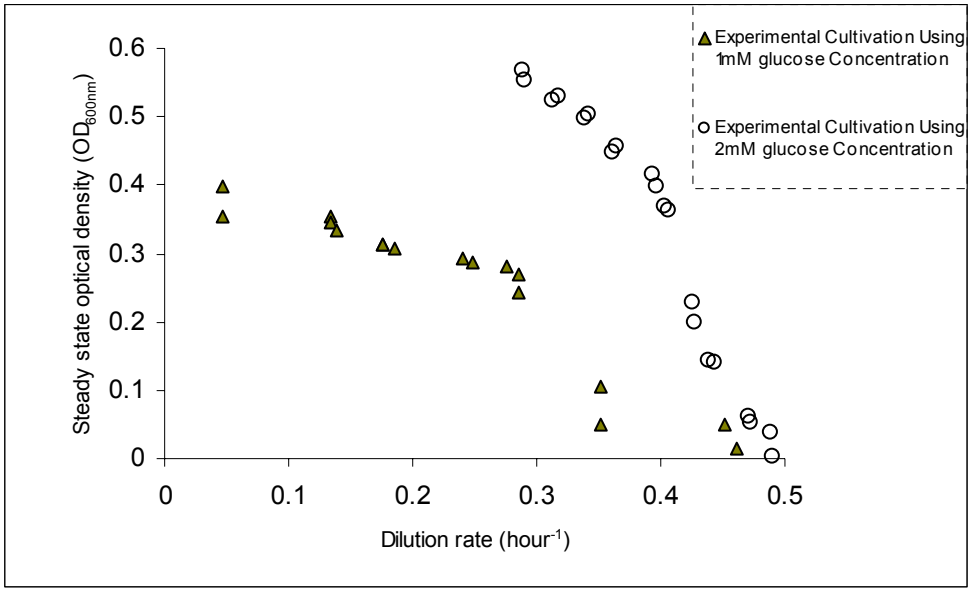

Figure 4.2: Steady state biomass concentration as a function of the dilution rate and medium glucose concentration. Decreasing biomass concentrations were observed with increasing steady state dilution rates. As expected a significant increase in biomass concentrations was observed when feeding glucose concentrations were doubled.

4.2 Stoichiometric parameters

For many applications, growth and degradation processes can be described satisfactory with three parameters; K_s, μ_{max} and the stoichiometric parameters $Y_{x/s}$ (growth yield) [6]. Growth yield quantifies the nutrient required by an organism for biomass formation. Monod showed that if a bacterial population were limited solely by the amount of carbon source available, the biomass concentration produced is proportional to the carbon source utilised [8]. And when the conditions are maintained constant, the growth yield is a constant, reproducible quantity [9].

In this study *S. cerevisiae* was cultivated with 1mM or 2mM glucose concentration under carbon-limited chemostat conditions, and conditions were maintained constant. Growth yields were calculated from the residual glucose concentrations and biomass optical densities measured at each steady state dilution rate. Contrary to what Monod postulated, the growth yield was not constant as shown in figure 4.3.

The observed bilinear change of biomass yields was reflected in the rate of glucose consumption, $Y_{-x/Glc} = \mu / q_{-Glc}$. Glucose uptake rates ($q_{-Glc}$) were calculated for different steady state dilution rates. When glucose uptake rate was plotted against dilution rate, the bilinear increase of glucose uptake was observed, as shown in figure 4.4. *S. cerevisiae* is a Crabtree positive yeast and is known to ferment glucose to ethanol, even under aerobic conditions at high external glucose concentrations. Our results suggest that above a dilution rate of $0.4h^{-1}$ a significant amount of glucose is fermented to ethanol, the lower efficiency of this pathway with respect to ATP formation can account for the increase in glucose consumption rate.

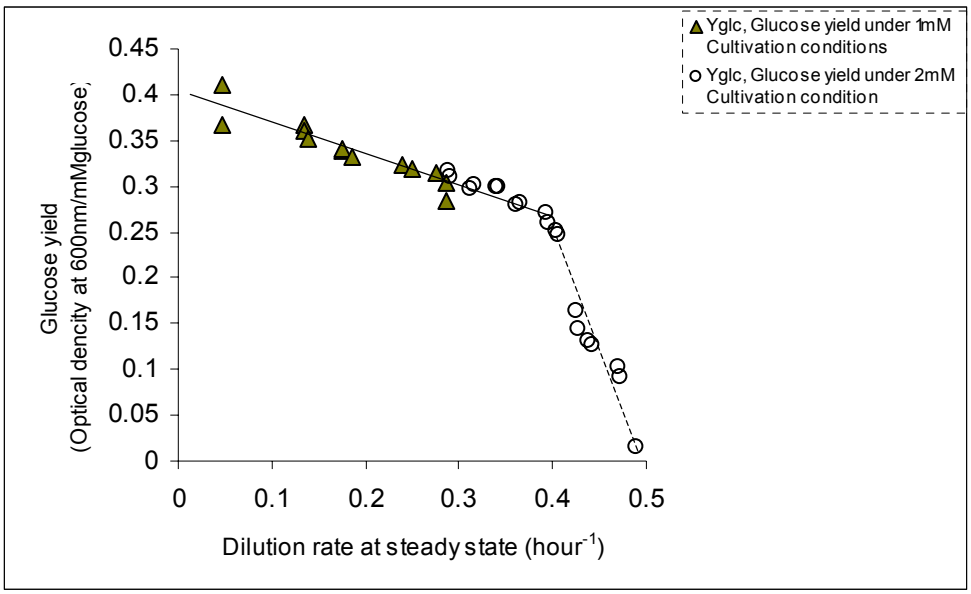

Figure 4.3: The change in biomass yield on glucose at different steady state dilution rates. Declining glucose yields were observed as steady state dilution rates were increased.

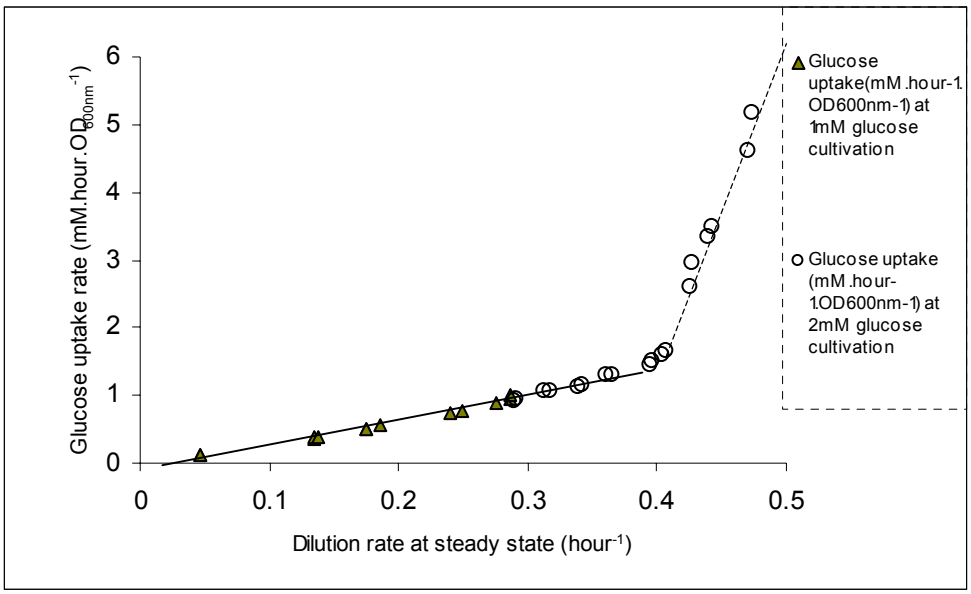

Table 4.4: The effect of changing the steady state dilution rates on glucose uptake rate. An increase in glucose uptake was observed when steady state dilution rates were increased from 0.05hour^{-1} to 0.49hour^{-1}

4.3 Monod parameters estimations

The Monod equation is often used for the description of microbial growth. Here it was investigated how well this equation can describe the data obtained in glucose limited chemostat cultures of *S. cerevisiae* VIN13 at different dilution rates. The best way to analyse microbial kinetic data would be to fit the data directly to the Monod equation using non-linear regression (by plotting substrate concentration against the specific growth rate of the micro organism) as shown in figure 4.5. Popular alternative ways to estimate half saturation constant (K_s) and maximum specific growth rate (μ_{max}) are via linear transformations. Four different linear transformation plots (direct-linear plot, Hanes Plot, Eadie-Hofstee plot, Lineweaver-Burk plot) were used as shown from figure 4.6 to figure 4.9 [2,4].

Five plots were used to estimate Monod parameters that could best fit the experimental data. Due to different nature of these transformation plots the estimated μ_{max} and K_s were slightly different. Table 4.1 lists the fitting results for the estimated parameters. Figure 4.10 shows how the Monod parameters (K_s and μ_{max}) obtained via different transformation plots could estimate the data. The estimation of residual glucose

concentrations at high dilution rate differed for parameters obtained from one-estimation plot to the other. It was deduced from the sum of squares of the difference between experimental values and model prediction (SSR) that the non-linear fit gave the best estimation and of the linear methods the direct linear plot was the best. This was in agreement with the theoretical understanding that the best way to analyze microbial kinetic data would be to fit the data directly to the Monod equation using non-linear regression software (by plotting substrate concentration against microorganism specific growth rate). The direct linear plot has been proposed to be the best of the alternative linear forms of rectangular hyperbolic equations such as the Monod equation or the Michaelis-Menten equation. This is because the direct linear plot is a non-parametric method and therefore does not assume a statistical distribution. This makes it particularly robust and insensitive to outliers [2, 4].

