Non-*Saccharomyces* wine yeast production in aerobic fed-batch culture

by

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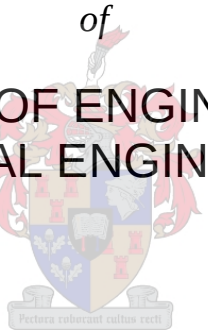

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Declaration

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Date: 25 November 2015

Abstract

The production of the non-*Saccharomyces* wine yeasts *Metschnikowia pulcherrima*, *Issatchenkia orientalis* and *Lachancea thermotolerans* was optimised in aerobic fed-batch cultures for commercial application. These non-*Saccharomyces* have been used in sequential wine fermentations where they are employed to enhance the organoleptic characteristics of wine. The primary goal was to produce these organisms at a maximum biomass yield ($Y_{x/s}$) through aerobic fed-batch cultivations where a dynamic feed regime was used to ensure accurate control over the specific growth rate (μ_{spec}) of each culture.

By controlling the μ_{spec} at preferred points during cultivation at 9 L scale, $Y_{x/s}$ maxima of 0.83 g g^{-1}, 0.68 g g^{-1} and 0.76 g g^{-1}could be achieved for *I. orientalis*, *M. pulcherrima* and *L. thermotolerans*, respectively. This was higher than the 0.51 g g^{-1} achieved in *Saccharomyces cerevisiae* cultures, due to the Crabtree-positive behaviour of the latter. When producing *L. thermotolerans* at 90 L pilot scale a maximum $Y_{x/s}$ of 0.54 g g^{-1} was achieved, which was significantly lower than the 0.76 g g^{-1} achieved at 9 L bench scale.

A secondary goal was to determine what effect different production growth rates has on the culture's subsequent fermentative performance or yeast quality. The fermentative performance of the yeasts produced under various culture conditions were evaluated by measuring the acidification power of the yeast and evaluating the yeast in synthetic wine fermentations. The tests indicated that the yeast can be produced at a growth rate where the $Y_{x/s}$ is at a maximum value without compromising the quality of the yeast culture. This allowed the selection of conditions where a maximum $Y_{x/s}$ is produced for industrial yeast production.

The *non-Saccharomyces* yeasts *M. pulcherrima*, *I. orientalis* and *L. thermotolerans* should be produced at 0.10 h^{-1}, 0.11 h^{-1} and 0.12 h^{-1}, respectively. These growth rates will ensure the

highest possible biomass yield on sugar with any compromise to the fermentative performance of the yeast product.

Opsomming

Die produksie van die nie-*Saccharomyces* wyngiste *Metschnikowia pulcherrima, Issatchenkia orientalis* en *Lachancea thermotolerans* is geoptimaliseer in aerobiese gevoerde-lotkulture vir kommersiële toepassings. Hierdie nie-*Saccharomyces* wyngiste toon potensiaal wanneer hulle in kombinasie met *Saccharomyces cerevisiae* gebruik word om die organoleptiese eienskappe van die wyn te bevorder. Die hoofdoel van die projek was om die giste by ‘n maksimale biomassa-opbrengs ($Y_{x/s}$) te produseer deur middel van aerobiese gevoerde-lotkulture waar ‘n dinamsie voermodel gebruik is om die kultuur by ‘n konstante spesifieke groeisnelheid (μ_{spec}) te handhaaf.

‘n Maksimum $Y_{x/s}$ van 0.83 g g^{-1}, 0.68 g g^{-1} en 0.76 g g^{-1} kon bereik word vir onderskeidelik *I. orientalis*, *M. pulcherrima* en *L. thermotolerans* deurdat die μ_{spec} by sleutelpunte gehandhaaf is tydens kulture by 9 L kon. Dit is hoër as die 0.51 g g^{-1} wat in *S. cerevisiae* kulture bereik is. Wanneer *L. thermotolerans* op proefskaal (90 L) geproduseer is, is ‘n maksimum $Y_{x/s}$ van 0.54 g g^{-1} bereik, wat noemenswaardig laer was as die 0.76 g g^{-1} wat op klein skaal (9 L) bereik is.

‘n Sekondêre doelwit was om die gistingsprestasie van die gis te toets na afloop van die afsonderlike kulture. Die gistingsprestasie is getoets deur die aansuringsvermoë van die afsonderlike kulture te toets. Die gis se gistingsprestasie is verder getoets in sintetiese wyngisting. Die toetse het gewys dat die gis by ‘n groeisnelheid gekweek kan word waar ‘n maksimum biomassa-opbrengs bereik kan word sonder dat die werkverrigting tydens wyngisting benadeel word. Hierdie bevinding bevestig dat die giste by lae groeisnelhede gekweek kan word op kommersiële vlak.

Die nie-Saccharomyces gis *M. pulcherrima*, *I. orientalis* en *L. thermotolerans* moet teen afsonderlike groeisnelhede van 0.10 h^{-1}, 0.11 h^{-1} and 0.12 h^{-1} produseer word om ‘n maksimale biomass opbrengs op suiker te lewer. By hierdie kondisies word die gistingsprestasie van die finale gis produk nie nadelig beïnvloed nie.

Dedication

I dedicate this thesis to the loved ones in my life, especially my wife Truidi-lee, who supported me throughout my studies.

Acknowledgments

I would like to express my gratitude to my supervisor, Prof Johann F Görgens, for his guidance throughout my master's studies.

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Finally, I would like to thank Emmanuel Anane for his mentorship. Without him this study would not have been concluded.

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Nomenclature

$Y_{x/s}$	:	Biomass yield on consumed sugar
x	:	Biomass concentration (g L^{-1})
X	:	Biomass (g)
s	:	Substrate concentration (g L^{-1})
s_f	:	Substrate feed concentration (g L^{-1})
S	:	Substrate (g)
ATP	:	Adenosine triphosphate
TCA	:	Tricarboxylic acid
P/O	:	Phosphate-to-Oxygen
PC	:	Pyruvate carboxylase
PDC	:	Pyruvate decarboxylase
PDH	:	Pyruvate dehydrogenase
μ	:	Growth rate (h^{-1})
μ_{spec}	:	Specific growth rate (h^{-1})
μ_{max}	:	Maximum growth rate (h^{-1})
μ_{crit}	:	Critical growth rate (h^{-1})
t	:	Time
t_D	:	Doubling time

F	:	Flow rate (L h^{-1})
D	:	Dilution rate (h^{-1})
q_{ss}	:	Quasi steady-state
DO	:	Dissolved oxygen
CER	:	Carbon dioxide evolution rate
OUR	:	Oxygen uptake rate
RQ	:	Respiratory quotient
OTR	:	Oxygen transfer rate
C	:	Oxygen concentration
k_La	:	Volumetric mass transfer coefficient
v_s	:	Superficial gas velocity (m s^{-1})
Fs	:	Superficial gas velocity (m^3 s^{-1})
P_g/V_L	:	Gassed power per unit volume
vvm	:	Volume of air per volume of broth per minute
V	:	Cultivation broth volume
YAN	:	Yeast assimilable nitrogen
AP	:	Acidification Power

Chapter 1

Introduction

In wine fermentation, the yeast *Saccharomyces cerevisiae* is responsible for the major part of the alcoholic fermentation of grape must into wine [1]. Other non-*Saccharomyces* yeasts that are present in wine fermentation were previously thought of as spoilage organisms [2]. It is now known that these yeasts, e.g. *Issatchenkia orientalis*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans*, actually contribute to the aroma and complexity of the final wine product [3,4]. These yeasts are active during the initial stages of fermentation, before ethanol concentrations becomes toxic and *Saccharomyces cerevisiae* takes over as sole fermenter. During the low-fermentative part of the fermentation process (initial stages of fermentation), important reactions take place that account for most of the aromas in wine [1]. For instance, glycosidases are enzymes produced by non-*Saccharomyces* yeasts that improve the aroma and flavour of wine by hydrolysing non-volatile precursors in grapes. The enzymes produced by non-*Saccharomyces* yeasts also contribute to other aspects of wine production, for example pectinases, which increases juice extraction and improves wine clarification. Non-*Saccharomyces* proteases hydrolyse peptide bonds in proteins, which improve the clarification process in winemaking [1]. Winemaking is a non-sterile process, but the growth of undesirable microbes is inevitable. Strategies to reduce the growth of spoilage organisms are readily employed because these spoilage yeasts have undesirable effects on the organoleptic profile of wine. There is, however, a great demand for the use of natural anti-microbials in the food and wine industry [2]. By producing killer toxins as a strategy to improve their own survival, non-*Saccharomyces* yeasts can present a natural way of controlling spoilage yeast growth [2]. The possible interaction between different species of yeast may also be valuable [2].

Recent studies show that the use of mixed starter cultures of *S. cerevisiae* with non-*Saccharomyces* yeast strains can be beneficial to winemaking and has received much attention in recent years [2]. In order to use *I. orientalis*, *M. pulcherrima* and *L. thermotolerans* as inocula, these non-*Saccharomyces* yeasts have to be produced on an industrial scale by means of an appropriate, effective production process. *In priori* knowledge of the physiology of these organisms is important to design such production processes since the processes can make use of yeast characteristics to achieve high biomass yields and the desired viability and fermentative activity of the yeasts are crucial factors.

The use of non-*Saccharomyces* yeast in mixed starter cultures is a new trend, therefore the production process of *I. orientalis*, *M. pulcherrima* and *L. thermotolerans* is not yet industrially established, whereas *S. cerevisiae* has a long history of industrial production. Not even the most basic information is available about the physiology of non-*Saccharomyces* yeasts under typical industrial production conditions, which makes the following research questions that are addressed in this study quite relevant:

a) What growth characteristics and physiology do the non-*Saccharomyces* yeast *I. orientalis*, *M. pulcherrima* and *L. thermotolerans* demonstrate in fed-batch cultures?
b) What is the maximum growth rate at which these non-*Saccharomyces* wine yeasts should be produced to get the highest biomass yield?
c) What effect does the selection of a growth rate for the fed-batch production process have on the subsequent fermentative performance of the yeast?
d) To what extent do typical scale-up effects influence the production of non-*Saccharomyces* yeasts at pilot scale, compared to bench-scale?

By answering these questions and combining the physiological and process information, the production process of *I. orientalis*, *M. pulcherrima* and *L. thermotolerans* can be optimised,

ensuring high biomass yields on sugar ($Y_{x/s}$) and high biomass concentrations (x) with a minimal loss in the fermentative performance of the yeast product.

The literature review presented in this study first summarises the basic physiology of yeast sugar metabolism that applies to the production of various yeasts. The second section focusses on the production process itself, summarising the basic procedure, process conditions and the kinetics of fed-batch cultures. The proposed experimental plan summarises the methods that were chosen based on the literature findings to best achieve the objectives of this study. The experimental design, result and discussion is then presented in the form of a manuscript comprising of the experimental work (Chapter 3).

Chapter 2

Theoretical Considerations

2.1 Yeast metabolism

Metabolism refers to the chemical processes involved in anabolism (assimilation) and catabolism (dissimilation) [5]. The anabolic pathway includes reductive reactions that produce new cellular material, leading to cell growth. Catabolic pathways, on the other hand, includes the oxidation reactions that produce energy. It is important to know that anabolism and catabolism are dependent on each other, since the former is fed by the latter. Depending on the physiochemical environment that the organism is exposed to (see sections 2.3 and 2.4), microorganisms can produce energy through different strategies [5].

The main energy source in *S. cerevisiae* comes from the oxidation of hexose, preferably glucose or fructose, to produce adenosine triphosphate (ATP) molecules [6,7]. The carbon source can be metabolised via two major strategies, namely aerobic respiration and anaerobic fermentation [6–8]. In terms of energy production, anaerobic fermentation produces 2 ATP molecules per glucose molecule compared to 36 ATP molecules per glucose molecule produced through respiration [8]. Aerobic respiration is therefore more efficient in producing energy that can be utilised for cell growth [9,10].

2.1.1 ***Substrate transport into yeast cell***

Substrates have to be taken up by the yeast cell before it can be metabolised. The transport of substrates across the cell membrane of *S. cerevisiae* is a well-studied system [11]. In *S. cerevisiae* glucose is transported via facilitated diffusion [11–13]. Facilitated diffusion is a form of passive diffusion that is facilitated by transport proteins, providing a means of substrate specificity [8]. Unlike free diffusion where there is a linear relationship between the transfer

gradient and transfer itself, facilitated diffusion can become saturated. In certain organisms other than *S. cerevisiae* an active transport system has been proposed where energy in the form of ATP is used to transport glucose across the membrane [11].

When more than one carbon source is available, yeast shows the ability to 'select' the more favourable source, usually glucose [14]. In the presence of glucose, the uptake of alternate carbon sources are repressed, which ensures that glucose is consumed first. This phenomenon, known as carbon catabolite repression, is sometimes also referred to as glucose repression because of the strong tendency towards glucose utilisation [15,16].

2.1.2 *Glycolysis*

Glucose entering the yeast cell is assimilated mainly through glycolysis, which consists of a series of enzymatic reactions occurring in the cytoplasm, ultimately leading towards pyruvate production [8]. The overall chemical reaction in glycolysis is [8]

$$glucose + 2\,ADP + 2\,NAD^{+} + 2\,Pi = 2\,pyruvate + 2\,ATP + 2\,NADH \qquad (1)$$

where a net amount of 2 ATP molecules is produced through substrate level phosphorylation. The fate of the resulting pyruvate is determined by the availability of oxygen.

2.1.3 *Aerobic Respiration*

When oxygen is available to serve as final electron acceptor, the oxidation of a carbon source through glycolysis is linked to the tricarboxylic acid (TCA) cycle or Krebs cycle [5,6]. In the TCA cycle, acetyl CoA is completely oxidised to carbon dioxide (CO_2), water and reducing equivalents. These reducing equivalents then act as electron donors in the electron transport chain of the mitochondria, where oxygen acts as final electron acceptor [8]. This electron transfer is coupled with the transfer of H^+ ions (protons) across the inner membrane of the mitochondria. The electrochemical gradient generated by this transfer is then used by the

enzyme ATP-synthase to create energy in the form of ATP through oxidative phosphorylation [5]. If a phosphate-to-oxygen (P/O) ratio of 3 is assumed, the overall reaction of respiration including glycolysis is [8]

$$glucose + 36\,Pi + 36\,ADP + 6\,O_2 = 6\,CO_2 + 6\,H_2O + 36\,ATP \quad (2)$$

The TCA cycle is a good example of how anabolism and catabolism co-exist, since many precursors for both amino acids and nucleotides are produced through the citric acid cycle [8]. The energy and intermediates produced by the TCA cycle are used to produce new biomass. With a P/O ratio of 3 the maximum biomass yield on glucose is suggested to be 0.68 – 0.72 g g^{-1} [17].

2.1.4 *Anaerobic Fermentation*

During anaerobic fermentation oxygen cannot act as an electron acceptor, which in principle eliminates the TCA cycle and electron transport chain as a means of generating energy [5,6]. In this case the organism relies solely on glycolysis for the production of ATP. The reducing equivalents produced during glycolysis are then re-oxidized in a two-step reaction that results in ethanol and CO_2, where acetaldehyde acts as the electron acceptor [18]. In some cases glycerol is also produced during anaerobic fermentation as an alternative way in which reducing equivalents are re-oxidised, especially in conditions of extreme oxygen-limitation [18].

2.2 The Crabtree effect

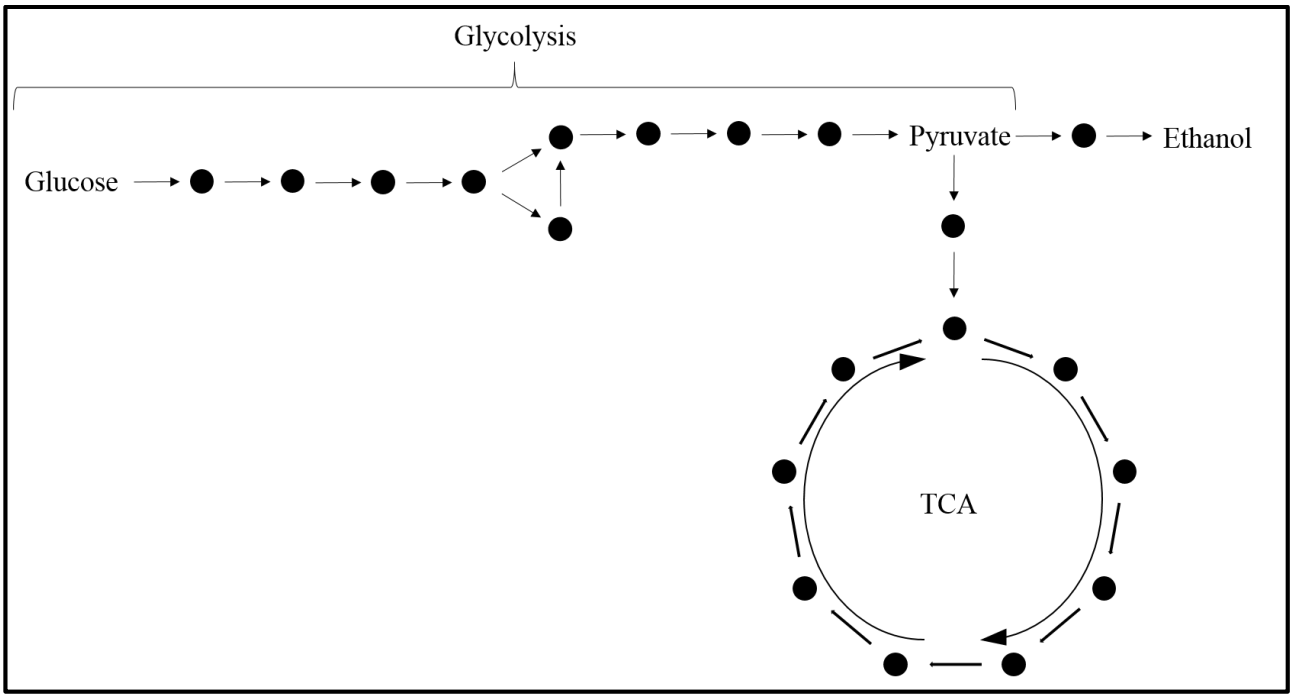

Figure 1: Representation of yeast metabolism redrawn from KEGG Metabolic pathways - *Saccharomyces cerevisiae* (budding yeast) [19]. Each dot indicates an intermediate of glycolysis and the tricarboxylic acid (TCA) cycle.