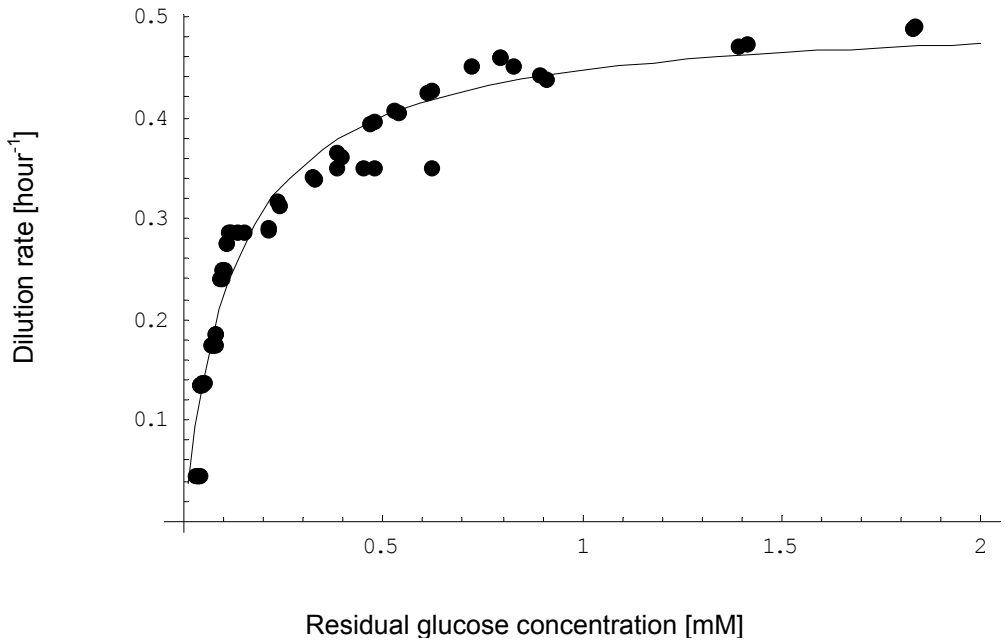

Figure 4.5: Non-linear estimation of Monod growth parameters (K_s and μ_{max}). Mathematica fitting software was used to estimate the K_s value of 0.13mM and μ_{max} 0.50h^{-1}.

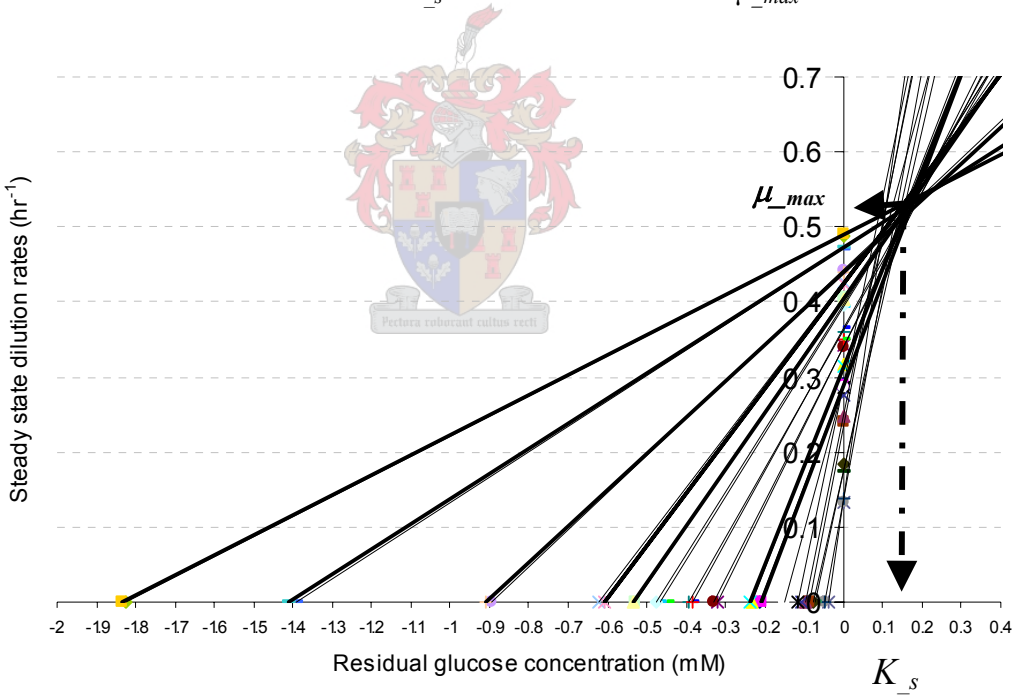

Figure 4.6: Linear transformation of data using the direct linear plot to estimate Monod growth parameters. Microsoft Excel was used to show how the direct linear method fitted the obtained data and estimated the K_s value of 0.13mM and 0.51h^{-1} μ_{max} [2,4]. The bold lines appeared as a result of more than one line drawn from similar coordinate values.

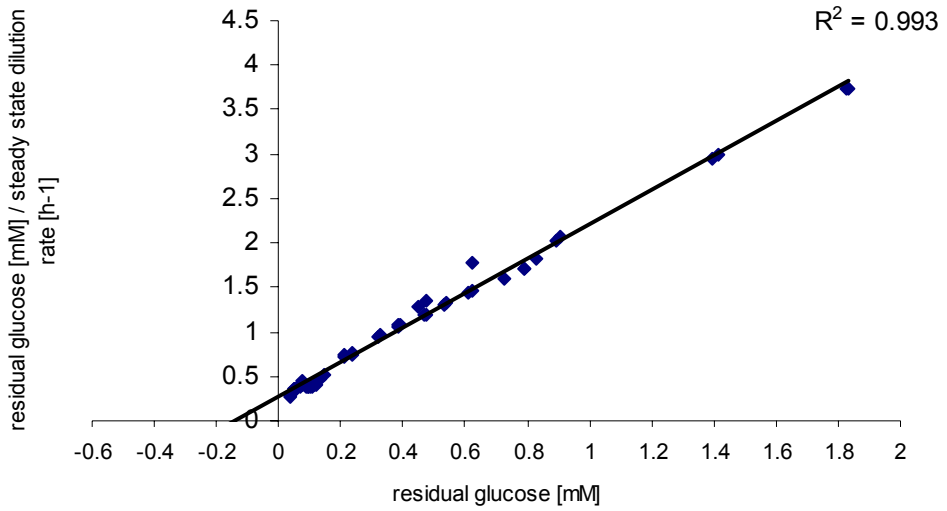

Figure 4.7: Linear transformation of data using Hanes plot (Woolf plot) to estimate Monod growth parameters. Using this transformation plot the K_s value of 0.12mM and μ_{max} value of 0.50h^{-1} were estimated [2,4].

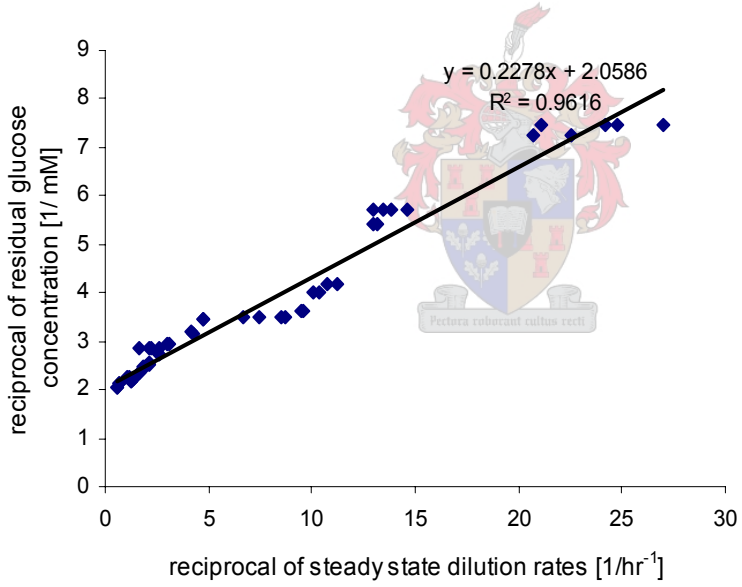

Figure 4.8: Linear transformation of data using Lineweaver-Burk plot to estimate Monod growth parameters. Using this transformation plot the K_s value of 0.11mM and μ_{max} value of 0.49h^{-1} were estimated [2,4].