Yeast glucose metabolism (Figure 1) is a diverse set of processes that are significantly more complex than the key characteristics discussed above. Many different pathways form a network of reactions that control yeast growth. Each reaction is furthermore subjected to various regulatory effects that respond to environmental conditions. The responses differ for each yeast species and strain. In terms of yeast biomass production, the Crabtree effect is the most significant phenomenon [13]. This effect is defined as the occurrence of alcoholic fermentation, even under aerobic conditions [18,20,21]. Because the amount of energy produced during alcoholic fermentation is significantly less than that the amount produced during fully aerobic respiration, the Crabtree effect has to be avoided in biomass-directed processes such as the industrial production of yeast biomass for use in wine fermentations.

2.2.1 *Rationale behind the Crabtree effect*

Yeast 'developed' the ability to ferment during aerobic conditions as a survival tool [22]. The rationale behind this can be explained by means of the growth characteristics of *S. cerevisiae* during aerobic batch growth. When high levels of glucose is available, *S. cerevisiae* increases the rate at which glucose is taken up from the environment [22]. The strategy behind an increase in substrate uptake is to deprive other organisms of substrate, thereby increasing the chances of survival [23,24]. Furthermore, because glucose is the first choice for most organisms, *S. cerevisiae* is able to repress the uptake of less desirable substrates through catabolite repression, thereby eliminating glucose even faster [22].

However, at high substrate uptake rates the rate of glycolysis exceeds that of respiration in Crabtree-positive organisms [22]. This is described as the "overflow metabolism", where the "surplus" uptake of glucose is converted into ethanol, which creates another level of competitiveness [22]. A high tolerance to ethanol allows *S. cerevisiae* to limit the growth of other organisms, while its own ability to proliferate is unaffected.

The Crabtree effect can be divided into a short-term Crabtree effect (overflow metabolism) and a long-term Crabtree effect (glucose repression) [18].

2.2.2 *The short-term Crabtree effect*

The short-term Crabtree effect occurs when the respiratory pathway becomes saturated at excess glucose levels. This becomes apparent in glucose-limited chemostat cultures where, because of a sudden glucose pulse, ethanol is produced via aerobic alcoholic fermentation [24].

The key point where the metabolic flux diverges between alcoholic fermentation and respiration is at the point of pyruvate, at the heart of metabolism [18]. The fate of pyruvate can, on a physiological level, be determined by three reactions. The first reaction is catalysed by the pyruvate dehydrogenase complex, which is situated in the mitochondrial matrix [18]. This

complex catalyses the oxidation of pyruvate to acetyl-CoA, which ultimately fuels the TCA cycle.

A second possible reaction is that of pyruvate decarboxylase. Alcohol fermentation results from the decarboxylation of pyruvate to acetaldehyde and CO_2. Acetaldehyde can then be converted to ethanol by alcohol dehydrogenase. Acetyl-CoA can also be generated by the pyruvate dehydrogenase by-pass [18]. This indirect route takes the order of pyruvate decarboxylase, acetaldehyde dehydrogenase and finally acetyl-CoA synthase [18]. In this case, acetyl-CoA is produced in the cytosol and enters the mitochondrion through the carnitine shuttle. Both alcohol fermentation and pyruvate dehydrogenase by-pass reactions start with the decarboxylation of pyruvate.

As previously discussed, the citric acid cycle is also an anabolic pathway that produces many building blocks for cell growth. The last molecule in the citric acid cycle is oxaloacetate, which has to be regenerated with each cycle. If the flux of metabolism is directed towards alcoholic fermentation due to the Crabtree effect, oxaloacetate still has to be generated in some way to produce precursors for biosynthesis. This is achieved by way of a third possible route for pyruvate, the reaction catalysed by pyruvate carboxylase that carboxylates pyruvate to oxaloacetate.

As is evident, these three enzymes (pyruvate dehydrogenase, pyruvate decarboxylase and pyruvate carboxylase) compete for the same substrate. Both pyruvate dehydrogenase and pyruvate carboxylase have much higher affinities (lower K_m values) for pyruvate than pyruvate decarboxylase [18]. This is why the flux is directed to respiration in low glucose concentrations. However, it is important to keep in mind that the pyruvate dehydrogenase complex is located in the mitochondrial matrix, which implies that pyruvate first has to cross the mitochondrial membranes via a mitochondrial pyruvate carrier [6]. This indicates that

pyruvate decarboxylase and pyruvate dehydrogenase do not directly compete for pyruvate. However, pyruvate carboxylase and pyruvate decarboxylase do compete for pyruvate directly, being located in the same yeast organelle.

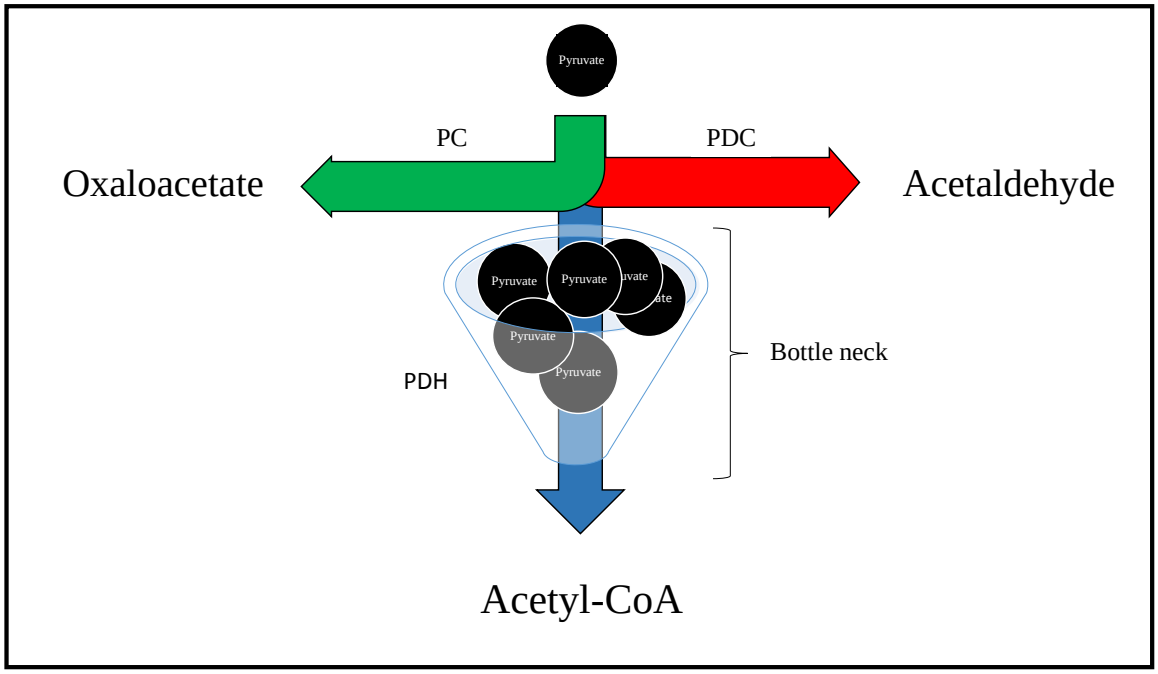

Figure 2: A basic representation of the bottleneck at the pyruvate branch-point that results in overflow metabolism. Pyruvate can be decarboxylated by pyruvate decarboxylase (PDC), carboxylated by pyruvate carboxylase (PC) or oxidised by pyruvate dehydrogenase (PDH). During overflow metabolism the metabolic flux is through pyruvate decarboxylase (PDC). Redrawn from Pronk et al. [18].

Overflow metabolism may be due to a bottleneck effect at pyruvate dehydrogenase (Figure 2) [18]. When *S. cerevisiae* is subjected to very high sugar concentrations it "bites off more than it can chew". The uptake of sugar into the cell is faster than the flux through respiration, which ultimately results in a build-up of intracellular pyruvate. This consequently decarboxylates by pyruvate decarboxylase to produce ethanol and CO_2. As mentioned, pyruvate decarboxylase has a much lower affinity for pyruvate than pyruvate carboxylase, but the K_m is not the only factor that determines the flux. Pyruvate decarboxylase has a much higher reaction capacity (V_{max}), which results in the flux not favouring pyruvate carboxylase, even though pyruvate carboxylase has a lower K_m [18].

The bottleneck may also be due to limited transport of pyruvate into the mitochondria [18]. Even though the mitochondria's affinity for pyruvate is more or less the same as that for pyruvate dehydrogenase (which is higher than that for pyruvate decarboxylase), the transport of pyruvate into the mitochondrial matrix occurs via the mitochondrial pyruvate carrier, which gets saturated at high levels of pyruvate (Figure 3). This will also cause a build-up of intracellular pyruvate, resulting in overflow metabolism.

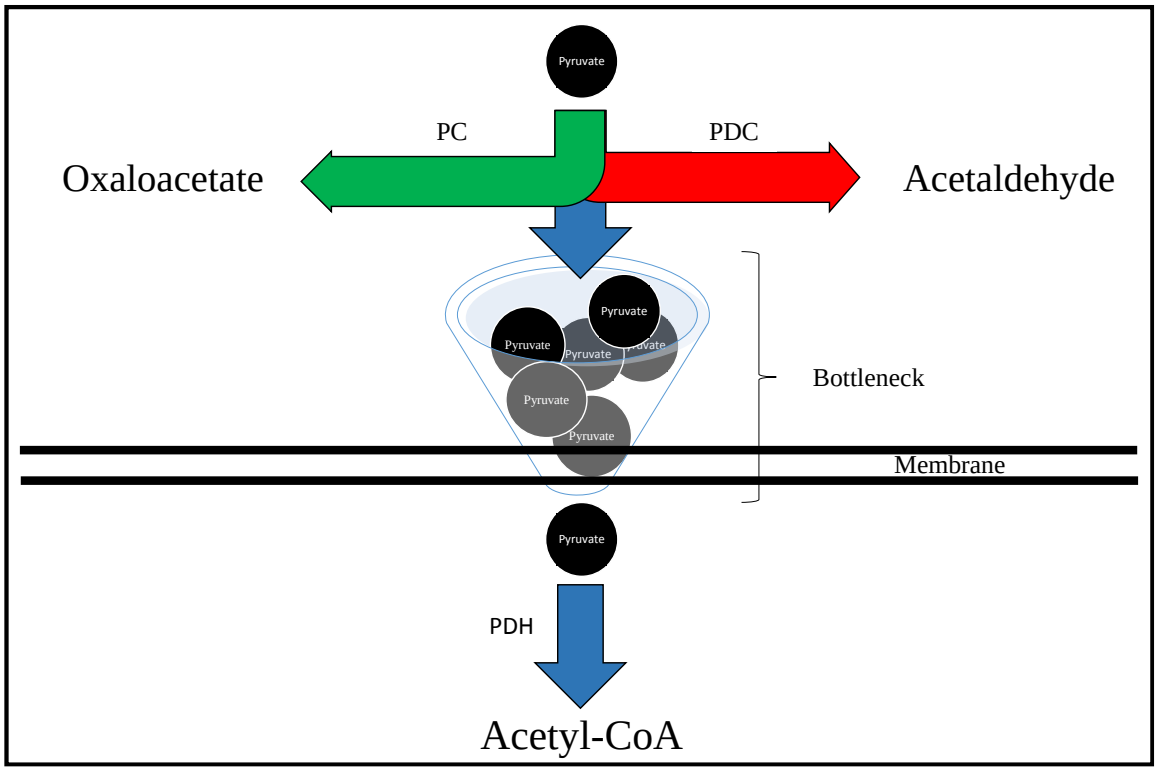

Figure 3: A basic representation of the bottleneck that may form as a result of saturated transport of pyruvate across the mitochondrial membrane. The enzymes pyruvate decarboxylase (PDC), pyruvate carboxylase (PC) and pyruvate dehydrogenase (PDH) are indicated. Redrawn from Pronk et al. [18].

2.2.3 *The long-term Crabtree effect*

Unlike the short-term Crabtree effect, which exists due to physiological constraints, the long-term Crabtree effect is a result of genetic regulation [25]. Carbon catabolite repression, used as a synonym for the long-term Crabtree effect, is defined as the repression of genes that encode for enzymes involved in respiration, mitochondrial function and the assimilation of fewer

desirable carbon sources at fast growth rates resulting from exposure to high glucose concentrations [25,26]. At high growth rates the fermentation pathway is fully expressed, while respiration is repressed in Crabtree-positive yeast [22]. During the growth of Crabtree-negative yeast the flow through glycolysis matches that of the TCA cycle, thereby avoiding overflow metabolism [22].

The transcriptional regulation is governed by glucose, which serves not only as substrate, but also as a regulator [25]. However, the respiration pathway of different yeasts is not repressed to the same extent when subjected to high glucose concentrations [22,27]. The transition from Crabtree-positive to Crabtree-negative is therefore not absolute, but relative because some yeast are more sensitive to glucose than others. In fact, when comparing the Crabtree-positive yeast *L. thermotolerans* to *S. cerevisiae*, the flux through the TCA cycle is ≈ 40 % higher than that of *S. cerevisiae* [27]. As in the case of Crabtree-negative yeasts, *L. thermotolerans* is therefore able to reach a biomass yield on glucose higher than the 0.51 g g^{-1} usually seen for S. *cerevisiae* [27].

2.3 Process kinetics for industrial yeast production

The metabolic state of a culture can be manipulated by the physical parameters of a process. The discussion above clearly reveals that the Crabtree effect can be avoided by employing a fed-batch culture so that the substrate is fed into the reactor at a limited rate. By controlling the substrate feed rate, the yeast can be forced to grow at a rate slower than the strain-specific critical growth rate (μ_{crit}) [28,29]. The strain-specific growth rate (μ_{spec}) is therefore a key parameter in yeast biomass production [28]. Van Hoek et al. kept the growth rate of a *S. cerevisiae* culture lower than 0.28h^{-1} to avoid ethanol production and typical biomass yields were as high as 0.49g g^{-1} of sugar [28].

The industrial production of *S. cerevisiae* for application in wine fermentation is often produced in baker's yeast production plants, involving a multistage process [30]. The process starts out in shake flask cultures known as a pre-culture. The cells are grown and inoculated into the batch reactor (the first reactor phase). During the lag phase, just after inoculation, the yeast cells produce the necessary enzymes to support growth in the new environment [7]. The presence of oxygen at the beginning of batch growth is necessary for lipid biosynthesis to proceed, which in its turn ensures that fermentation can proceed efficiently [30].

2.3.1 *Inocula production*

The physical status of the inoculum has a significant effect on the duration of the lag phase [8]. Therefore, cells are used as inocula while they are still in their exponential growth phase and actively growing. Inoculating with exponential phase cells will result in a shortened lag phase or even the absence of a lag phase [7], especially if the conditions in the reactor is more or less the same as that in the shake flask. The same medium is therefore used to generate pre-cultures. The age of the inocula is also an important factor to consider since older cells are less active. In the yeast production industry, cells produced in one process are used immediately to inoculate the next production process. The status of these recycled cells are closely monitored to ensure that the process is not compromised. Apart from the status of the inoculum, the size of the inoculum is importance. Inoculation with a small volume will result in an extended lag phase, ultimately increasing the total fermentation time. Inoculation size should be more or less 5-10% (v/v) of the total batch growth medium [8].

2.3.2 *Batch medium considirations*

Molasses is often used as a carbon source in industrial processes where *S. cerevisiae* is produced, for example in baker's yeast production [30,31]. Because molasses is a waste product from sugar production, it is not expensive and therefore economically viable. It

comprises about 70% sugars, mainly the disaccharide sucrose, which is hydrolysed by the yeast to give glucose and fructose [30]. This is achieved by the enzyme invertase, which can be either extracellularly active or produced inside the yeast cell. This implies that sucrose has to be transported into the cell before hydrolysis can occur [32]. The resulting monosaccharides are assimilated by way of the Embden-Meyerhof pathway (glycolysis).

However, the chemical composition of molasses is highly variable because of different sugar production procedures. In some cases variability can even result from different weather conditions [30]. Some toxins that can be detrimental to the yeast cells can also be present. Shima et al. [33] have shown the expression of FDH1 and FDH2 genes by yeast grown in molasses, which is a consequence of toxins in the molasses. The SUL1 gene was also expressed due to low levels of sulphate [33]. Some vitamins (thiamine, pantothenic acid and biotin) also had to be supplemented [30]. If the salt (i.e. NaCl) concentration of molasses is too high, the molasses has to be diluted. High salt concentrations can have detrimental effects on the growth rate, biomass yield and length of the lag phase [34]. One other shortcoming of molasses is the low assimilable nitrogen concentrations. As discussed in section 2.3.5, nitrogen is beneficial to cell growth and can have a substantial effect on biomass yields. It is therefore necessary to supplement the molasses growth medium with nitrogen, organic or inorganic [30,35].

2.3.3 *Batch phase*

In a batch culture, *S. cerevisiae* shows a diauxic growth that is indicative of a culture utilising more than one carbon source, for instance glucose and ethanol [36]. Ethanol is formed in batch cultures containing high sugar concentrations when *S. cerevisiae* grows at a maximum growth rate (μ_{max}) during the exponential growth phase. The high growth rates and high glucose concentration results in the production of ethanol due to the Crabtree effect. After the available glucose is exhausted, *S. cerevisiae* starts to metabolise the ethanol as an alternate carbon source,

resulting in the second growth spurt that is seen in diauxic growth [8]. The cells are able to fully assimilate the ethanol because oxygen is present. This transition triggers the yeast's metabolism to change from respiro-fermentative to fully respiratory, all the while eliminating ethanol from the medium [30].

The exposure to high sugar concentrations also ensures that the final product has some degree of fermentative capacity by producing reserve carbohydrates that can be used during fed-batch cultures [30]. Therefore, the batch phase during production is important. Jansen et al. has shown that prolonged exposure to glucose-limited growth that did not include a batch phase, caused a partial loss of glycolytic capacity [37]. The fermentative and glycolytic capacity of yeasts produced under conditions of limited carbon source availability through fed-batch culture to maximise biomass yields and to avoid ethanol production, should therefore be monitored.