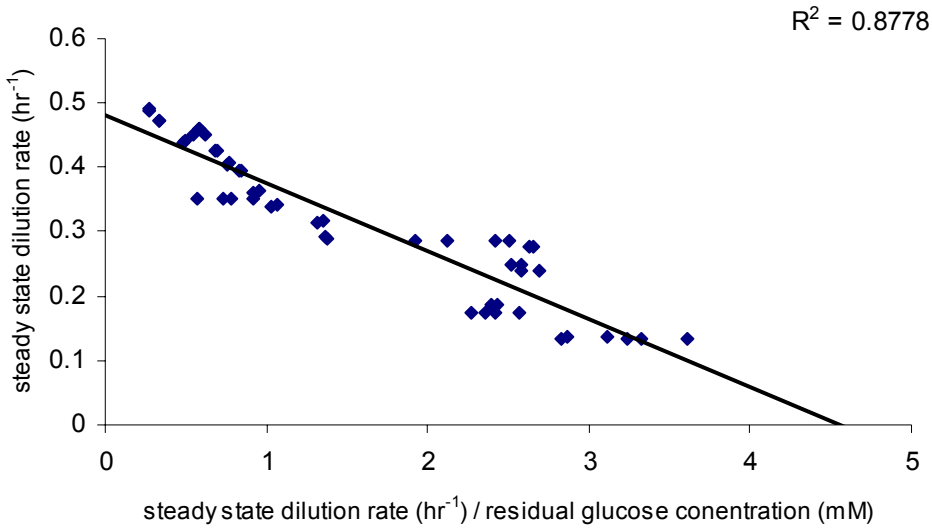

Figure 4.9: Linear transformation of data using the Eadie-Hofstee plot to estimate Monod growth parameters. Using this transformation plot the K_s value of 0.11mM and μ_{max} value of 0.48h^{-1} were estimated [2,4].

Table 4.1: The μ_{max} and K_s parameter values obtained via the different estimation plots.

Parameter	Non-Linear estimation Plot	Direct linear Plot	Hanes / Woolf Plot	Lineweaver – Burk Plot	Eadie-Hofstee Plot
μ_{max} (h^{-1})	0.50	0.51	0.50	0.50	0.50
K_s (mM)	0.13	0.13	0.12	0.11	0.11
Sum of R residual squares	0.04	0.04	0.05	0.05	0.05

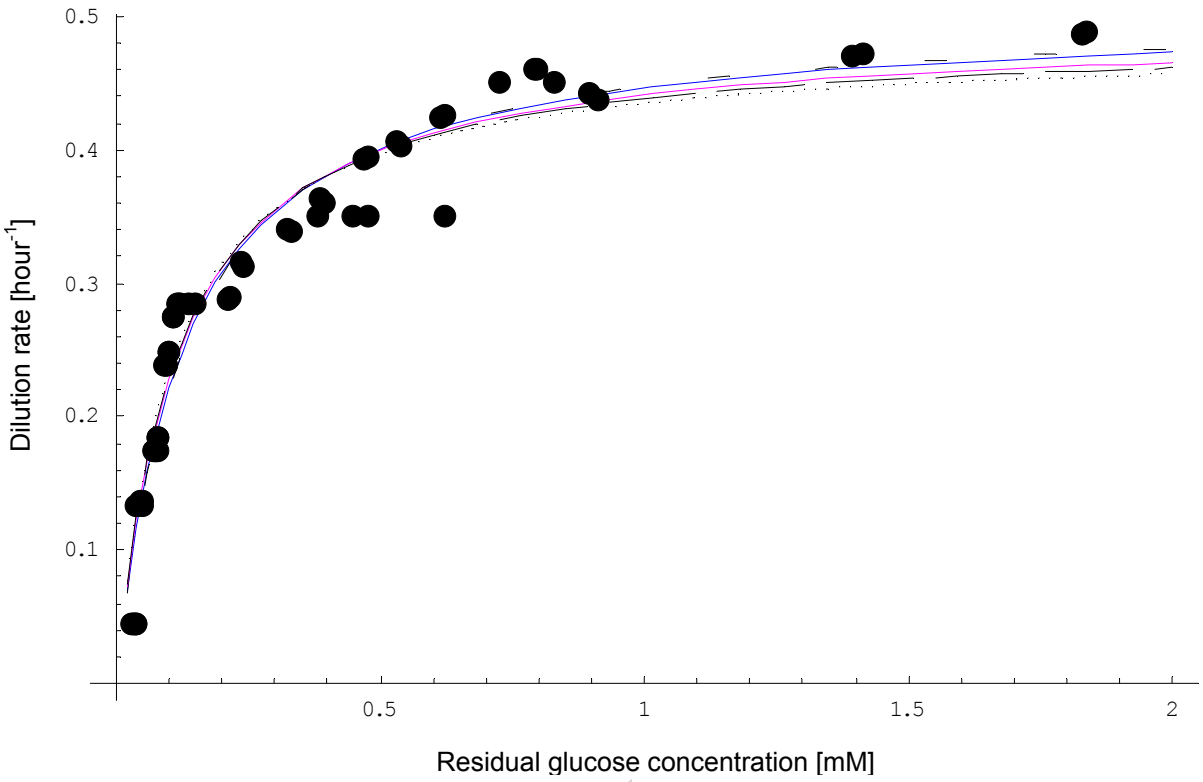

Figure 4.10: Monod predictions of the steady state glucose concentrations at different dilution rate of *S. cerevisiae* in carbon limited chemostat culture using K_s and μ_max estimated from five different estimation plots. (————) Non-linear estimation, (----------) Direct-linear plot, (–··–··–··–··) Hanes plot, (————) Lineweaver – Burk and (··········) Eadie-Hofstee. ● Experimentally measured steady state glucose concentrations.

4.4 Model predictions of the steady state residual glucose concentrations at different dilution rates.

The predictions of the experimentally determined steady state glucose concentrations by various unstructured growth models were compared as shown in figure 4.11. Only the models that relate μ to substrate concentrations were fitted such as; Monod, Teissier, Blackman and logarithmic growth model. Non-linear fitting was used to estimate the kinetic parameters of the represented models (table 4.2). The quality of the fit to the experimentally determined data points was evaluated by quantifying SSR (sum of squares of the deference between experimental values and model prediction). The estimated K_s for all four models were similar, but the estimated μ_max varied from one model to the other (table 4.2).

The RSS were used to determine which model best fitted the data points. The Westerhoff model showed a slightly better fit than Monod. The residuals of model fit were plotted against the dilution rate to follow the predictions of the models (figure 4.12). The distribution of residuals showed that Blackman estimated the data relatively good below the dilution rate of $0.3h^{-1}$. Above 0.3 h^{-1} up to 0.44 h^{-1} Westerhoff model provide a good estimation and Monod could best estimate the flattening of growth rate i.e. above 0.4 h^{-1}.

Table 4.2: Comparison of estimated microbial growth parameters ($K\alpha$ and μ_{max}) of four different unstructured models. The *S. cerevisiae* VIN13 affinity constant is represented by $K\alpha$ while maximum specific growth rate is represented by μ_{max}.

Types of models	**Saturation constant, K_{α} (mM)**	**Maximum growth rate constant, μ_{max} (h^{-1})**	**Sum of R squares**	**Estimation plots**
Monod model	0.13	0.50	0.044	Mathematica 5.0 Software
Tiessier model	0.14	0.43	0.07	Mathematica 5.0 Software
Blackman model	0.17	0.40	0.12	Mathematica 5.0 Software
Logarithmic model	0.11	0.46	0.043	Mathematica 5.0 Software

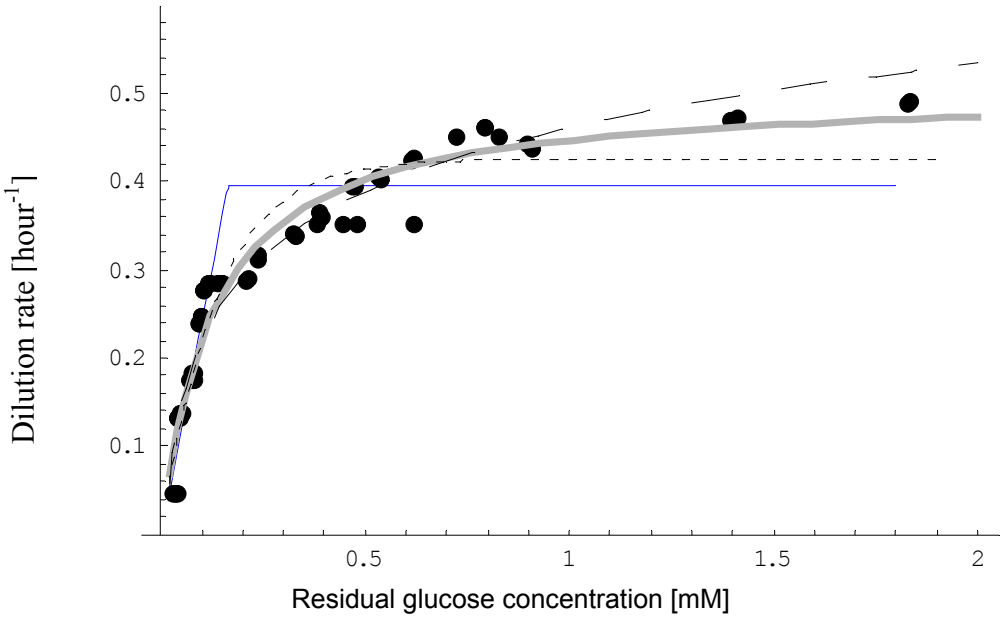

Figure 4.11: Predictions of different models for the steady state glucose concentrations at different dilution rate of S. cerevisiae in carbon limited chemostat culture. (————) Monod, (————) Blackman, (·····) Tiessier, (----------) Westerhoff model. All fits were minimized for SSR. ■ Experimentally measured steady state glucose concentrations.

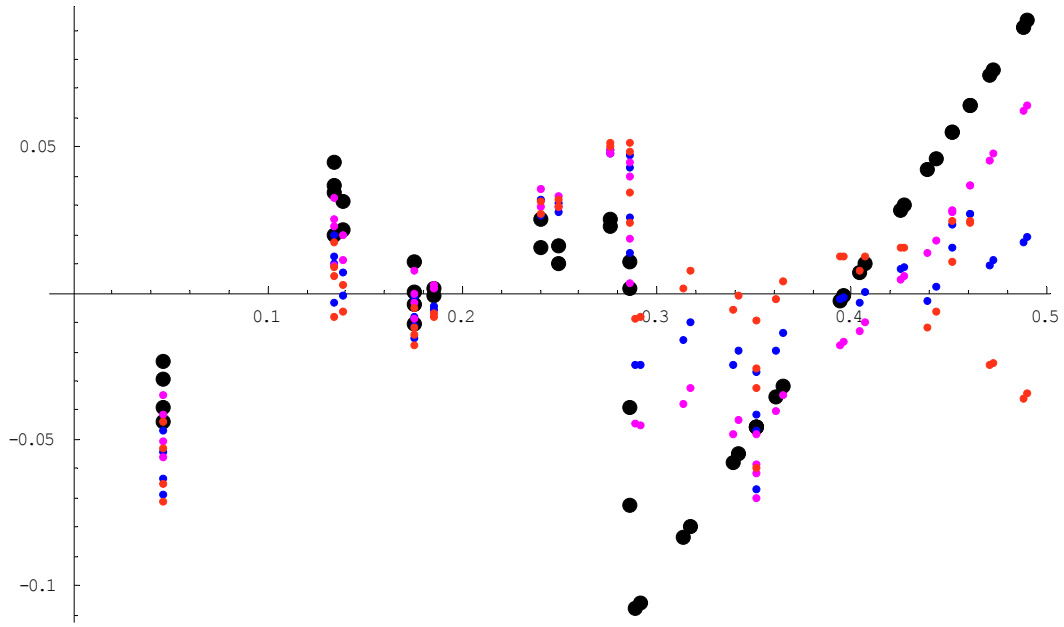

Figure 4.12: Representation of fit residuals for different model. Each dot shows the deviation of the estimated μ from the experimental μ. Monod is represented by blue dots, Tiessier by pink, Blackman model by black dots, and the logarithmic model by red dots.

4.5 Relating K_s of *S. cerevisiae* on glucose to K_m of the glucose transporter

The Monod is empirical and no mechanistic interpretation can be given for the saturation constant K_s. However in several studies the Monod constant has been used as if it is equal to K_m of the transporter of the limiting substrate ([5], also noted by [6]). Such confusion might stem from the analogy of the two equations. As shown in table 4.3 the estimated K_s is approximately ten-fold lower than K_m of the highest affinity transporter determined in any *S. cerevisiae* strain. K_s values that are lower than K_m values of the limiting substrate transporter have also been found in other studies (Table 4.3). Integration of theory and kinetic modeling will be used to gain insight into the relation between K_s and K_m in chapters 5 and 6.

Table 4.3: Comparison of Monod affinity constant obtained from our study with those obtained from other studies in relation to transporters affinity constants obtained by means of transport assays.

Strain	**Cultivation Conditions**	**Maximum specific growth rate, μ_{max} (hr^{-1})**	**Monod Constant, K_s (mM)**	**Transporter affinity constant, K_m (mM)**	**Reference**
S. cerevisiae VIN13	Aerobic glucose-limited chemostat, 2mM	0.51	0.11	Not measured	Current study
S. cerevisiae CBS 8066	Glucose-limited chemostat, 83,3mM	0.49	0.11 0.39	1 20	1
S. cerevisiae MC996A	Batch cultivation 2mM glucose	Not measured	Not measured	1–2	7
S. cerevisiae CEN.PK113-7D	Aerobic glucose-limited chemostat cultures at various dilution rates.	Not measured	Not measured	***D*** **(h^{-1})** / **Value (mM)** 0.05 / 1.6 0.1 / 1 0.15 / 0.78 0.2 / 0.76 0.25 / 0.74 0.28 / 0.64 0.3 / 1 0.33 / 0.73 0.35 / 0.73 0.38 / 2.1	3

4.6 References

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CHAPTER 5

5 Theoretical Approach

5.1 Introduction

In a microbial cell there are thousands of processes that occur simultaneously. It is necessary that the rates at which these processes run be well adjusted to each other to maintain cell fitness. In order to understand these adjustments cell physiologists study control and regulation of these processes at cellular and molecular levels. The complexity of integrating molecular and cellular levels has led to the development of methods strongly relying on mathematics, for example metabolic control analysis (MCA).

MCA provides both a strategy of investigating control of metabolic processes and a quantitative description of steady state conditions. In what way does MCA provide a quantitive description of steady-state conditions? MCA provides a quantitative description of the degree of control that any step has on a steady-state variable and how this relates to the local kinetics of all the steps (measured as elasticities). MCA enables local kinetic properties of enzymes to be related to their global properties, with respect to control of variables such as flux and intermediary metabolite concentrations. It is important to distinguish between parameters and variables. Parameters are those factors that are set by the experimenter (temperature, pH, clamped substrate concentrations) or by the system itself (K_m, K_i and V_{max}) and are constant during the course of the experiment. Variables are those factors that attain a constant value only when a system attains a steady state. Flux (J) and concentrations of intermediary metabolites are the most important variables; variables are controlled and can have no control themselves.

Formulating a pathway of a metabolic process enables the MCA concept to be understood relatively easy. Figure 5.1 is a schematic representation of a general microbial chemostat cultivation process and will be referred to as an example throughout the chapter.

Studying a metabolic pathway with the aim to either understand what regulates it and how is it regulated or even to increase its flux often provokes a traditionally asked question, such as 'which enzyme is rate limiting'? MCA shows that the contribution of an individual enzyme to the control of the flux through a pathway is a systemic property that can be expressed in quantitative terms as the ratio of the relative changes in flux to the relative change in the activity, $v_{\_i}$ of the step that caused the change in flux. This dimensionless number is called flux-control coefficient ($C^J_{v_{\_i}}$) of step *i*. The flux control coefficient $C^J_{v_{\_i}}$ (or response coefficient R^J_s) quantifies the percentage change in steady state value of the systemic flux (*J*) upon a 1% of a particular enzyme activity v_i, (or *S*, external (starting) substrate concentration) defined as:

$$C^J_{v_{-i}} = \left(\frac{\frac{dJ}{J}}{\frac{dv_{-i}}{v_{-i}}} \right)_{ss} \equiv \left(\frac{d \ln J}{d \ln v_{-i}} \right)_{ss} \qquad \text{Or} \quad 5.1 \qquad R^J_s = \left(\frac{\frac{dJ}{J}}{\frac{dS}{S}} \right)_{ss} \equiv \left(\frac{d \ln J}{d \ln S} \right)_{ss} \qquad 5.2$$

The change in enzyme activity v_i, is brought about by changing parameters that affect that activity. The sum of the flux control coefficients of the enzymes in a pathway equals 1. This relationship is referred to as the flux summation theorem, implying that if the flux control coefficient of an enzyme is less than 1 other enzymes must have control too. The system can also be understood in terms of the control of an intermediary metabolite concentration by enzyme activities; this is called a metabolite concentration-control coefficient. The metabolite concentration-coefficient ($C^X_{v_{-i}}$) quantifies the percentage change in the steady state value of an intermediary metabolite concentration ([*X*]) upon a 1% change of a particular enzyme activity v_i as defined below. The sum of metabolite concentration-coefficients of the enzymes in a pathway equals zero.

$$C^X_{v_{-i}} = \left(\frac{\frac{dX}{X}}{\frac{dv_{-i}}{v_i}} \right)_{ss} \equiv \left(\frac{d \ln X}{d \ln v_{-i}} \right)_{ss} \qquad 5.3$$

Enzyme activities can be affected by concentrations of metabolites (i.e. substrate, products), and MCA describes these interactions in terms of elasticity coefficients or elasticities. The elasticity ($\varepsilon^{v_i}_x$) quantifies the percentage change in enzyme activity v_i upon a 1% change in an effector *X*.

$$\varepsilon_x^{v_i} = \left(\frac{\frac{\partial v_i}{v_i}}{\frac{\partial x}{x}} \right) \equiv \left(\frac{\partial \ln v_i}{\partial \ln x} \right) \qquad 5.4$$

Control coefficients and elasticity coefficients respectively describe quantitatively the global and local properties of the metabolic system. These coefficients are related by so-called connectivity theorems. Response coefficients can be thought of as quantifying the control by an external effector on the system.