When the sugars present from the start of the batch culture are completely assimilated, the fed-batch phase starts [30]. A spike in the dissolved oxygen concentration usually serves as an indication that carbon sources are exhausted. The inverse is true for carbon dioxide [9,38]. Initiating the feed profile before the sugars present in the batch medium are completely assimilated may result in overfeeding and essentially an increased batch phase. This will in turn be detrimental to the productivity of the fed-batch phase.

2.3.3.1 Cell growth in the batch phase

When yeasts are grown in a reactor different phases can be observed [7,8]. Initially, in the lag phase, the growth rate (μ) of the organisms are equal to zero since the cells do not grow, but adapt to the environment. After the cells have prepared the appropriate metabolic pathways, they enter the accelerated growth phase where the μ increases up to the point of the exponential growth phase, where $\mu = \mu_{max}$, the maximum specific growth rate of the yeast [7].

During the exponential growth phase, cell growth can be defined by [8]

$$lnX = ln\,X0 + \mu t \tag{3}$$

or in differential form [8]

$$dX/dt = \mu X \tag{4}$$

where X is biomass (g), X_0 the initial biomass (g), μ the growth rate (h^{-1}) and t the time (h) at any given point.

This would mean that the growth rate can be expressed as

$$\mu = \frac{d(lnX)}{dt} \tag{5}$$

A plot of ln$X$ versus time will give a straight line with a slope of μ_{max} based on equation 5

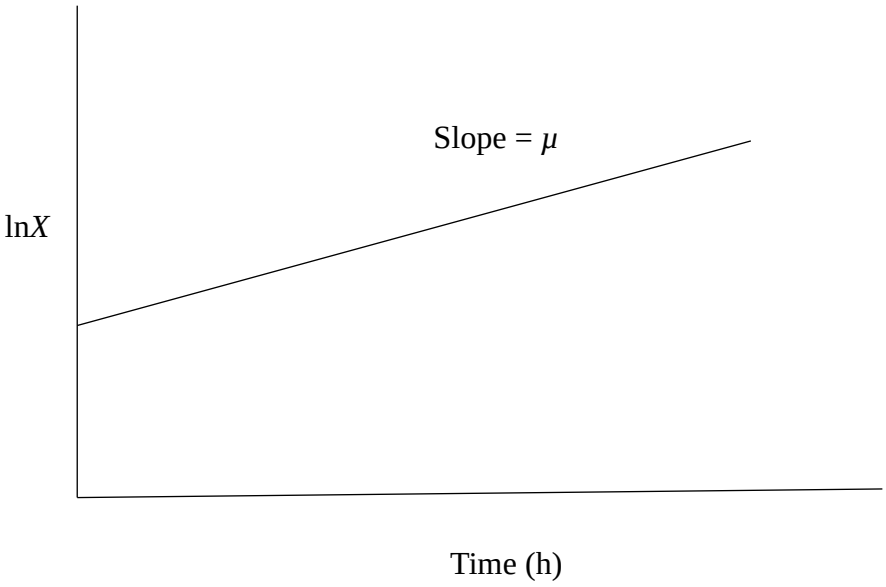

Figure 3 – Plot of ln$X$ versus time with a slope equal to the growth rate (μ, h^{-1}).

By integrating equation 5, the biomass at any given time can be calculated with [7]

$$X = X_0 e^{\mu t} \quad (6)$$

where μ is the specific growth rate μ_{spec} at time t.

The time required for the cells to double in number is given by [8]

$$t_D = ln2/\mu_{spec} \quad (7)$$

where t_D is the doubling time and μ_{spec} the specific growth rate (h^{-1}).

2.3.4 *Feed medium considerations*

In practice, the occurrence of respiro-fermentative metabolism is avoided by forcing the cells to grow below the μ_{crit}. This is achieved by maintaining an appropriate level of carbon source limitation in a fed-batch process [8,39] through control of the substrate concentration in the reactor while supplying the cells with enough oxygen to support aerobic respiration at a specific growth rate (μ_{spec}) slower than the μ_{crit}. Under substrate-limiting conditions cell growth is directly related (first order) to the substrate concentration in the reactor. The relationship between the substrate concentration (S) and μ_{spec} of an organism can be described by the Monod saturation kinetics

$$\mu_{spec} = \frac{\mu_{max}\, s}{Ks + s} \quad (8)$$

where s is the substrate concentration and K_s the saturation constant (substrate concentration at $1/2\mu_{max}$) [40]. At high substrate concentrations the relationship between the substrate concentration and μ_{spec} is no longer linear due to saturation.

The feed composition and concentrations during the fed-batch phase are also important factors to consider. In order to maximise the volumetric productivity of fermenters used for fed-batch culture, the feed is highly concentrated [39]. In fed-batch cultures the fermentation broth volume is allowed to change, which implies that the feed volume has a direct effect on the

fermentation time [39]. If a non-concentrated feed is used, the feed rate has to be higher to meet the substrate demand, meaning the maximum working volume of the reactor will be reached in a relatively short time. If, on the other hand, a very concentrated feed is used, the feed rate will be slow to avoid the build-up of residual substrate in the reactor. A longer fermentation as a result of a concentrated feed can increase the biomass concentration significantly [10].

2.3.5 *Nitrogen in the feed medium*

Nitrogen is another important substrate that is essential for yeast growth [30]. Yeast utilises a number of different nitrogen sources, both organic or inorganic, which are incorporated into the synthesis of cell components, for example amino acids and therefore proteins [8]. Nitrogen is often incorporated into the feed medium as not to become limiting and inhibit biomass formation. In glucose-limited cultures, the μ_{crit} is also dependent on the assimilable nitrogen concentration [35,41,42]. Compared to low concentrations of nitrogen, higher nitrogen concentrations can be allowed for a twofold increase in the μ_{crit} [35]. This shows that nitrogen limitation can result in an anabolic constraint on the cell, unlike glucose-limitation, which is a catabolic restraint [35].

During the industrial production of fermentative yeasts, the yeasts are subjected to nutrient limitations to induce the formation of stress-related molecules [43]. If the cells are starved of nitrogen, they produce trehalose and glycogen as storage carbohydrates [41,42]. Apart from serving as a source of carbon during substrate limiting conditions (i.e. storage), glycogen, and especially trehalose, is presumed to protect the cells from harsh conditions such as low temperatures and high osmatic pressures encountered during storage of yeast biomass [41,43]. However, to maximise the biomass yield during production of a Crabtree-positive yeast, it is important to adjust the carbon feed rate to avoid the formation of ethanol during the nitrogen-limiting phase [41].

2.3.6 *Fed-batch phase*

If the only aim of the process is to have the highest possible biomass yield ($Y_{x/s}$), the μ_{spec} should be as close as possible to the μ_{crit}. This will ensure the maximum growth rate without the onset of respiro-fermentative metabolism [44]. This is achieved by having good control over the sugar concentration, aeration, and especially μ_{spec} [9]. The system for control of the substrate feed should be designed according to either the quasi steady-state (*qss*) or dynamic control methods [45,46] to have control over μ_{spec} of the yeast during fed-batch culture.

2.3.6.1 Quasi steady-state method

A system is in *qss* when both the biomass and volume in the reactor increase in equal increments. This results in a constant biomass concentration in fed-batch culture and process conditions that approach those obtained during steady-state conditions during continuous cultivation (quasi steady-state implies a culture that approaches steady-state). During *qss* the dilution rate ($D = F/V$) is used to control (through adjusting the substrate feed pump) the μ_{spec} (a biological parameter). The following derivation can be used to explain this mathematically.

Firstly, the change in volume of a fed-batch reactor can be defined as [8]

$$\frac{dV}{dt} = F \tag{9}$$

where F is the flow rate (L h^{-1}) of the feed medium into the reactor.

The change in biomass in a fed-batch reactor is defined by

$$\frac{dX}{dt} = \mu X - k_d X \tag{10}$$

where μX is biomass accumulation (g h^{-1}) and $k_d X$ is cell death (g h^{-1}). If cell death is neglected ($k_d X << \mu X$) and a mass-concentration conversion is made ($X = xV$), then

$$\frac{d(xV)}{dt} = \mu xV \tag{11}$$

where x is the biomass concentration (g L^{-1}) and V the broth volume (L). Therefore

$$\frac{Vdx}{dt} + \frac{xdV}{dt} = \mu xV$$

and if substituted with $dV/dt = F$ and dividing the entire equation by V

$$\frac{dx}{dt} + x\frac{F}{V} = \mu x$$

Removing x as a communal factor generates Equation 12

$$\frac{dx}{dt} = x(\mu - \frac{F}{V}) \tag{12}$$

As mentioned, at *qss* there is no change in the biomass concentration with time [8]. Therefore it can be assumed that

$$\frac{dx}{dt} = 0$$

and if $dx/dt = 0$ is substituted into Equation 12

$$0 = x(\mu - \frac{F}{V})$$

recall $D = F/V$, then

$$0 = x(\mu - D)$$

$$xD = x\mu$$

and ultimately

$$D = \mu \tag{13}$$

This implies that at *qss* the μ_{spec} can be controlled by controlling *D*, which in turn can easily be manipulated by controlling *F*.

However, there are significant limitations to the *qss*-assumptions and method when applied to fed-batch culture. According to Dragosits et al. [47], a typically microbial culture requires 5 residence times to reach a true steady-state, while a *qss* should be close to this. In fed-batch culture, the total culture time, and therefore the number of residence times available for feeding, is limited by the total bioreactor volume available. If for example, a feed is started (after a batch phase of 4L) to achieve a $D = \mu = 0.1h^{-1}$, in a reactor with a working volume of 9 L, the *F* should be

$$F = DV$$

$$F = (0.1h^{-1})(4L)$$

$$F = 0.4L\,h^{-1}$$

Thus, the remaining 5 L will be filled in 12.5 h if a constant *F* of 0.4 L h^{-1} is maintained. Since residence time is the inverse of μ and it takes 4-6 residence times to reach *qss*, a total residence time (τ) of

$$\tau = \frac{1}{0.1h^{-1}}.4$$

$$\tau = 40h$$

is required to reach *qss*. This implies that even if sufficient total reactor time is available to reach a *qss* by the end of the fed-batch culture, the majority of the fed-batch run will still be conducted far from a true steady-state, implying that the *qss*-assumption will not hold true and that there will be no control over the μ.

The lack of control over μ during the time before *qss* leads to the second limitation of the *qss*-method. During the time before *qss* is reached, the μ may fluctuate. In yeast cultivations this may lead to a loss in productivity, for instance ethanol production in the case of Crabtree-positive yeast.

2.3.6.2 Dynamic methods

Dynamic feeding strategies can be divided into two major parts: i) predetermined or ii) feed-back [44]. A feed profile can be predetermined based on *in priori* knowledge of the system. It is usually necessary to adapt the feeding profile as the fermentation proceeds, because a predetermined feed profile is based on certain assumptions. Another way of feeding is to base the feeding on feedback information of the process, either direct or indirectly. Direct feedback is defined as the control of feeding based on online substrate concentrations [44], whereas indirect feedback is based on any measurement other than the substrate concentration, for instance pH, dissolved oxygen (DO), carbon dioxide evolution rate (CER), oxygen uptake rate (OUR), biomass concentration or respiratory quotient (RQ) [31,44,48].

2.3.6.3 Feeding with feedback control (direct and indirect)

There are a number of online measurements that can be used to control the feed in a fed-batch process. DO concentration is most frequently used as a means of controlling the feed rate [39,44]. Such operations are known as DO-stat fermentations. In a DO-stat a set value for oxygen is defined and should be maintained, for example 20% of saturation. If the DO drops below this set value, indicating oxygen limitation, the feed rate is lowered, allowing the DO to rise. If the DO rises above the set value, the feed rate can be increased again. A pH-stat operation can also be used because pH is affected in the same way as DO with changes in metabolism of the organism. Control using pH is not generally used because changes in pH is not as responsive to changes in metabolism compared to DO [44]. Exhaust gasses can also be

analysed as an indirect measurement of the metabolic activity of the culture. These measurement include CER, OUR, RQ, and in some modern fed-batch processes ethanol [49].

Online measurements of the substrate (direct feedback) can be achieved by incorporating high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS) and also some commercially available enzyme kits, which can give a quick indication of the substrate concentration [44]. Online measurements of carbon and nitrogen substrate concentrations can also be incorporated into the fermentation control system. These direct or indirect methods where however, not available in the current study. Therefore, a method which allowed for control of the system without feedback was incorporated.

2.3.6.4 Feeding without feedback control

In constant rate feeding, the limiting substrate is fed at a constant predetermined rate. At some point during the process when the cell concentration becomes high, the carbon supply will become a limiting factor. This will be detrimental the total biomass production. Therefore, although less complex, a constant feed rate results in a lower biomass yield compared to an exponential feed [44].

A step-wise feeding approach can also be employed. Pulse feeding may cause the occurrence of overflow metabolism. This is especially apparent in large reactors or in high cell densities because of limited oxygen transfer. Some studies have found that stepwise feeding may even cause DO fluctuations [41].

Having an exponential feed rate, sometimes referred to as specific growth rate control or the glucose flux method, can keep the growth rate at a constant rate [45,46,50]. The feed rate is often predetermined and adjusted throughout the cultivation as measurements are taken. The principle underlying the control of μ with D (as in the *qss*-method) is that the D governs the amount of glucose supplied to the reactor [50]. However, in the dynamic method there is no

qss assumption, and D does not equal μ. Instead, μ is controlled directly by supplying glucose at a feed rate (g h^{-1}) equalling the glucose flux demand that is required through the EMP to maintain a particular μ. Therefore μ can be controlled right from the start of the fed-batch phase without any of the loss in productivity one would find in the initial stages of the feeding method based on the *qss*-assumption. To calculate the required glucose feed rate for a particular μ the following derivation can be made:

The substrate (S) balance in a fed-batch fermentation is

$$\frac{dS}{dt} = s_f F - q_s X \tag{14}$$

where S is the amount of substrate (g) in the culture medium, s_f is the substrate concentration of the feed (g L^{-1}) and q_s the specific substrate consumption rate ($\mu/Y_{x/s}$). If a mass-concentration conversion is made ($S = sV$)

$$\frac{d(sV)}{dt} = s_f F - q_s x V \tag{15}$$

and therefore

$$\frac{sdV}{dt} + \frac{Vds}{dt} = s_f F - \frac{\mu X}{Y_{\frac{x}{s}}}$$

Assuming the residual substrate concentration is zero (or close to zero compared to S_f) then substituting with $F = dV/dt$, $ds/dt = 0$ and $sF = 0$ results in

$$0 + V(0) = s_f F - \frac{\mu X}{Y_{\frac{x}{s}}}$$

Rearranging the equation result in Equation 16

$$s_f F = \frac{\mu X}{Y_{\frac{x}{s}}} \tag{16}$$

and because $X = X_0 e^{\mu t}$ (Equation 6)

$$s_f F = \frac{\mu X_0 e^{\mu t}}{Y_{\frac{x}{s}}} \tag{17}$$

where $s_f F$ is ultimately the glucose demand (feed rate in g h^{-1}) of the biomass at a specific time t to grow at a set μ.

A feed pump can then be set at an F determined by

$$F = \frac{\mu X_0 e^{\mu t}}{s_f Y_{x/s}} \tag{18}$$

where F is the pump set point (flow rate) in L h^{-1} to achieve a feed rate of $s_f F$ (Equation 17).

In practice a mixed feeding profile is often used [44]. The fed-batch phase is started off with a predetermined exponential feed to accelerate cell growth. In order to avoid oxygen limitation the feed is decreased or kept constant during the last part of fermentation [9,28,41]. Mixed feeding is used especially in enzyme production processes if the product is not growth-associated. In this case the process is also initiated with an exponential feed to achieve an appropriate cell concentration, but when there are enough cells the feed is adjusted to maximize product formation [44].

To maximise the biomass production in the current study, a predetermined exponential feed profile was employed throughout the entire fed-batch culture. The agitation cascade that was used ensured that no oxygen limitation occurred at high cell densities.

2.3.7 *Fermentative performance*

The fermentative performance is defined as the ability of the yeast produced under particular (often aerobic) conditions to ferment sugars to ethanol, upon transfer/application to fermentative (often anaerobic or oxygen-limited) conditions. It is an important factor to consider when producing yeast for application in fermentative conditions [28]. As mentioned, during fed-batch culture the yeasts are produced at limited growth rates by feeding the sugar substrate at a limited rate. These limiting conditions are in conflict with the fermentative conditions that the organism will be introduced to after production, such as wine must, where there is an excess of carbon source, limited oxygen and the expectation of maximum ethanol production, rather than yeast biomass production (Table 1) [43]. Even before the yeast is introduced to the stress-inducing grape must (high glucose concentrations and osmotic stresses), the yeast will have been stored (rapid freezing or lyophilisation) and rehydrated (in the case of active dry yeast). These processes have detrimental effects on both yeast vitality and viability, and thus also the fermentative activity [30,43].

The presence of trehalose is thought to be one of the most important factors ensuring the survival of yeast throughout these post-production processes [30]. Reserve carbohydrates such as trehalose protect the yeast cells during storage at low temperatures because of their cryoprotectant properties. Furthermore, during wine fermentation the reserve carbohydrates trehalose and glycogen are metabolised as soon as the yeast is introduced to the new environment, even in the presence of high residual sugar concentrations [51]. This suggests that glycogen and trehalose play a major role during stress situations [43,51]. An increase in the amount of trehalose stored inside yeast cells will increase the rate at which the cells adapt to the grape must, and thus the onset of ethanol-fermentation [51]. Therefore, producing yeast with a high trehalose content may improve the quality of the product.

In terms of the fed-batch process for yeast production, a linear relationship has been reported between the fermentative performance of the organism and the growth rate at which it is produced [9,28]. This suggests that increasing the growth rate during fed-batch culture while still avoiding ethanol production by maintaining a growth rate below μ_{crit} will also maximise the fermentative capacity during subsequent wine fermentations with the produced yeast biomass.

In theory, the fermentative performance may also be directly related to the concentration of the enzymes active during fermentative metabolism [31]. As a result, the fermentative performance of yeast cells might also be effected by the protein content of a cell. This implies that producing yeast with a high protein content should be an important consideration [41].