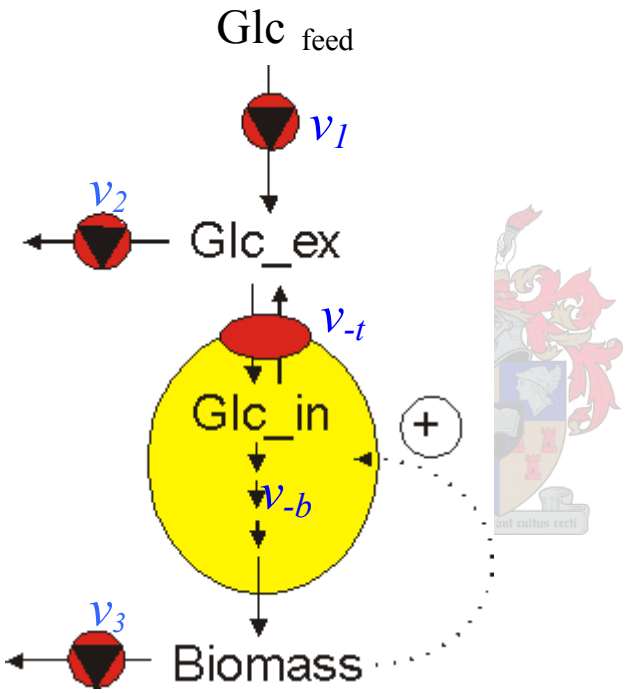

Figure 5.1: Shows a schematic representation of a chemostat cultivation that is used for theoretical derivations. The feed rate is represented by v_1 and the washout rate represented by v_2 and v_3 were linear dependent on a pump speed. The glucose uptake rate is represented by $v_{\_t}$, and the residual glucose and intracellular glucose represented by $Glc_{\_ex}$ and $Glc_{\_in}$ respectively. The rate at which intracellular glucose is metabolized to synthesize biomass is represented by v_b

5.2 Theoretical argument

The Monod equation is often used for the description of microbial growth. This model describes the specific growth rate (μ) of a microbial population as a function of the substrate concentration (S) via two kinetic parameters, μ_max (maximum specific growth rate) and K_s (half saturation constant, often referred to as substrate affinity constant). The interpretation of μ_max as maximum specific growth rate is straightforward, whereas the biological meaning of

K_s is less obvious. The Monod equation is mathematically analogous to the formula that was proposed by Michaelis and Menten to describe enzyme kinetics. However, the two are different in the sense that the Monod equation describes the kinetics of a system constituted by thousands of enzymes, while the Michaelis Menten equation describes the kinetic of a single enzyme.

Can we relate the half saturation constant (K_s) of *S. cerevisiae* VIN13 to the Michaelis constant (K_m) of the *S. cerevisiae* glucose transporter? In this chapter we will define assumptions under which *Ks* can be related to K_m of the transporter. The approach will be made explicit for the system shown in figure 5.1, and is designed in such a way that it represents the experimental set-up.

The rate at which the culture is diluted and is washout is linearly dependent on the pump speed, and denoted by v_1 and v_2 respectively on figure 5.1. On the derived equations below these two rates are both represented as a function of (p). The net glucose uptake rate by the transporter is denoted by v_t and biomass formation rate denoted by v_b. Using MCA, specific growth rate (μ) is described as a function of the pump rate. In order to understand the response of μ to the pump, it is essential to follow the effect a pump has on properties that contribute to cell growth; such as residual glucose concentration, transporter activity, biomass concentration and the activity of the enzymes involved in biomass formation. So the response of μ to the pump can be quantified following the response of residual glucose concentration (Glc_ex) to the pump (p) and the subsequent effect of Glc_ex to the transporter activity (v_t) and how v_t will affect the yeast growth. In addition the response of biomass concentration (*Biomass*) to p, and the effect of biomass on v_t and v_b and the control of these enzyme activity on growth have to be taken into account:

$$R_p^{\mu} = \left(R_p^{Glc\_ex} \cdot \varepsilon_{Glc\_ex}^{v_t} \cdot C_{v\_t}^{\mu}\right) + \left(R_p^{Biomass} \cdot \varepsilon_{Biomass}^{v\_t} \cdot C_{v\_t}^{\mu}\right) + \left(R_p^{Biomass} \cdot \varepsilon_{Biomass}^{v\_b} \cdot C_{v\_b}^{\mu}\right) \qquad 5.5$$

The response of specific growth rate (μ) to the pump is expressed as the sum of three products. The first product is the response of substrate to the pump ($R_p^{Glc\_ex}$) of a flux-driven system multiplied by the effect of the substrate to the activity of the substrate transporter

($\varepsilon_{Glc\_ex}^{v_t}$) multiplied by the effect of transporter activity on cell growth ($C_{v\_t}^{\mu}$) of a clamped substrate system. The second product is the response of biomass concentration to the pump ($R_p^{Biomass}$) multiplied by the effect of biomass concentration to the activity of the transporter ($\varepsilon_{Biomass}^{v\_t}$) multiplied by the effect of the transporter activity on cell growth ($C_{v\_t}^{\mu}$). The third product is the response of biomass concentration to a pump ($R_p^{Biomass}$) multiplied by the effect of biomass concentration to the activity of the enzyme of biomass forming step ($\varepsilon_{Biomass}^{v\_b}$), multiplied by the effect of the enzyme activity catalysing biomass forming on cell growth ($C_{v\_b}^{\mu}$). In this analysis we distinguished between response coefficients denoted with R, defined for the complete system, and control coefficients denoted with C, defined for a subset of the system, namely the isolated bacteria, with clamped substrate, product and biomass concentration.

Under steady state conditions the dilution rate dictates the cell's specific growth rate, which is a function of the pump. That implies that the pump has full control over cell growth $R_p^{\mu}=1$.

$$1=\left(R_p^{Glc\_ex}\cdot\varepsilon_{Glc\_ex}^{v_t}\cdot C_{v_t}^{\mu}\right)+\left(R_p^{Biomass}\cdot\varepsilon_{Biomass}^{v\_t}\cdot C_{v\_t}^{\mu}\right)+\left(R_p^{Biomass}\cdot\varepsilon_{Biomass}^{v\_b}\cdot C_{v\_b}^{\mu}\right) \qquad 5.6$$

Say, we make an assumption that the transport of glucose across the membrane has full control over the rate at which biomass is formed ($C_{v\_t}^{\mu}$=1). This means the rate of biomass production has zero control ($C_{v\_b}^{\mu}$=0).

$$1=\left(R_p^{Glc\_ex}\cdot\varepsilon_{Glc\_ex}^{v\_t}\right)+\left(R_p^{Biomass}\cdot\varepsilon_{Biomass}^{v\_t}\right) \qquad 5.7$$

In this case, transporter activity is assumed to be proportional with biomass. So the elasticity coefficient of biomass to the transporter would be equal to 1 ($\varepsilon_{Biomass}^{v_t}$=1).

$$1=\left(R_p^{Glc\_ex}\cdot\varepsilon_{Glc\_ex}^{v\_t}\right)+R_p^{Biomass} \qquad 5.8$$

$$R_p^{Biomass}=1-\left(R_p^{Glc\_ex}\cdot\varepsilon_{Glc\_ex}^{v\_t}\right) \qquad 5.9$$

The response coefficient of biomass concentration to a pump would be expressed as change in biomass concentration as the result of a small change in the pump rate.

$$R_p^{Biomass} = \frac{d[Biomass]}{dp} \cdot \frac{p}{Biomass} \qquad 5.10$$

Biomass is the function of cell concentration yielded per glucose molecule multiplied by the consumed glucose:

$$Biomass = x - x_0 = Y_{\_x/\_s} Glc_{\_feed} - Glc_{\_ex} \qquad 5.11$$

In the equation that express response coefficient of biomass to the pump (eqn. 5.10), the biomass concentration will be substituted by the yield times consumed glucose concentration (eqn. 5.11).

$$R_p^{Biomass} = Y \frac{d(Glc_{\_feed} - Glc_{\_ex})}{dp} \cdot \frac{p}{Y(Glc_{\_feed} - Glc_{\_ex})} \qquad 5.12$$

$Glc_{\_feed}$ is constant, and the differentiation of a constant is zero.

$$R_p^{Biomass} = Y \frac{-d(Glc_{\_ex})}{dp} \cdot \frac{p}{Y(Glc_{\_feed} - Glc_{\_ex})} \qquad 5.13$$

The differentiation of extra cellular glucose with respect to the pump is equivalent to the response coefficient of extra cellular glucose to the pump. The response of extra cellular glucose concentration to pump can thus be expressed as:

$$R_p^{Glc\_ex} = \frac{d(Glc_{\_ex})}{dp} \cdot \frac{p}{Glc_{\_ex}} \qquad 5.14$$

$$\frac{d(Glc_{\_ex})}{dp} = \frac{Glc_{\_ex}}{p} \cdot R_p^{Glc\_ex} \qquad 5.15$$

In order to simplify the expression in eqn. 5.13 $\frac{dGlc_{\_ex}}{dp}$ is substituted by $\frac{Glc_{\_ex}}{p} \cdot R_p^{Glc\_ex}$:

$$R_p^{Biomass} = Y \cdot \frac{-Glc_{\_ex}}{p} \cdot R_p^{Glc\_ex} \cdot \frac{p}{Y(Glc_{\_feed} - Glc_{\_ex})} = R_p^{Glc\_ex} \cdot \frac{-Glc_{\_ex}}{Glc_{\_feed} - Glc_{\_ex}} \qquad 5.16$$

Now in eqn. 5.9, the response coefficient of biomass to the pump change could be substituted with eqn.5.16. This yields:

$$R_p^{Glc\_ex} \cdot \frac{-Glc\_ex}{Glc\_feed - Glc\_ex} = 1 - R_p^{Glc\_ex} \cdot \varepsilon_{Glc\_ex}^{v\_t} \quad 5.17$$

$$R_p^{Glc\_ex} \times \frac{-Glc\_ex}{Glc\_feed - Glc\_ex} + R_p^{Glc\_ex} \cdot \varepsilon_{Glc\_ex}^{v_t} = 1 \quad 5.18$$

$$R_p^{Glc\_ex} \varepsilon_{Glc\_ex}^{v_t} - \frac{Glc\_ex}{Glc\_feed - Glc\_ex} = 1 \quad 5.19$$

$$R_p^{Glc\_ex} = \frac{1}{\varepsilon_{Glc\_ex}^{v_t} - \frac{Glc\_ex}{Glc\_feed - Glc\_ex}} \quad 5.20$$

The elasticity coefficient of transporter activity to extra cellular glucose concentrations is given by:

$$\varepsilon_{Glc\_ex}^{v\_t} = \frac{dv\_t}{d(Glc\_ex)} \cdot \frac{Glc\_ex}{v\_t} \quad 5.21$$

Equation 5.8 is substituted in equation 5.20:

$$R_p^{Glc\_ex} = \frac{1}{\frac{dv\_t}{dGlc\_ex} \cdot \frac{Glc\_ex}{v\_t} - \frac{Glc\_ex}{Glc\_feed - Glc\_ex}} \quad 5.22$$

The rate of the transporter is now assumed to follow the irreversible Michaelis-Menten model:

$$v_t = \frac{V\_\max \cdot Glc\_ex}{K\_m + Glc\_ex} \quad 5.23$$

The rate of the transporter (equation 5.23) is substituted to equation 5.22:

$$R_p^{Glc\_ex} = \frac{1}{\frac{d}{dGlc\_ex}\left(\frac{V\_\max \cdot Glc\_ex}{K\_m + Glc\_ex}\right) \cdot \frac{Glc\_ex}{\frac{V\_\max \cdot Glc\_ex}{K\_m + Glc\_ex}} - \frac{Glc\_ex}{Glc\_feed - Glc\_ex}} \quad 5.24$$

$$R_p^{Glc\_ex} = \frac{1}{\frac{K\_m}{K\_m + Glc\_ex} - \frac{Glc\_ex}{Glc\_feed - Glc\_ex}} \quad 5.25$$

At steady state conditions of low dilution rate the concentration values of Glc_ex are so low that (Glc_feed-Glc_ex) could be equivalent to Glc_feed. A fraction of Glc_ex to Glc_feed would be a very small value that can be considered as zero (insignificant).

$$R_p^{Glc\_ex} = \frac{1}{\frac{K\_m}{K\_m + Glc\_ex}} = \frac{K\_m + Glc\_ex}{K\_m} \qquad 5.26$$

In summary eqn. 5.11 defines the response of the extra cellular glucose concentration to the pump if the latter is assumed to have full control on cell growth. The extra cellular glucose concentration response to a pump rate change depends on the affinity of the transporter to extra cellular glucose concentration. How can we relate the response of extra cellular glucose concentration to the specific growth rate of yeast cells? Co-response analysis can be useful here because it would account for the response of specific growth rate to the extra cellular glucose concentration as a result of pump fractional changes, defined as:

$$O_p^{\mu:Glc\_ex} = \frac{R_p^{\mu}}{R_p^{Glc\_ex}} = \frac{d(\ln \mu)}{d(\ln Glc\_ex)} \qquad 5.27$$

If the Monod model is assumed as the description of cell growth, this would imply a specific growth change as a result of substrate concentration change:

$$\frac{d \ln \mu}{d \ln Glc\_ex} = \frac{d\mu}{dGlc\_ex} \cdot \frac{Glc\_ex}{\mu} = \frac{d(\mu_{\max} \cdot \frac{Glc\_ex}{K\_s + Glc\_ex})}{dGlc\_ex} \cdot \frac{Glc\_ex}{\mu\_\max \cdot \frac{Glc\_ex}{K\_s} + Glc\_ex} \qquad 5.28$$

$$O_p^{\mu:Glc\_ex} = \frac{d(\ln \mu)}{d(\ln Glc\_ex)} = \frac{K_s}{K_s + Glc\_ex} \qquad 5.29$$

So in equation 5.27 the ${}^{p}O_{Glc\_ex}^{\mu}$ can be substituted by $\frac{K\_s}{K\_s + Glc\_ex}$ yielding:

$$\frac{K\_s}{K\_s + Glc\_ex} = \frac{R_p^{\mu}}{R_p^{Glc\_ex}} \qquad 5.30$$

The response coefficient of specific growth rate on the pump rate is 1, since the pump is has full control on specific growth rate of cells in the chemostat.

$$\frac{K\_s}{K\_s + Glc\_ex} = \frac{1}{R_p^{Glc\_ex}} \qquad 5.31$$

From equation 5.26, the response coefficient of extra cellular glucose to the pump was simplified to be equal $\frac{K\_m + Glc\_ex}{K\_m}$

$$\frac{K_{\_s}}{K_{\_s}+Glc_{\_ex}}=\frac{1}{\frac{K_{\_m}+Glc_{\_ex}}{K_{\_m}}} \quad 5.32$$

$$\frac{K_{\_m}}{K_{\_m}+Glc_{\_ex}}=\frac{K_{\_s}}{K_{\_s}+Glc_{\_ex}} \quad 5.33$$

$$K_{\_m}=K_{\_s} \quad 5.34$$

Thus, it has been deduced that if the transporter has full flux control the half saturation constant of a cell would be numerically equal to the substrate affinity constant of the transporter. This principle will only be true if the following listed assumptions hold:

Formulated assumptions

- The pump rate dictates the specific growth rate of the cells, and the response coefficient of cell growth rate to pump rate is 1 $(R_p^{\mu}=1)$.
    - R_p^{μ} represents the control of growth by a pump in a chemostat system (i.e. a flux driven system), and the pump determines the specific growth rate. Note that the pump was assumed to have full flux control, $(R_p^{\mu}=1)$.
    - $C_{v\_t}^{\mu}$ represents the growth control by the activity of the transporter in a biological environment, where the extracellular substrate concentration is constant, and is referred to as clamped substrate system. Note that the glucose transporter is assumed to have full flux control $C_{v\_t}^{\mu}$=1.
- Glucose transporter is assumed to have full flux control; the biomass formation steps have no control $C_{v\_b}^{\mu}=0$.
- Transport activity is assumed to be proportional with biomass, $\varepsilon_{Biomass}^{v\_t}$=1.
- Low residual glucose concentrations.
- The transporter was assumed to follow irreversible Michaelis-Menten kinetics
- Cell growth was assumed to follow Monod model.

5.3 Discussion

The question is, can we relate the K_s that which a system property potentially dependent on characteristics of with thousands of enzymes to K_m, a characteristic of a single reaction? This of course seems impossible, but metabolic control analysis has been used as a tool to formulate a link between the two parameters. A chemostat yeast cultivation under glucose limitation conditions as described in chapter 4 served as a model. Our MCA analysis followed the response of yeast specific growth rate to the pump used to set medium feed rate. The medium had a single substrate limitation. The factors that were affected by the pump; extra cellular substrate, activity of the transporter, biomass concentration, the activity of an enzyme involved in biomass formation step and their link to specific growth rate were formulated. MCA was then applied to connect these pump-affected factors in order to understand the role the pump plays in the system. Several assumptions were made in the analysis: the transporter had full control on cell growth, glucose transportation was assumed to follow Michaelis and Menten kinetics and cell growth was described using Monod kinetics. Under these specific conditions K_s was deduced to be equal to the K_m of the transporter. To illustrate this principle we shall design a core model in the next chapter. The kinetic model will be set in such a way that the transporter has full control over growth rate as formulated in the assumptions. Subsequently another model will be constructed where the transporter has no control on growth.

5.4 References

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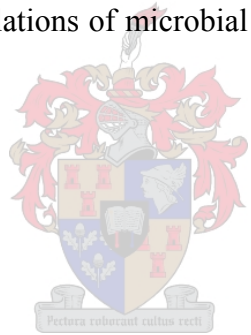

CHAPTER 6

6 Kinetic Model

6.1 Introduction

Kinetic models are used to simulate the behavior of biological systems. Often such models are analysed numerically using computers. One of the earliest applications of computer modeling of biological systems was the modeling of metabolic pathways. A simulation can resemble an experiment: by using sets of metabolites with initial concentrations, software will produce time evolution and/or the steady state concentrations metabolites. In addition to the initial metabolites concentrations one also needs to know the differential equations describing the kinetics of the pathway and values for all the parameters involved in these equations. There are programs (software) that minimize the mathematical effort of setting up and analysing these models, such as Gepasi [1,2].

Kinetic models can be characterised as either detailed models or core models. Detailed models simulate the behaviour of a biological system and are commonly referred to as realistic models. To develop these models requires detailed information about the metabolic pathway modelled. Core models are used to illustrate a principle, and are normally set up as simple as possible.

Using a simple kinetic model on *Saccharomyces cerevisiae* growth in substrate-limited chemostat we will test the hypothesis postulated in chapter 5. The aim is to provide insight to our experimental findings. It has been postulated using MCA that when the transporter activity of yeast has full flux control over glucose metabolism for biomass synthesis, then the half saturation constant of the transporter (K_m) will be numerically equal to the half saturation constant of the whole yeast cell (K_s), and when the transporter has no control then K_s has no functional relationship to the K_m of the transporter. Three types of models are designed to illustrate this principle.