Table 1 – Some of the differences between the production conditions and fermentation conditions an organism is subjected to. Reconstructed from Bauer 2000 [43].

Production Process	**Wine Fermentation**
Low sugar concentration (carbon limiting)	High sugar concentration (>200g/L)
High oxygen concentrations (>30% of saturation)	Low oxygen concentrations
Low ethanol concentrations	High ethanol concentrations (> 10% v/v)
Constant nitrogen supply	Variable nitrogen concentrations
Sterile environment	Competing organisms present
Physical environment adjusted to be optimal	Physical environment changing
Pressure constant	Hyperosmotic pressure
Temperature optimal (30°C)	Temperature mostly below optimal
pH optimal (+/- 5)	pH below optimal (3-3.7)
Respiratory metabolism	Fermentative metabolism

2.4 Process conditions

2.4.1 *Oxygen supply in aerobic cultivations*

2.4.1.1 Oxygen transfer rate (OTR)

Another important nutrient that is of absolute importance during aerobic fed-batch processes for yeast biomass production is oxygen, in particular the dissolved oxygen (DO) concentration, which can greatly influence the growth rate of the microbe on a first order basis [8,39]. In some cases the DO is kept between 60% and 80% of air saturation to ensure aerobic conditions [12]. The limitation of this substrate should be avoided if maximum flux through aerobic metabolism is desired.

Before oxygen can be utilised by the organism, it has to pass through three distinct phases [39]. First, the oxygen supplied to the reactor is in the gas phase and has to be dissolved in the liquid, implying that the oxygen molecules must migrate to the surface of the gas bubbles, pass the gas-liquid boundary and dissolve into the liquid. Once dissolved in the liquid medium, the oxygen molecules have to migrate to the yeast cell surface before it can finally be taken up by the organism.

The oxygen transfer rate (OTR) is defined as the rate at which oxygen is transferred from the gas phase to the liquid phase, encompassing all three phases described above, and can be described by the equation:

$$OTR = k_L a\,(C^* - C) \tag{19}$$

where C^* is the oxygen saturation concentration, C is the concentration of oxygen in the medium, "k_L" the mass transfer coefficient and the term "a" is the specific surface area of the sparged air bubbles or interfacial area [52]. The term $(C^* - C)$ in Equation 19 can be seen as the

driving force that drives the transfer of oxygen into the liquid phase. The equation can also be written as:

$$OTR = \frac{(C^* - C)}{(k_L a^{-1})} \quad (20)$$

In this form it becomes apparent that "k_L" is the resistance of transfer of oxygen to liquid. Because it is difficult to calculate the specific surface area of air bubbles (a) and the transfer coefficient (k_L) these factors are usually combined to give the volumetric transfer coefficient ($k_L a$). When combined the $k_L a$ is an indication of the aeration capacity of a reactor.

The overall change in oxygen concentration (dC/dt) is given by:

$$\frac{dC}{dt} = OTR - OUR \quad (21)$$

where *OUR* is defined as the oxygen uptake rate by the culture. The *OUR* is defined by:

$$OUR = Q_{Oxygen} X \quad (22)$$

where Q_{Oxygen} is the oxygen utilisation rate and X is the biomass concentration. Therefore, substituting equation 22 and 20 into equation 21 yields

$$\frac{dC}{dt} = k_L a\,(C^* - C) - Q_{Oxygen} X \quad (23)$$

From equation 23 it is clear that the oxygen concentration in a reactor is affected by the dissolved oxygen concentration (DO), oxygen transfer rate, the biomass and its demand for oxygen. If the fermentation is governed by aerobic metabolism the oxygen demand, and therefore Q_{Oxygen}, will be high [10].

2.4.1.2 Oxygen solubility

The solubility of oxygen itself is affected by other factors such as the temperature of the medium and the medium rheology [39]. The solubility of pure oxygen in water at 10°C is about

55 ppm and as the temperature increases to 30 °C, which is usually the operation temperature of yeast fermentations, the solubility drops to more or less 38 ppm [53]. The content of solids and dissolved salts also alter the solubility of oxygen in the fermentation broth.

2.4.1.3 The volumetric oxygen transfer coefficient (k_La)

The k_La of a reactor containing a particular fermentation medium/broth is influenced mainly by aeration, the design of the impeller (impeller design is not discussed), antifoam, viscosity, and most significantly, by the agitation rate [39]. These factors affect the aeration capacity by either altering the resistance to transfer (k_L) or by changing the specific surface area, number and residence time of the air bubbles in the medium. Larger air bubbles rise more rapidly than smaller bubbles, allowing less time for oxygen transfer from bubbles to medium [53] .

The air flow rate affects the k_La only to a certain extent because an oxygen sparger breaks up air bubbles insufficiently when sparged at extreme rates. Very high air flow rates can even be detrimental to the OTR due to flooding, which occurs when the impeller is unable to disperse the incoming air [39]. The dispersion of air bubbles is a direct function of the agitation rate for a particular impeller design. As with air flow rate, high agitation rates also have detrimental effects. This can be seen with shear sensitive cells, which are damaged by high agitation rates. A high level of agitation also generates foam, especially if the medium contains high protein concentrations as in cultivations where yeast extract and corn steep liquor is used as the nitrogen source [39]. If the reactor has insufficient head space, the foam can exit through the exhaust line, damaging filters and increasing the risk of contamination. A continued loss of foam may even lead to a significant loss of pressure inside the vessel [39]. Mechanical breakers or chemical antifoams can be used to control foam formation. However, all chemical antifoams are surface-acting surfactants, which, if present in high concentration, lower the k_La and therefore the aeration capacity of the reactor [8,39].

2.4.2 *Effects of Temperature*

Many factors other than the medium concentration and composition can affect microbial growth. As the operation temperature increases towards the optimal temperature, the growth rate of the organism increases exponentially for every 10°C [8]. High temperatures has a negative effect on the growth rate of yeast, but an even greater effect on the microbial thermal death rate [8]. At high temperatures the rate of death exceeds that of growth, resulting in an absolute decrease in viable cells. As discussed, the operation temperature can also have a detrimental effect on the solubility of oxygen (section 2.4.1.2).

2.4.3 *Effects of pH*

The pH of a growth medium has a direct influence on the enzyme activity of an organism, thus affecting the metabolic activity of the organism [8,39]. Fluctuating pH levels also increase the maintenance energy required for growth and thereby decreases the biomass yield. Ironically, the metabolic activity of the organism is the main reason for pH fluctuations. When excess sugar is present and metabolic flux is high, the organisms produce organic acids that lower the pH of the environment. When glucose is exhausted the pH rises again.

Different nitrogen sources also affect the pH of the growth medium. When ammonium is used as a nitrogen source, hydrogen ions (H^+) are released, resulting in a decrease in pH. However, when nitrate is used, the pH increases [8].

Medium pH can also be used to limit the growth of undesired bacteria. The optimum pH for most yeasts is 3 – 6, which is lower than the optimum for most bacteria [8]. Therefore, if the fermentation is run at for example a pH of 5, the growth of contaminants is limited, whereas the growth of the cultivated yeast is encouraged.

2.4.4 *Mixing Considerations*

Mixing in a reactor is achieved by means of agitation. As discussed earlier, the level of agitation has a great influence on the OTR in the reactor (section 2.4.1.3). This is because the agitator disperses the air throughout the medium. If mixing did not occur, aerobic conditions would only exist at the point of sparging.

Mixing is also important for the heat dispersion in the reactor and of cardinal importance for the dispersion of substrate [54]. In larger reactors the existence of substrate gradient are almost inevitable due to less efficient mixing than in smaller bioreactors. The substrate concentration is highest at the point of supply and decreases as the distance from the feed point increases. Therefore, cells will be exposed to fluctuating substrate concentrations. The existence of high concentration zones may cause a loss in biomass yield due to ethanol production by Crabtree-positive microbes. This is likely due to oxygen-limitation and ultimately the occurrence of overflow metabolism when cells are exposed to high substrate concentrations [31]. The compromised biomass yield may also result from the need for cells to continuously adapt their metabolism to different zones in the medium. [40]. The loss in biomass yield may be reduced by feeding at more than one point or by decreasing the concentration of the feed medium [40].

2.4.5 *Vessel pressure*

In practice a positive vessel pressure (pressure inside vessel higher than outside vessel) is used to 1) aid in oxygen transfer by increasing the solubility of oxygen [9,29], and 2) to decrease the chance of contamination because materials are forced out of reactor rather than into the reactor.

2.4.6 *Process conditions required for non-Saccharomyces yeast production*

Literature regarding the required conditions for *M. pulcherrima* [55,56], *I. orientalis* [36,55] and *L. thermotolerans* [55] biomass production is scarce. According to the study of Schnierda

et al. [55], it can be assumed that these non-*Saccharomyces* yeasts require similar parameters to that of *S. cerevisiae* [30,52]. Therefore, these yeasts can be produced in an aerobic culture with a DOT maintained at 30 % of saturation and a pH of 5, all the while maintaining a temperature of 30 °C [8,52,55].

2.5 Scale-up procedures

The physiochemical conditions inside the bioreactor has a significant effect on the culture and therefore on the productivity of the production process. When a production process is scaled up, for instance from bench scale to pilot scale, it is important that these process conditions remain within acceptable ranges. The so-called "scale-up" effect describes the limitation in mass transfer that occurs in large scale cultures [57]. Due to the increased volume, limitations in the distribution of substrate, heat and oxygen may have a detrimental effect on the biomass yield and quality of the microbial product. Different scale-up strategies have been employed to ensure the successful scale-up of a production process, which mainly includes 1) constant impeller tip speed, 2) constant gassed power per volume ratio (P_g/V_L), and 3) constant k_La [52,58,59].

For the scale-up of aerobic cultures, such as fed-batch production of yeast biomass in the present study, a constant k_La strategy seems to be the most appropriate [8,38,52]. The process starts by determining the k_La, usually by employing the dynamic gassing-out method where an actively growing culture is used instead of a gas [38,52]. The required aeration and agitation rates to achieve the same k_La at a different scale can then be determined using the transfer equation [38]

$$k_L a = \alpha \left(\frac{P_g}{V_L}\right)^a {v_s}^b \tag{24}$$

where α, a and b are empirical coefficients describing the relationship between the mixing intensity of the impeller (P_g/V_L, W m^{-3}), the gas flow rate v_s (m h^{-1}) and k_La. The determined parameters can then be implemented at a different scale to ensure that the culture is aerobic.

In many cases a down-scaling strategy was implemented to simulate the conditions of industrial scale fermentations [40,60,61]. This approach is especially useful when the large scale equipment is already in place and no alteration can be done. The k_La is then determined at large scale and any limitations can then be enforced on the bench scale cultures. By considering the limitations of the production equipment the culture can be simulated at bench scale and any optimisation thereof will be viable.

2.6 Experimental plan and objectives

As discussed in the previous sections, the main physiological response to be avoided during yeast biomass production is the Crabtree effect, which can occur due to high growth rates and/or in the presence of excess glucose concentrations [30]. These are the conditions that have to be avoided since the aim of this project is to achieve high yields of yeast biomass. If the only aim of the process is to have a highest possible biomass yield ($Y_{x/s}$), the specific growth rate (μ_{spec}) should be as close as possible to the critical growth rate (μ_{crit}), but not higher. This will

ensure the maximum growth rate without the onset of respiro-fermentative metabolism and therefore ethanol production [30]. However, the fermentative performance of the product should also be optimised by determining the effect of different growth rates on the subsequent fermentative performance.

A fed-batch culture offers a practical process strategy where the growth-limiting substrate is fed into the reactor at a specific rate, providing control of the culture's growth rate. It seems most promising to control the growth rate just below the μ_{crit}, a dynamic control approach based on the relationship between glucose flux and growth rate [44]. However, the Crabtree-physiology and μ_{crit} values for *I. orientalis*, *M. pulcherrima* and *L. thermotolerans* are not known and have to be determined in this project to allow for the design of an industrial yeast production method. Therefore the following aims and objectives were set out:

i. Determination of the growth rate of *I. orientalis*, *M. pulcherrima* and *L. thermotolerans* where a maximum biomass yield is reached in aerobic fed-batch cultures at 9 L scale (Chapter 3).

   For each organism the μ of interest was determined by employing a fed-batch process where the limiting-substrate feed rate (g h^{-1}) was adjusted over a range of growth rates. The biomass yield and ethanol production during each cultivation were be monitored. The experiments were compared to a typical aerobic fed-batch culture with *S. cerevisiae*. Cultivations were conducted at 9 L in a bioreactor.

ii. Evaluation of the yeast biomass quality according to its fermentative performance, corroborated by the acidification power, after production at 9 L scale (Chapter 3).

   The fermentative performance of each yeast was evaluated by determining the acidification power (AP) of each respective culture. The AP of a culture gives an indication of its viability and vitality.

iii. Scale-up of yeast production to 90 L employing the best conditions for biomass production as determined by the 9 L cultivations and acidification power tests (Chapter 3).

A constant k_La scale-up strategy was used to scale the production of at least one non-*Saccharomyces* yeast up to 90 L. A dynamic k_La determination was used where an active growing culture was used to remove oxygen from the system.

iv. Validation of the final yeast biomass product according to the fermentative performance during anaerobic synthetic wine medium fermentations after being produced in 9 L aerobic fed-batch cultures.

The final yeast product was evaluated according to its fermentative performance. The fermentation kinetics of *I. orientalis*, *M. pulcherrima* and *L. thermotolerans*, based on weight-loss experiments, were determined in synthetic wine medium. Evaluation of the product was done in collaboration with the Institute of Wine Biotechnology (IWTB, Stellenbosch University).

Chapter 3

Non-Saccharomyces wine yeast production in aerobic fed-batch culture

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3.1 Abstract

Non-*Saccharomyces* yeasts were associated with wine spoilage until some strains emerged as important agents to enhance the organoleptic properties of wine. The biomass yields ($Y_{x/s}$) and subsequent fermentative performances in synthetic grape must of *Lachancea thermotolerans* Y1240, *Issatchenkia orientalis* Y1161 and *Metschnikowia pulcherrima* Y1337 were optimised in aerobic fed-batch cultures for seed culture production in commercial wine fermentations in comparison to *Saccharomyces cerevisiae* (Lalvin EC1118). Similar to *S. cerevisiae*, *L. thermotolerans* exhibited Crabtree positive behaviour at growth rates greater than 0.21 h^{-1} (μ_{crit}), with the $Y_{x/s}$ of 0.76 g g^{-1} recorded at 0.21 h^{-1} (46% of μ_{max}) during 9 L cultivation. Producing *L. thermotolerans* at 0.133 h^{-1} (36 % μ_{max}) at 90 L scale resulted in unwanted ethanol concentrations of up to 10.61 g L^{-1}, possibly due to substrate gradients that resulted in overflow metabolism, lowering the biomass yield to 0.54 g g^{-1}. On the other hand, *I. orientalis* and *M. pulcherrima* exhibited Crabtree-negative behaviour with $Y_{x/s}$ values of 0.83 g g^{-1} and 0.68 g g^{-1} respectively, both recorded at specific growth rates below 32 % of their maximum specific growth rate (μ_{max}). This is the first study to explore the effects of fed-batch production growth rates on $Y_{x/s}$ and fermentation performance of these non-*Saccharomyces* yeast at bench- and pilot scale.

Keywords: Fed-batch Culture, Wine Yeast, Fermentation performance, Scale-up Cultivation, non-*Saccharomyces* yeast, Crabtree effect.

3.2 Introduction

The use of non-*Saccharomyces* yeasts, mostly associated with wine spoilage [62,63], has been receiving increasing attention in the wine industry [1,2,64] due to their intriguing ability to impart unique organoleptic properties to wine [1]. Additional benefits of using these yeast species in wine fermentations include: (i) decreased final alcohol concentrations [65,66], (ii) improved wine acidity [66], and (iii) improved downstream filtration by facilitating the degradation of proteins through proteolytic enzyme production [1]. Furthermore, conventional methods for controlling the growth of undesirable organisms make use of chemicals such as sulphur dioxide [2], while non-*Saccharomyces* yeasts produce natural killer toxins that limit the growth of undesirable organisms [2] in a natural and environmentally safe manner.

Generally, non-*Saccharomyces* yeasts are used in sequential fermentation strategies through inoculation at the start of the wine fermentation process, prior to inoculation with *Saccharomyces cerevisiae* [1]. Low tolerance to ethanol and inferior sugar uptake rates limit the ability of non-*Saccharomyces* yeasts to compete with *S. cerevisiae* for survival during later stages of the fermentation when *S. cerevisiae* takes over the role as main fermenting organism [1,67,68]. Even though these yeasts do not contribute to ethanol production during later stages of wine fermentation, they remain metabolically active [4] and continue to improve the wine quality through their synergistic interaction with *S. cerevisiae* [69] or by producing metabolites such as glycerol [69,70]. Since these non-*Saccharomyces* yeasts are primarily used for their organoleptic contribution and metabolite production, it is important to evaluate the fermentation performance of non-*Saccharomyces* yeasts not solely in terms of fermentation vigour (ethanol production), but also the metabolic state of the yeast. The term ‘fermentation performance’ is used in this article to describe the overall ability of a yeast to adapt to, and fulfil the function it is used for in grape must fermentations.

The production of *S. cerevisiae* for the wine, brewing and baking industry is a well-established industrial process [30] based on the unique physiology of this yeast [18,42]. The Crabtree effect is a key characteristic of this organism that can be detrimental to the biomass yield due to the onset of ethanol production at high growth rates (long-term Crabtree effect) or high residual sugar concentrations (short-term Crabtree effect) [13,20,71]. In yeast the Crabtree effect is thought to have occurred as a means of survival when competing for substrates [22,24]. In *Saccharomyces* lineages the preference to ferment during aerobic conditions is even further enhanced due to the rewiring of the genes' encoding for the alcohol dehydrogenase pathway in *Saccharomyces* lineages [72]. Blank et al. [27] have come to the conclusion that the Crabtree effect is not absolute, but rather gradual when comparing different yeasts, implying that some yeasts are more sensitive to glucose than others. Indeed, Hagman et al. [24] have found that when comparing the Crabtree effect of aerobic ethanol producing yeast, *S. cerevisiae* is superior.

In *S. cerevisiae*, the transition from respiration to a respiro-fermentative metabolism is governed by the glucose concentration in the culture medium through a series of transcriptional changes in gene expression resulting from catabolite repression [25,73]. Dikicioglu et al. [25] have defined catabolite repression as the ability of glucose to regulate the metabolism, resulting in the repression of genes expressing coding for enzymes associated with the utilisation of carbon, respiration, mitochondrial development and gluconeogenesis. Under these conditions, the rate of glycolysis exceeds that of respiration, resulting in a bottleneck at the pyruvate junction [18]: Pyruvate is then diverted to ethanol production, resulting in a decrease in the biomass yield [20]. Apart from *S. cerevisiae*, Crabtree or Crabtree-like behaviour has been described for various other yeasts [27,74,75], including *L. thermotolerans* [22,27], and even bacteria [76]. However, in the case of *L. thermotolerans*, the Crabtree effect seems less pronounced than in *S. cerevisiae* [26], apparently due to less severe repression of the respiratory

pathway in response to high glucose concentrations [26,27], or a more efficient oxidative phosphorylation [12]. The development of an industrial fed-batch process for production of the Crabtree-positive *L. thermotolerans*, and the Crabtree-negative, *I. orientalis* and *M. pulcherrima*, yeasts for maximum biomass yields is therefore required.

To ensure high biomass yields, *S. cerevisiae* is produced using aerobic fed-batch culture with molasses as a low cost carbon source [30] and an exponential feed strategy to maintain a low growth rate. Fed-batch culture allows the Crabtree effect to be avoided by (i) ensuring the culture remains carbon-limited (medium formulated with all other medium components in excess), thereby avoiding high sugar concentrations, and (ii) limiting the rate at which carbon is supplied to the culture, thereby ensuring that culture growth is maintained at a specific growth rate lower than μ_{crit} [30,38]. However, the growth rate at which the yeast is produced could also affect the metabolic state of the yeast, which directly impacts on the fermentative performance during subsequent grape juice fermentation to wine [9,28]. It is therefore not only important to produce yeast at a growth rate that will result in high biomass yields, but also to ensure a high fermentation performance of the yeast biomass.

Limited information is available on the commercial production of non-*Saccharomyces* wine yeasts [36,55]. Attaining high biomass yields on an industrial scale with these yeasts relies on understanding the growth characteristics of these non-*Saccharomyces* yeasts in high density fed-batch culture. To the knowledge of the authors no information is in existence on (i) where the fed-batch production of these novel non-*Saccharomyces* wine yeasts has been investigated, (ii) how the transient conditions in fed-batch culture might affect production performance, and (iii) to what extent the cultivation conditions might impact on the subsequent fermentation performance of the final yeast product. This study aims to address this limitation in available process and yeast physiology. The study is done in comparison to a typical *S. cerevisiae* wine yeast.

3.3 Materials and methods

3.3.1 *Yeast strain and inoculum preparation*

Stock cultures of the wine yeasts *Saccharomyces cerevisiae* Lalvin EC1118 (supplied by Lallemand Inc., South Africa), *Lachancea thermotolerans* IWBT Y1240, *Issatchenkia orientalis* IWBT Y1161 and *Metschnikowia pulcherrima* IWBT Y1337 (deposited in the yeast culture collection of the Institute for Wine Biotechnology, Stellenbosch University, South Africa) were stored in 2 mL aliquots containing 40 % (v/v) glycerol as cryoprotectant at -85 °C. The cells were activated for 12 h in an Erlenmeyer flask containing 40 mL YD broth consisting of (per litre): 20 g glucose and 10 g yeast extract (all Merck, Gauteng Province, South Africa), before transferring the whole culture volume to 360 mL YD medium in a 2 L Erlenmeyer flask. This inoculum culture was incubated at 30 °C for 10 to 11 hours, which coincided with the late exponential growth phase, depending on the organism, in an orbital shaker adjusted to 150 RPM (Gallenkamp, Leicestershire, UK). The whole volume of this culture was used to inoculate the bioreactor, accounting for 10 % (v/v) of the volume during the batch phase. This amounted to a volume of 4 L.

3.3.2 *Medium preparation*

Molasses donated by NCP Alcohols (Durban, South Africa) was used as carbon source during all fermentations. It consisted of 390.27 ± 16.55 g L^{-1} sucrose, 86.47 ± 2.82 g L^{-1} fructose and 55.65 ± 1.71 g L^{-1} glucose. The molasses, stored at 18 °C, was diluted in a 1:3 ratio using reverse osmosis (RO) water before removing the solids ($22.8\,\% \pm 0.52$, w/w) by centrifugation at 10 020 rcf for 30 min. The batch cultivation medium consisted of diluted molasses with a total sugar concentration of 15 g L^{-1}, representing the combined concentrations of sucrose, fructose and glucose. Based on the method followed by Schnierda et al. [55], the medium was supplemented with 10.25 g L^{-1} yeast extract, which amounted in a yeast

assimilable nitrogen (YAN) concentration of 500 mg L^{-1}. The feed medium during the fed-batch phase had a total sugar concentration of 70 g L^{-1} and a YAN concentration of 500 mg L^{-1}. A feed concentration of 70 g L^{-1} was chosen to best accommodate the dynamic feed strategy and flow rate capacity of the feed pumps. Based on the dynamic feed method employed (section 3.3.4), a higher feed concentration would result in a flow rate below the minimum setpoint of the pump, while the opposite will be true at low feed concentration.

Since *I. orientalis* and *M. pulcherrima* lack invertase activity, Max Invertase®, donated by DSM (Heerlen, The Netherlands) was added aseptically at a concentration of 200 µL L^{-1}. The Max Invertase® was filter sterilised using a syringe filter (0.20 µm Syringe filter, Gema Medical S.L., Barcelona, Spain) before it was added to the medium. The appropriate invertase concentration was determined in separate experiments (data not shown). Hydrolysis was conducted after medium sterilisation at 60 °C for 12 hours, agitating at 150 RPM prior to inoculation with yeast.

3.3.3 *Cultivation control*

All bench scale cultivations were carried out in BioFlo® 110 Modular bench top bioreactors (New Brunswick Scientific-Eppendorf, NBS, Enfield, USA) with a total volume of 14 L. The bioreactor was equipped with two Rushton impellers and an exhaust condenser to limit evaporation. The pH was monitored with a gel-filled pH probe (Mettler-Toledo Ingold, Columbus, Switzerland) and the culture pH was maintained at pH 5.0 by automatic titration using 3 N KOH. Aeration was supplied with a submerged sparger at a volumetric air flow rate of approximately 1 vvm (L/L m^{-1}), corresponding to a rate of 4 – 9 L m^{-1} by manually changing the air flow rate using a rotameter during the course of fed-batch fermentations. Dissolved oxygen (DO) was measured using a polarographic oxygen sensor (InPro® 6000 Mettler-Toledo, Columbus, Switzerland) and the DO of the culture was maintained at a minimum of 30 %

saturation using an agitation control cascade with an agitation range of 150 RPM to 1200 RPM. A constant temperature of 30 °C was maintained throughout all cultivations. The formation of foam was limited by supplementing the batch- and feed medium with 200 µL L^{-1} antifoam (Antifoam 204, Sigma–Aldrich, Kempton Park, South Africa).

All large scale (90 L) cultivations were carried out in a 150 L bioreactor (New Brunswick Scientific, Enfield, CT, USA). The reactor was equipped with two Rushton impellers with a maximum agitation rate of 450 RPM. The pH and DO were monitored using a dissolved oxygen probe (polarographic) and a temperature compensated glass pH electrode (all Endress Hauser, Reinach, Switzerland). The volumetric air flow rate was controlled with an EL-FLOW® air flow meter (Bronkhorst High-Tech B.V., Ruurlo, The Netherlands). An Indusoft® Supervisory Control and Data Acquisition (SCADA) system (Indusoft Inc., Austin, TX, USA) was used for control over the system which included the substrate feed rate. The cultivation parameters were maintained as in the case of the bench scale cultivations and the medium fed into the reactor was tracked gravimetrically.

3.3.4 *Exponential feed profile*

The fed-batch phase commenced upon sugar depletion at the end of the batch phase. Sugar depletion was identified from a sudden increase in the dissolved oxygen levels, which was confirmed with subsequent sugar analyses (see section 3.3.9). The feed for all bench scale fermentations was administered at two separate entry points (to reduce substrate gradient formation) using a single peristaltic pump (Gilson Minipuls® 3, Middleton, USA), followed by a split in the feed line.

The pump was controlled by the supervisory software NBS BioCommand® v3.30 Plus to maintain a constant growth rate, using a pre-determined exponential feed profile as input. This profile was calculated using Equation 25 [38,46]

$$F = \frac{\mu X_0 e^{\mu t}}{s_f Y_{\frac{x}{s}}} \tag{25}$$

were F is the flow rate (L h^{-1}), μ the desired specific growth rate (h^{-1}), X_0 the biomass at the end of batch phase (g), t the time (h), s_f the sugar concentration (g L^{-1}) of the feed medium and $Y_{x/s}$ the biomass yield on sugar (g g^{-1}). The $Y_{x/s}$ used in Equation 25 was that determined by Schnierda et al. [55]. The numerator in Equation 25 was used to predict the growth of the organism at any point in time (t), including the time at which the feed was started.

Equation 25 was derived by calculating the required feed rate (g h^{-1}) to sustain a biomass of X_0 at a growth rate of μ. The following derivation can be made to calculate the required feed rate:

The substrate (S) balance in a fed-batch fermentation is [52]

$$\frac{dS}{dt} = s_f F - q_s X \tag{26}$$

Where s_f is the substrate concentration of the feed (g L^{-1}), F the flow rate (L h^{-1}), S the amount of substrate (g) in the culture medium and q_s the specific substrate consumption rate ($\mu/Y_{x/s}$). If a mass-concentration conversion is made

$$\frac{d(sV)}{dt} = s_f F - q_s X \tag{27}$$

and therefore

$$\frac{sdV}{dt} + \frac{Vds}{dt} = s_f F - \frac{\mu X}{Y_{\frac{x}{s}}} \tag{28}$$

where s is the substrate concentration of the culture medium, and V the culture volume (L). Assuming the residual substrate concentration (s) is zero (or close to zero compared to s_f) then

$$s_f F = \frac{\mu X}{Y_{\frac{x}{s}}} \quad (29)$$

and by substituting with $X = X_0 e^{\mu t}$

$$s_f F = \frac{\mu X_0 e^{\mu t}}{Y_{\frac{x}{s}}} \quad (30)$$

where $s_f F$ is ultimately the required feed rate (g h^{-1}) of the biomass X_0 (g) at a specific time t (h) to grow at a set μ (h^{-1}). By dividing Equation 30 with the feed concentration, the flow rate F (L h^{-1}) is determined (Equation 25). Sugar analysis during fed-batch confirmed that no over-feeding occurred, while yeast growth rates were determined from actual biomass measurements during fed-batch culture (see below).

3.3.5 *Scale-up cultivations*

The scale-up from bench scale to pilot scale was based on a constant volumetric mass transfer coefficient ($k_L a$) to ensure that the oxygen transfer is maintained at a sufficient level [38]. The dynamic gassing-out method was used to determine the $k_L a$ achieved in bench scale culture, which was then used as a benchmark for pilot scale cultures [8,38,52]. The dynamic gassing-out method is advantageous over the static gassing-out method in that an actively growing culture is used to remove oxygen from the system instead of a gas, like for instance nitrogen [8,52]. The $k_L a$ was determined by the equation [38,52]

$$\ln\left(\frac{Ce-C1}{Ce-C2}\right) = k_L a(t_2 - t_1) \quad (31)$$

where C_e is the equilibrium DO concentration in the culture at the prevailing aeration and agitation rates before gassing-out commenced, and C_1 and C_2 are respective DO concentration at t_1 and t_2 during re-oxygenation. The $k_L a$ was then calculated from the slope of a curve where ln (C_e-C_1/C_e-C_2) was plotted as a function of (t_2-t_1) as described by Doran [52].

The maximum $k_L a$ achieved at bench scale was used to determine the required agitation and

aeration rates needed to achieve the same k_La at pilot scale by considering the relationship between k_La, agitation and aeration described by transfer Equation 32 [38]

$$k_La = \alpha \left(\frac{P_g}{V_L}\right)^a {v_s}^b \tag{32}$$

where α, a and b are empirical coefficients describing the relationship between the mixing intensity of the impeller (P_g/V_L, W m^{-3}), the gas flow rate v_s (m h^{-1}) and k_La. The empirical coefficients were calculated by determining k_La at different values of P_g/V_L and v_s and using simultaneous division to solve the unknown coefficients in Microsoft Excel Solver (Microsoft, Redmond, WA, USA). The gassed power per unit volume (P_g/V_L) that is used in Equation 32 was calculated using Equation 33 [38,77]

$$\left(\frac{P_g}{V_L}\right) = \frac{\frac{Pg}{Po}\rho N^3 {D_i}^5}{{D_i}^3} \tag{33}$$

where P_g/P_o is the ratio of the gassed power to un-gassed power [52], ρ the broth density (kg m^{-3}), N the impeller speed (Hertz) and D_i the impeller diameter (m^3). The gassed-power-to-ungassed-power ratio was calculated from Equation 34, as described by Doran et al. [52]

$$\frac{P_g}{P_o} = 0.10 \left(\frac{F_g}{NV}\right)^{-0.25} \left(\frac{N^2 {D_i}^4}{9.82 W_i V^{\frac{2}{3}}}\right)^{-0.20} \tag{34}$$

where F_g is the superficial gas velocity (m^3 s^{-1}), V the volume (m^3) and W_i the impeller width (m^3).

3.3.6 *Specific growth rate calculations*

In an effort to verify that a constant specific growth rate was maintained in fed-batch cultures, actual measurements of biomass concentrations during fed-batch was used to calculate actual growth rates for each culture, using the equation [38]

$$\mu = \left(\frac{1}{X}\right)\left(\frac{dX}{dt}\right) = \frac{d(lnX)}{dt} \tag{35}$$

where X is the number of cells (g) at time t. The specific growth rate was determined by plotting ln X as a function of time (Figure 4). A minimum of four data points were used to calculate μ from the slope of the curve using Equation 35. For accurate estimation of the total culture volume (V), the volume of the feed medium added to the reactor vessel up to the specific sampling point, the total volume of KOH added for pH control as well as the sampling volumes withdrawn from the vessel were monitored. The amount of medium added to the culture was monitored gravimetrically throughout the duration of the fed-batch phase.

3.3.7 *Yield calculations*

The biomass yield on consumed sugar ($Y_{x/s}$), ethanol yield on consumed sugar ($Y_{eth/s}$) and ethanol yield on biomass ($Y_{eth/x}$) was determined at the end of the fed-batch phase. For instance, $Y_{x/s}$ was calculated by Equation 36

$$Y_{x/s} = \frac{X - X_0}{S_0 - S_R} \tag{36}$$

where X is the biomass (g) at the end of the cultivation, X_0 the biomass (g) at the beginning of the fed-batch phase, S_0 the amount of sugar (g) at the beginning of the fed-batch phase and S_R the residual sugar (g) at the end of the cultivation.

The $Y_{x/s}$ was also confirmed by linear regression as the slope of at least four data points for biomass plotted as a function of consumed sugar ($R^2 > 0.90$) over the course of the fed-batch culture.

3.3.8 *Acidification Power test*

The Acidification Power (AP) of the produced yeast biomass, indicating the effectiveness with which an carbon source is utilised and thus the yeast fermentative activity, was estimated according to the method of Opekarová and Sigler [78], using a 1.12 M (20.2% m/v) glucose solution [79]. When carbon is metabolised, hydrogen ions are released into the environment through ATPase activity [79]. The test was conducted in a 50 mL conical tube and agitated using a magnetic stirrer. The test commenced by suspending 1 g washed (with distilled water, dH_2O) cells in 10 mL dH_2O, pre-heated to 30 °C. After 10 min, 500 µL of the glucose solution, also pre-heated to 30 °C, was added and the reaction was left for another 10 min. The pH was monitored at 1 min intervals throughout the entire test using a Crison® (Barcelona, Spain) GLP 21 combination pH probe. The AP was calculated using the equation [78,80]

$$AP = pH_{init} - pH_{20} \tag{37}$$

where pH_{init} and pH_{20} represented the pH values of the reaction mixture at times 0 and 20 min respectively. A pH_{init} of 6.3 was used by adjusting the dH_2O with 0.05 N NaOH to allow comparison of results to that of other studies [81]. The AP value was then used as a tool to compare the fermentative capacity or metabolic state of yeast produced in different cultivations. Cells with an AP < 0 were considered damaged, whereas cells with an AP value between 0 and 1 were partially damaged and cells with an AP > 1 were considered to be metabolically active [78].

Samples were taken at the end of each fed-batch culture and stored in 40% glycerol (v/v) at -85 °C for 7 days before testing the AP.

3.3.9 *Analytical methods*

The dry cell weight (DCW) was determined by filtering a 5 – 0.5 mL sample, depending on the biomass density, through a pre-weighed 0.45 µm cellulose acetate filter (Sartorius Stedim Biotech, Germany) before rinsing the pellet using 10 mL dH_2O. The filter together with the filter cake was then heated for 8 min in a microwave oven at 330W, followed by cooling in a desiccator for 10 min before subtracting the mass of the filter from the combined mass.

Residual sucrose, glucose and fructose were analysed using high performance liquid chromatograph (HPLC) equipped with an Xbridge Amide column (Waters, Milford Massachusetts, United States of America) and an ELSD detector. A gradient elution strategy, using H_20 and 85 % acetonitrile as eluent, was used at a maximum flow rate of 0.7 mL m^{-1} and a column temperature of 30 °C. Residual ethanol and glycerol were analysed using an HPLC (Thermo Scientific, MA, United States of America) equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa) and an Aminex HPX-87H Column (Bio-Rad, Johannesburg, South Africa) which was maintained at 65 °C. The RI detector (Shodex, RI-101) used was maintained at 45 °C. A 0.005 M H_2SO_4 solution with a flow rate of 0.6 mL m^{-1} was used as eluent.