6.2 Model description

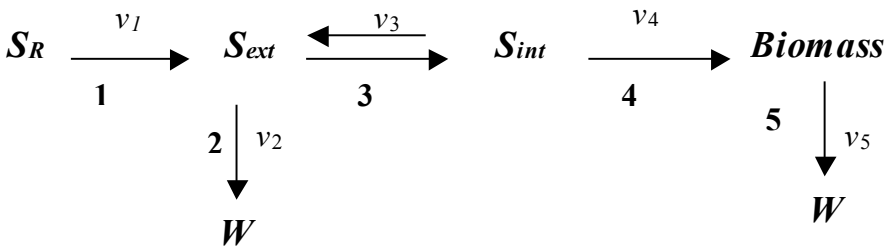

Figure 6.1: Pathway scheme used to illustrate the model structure. In the description of all subsequent reactions the subscripts *R*, *ext* and *int* refer to the medium feed (reservoir), extracellular glucose (chemostat compartment) and intracellular glucose (combined internal cellular compartment) used to synthesize biomass respectively. The rate equations of reaction 1 to 5 are given by equations 6.4-6.8.

A core model that simulates glucose-limited growth of *S. cerevisae* in a chemostat with feed medium concentration (reservoir), $S_{\_R,}$ was derived. The residual concentration of glucose in a culture vessel (chemostat compartment) was referred as extra cellular concentration, $S_{\_ext.}$ The transported $S_{\_ext}$ across the cell membrane via the transporter into the cell was referred as intracellular concentration, $S_{\_int}$ (internal cellular compartment). Which, when further metabolized by the cell resulted to formation of biomass. This process was modeled by three differential equations describing the rates of change of extra cellular glucose $S_{\_ext,}$ intracellular glucose $S_{int,}$ and biomass.

$$\frac{d(s_{\_ext(t)})}{dt} = v_1 - v_2 - v_3 \qquad (6.1)$$

$$\frac{d(s_{\_\mathrm{int}(t)})}{dt} = v_3 - v_4 \qquad (6.2)$$

$$\frac{d(Biomass)}{dt} = v_4 - v_5 \qquad (6.3)$$

The v_1, v_2 and v_5 were linked by k, which was represented by` the steady state dilution rate (D) as a function of the pump. The S_{ext} transportation (v_3) and biomass formation (v_4) steps were the only steps that were enzyme catalyzed. Two different enzymatic rate equations were assumed; for v_3 a reversible Michaelis Menten rate equation was assumed to describe the rate of glucose uptake via

the transporter and for v_4 an irreversible Michaelis Menten rate equation was assumed to describe the biomass formation steps.

$$v_1 = D \cdot S_{\_R} \cdot V_{\_t} \tag{6.4}$$

$$v_2 = D \cdot S_{\_ext,(t)} \tag{6.5}$$

$$v_3 = \frac{\frac{V_{\_\max}}{K_{\_m,S_{ext}}} \cdot Biomass_{(t)} \cdot \left(\frac{S_{\_ext(t)}}{V_{\_t} - \alpha_{\_V_i} \cdot Biomass_{(t)}} - \frac{S_{\text{int}(t)}}{\alpha_{\_V_i} \cdot Biomass_{(t)} \cdot K_{\_eq}} \right)}{1 + \frac{S_{\_ext(t)}}{(V_{\_t} - \alpha_{\_v_i} \cdot Biomass_{(t)}) \cdot K_{\_m,S_{ext}}} + \frac{S_{\_\text{int}(t)}}{\alpha_{v_i} \cdot Biomass_{(t)} \cdot K_{\_m,S_{\text{int}}}}} \tag{6.6}$$

$$v_4 = \frac{V_{\_\max} \cdot Biomass_{(t)} \cdot \left(\frac{S_{\_\text{int}(t)}}{\alpha_{\_V_i} \cdot Biomass_{(t)}} \right)}{K_{\_m,S_{\text{int}(t)}} + \left(\frac{S_{\_\text{int}(t)}}{\alpha_{\_V_i} \cdot Biomass_{(t)}} \right)} \tag{6.7}$$

$$v_5 = D \cdot Biomass(t) \tag{6.8}$$

Table6.1: Parameter values of the models.

Models	Steps	Parameter	Value	Units	Comments
Model 1	**Transporter Step, v_3**	V_{max}	90	mmol/h	activity of the transporter
		$K_{m,glcc}$	0.9	mM	hxt affinity constant for external gluc.
		$K_{m,glci}$	0.9	mM	hxt affinity constant for internal gluc.
		α_{Vi}	5 X 10^{-6}	L	internal volume (ml)/mg dry mass
		V_t	1	L	total working volume
		K_{eq}	1	-	equilibrium constant
	Biomass Formation Step, v_4	V_{max}	0.52	mmol/h	activity of the biomass forming enzyme
		α_{Vi}	5 X 10^{-6}	L	internal volume
		$K_{m,glci}$	2	mM	affinity for intracellular glucose
Model 2	**Transporter Step, v_3**	V_{max}	0.52	mmol/h	activity of the transporter
		$K_{m,glcc}$	1	mM	hxt affinity constant for external gluc.
		$K_{m,glci}$	1	mM	hxt affinity constant for internal gluc.
		α_{Vi}	5 X 10^{-6}	L	internal volume (ml)/mg dry mass
		V_t	1	L	total working volume
		K_{eq}	1	-	equilibrium constant
	Biomass Formation Step, v_4	V_{max}	90	mmol/h	activity of the biomass forming enzyme
		α_{Vi}	5 X 10^{-6}	L	internal volume
		$K_{m,glci}$	0.001	mM	affinity for intracellular glucose
Model 3	**Transporter Step, v_3**	V_{max}	90	mmol/h	activity of the transporter
		$K_{m,glcc}$	0.9	mM	hxt affinity constant for external gluc.
		$K_{m,glci}$	0.9	mM	hxt affinity constant for internal gluc.
		α_{Vi}	5 X 10^{-6}	L	internal volume (ml)/mg dry mass
		V_t	1	L	total working volume
		K_{eq}	1	-	equilibrium constant
	Biomass Formation Step, v_4	V_{max}	0.52	mmol/h	activity of the biomass forming enzyme
		α_{Vi}	5 X 10^{-6}	L	internal volume
		$K_{m,glci}$	0.1	mM	affinity for intracellular glucose

The feed glucose concentration (S_R) had a fixed value of 20.0 mM, and the initial biomass concentration was 0.03mmol per compartment volume. The initial glucose concentrations of extracellular and intracellular compartment were set to 0.001mmol per compartment volume. The rate constant of step 1 (k_1) was set to range between 0.05h^{-1} to 0.5h^{-1} and was linked to k_2 and k_5.

6.3 Results

The rate constant (k) was scanned between 0.05hr^{-1} and 0.5hr^{-1}. At steady state the rate constant was numerically equivalent to the specific growth rate function value. The specific growth rate was plotted against the steady states residual glucose concentrations. An inverse hyperbolic increase of residual glucose with increasing specific growth rate was observed. Non-linear fit was used to estimate Monod half saturation (K_s) constant as well as maximum specific growth rates (μ_{max}) as shown in figure 6.2 using Mathematica software. This was done to illustrate the relationship between the estimated K_s and K_m of the transporter. This was achieved by comparing the estimated K_s to K_m of the transporter and also with a K_m value of the biomass formation step (table 6.1).

The three models were set up such that the transporter was expected to have full flux control in model 2 and no flux control in model 1 and 3. The step was derived to have no flux control by assigning a high enough V_{max} value for the step to loss control, while the step with smaller V_{max} value gained full flux control. It was postulated that the K_m of the step with full flux control will determine the K_s of the cell. In the latter two models the K_m of the biomass formation step was varied to test whether K_s would be equal to this value and whether this could potentially be lower than the K_m of the glucose transporter.

For all three models the extra cellular glucose concentration (residual glucose concentrations) were obtained at specific growth rates between 0.05h^{-1} and 0.5h^{-1}, as shown in figure 6.1. The microbial growth parameters, K_s and μ_{max} were estimated by non-linear fit using Mathematica software.