The residual yeast assimilable nitrogen (YAN) concentration was determined after each cultivation using the formol titration method adapted from Gump et al. as modified by Schnierda et al. [55,82] to confirm that nitrogen was not limiting. The method is readily used in the wine industry and determines the amount of residual nitrogen (mg L^{-1}) available for assimilation by the organism. A 4 mL sample of the fed-batch growth medium was diluted to a final volume of 12 mL using RO H_2O, whereafter the solution was adjusted to pH of 8 using 0.05 N NaOH. Subsequently, 2 mL of a 37% (v/v) formaldehyde solution (Sigma-Aldrich, Kempton Park, South Africa), also adjusted to pH 8 using 0.05 N NaOH, was added to the

solution. Formaldehyde reacts with free nitrogen groups of α-amino acids, releasing hydrogen ions in proportion to the amount of available nitrogen [82]. As soon as the reaction was complete, as evident from a stable pH reading, the solution was titrated back to pH 8 using 0.05 N NaOH. The titrated volume was recorded and the YAN concentration determined using the equation

$$N = n \times 175 \quad (38)$$

where N is the YAN in mg L^{-1} and n the titration volume (mL).

Analysis of variance (ANOVA) for batch cultures was carried out using MS Excel (Microsoft, Redmond, WA, USA), and a P-value < 0.05 was considered significant.

3.3.10 *Synthetic wine fermentation sample preparation*

A yeast sample was harvested at the end of each fed-batch culture and washed using distilled water before re-suspending the yeast in glycerol with a final concentration 40 % (v/v). The samples were then stored at 85 °C for 7 consecutive days before synthetic wine fermentations were conducted. Samples from *Saccharomyces cerevisiae* fed-batch cultures were used as a control.

3.3.11 *Synthetic wine fermentation*

The synthetic wine medium was that of Henschke & Jiranek [83], combined with the amino acid mixtures described by Bely et al. [84]. Glucose and fructose were each supplemented at 100 g L^{-1} to 100 mL fermentation medium, in 250 mL Erlenmeyer flasks and anaerobically incubated for 7 consecutive days at 20 °C without agitation.

3.3.12 *Synthetic fermentation: kinetics and analytic methods*

Daily gravimetric measurements allowed estimation of the fermentation rate by weight-loss as a result of CO_2 evaporation. To further evaluate the fermentation performance of the yeast,

residual glucose, fructose, ethanol and acid content were analysed after each fermentation by means of Fourier-transform mid-infrared (FT-MIR) spectroscopy using a FOSS WineScan™ (Hillerød, Denmark) instrument.

The fermentation rate gives an indication of the efficiency with which the specific organism will complete the fermentation. The fermentation rate was calculated as the average amount of CO_2 produced per day over a 7-day period [85,86]. The fermentation purity was determined as the volatile acids (in this case acetic acid in g L^{-1}) divided by the total amount of ethanol (v/v) produced at the end of the 7-day fermentation [85,86]. A high value indicates the ability of an organism to produce low quantities of this undesirable by-product during the fermentation process. Fermentation vigour was defined as the total amount of ethanol (v/v) produced at the end of the fermentation [85].

3.4 Results

3.4.1 *Batch cultures*

The average values for maximum growth rate, biomass yield, productivity and ethanol yield achieved during the batch phase foregoing each fed-batch phase are shown in Table 2.

Table 2: Batch cultures of *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae* at bench scale. Average values are shown with standard deviations.

Organism	Maximum specific growth rate (μ_{max}; h^{-1})	Biomass Yield ($Y_{x/s}$; $g.g^{-1}$)	Volumetric Productivity (mg L^{-1}/h)	EtOH yield ($Y_{eth/s}$; $g.g^{-1}$)
M. pulcherrima	0.46±0.06	0.59±0.19	0.6±0.06	0.034±0.057
I. orientalis	0.38±0.15	0.84±0.16	0.5±0.20	0.042±0.061
L. thermotolerans	0.31±0.04	0.30±0.10	0.4±0.00	0.190±0.037
S. cerevisiae	0.33±0.05	0.21±0.08	0.3±0.10	0.220±0.150
p - value†	-	< .001	< .001	0.0026

† One-way ANOVA.

The Crabtree-positive yeast *M. pulcherrima* and *I. orientalis* reached biomass yields significantly higher than that of *L. thermotolerans* and *S. cerevisiae*. The Crabtree-negative

yeast *I. orientalis* reached the highest biomass yield of all yeasts at 0.84 g g^{-1}, with a maximum specific growth rate of 0.38 h^{-1}. Due to a higher μ_{max} of 0.46 h^{-1}, the volumetric productivity of *M. pulcherrima* batch cultures exceeded that of *I. orientalis*. As expected, *S. cerevisiae* cultures achieved the highest ethanol yield with a value of 0.22 g g^{-1} at an average growth rate of 0.33 h^{-1}. Ethanol yields of the non-*Saccharomyces* yeasts were lower, indicating lower sensitivity to glucose excess compared to *S. cerevisiae*. The biomass yield and volumetric productivity was also significantly higher for Crabtree-positive yeast ($p = 0.005$ and $p < .001$, respectively).

3.4.2 *Exponential feed validation*

The effectiveness of the dynamic fed-batch strategy was corroborated in plots of ln *X* as a function of time (h) over the fed-batch phase of the different strains at all growth rates tested (Figure 4). The growth rates of the *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae* yeasts were effectively controlled in ranges from 0.08-0.2 h^{-1}, 0.11-0.24h^{-1}, 0.09-0.24h^{-1} and 0.09-0.31h^{-1} respectively. The linearity of the plot ($R^2 \geq 0.941$) confirmed that the specific growth rates remained constant during the exponential feeding phase. To further confirm that exponential growth at a constant growth rate was indeed maintained, representative plots of the predicted biomass in correlation with the actual biomass measured is shown in Figure 5. The dynamic exponential feed strategy therefore succeeded in maintaining exponential growth at constant growth rates, while also maintaining acceptable amounts of residual glucose (Figure 7).

The effect of different growth rates on the duration of the culture can also be seen in Figure 4, where slow growth rates resulted in longer cultures and fast growth rates in shorter cultures.

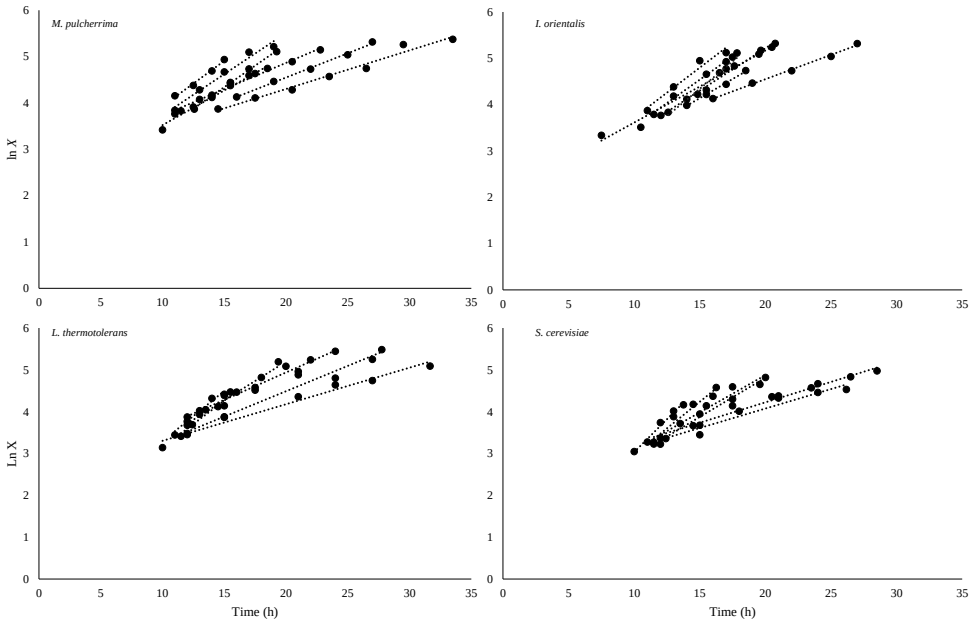

Figure 4: Linear curves of ln *X* as a function of time (hours) for the entire range of growth rates (h^{-1}) maintained during respective fed-batch phases of *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae* fed-batch cultures with minimum R^2 values of 0.97, 0.96, 0.94 and 0.95 respectively. Dotted lines are fitted and do not represent data points.

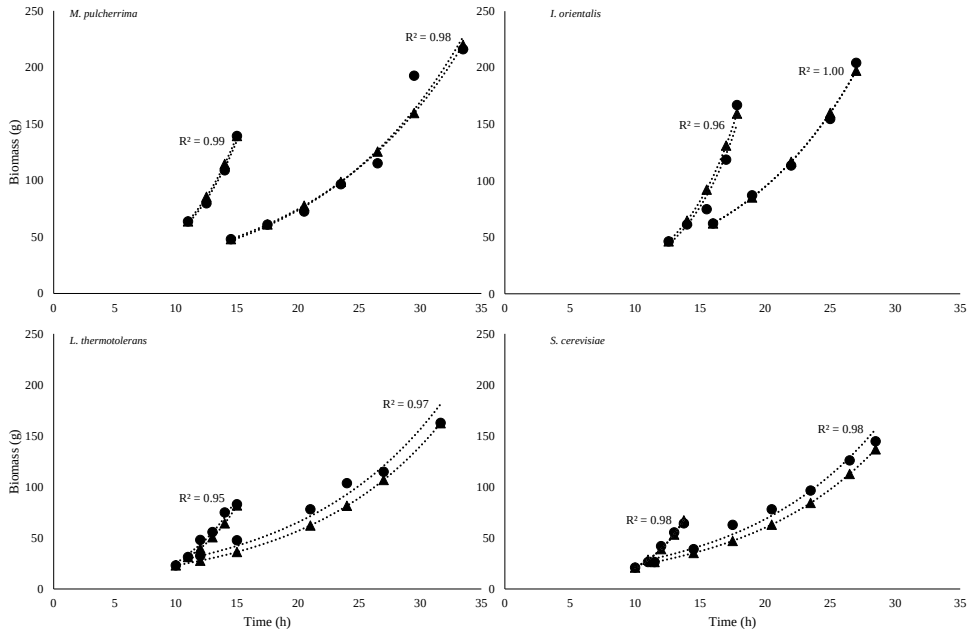

Figure 5: The predicted (▲) and actual (●) amount of biomass (g) as a function of cultivation time (h) for *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae* during fed-batch cultures. For each yeast a slow growth rate and a fast growth rate is shown. R^2 values represents the correlation between actual values and predicted values. Dotted lines are fitted and do not represent data points.

3.4.3 *Fed-batch culture at 9 L scale*

The total amounts of biomass and ethanol accumulated in the fermenter during the combined batch and fed-batch cultures (g) were plotted as a function of the specific growth rate (Figure 6). Generally, the highest biomass accumulation was recorded at the lowest growth rate tested, reaching values of 216 g, 204.6 g and 144.1 g for *M. pulcherrima*, *I. orientalis* and *S. cerevisiae* respectively (Figure 6). On the other hand, the biomass production of *L. thermotolerans* decreased at growth rates above 0.12 h^{-1} (32.4 % of μ_{max}), where a total amount of 241 g was recorded (Figure 6).

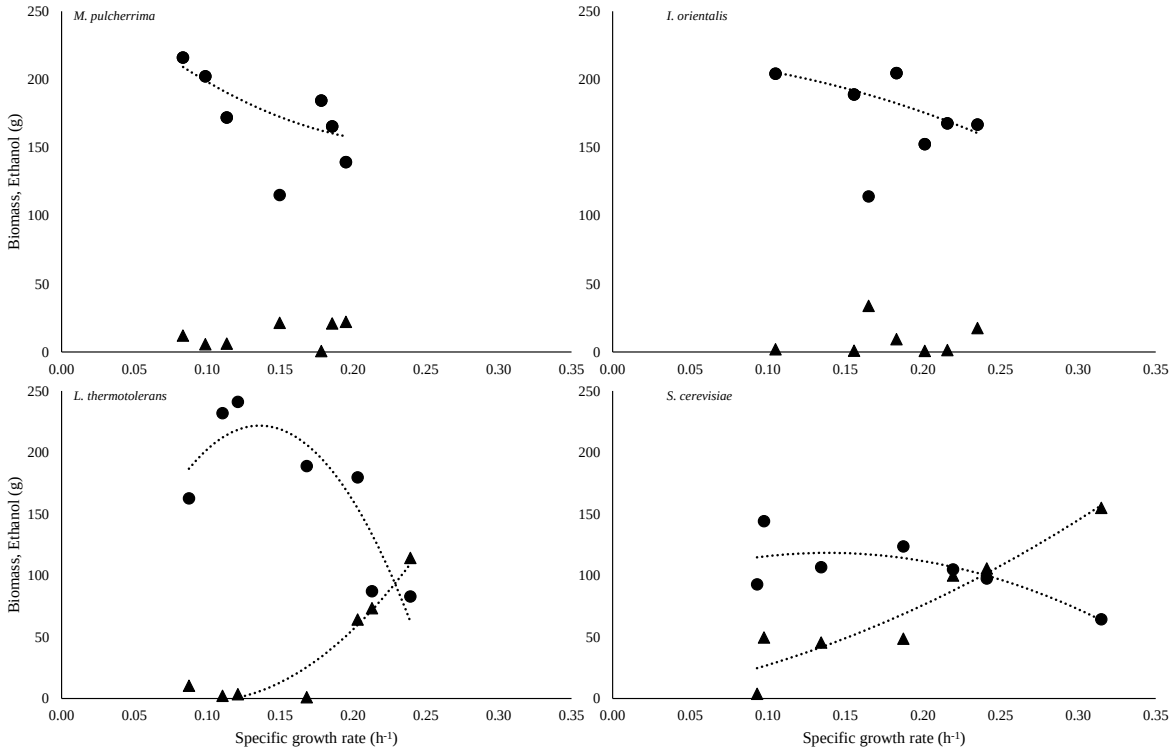

Figure 6: Biomass (●) and ethanol (▲) production as a function of specific growth rate (h^{-1}) during bench scale fed-batch cultures of *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae*. Each data point represents a single fed-batch culture. Dotted lines are fitted and do not represent data points.

Residual sugar as a function of the specific growth rate is shown in Figure 7. Generally, residual glucose concentrations remained below the detection limit, which indicated that the fed-batch

culture was conducted at glucose-limiting conditions, especially at growth rates lower than 0.20 h^{-1}. Measurable levels of residual glucose were observed at growth rates approaching μ_{max} during *S. cerevisiae* and *L. thermotolerans* cultures. The non-*Saccharomyces* yeast *I. orientalis* was an exception with glucose concentrations of up to 8.2 g L^{-1} at the end of each culture, which may indicate the limitation of a nutrient other than glucose; *I. orientalis* may require a more complex growth medium [36]. On the other hand, residual fructose was detected in most of the cultures, especially in cultures of the Crabtree-negative yeasts *M. pulcherrima* and *I. orientalis*. Fructose was detected even at low μ values, which indicates the preferential utilisation of glucose as carbon source. Both glucose and fructose are produced by the inversion of sucrose present in molasses. No specific pattern was observed for residual sucrose concentrations.

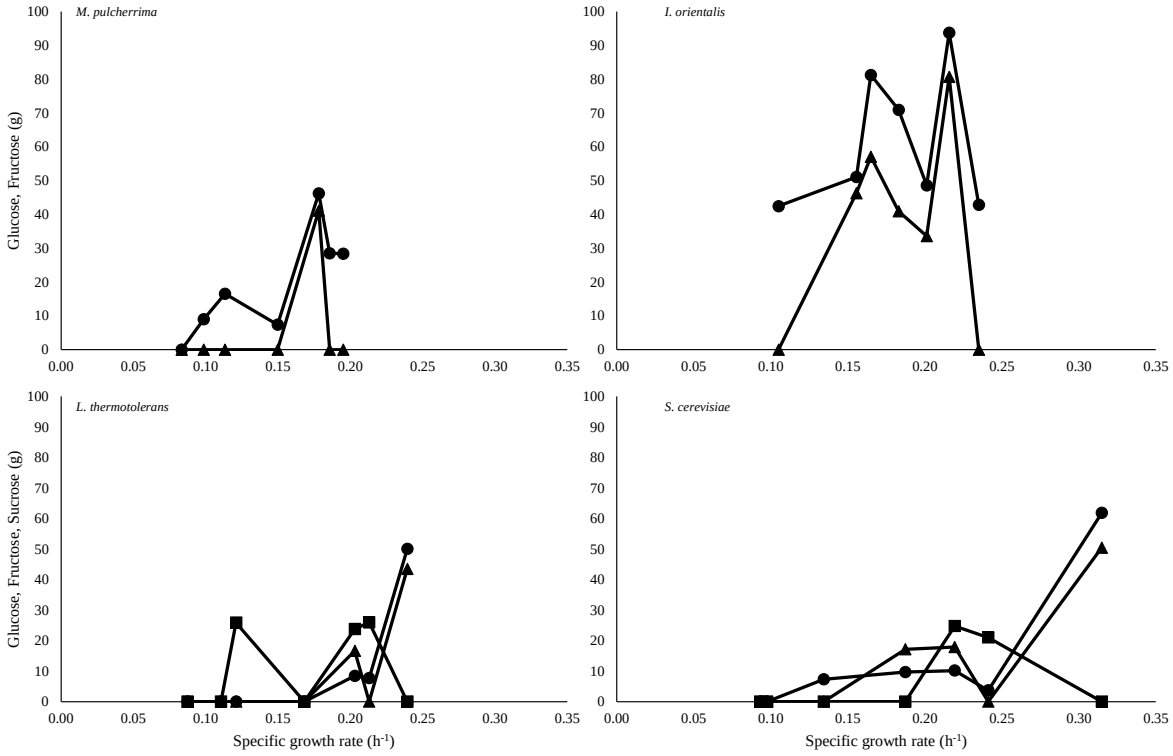

Figure 7: Residual glucose (▲), fructose (●) and sucrose (■) as a function of specific growth rate (h^{-1}) of *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae*. Since both *M. pulcherrima* and *I. orientalis* could not metabolise any residual sucrose present due to incomplete sucrose hydrolyses, sucrose utilisation by these organisms were not considered. Each data point represents a single fed-batch culture.

The residual YAN (mg) is shown as a function of the specific growth rate in Figure 8. The YAN remained in excess in all of the cultures over the range of growth rates tested, confirming that nitrogen did not become limiting, even at growth rates close to μ_{max}.

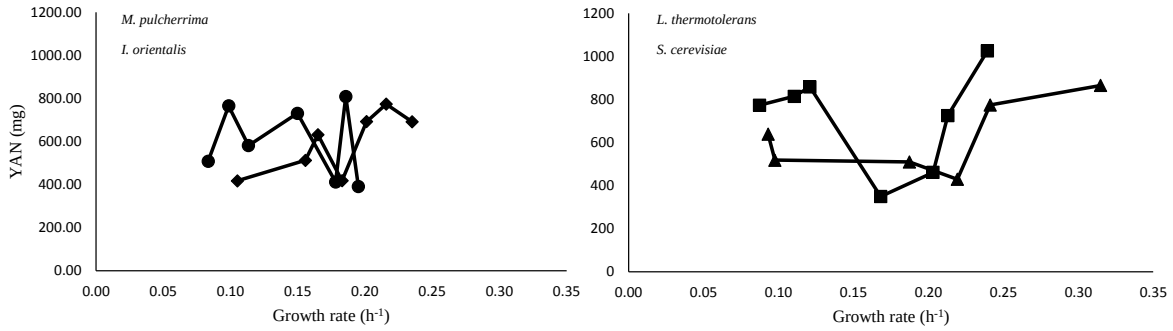

Figure 8: Yeast assimilable nitrogen (mg) as a function of the specific growth rate (h^{-1}) for *M. pulcherrima* (●), *I. orientalis* (♦), *L. thermotolerans* (■) and *S. cerevisiae* (▲) during fed-batch cultures.

For all of the organisms investigated, an increase in μ resulted in a decrease in $Y_{x/s}$ (Figure 9), which was more evident for Crabtree-positive strains due to the associated ethanol production The higher $Y_{eth/s}$ for *S. cerevisiae* indicated the prolific fermentation capability of this organism when compared to *L. thermotolerans* and the other yeasts (Figure 9). The apparent accelerated decline in $Y_{x/s}$ for *L. thermotolerans* is probably due to a lower μ_{max}. The estimated μ_{crit} values where overflow metabolism commenced in Crabtree-positive yeasts were 0.17 h^{-1} and 0.19 h^{-1} for *L. thermotolerans* and *S. cerevisiae*, respectively. In *S. cerevisiae* cultures, overflow metabolism occurred even at the lowest values of μ tested, resulting in measurable levels of ethanol production. High $Y_{x/s}$ values were recorded for all three non-*Saccharomyces* yeasts, especially *I. orientalis*, which reached an $Y_{x/s}$ of 0.83 g g^{-1} at a growth rate of 0.1 h^{-1}. The decrease in $Y_{x/s}$ at increased values of μ was more gradual than that of *M. pulcherrima*. In *L. thermotolerans* cultures a maximum $Y_{x/s}$ (0.76 g g^{-1}) was reached, which was 1.5 times higher than that of *S. cerevisiae* where a value of 0.51 g g^{-1} was reached at μ = 0.09 h^{-1}. Reaching a

biomass yield equal to the theoretical maximum is obviously impossible, suggesting that some experimental error does exist.

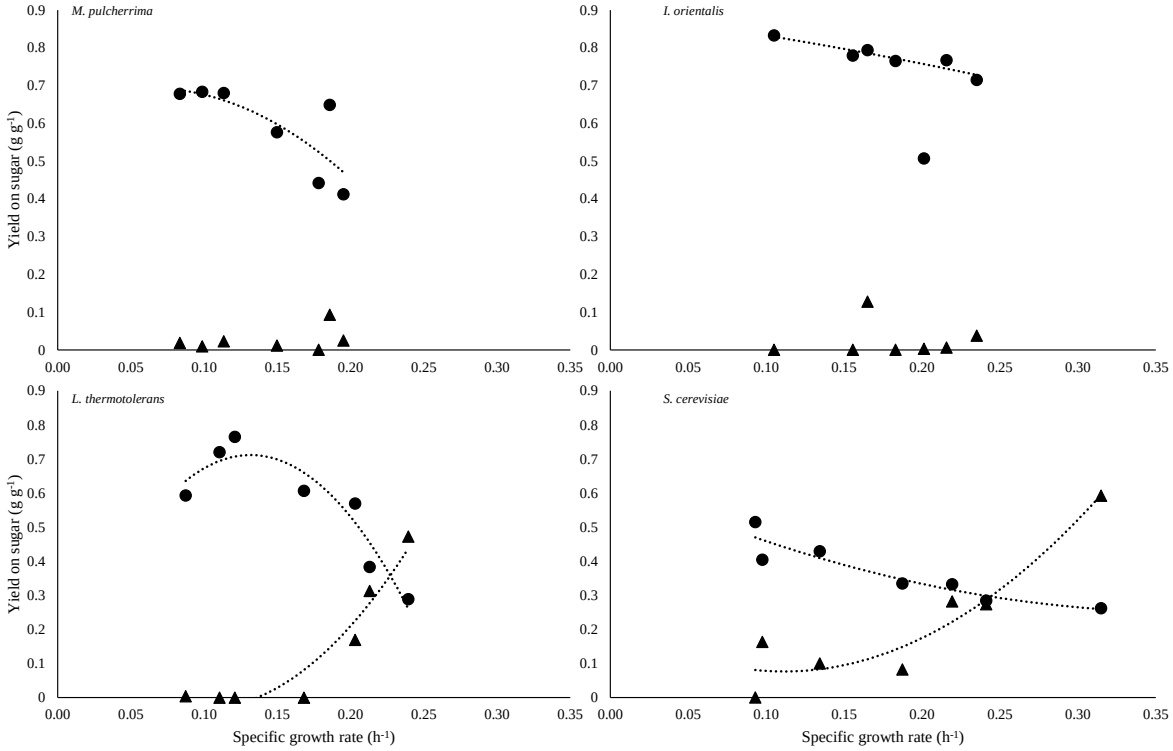

Figure 9: Biomass yield (●) and ethanol yield on consumed sugar (▲) as a function of specific growth rate (h^{-1}) for (A) *M. pulcherrima*, (B) *I. orientalis*, (C) *L. thermotolerans* and (D) *S. cerevisiae* during fed-batch cultures using molasses as carbon source. The data represents the fed-batch phase only. Dotted lines are fitted and do not represent data points.

The volumetric productivity (g L^{-1}/h) of biomass production during the fed-batch phase of respective cultures is shown in Figure 10 to investigate any correlation to biomass yield.

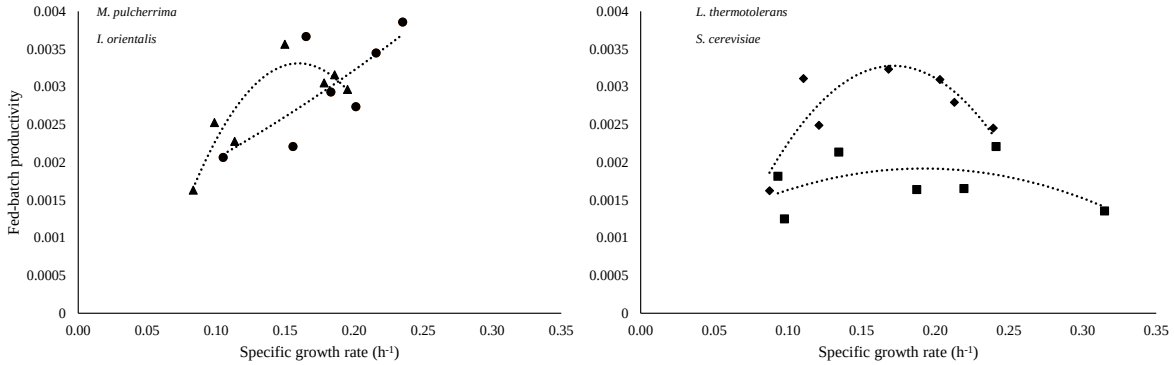

Figure 10: Volumetric productivity of yeast biomass production (g L^{-1}/h) of *M. pulcherrima* (▲), *I. orientalis* (●), *L. thermotolerans* (♦) and *S. cerevisiae* (■) as a function of specific growth rate (h^{-1}) during the fed-batch phase. Dotted lines are fitted and do not represent data points.

No significant difference was observed between the productivity of Crabtree-negative yeast and *L. thermotolerans*. The productivity of *S. cerevisiae* was not as sensitive to changes in growth rate when compared to the non-*Saccharomyces* yeast.

3.4.4 *Acidification Power (AP)*

The metabolic state and fermentative capability of yeast biomass from each culture was quantified from the ability of the yeast to produce acid from glucose, evident from a drop in pH. The Acidification Power (AP) of respective fed-batch cultures over the whole range of growth rates tested is shown for the different species in Figure 11.

The metabolic robustness of Crabtree-positive yeasts were clearly superior to that of the Crabtree-negative yeasts, evident from the constant AP over the whole growth rate range test for Crabtree-positive. The AP of Crabtree-negative yeasts was negatively affected by increased growth rates applied during yeast production. Furthermore, the AP of Crabtree-positive *S. cerevisiae* yeasts was 1.6 times higher than the Crabtree negative *I. orientalis* yeasts at the lowest growth rates tested. The data suggests that in commercial fed-batch cultures, the fermentative capacity of Crabtree-negative yeasts will be maximised at lower growth rates, which is contrary to previous reports on Crabtree-positive *S. cerevisiae* [9].

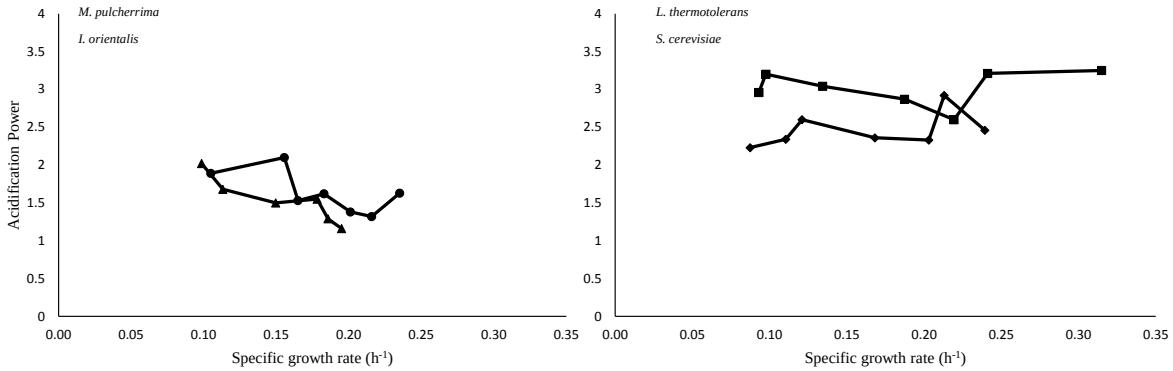

Figure 11: Acidification power of *M. pulcherrima* (▲), *I. orientalis* (●), *L. thermotolerans* (♦) and *S. cerevisiae* (■) as a function of specific growth rate (h^{-1}).

3.4.5 *Synthetic wine fermentations*

Synthetic wine fermentations were carried out to investigate the correlation between the specific growth rate (during fed-batch production) and subsequent synthetic wine fermentation performance. Fermentation vigour (EtOH %, v/v) and residual sugar concentration (g L^{-1}) of cells harvested from each respective fed-batch fermentation is shown in Figure 12 as a function of growth rate. *L. thermotolerans* and *S. cerevisiae* reached maximum ethanol production at respective growth rates of 0.20 h^{-1} and 0.22 h^{-1}, resulting in an ethanol percentage (v/v) of 9.64 % and 11.34 %, respectively (Figure 12). *M. pulcherrima* and *I. orientalis* did not ferment to the same extent, reaching an average EtOH % of 1.52 ± 0.45 % and 1.89 ± 0.45 %, respectively, with no correlation to specific growth rate (Figure 12). The inability of *M. pulcherrima* and *I. orientalis* to ferment glucose and fructose to ethanol is also seen from high levels of residual glucose and fructose after each synthetic wine fermentation. *M. pulcherrima* and *I. orientalis* could only ferment 17.2 ± 4.52 % and 23.9 ± 4.65 % of the original glucose and 17.9 ± 2.97 % and 13.7 ± 2.40 % of the original fructose respectively (Figure 12). *L. thermotolerans* and *S. cerevisiae* on the other hand, assimilated 76.2 ± 6.56 % and 94.6 ± 2.29 % of the original glucose and 63.5 ± 6.55 % and 84.7 ± 6.47 % of the original fructose respectively (Figure 12).

I. orientalis, *L. thermotolerans* and *S. cerevisiae* showed a discrepancy between glucose and fructose utilisation, where glucose was consumed first during synthetic wine fermentations.

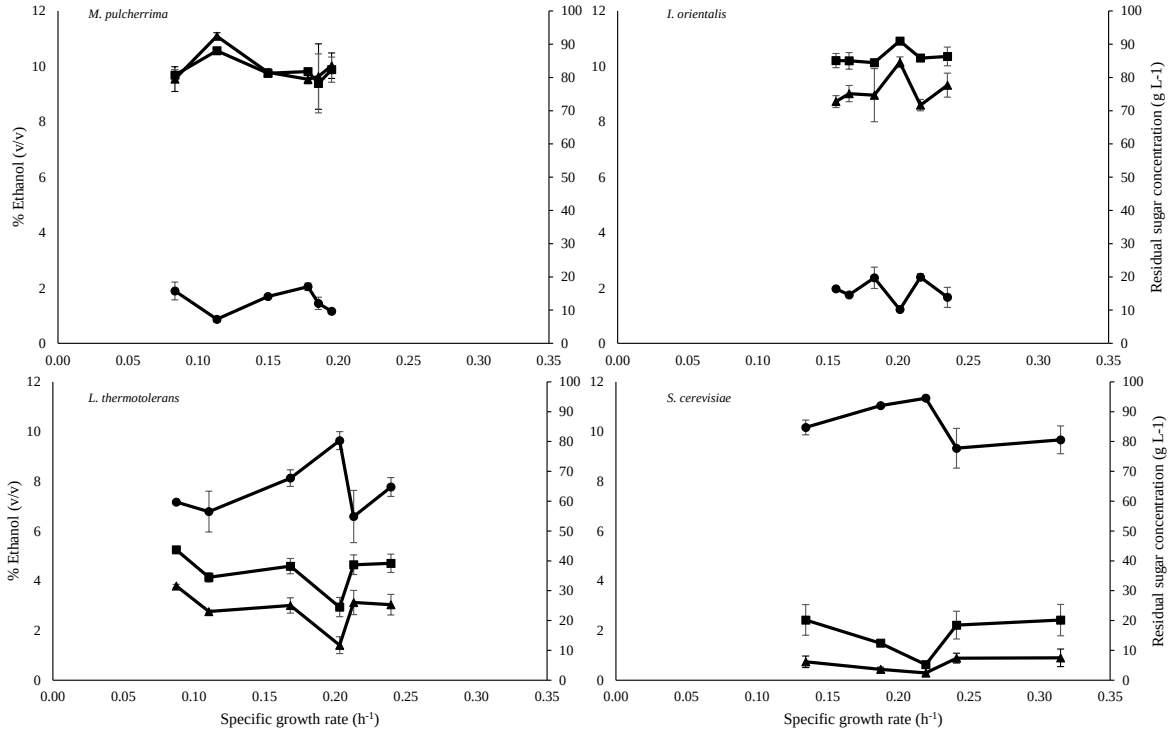

Figure 12: Ethanol (v/v) (●), and residual glucose (g L^{-1}) (▲) and fructose (g L^{-1}) (■) concentrations as a function of specific growth rate (h^{-1}) during *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae* synthetic wine fermentations.

Glycerol production (g L^{-1}) and total acid production (g L^{-1}), which is a combination of malic acid and the volatile acids as a function of specific growth rate, are shown in Figure 13. *M. pulcherrima* and *I. orientalis* produced high levels of glycerol throughout the entire growth rate range, reaching maximum concentrations of 18.0 ± 0.11 g L^{-1} and 16.7 ± 0.2 g L^{-1} respectively. *L. thermotolerans* and *S. cerevisiae* produced significantly lower levels, reaching only 9.3 ± 0.05 g L^{-1} and 5.13 ± 0.36 g L^{-1} respectively. No significant difference in total acid production was observed between any of the yeasts, with an average of 4.85 ± 0.30 g L^{-1} for all yeast.

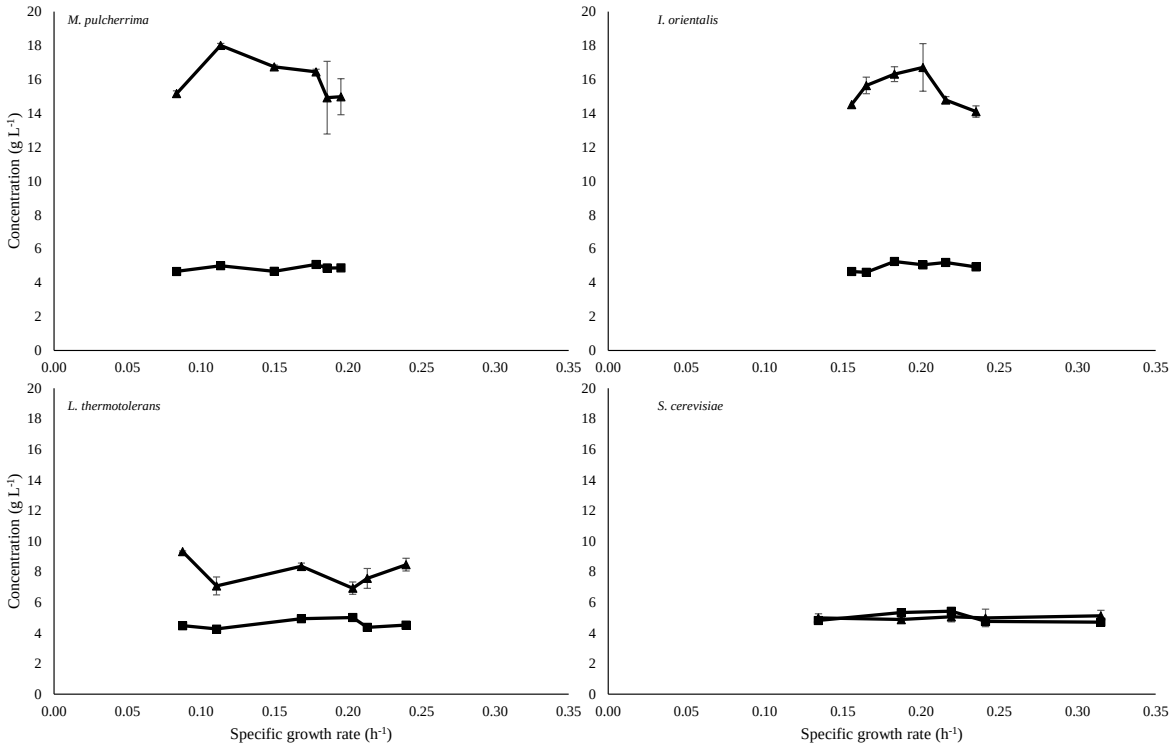

Figure 13: Residual concentrations (g L^{-1}) of glycerol (▲) and total acid (■) during synthetic wine fermentations as a function of specific growth rate (h^{-1}) for *M. pulcherrima*, *I. orientalis*, *L. thermotolerans* and *S. cerevisiae*.