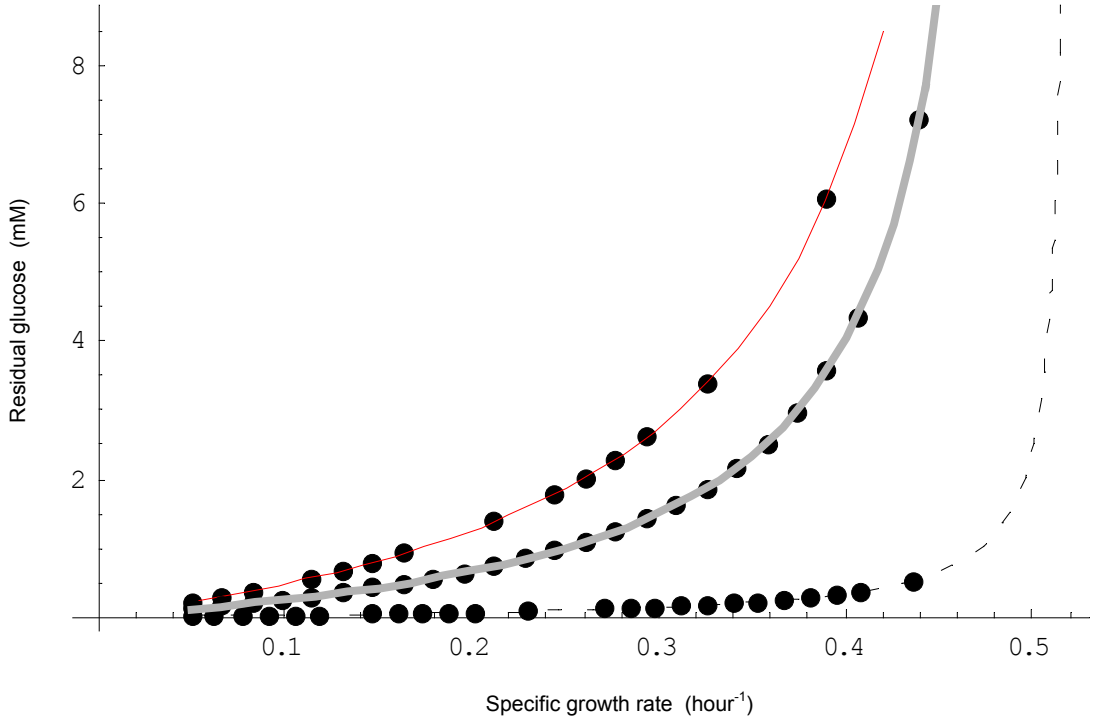

Figure 6.2: Non-linear estimation of Monod growth parameters (K_s and μ_{max}) for three designed models. Mathematica fitting software was used to estimate the K_s values and μ_{max} values for three models. (●) Data points of steady state residual concentrations at different specific growth rate, (————) Model 1 ($K_{m,T}$ is less than K_s), (▬▬▬) Model 2 ($K_{m,T}$ is equal to K_s) and (– – – – – – –) Model 3 (when $K_{m,T}$ is greater than K_s).

Table 6.2: The relationship between the model parameters and estimated growth parameters

	Model 1		*Model 2*		*Model 3*	
	V_{max} **(mM/second)**	K_m **(mM)**	V_{max} **(mM/second)**	K_m **(mM)**	V_{max} **(mM/second)**	K_m **(mM)**
Transport step, v_3	90.0	0.9	0.52	1	90.0	0.9
Biomass formation step, v_4	0.52	2	90.0	0.001	0.52	0.1
Estimated K_s (mM)	2.01		1.00		0.10	
Estimated μ_{max} (h^{-1})	0.52		0.52		0.53	

6.4 Discussion

Can we relate the K_s to K_m? Three core models were designed to relate the K_m of the glucose transporter to K_s of *S. cerevisiae* growth on glucose. Two models were designed in such a way that glucose transporter activity was so high that full control over cell growth flux reside in the sugar metabolism step (biomass formation step). For one of the two models, the transport step was assigned the K_m value that was two fold lower than the K_m of glucose metabolizing step and for the second model the transporter was assigned K_m value that was nine fold higher than the K_m value assigned for glucose metabolizing step. The third model (model 2) was designed in such a way that the glucose transporter activity was so low that full control resides in the transporter step.

In models 1 and 3 the estimated K_s value was numerically equally to the K_m value assigned for sugar metabolizing step, independent of whether K_m value assigned for glucose metabolizing step was lesser or higher than K_m value assigned for glucose transporter.

For the second model, when glucose transporter activity was so low that full control over cell growth resided on glucose transportation across the cell membrane, the estimated K_s was numerically equal to the K_m value assigned for glucose transportation step. The K_s of the system can be related to the K_m of a step that controls the growth rate.

This study clearly illustrates that the estimated K_s for the cell can be smaller or larger than the K_m of the transporter. The K_s value is equal to the K_m value of the enzyme that holds full control over the growth of cell. In a system where control of growth rate is distributed, no such a simple link between the K_s and the enzyme characteristics can be made.

6.5 Conclusion

It is possible to use a core model to illustrate that half-saturation constant of a cell on single substrate (K_s) can be understood in terms of the K_m of an enzyme that holds full control on growth rate. This principle states that the enzyme that has full control on specific growth of the cell determines the K_s of the cell by its K_m.

6.6 Reference

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CHAPTER 7

7 General Discussion

Growth of a microbial culture follows certain general rules and once these rules are known, it should become predictable. This way of thinking is reflected in the central concept of microbial growth kinetics, i.e., that the specific rate of growth (μ) is dependent on the extra cellular concentration of a substrate. For the past fifty years, efforts have been made to confirm the exact dependence of μ on S. This led to various mathematical models to describe the μ-S relationship. However, it is reported that verification of these models as well as discrimination between the many models was hampered by experimental difficulties. Two challenges are raised in the literature. Firstly, scattering of the experimental data does not allow distinguishing between competing models. Secondly, the kinetic constants reported in the literature for a given micro-substrate combination are inconsistent [11].

Already Monod was aware of the inadequate quality of his data and commented in a later review that 'several different mathematical formulations could be made to fit the data. But it is convenient and logical to adopt a hyperbolic equation" [7]. As a result other researchers tried to improve experimental techniques to produce quality results, while others came up with alternative models to fit the data better. And this required statistical evaluation for the goodness of fit of these model [11].

The current study has two clear goals: firstly, to verify that the hyperbolic description of cell growth on substrate concentration also holds for *S. cerevisiae* on glucose. The reason for choosing VIN13 is because it is an important strain widely used by industry for wine making. The second aim was to formulate means of understanding the mechanism of Monod's description for growth.

The data set collected in this work for glucose-limited growth of *S. cerevisiae* in continuous culture could be well described by the Monod rate equation. The metabolism of yeast was found to shift from a respiratory to a respirofermentative mode at dilution rates above $0.4h^{-1}$. In general for some yeast species (known as Crabtree positive), there is a critical dilution rate value, D_c, above which one observes a striking yield coefficient decrease as it was in this study. The Crabtree effect has been known for a long time [5,13]. However, even at dilution rates below the D_c a slight decrease in biomass yields was observed. Monod rate equation assumed the biomass formation to be proportional to substrate consumption, i.e. $Y_{x/s}$ to be a constant. Pirt (1982) observed, "The concept of 'maintenance energy' is essential to understand the energy requirements for microbe and cell growth" [10]. His analysis made estimation of the maintenance energy demand of cells technically feasible in the chemostat [3]. The existence of this concept can be explained by thermodynamic reasoning [6]. In a comprehensive analysis of microbial growth Westerhoff and co-workers [14] have put forward arguments based on non-equilibrium thermodynamics and proposed that the rates of catabolism and anabolism are governed by the differences in free energy between substrate and products and hence, that a logarithmic relationship should exist between the specific growth rate and the growth-limiting substrate concentration [11]. The growth dependence of maintenance energy proposed by Neijessel et al. [9] and Stouthamer et al. [12] could explain the slight reduction in yield.

It was also observed when Monod was fitted to the data along with other unstructured models, that the logarithmic model of Westerhoff could describe growth of S. cerevisiae equally well as the Monod model. The SSR value of 0.044 and 0.043 for the Monod and Westerhoff model respectively, is did not show significantly different. However, the distribution of specific growth rate residuals when plotted against dilution indicated that Westerhoff model could describe growth of S. cerevisiae slightly better than Monod. Still Monod showed a better fit than the Tiesseir model (assumes μ-S exponential relationship) and Blackman model (assumes a μ-S bilinear relationship).

Monod is the most commonly used description of microbial growth. The Monod equation is mathematically analogous to the formula that was proposed by Michaelis and Menten [4] to describe enzyme kinetics; the meaning of the two saturation constants K_s and K_m is

different. Regardless of the fact that Monod had stressed [7] that there is no relationship between the *Ks* (affinity constant used in his growth model, which represents the substrate concentration at μ=0.5μ_{max}) and the Michaelis-Menten constant K_m. There is a common representation of K_s as though it is the K_m of the transporter. In contrast to Michaelis-Menten kinetics, which describes a process catalysed by a single enzyme, Monod kinetics describes processes (both growth and growth linked biodegradation) of a more complex nature in which many enzymes are involved [6].

The question that was investigated in this study is whether we can relate K_s to K_m of the transporter, given the differences that have been presented above? Kovárová-kova and Egli incompletely answered this question by saying only in some special cases, when cell growth is controlled by the rate of active transport of a substrate, may *Ks* be considered to be similar to the Michaeli-Menten constant (K_m) for the permease carrier [1,2,6].

In order to understand what was referred as "special case" metabolic control analysis was used to explain what permease carrier having control on growth of the cell means. During the analysis we defined assumptions that can be experimentally tested and could show that when the control coefficient of the substrate permease activity on specific growth rate is 1, K_s is equal to the K_m of the transporter (chapter 5). What about when the control of the transporter is not 1? A simple kinetic model was used to illustrate this principle and to show how to understand K_s even when control is not on the transporter. When the K_m of the enzyme that controls growth is less than the K_m of the transporter, the organism will have a K_s value that is smaller than the K_m of the transporter. This could explain the counter intuitive results obtained from the experimental data, in which *S. cerevisiae* showed a higher affinity for the substrate than the transporter.

The success of this study was that it provides a better understanding of the Monod parameters by using an integrated approach to answer scientific questions.

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