Both *M. pulcherrima* and *I. orientalis* showed decreased fermentation rates (gCO_2 day^{-1}) at increased growth rates. The fermentation rate of *L. thermotolerans* and *S. cerevisiae* reached a maximum at μ = 0.20 h^{-1} and μ = 0.22 h^{-1} respectively. In general, the fermentation purity of *M. pulcherrima* and *I. orientalis* was higher than that of *L. thermotolerans* and *S. cerevisiae*.

3.4.6 ***Fed-batch cultivations at pilot scale***

Scale-up cultures of *L. thermotolerans* were conducted to investigate the influence of scale-up on the growth parameters of the non-*Saccharomyces* yeasts. *L. thermotolerans* was chosen because the Crabtree-positive nature of the yeast posed the most challenges for successful scale-up.

During the exponential growth phase of bench scale batch cultures of *L. thermotolerans*, conducted at a volume of 4 L, a k_La value of 130.68 h^{-1} was achieved by maintaining an aeration rate of 1 vvm (40 L m^{-1}) and an agitation rate of 700 RPM. The k_La was determined during the exponential growth phase in batch cultures because the culture grows at a maximum rate, requiring the most oxygen, and the conditions are more constant than in the fed-batch phase. Based on the parameters used, the relationship between k_La, agitation and air flow can be expressed by transfer Equation 39 on a per hour basis.

$$k_La = 3.64\left(\frac{P_g}{V_L}\right)^{0.15}(v_s)^{1.17} \quad (39)$$

According to this equation, achieving a k_La value of 130.68 h^{-1} at 40 L scale, which represents the batch phase of the pilot scale culture, with an air flow rate of 1 vvm would require an agitation rate of at least 61.2 RPM. The model was validated in 40 L culture with the k_La determined during exponential growth phase of *L. thermotolerans* to confirm that a k_La of 130.68 h^{-1} could indeed be maintained. A comparison between the bench scale and pilot scale k_La measurements is shown in Table 3. At bench scale a maximum k_La of 130.68 h^{-1} was achieved at an agitation rate of 700 RPM, corresponding to P_g/V_L = 4896 W m^{-3}, and a volumetric air flow rate of 1 vvm, corresponding to v_s = 6.93 m h^{-1}. At pilot scale a k_La of 121.32 h^{-1} was achieved at 450 RPM, corresponding to P_g/V_L = 5488.8 W m^{-3}, and volumetric air flow rate of 0.75 vvm, corresponding to v_s = 11.54 m h^{-1}. The k_La determination at pilot scale could not be conducted at 61.2 RPM because an equilibrium oxygen concentration (*Ce*) could not be maintained at such a low agitation.

Table 3: k_La measurements at 4 L scale and 40 L scale during late exponential growth of *L. thermotolerans* during batch phase cultures.

Bench scale				Pilot scale			
Agitation (RPM)	$\frac{P_g}{V_L}$ (W m^{-3})	v_s (m h^{-1})	k_La (h^{-1})	Agitation (RPM)	$\frac{P_g}{V_L}$ (W m^{-3})	v_s (m h^{-1})	k_La (h^{-1})
700	5109.7	5.20	93.96	300	1728.7	11.54	89.64
800	10586	5.20	105.12	450	5488.8	11.54	121.32
700	4896.0	6.93	130.68	450	5103.8	15.38	119.52

The production data of pilot scale *L. thermotolerans* fed-batch cultures are shown in Table 4. The aim of the pilot scale fed-batch cultures was to grow the culture at μ = 0.13 h^{-1} where, at bench scale cultures, an $Y_{x/s}$ value of 0.76 g g^{-1} was obtained (Figure 9).

Table 4: Fed-batch *L. thermotolerans* cultures at pilot scale. The data represents duplicate runs shown in two respective columns.

L. thermotolerans		
Growth rate (h^{-1})	0.117	0.133
Biomass accumulation (g)	1670.33	2066.03
Biomass Yield on Sugar (g g^{-1})	0.54	0.42
Volumetric Productivity (g L^{-1}/h)	0.00	0.00
Ethanol Accumulation (g)	145.62	953.20
Ethanol Yield on Sugar (g g^{-1})	0.00	0.20
Ethanol Yield on Biomass (g g^{-1})	0.00	0.47
Glycerol Accumulation (g)	86.35	94.18
AP[a]	2.40	2.05
YAN[b] (mg)	3457.58	4385.50

a- Acidification Power
b- Yeast assimilable nitrogen (mg) at the end of the culture

During both fed-batch pilot scale cultures a DO > 20% of saturation could be maintained throughout the entire cultivation, which indicates that the constant k_La scale-up strategy was indeed successful. However, achieving μ = 0.13 h^{-1} was not possible for both of the duplicate

runs. At a growth rate of 0.117 h^{-1} a $Y_{x/s}$ of 0.54 g g^{-1} was achieved, without the onset of ethanol production. Even though the culture was maintained in a respiratory state, the biomass yield was lower than the corresponding biomass yield at 9 L scale. However, at μ = 0.133 h^{-1} ethanol production did occur, reaching a total of 953.2 g that corresponded to $Y_{eth/s}$ = 0.20 g g^{-1} and $Y_{eth/x}$ = 0.47 g g^{-1}. Consequently the biomass yield dropped by 22% to a value of 0.42 g g^{-1}.

The total residual sugar concentration of both cultures were low, undetectable and 1.6 g L^{-1} (0.50 g L^{-1} glucose, 0.54 g L^{-1} fructose and 0.61 g L^{-1} sucrose) for cultures of μ = 0.177 h^{-1} and μ = 0.133 h^{-1} respectively. The YAN measurement confirmed that nitrogen was not limiting.

3.5 Discussion

The fed-batch production of *L. thermotolerans* IWBT Y1240, *I. orientalis* IWBT Y1161 and *M. pulcherrima* IWBT Y1337 yeast biomass on molasses at 9L scale was optimised by investigating the effect of growth rates maintained by exponential substrate feeding on the growth characteristics of each yeast, with *Saccharomyces cerevisiae* Lalvin EC1118 as control strain. This was achieved by conducting fed-batch cultures over a range of different growth rates and evaluating the biomass production as a function of specific growth rate by using regression analysis. Furthermore, the influence of growth rates on the subsequent metabolic state of the yeast was investigated through measurement of the acidification power of yeast biomass samples [78,80,81]. Finally, the production of *L. thermotolerans* was scaled up to 90 L in aerobic fed-batch culture, using an exponential feed and constant growth rate to assess culture behaviour and process performance at pilot scale. Scale-up was based on a constant volumetric mass transfer coefficient (k_La) strategy [38,52,77].

3.5.1 *The Crabtree effect*

Based on the growth characteristics the yeast could be divided into two main groups where *S. cerevisiae* EC1118 and *L. thermotolerans* Y1240 could be classified as Crabtree-positive and, *I. orientalis* Y1161 and *M. pulcherrima* Y1337 were classified as Crabtree-negative due to restricted ethanol production, which is in agreement with the findings of Schnierda et al. [55]. Hagman et al. have classified yeast as belonging to the *Lachancea* family as moderately Crabtree-positive when compared to *S. cerevisiae* [24]. The same conclusion can be drawn from the study of Schnierda et al., where a final ethanol concentration of 8.00 g L^{-1} and 5.51 g L^{-1} was achieved during *S. cerevisiae* and *L. thermotolerans* aerobic batch cultures, respectively [55]. This is in agreement with our findings, showing that ethanol production in *S. cerevisiae* cultures could not be avoided, even at a growth rate of μ = 0.1 h^{-1}, opposed to ethanol production commencing only at a growth rate of 0.17 h^{-1} in *L. thermotolerans* cultures (Figure 9). This may be due to the fact that the respiration pathway of *Lachancea* yeast is not repressed to the same extent when glucose is present, as Hagman et al. [24] have concluded, even though the yeast is also sensitive to glucose [27].

3.5.2 *Dynamic fed-batch control*

By considering the specific biomass concentration at the time the feed profile was started, the dynamic equation (Equation 25) allowed us to control μ from the start of the fed-batch phase (Figure 4) [38,46]. This differs from the conventional quasi-steady state method where μ is controlled by the dilution rate (D) without considering the initial biomass concentration under quasi-steady state conditions, which is only achieved after 4-6 residence times [8,38,52]. This method of control also ensured that sugar did not accumulate to unacceptable amounts.

3.5.3 *Biomass yield*

According to Verduyn et al. [12], the biomass yield of a culture is affected by a number of factors that include the substrate transport mechanism, the cell composition and the ATP yield of respiration (P/O ratio) of the specific organism. Verduyn has further proposed that a biomass yield on sugar of 0.68–0.72 g g^{-1} may be possible in the case where an organism possesses an efficient electron transport phosphorylation system [12]. For instance, organisms such as *S. cerevisiae* that do not possess a phosphorylation site in the mitochondrial electron transport chain has a lower P/O-ratio and will not reach biomass yield values of 0.68-0.72 g g^{-1}. In fact, the theoretical maximum biomass yield for *S. cerevisiae* is 0.51 g g^{-1} [12]. This was also found in the current study where *S. cerevisiae* reached a biomass yield of 0.514 g g^{-1} (Figure 9). The same trend is also seen in most studies where biomass yields close to the theoretical maximum are observed for *S. cerevisiae* [32,87,88]. It should be noted that reaching a biomass yield equal to or higher than the theoretical maximum is practically impossible, suggesting that some experimental error does exist.

The relatively low biomass yield of *S. cerevisiae*, when compared to other organisms tested, is obviously not only as a result of the lack in phosphorylation capacity, but can be coupled with the sensitivity to glucose, as discussed earlier. The opposite is also not true, as has recently been confirmed by Leite et al. [32] who have reported a biomass yield on glucose of 0.62 g g^{-1} for the Crabtree-positive yeast *Dekkera bruxellensis*. The same was found for *L. thermotolerans*, which reached a biomass yield value of 0.76 g g^{-1} even though it is also Crabtree-positive (Figure 9). As with *D. bruxellensis*, the presence of glucose coincided with ethanol production, which is indicative of the short-term Crabtree-positive nature of these organisms [32]. However, when excessive glucose concentrations were not present, both *D. bruxellensis* [32] and *L. thermotolerans* (Figure 9) reached biomass yields higher than *S. cerevisiae*.

Meijer et al. [89] have proposed that catabolite repression is triggered by the glucose concentration rather than the glucose flux into the cell. In the aerobic chemostat cultures performed by Leite, catabolite repression also occurred as a result of a glucose perturbation [32]. This was also the case during the aerobic fed-batch cultures of the current study, where *L. thermotolerans* cultures showed catabolite repression when extracellular glucose concentrations increased (Figure 7 and Figure 9). *L. thermotolerans* was, however, not as sensitive to glucose as *S. cerevisiae*, seen in negligible ethanol production by *L. thermotolerans* for growth rates up to 0.17 h^{-1} (Figure 9). The observed difference in growth characteristics between *L. thermotolerans* and *S. cerevisiae* coincides with reports by Hagman et al. [24] and Blank et al. [27], who have found that yeast belonging to the *Lachancea* family is not as sensitive to glucose as *Saccharomyces* yeast. Hagman et al. [24] have found that *Lachancea* yeast exhibited a less drastic response to a sudden glucose pulse (8–10 g L^{-1}) when grown in an aerobic glucose-limited continuous culture. This 'tolerance' to transient conditions also existed in fed-batch cultures where, at low feed rates, no ethanol was produced. *S. cerevisiae* was more sensitive to transient conditions in fed-culture, which resulted in ethanol production even at the lowest feed rate.

The fact that *L. thermotolerans* cultures reached biomass yields higher than that of *S. cerevisiae* may therefore be a consequence of a more efficient oxidative phosphorylation system (higher P/O-ratio), a lower degree of short term Crabtree behaviour and a regulatory system that is less prone to catabolite repression in the presence of glucose.

The Crabtree-negative yeasts, *I. orientalis* and *M. pulcherrima*, also reached biomass yields higher than that of *S. cerevisiae* (Figure 9). The biomass yields were in agreement with the yields found by Schnierda et al. [55] who have observed biomass yield values of 0.699 g g^{-1} and 0.807 g g^{-1} during aerobic batch cultures of *M. pulcherrima* and *I. orientalis*, respectively, during shake flask cultivation. Biomass yields higher than the theoretical maximum suggested

for *S. cerevisiae* have been observed for a number of other organisms [32,90]. This may be due to the complete absence of catabolite repression and, as with *L. thermotolerans*, and/or due to an efficient oxidative phosphorylation system. The use of yeast extract as a carbon source, as in the study of Schnierda et al., may also account for the high biomass yields found for the non-*Saccharomyces* yeast [55,91]. Unfortunately, literature regarding the metabolism of these non-*Saccharomyces* yeast in terms of biomass production is limited.

3.5.4 ***Sugar utilisation***

It has been proposed that Crabtree-negative yeasts may be restricted in the transport of sugars, including glucose, into the cell to avoid overflow metabolism. This may explain the accumulation of sugars during *I. orientalis* and *M. pulcherrima* cultures, especially at high feed rates (Figure 7) [11]. This was especially apparent in *I. orientalis* cultures where residual fructose, sucrose and even glucose was present throughout all the respective cultures. An increase in residual glucose, fructose and sucrose was also observed at high growth rates of *L. thermotolerans* and *S. cerevisiae* cultures. This may be due to (i) overfeeding, (ii) a nutrient limitation, (iii) the ongoing release of monomers by invertase hydrolysis or, (iv) a competitive effect between the different substrates. When more than one carbon source is available, yeast will always consume glucose first [14]. This was also observed in the fed-batch cultures of *M. pulcherrima*, *L. thermotolerans* and *S. cerevisiae* (Figure 7). The preferential use of glucose may be achieved by (i) the selective active transport of glucose across the membrane [11], or (ii) through catabolite repression where, because of the regulatory action of glucose, the genes encoding the transport of other sugars are repressed [15]. It might be that the presence of glucose in the feed medium restricted the uptake of fructose and sucrose [22,73]. Overfeeding of glucose will therefore augment the accumulation of fructose and sucrose as seen in the current study (Figure 7). Also, the increased residual sugar occurred despite the fact that DO was maintained at 30 % of saturation and that nitrogen was never limiting for any of the cultures

(Figure 8). Any limitation in such a substrate would have been reflected in the growth rate of the culture. Therefore it can be assumed that the accumulation of sugars, especially fructose and sucrose, is due to overfeeding and the ongoing release of monomers by invertase hydrolysis.

Whatever the case may be, no residual sugars were detected at growth rates where high biomass yields were achieved. The sugar was therefore fed at a rate which allowed the yeast to utilise the sugar efficiently, an important aspect in terms of commercial production.

3.5.5 *Transient effects and overfeeding in fed-batch cultures*

Unlike continuous cultures at steady-state, the environment in fed-batch cultures is ever-changing (transient), especially in terms of substrate concentration. Seeing that yeasts respond to changes in the extracellular environment within seconds [73,92], it can be assume that the transient condition will inflict some stress on the culture. The stress is caused due to a continuous need for the culture to adapt to fluctuations in the culture environment, especially at high substrate feed rates and high cell densities. This is especially apparent in large scale cultivation where the mixing efficiency is limited [54]. It is however, difficult to distinguish between transient effects and overfeeding, especially in Crabtree-positive cultures where overfeeding will have a greater effect. However, the fact that the growth rates of the respective fed-batch cultures remained constant (Figure 4), indicating that the biomass yield did not change significantly, shows that overfeeding did not occur at unacceptable amounts.

Compared to *S. cerevisiae*, *L. thermotolerans* was more resistant to overfeeding and possibly also transient effects, therefore the higher critical growth rate of 0.19 h^{-1}. This was also found in cultures of *S. kluyveri*, a Crabtree-positive organism, were ethanol accumulated only 20-50 min after a sudden glucose perturbation to a fully respiratory culture [93]. The ethanol produced by *S. cerevisiae* cultures growing below the critical growth rate of 0.17 h^{-1} may therefore be

due to the transient nature of fed-batch cultures, for instance slight fluctuations in substrate availability or high localised sugar concentration at the point where the feed enters the culture medium, rather than overfeeding since no accumulation of substrate was observed (Figure 7).

In *I. orientalis* and *M. pulcherrima* cultures the biomass yield dropped as the substrate feed rate was increased, even though no overflow into ethanol production occurred. This was possibly due to the increasing transient nature of fed-batch cultures at higher feed rates and not overfeeding, since the specific growth rate of these cultures remained constant (Figure 4). The increased transient state may be why a decrease in metabolic state was observed at increased growth rates (see section 3.5.6). Shima et al. [33] have also identified stress-related gene expression in cultures where molasses is used as carbon source due to toxins in the molasses, which may lead to a decrease in biomass yield and metabolic state when these toxins are fed to the culture at high rates.

3.5.6 *Acidification Power*

The metabolic state of the yeast was validated by the acidification power after each cultivation. The relationship between acidification power and specific growth rate differed for Crabtree-positive and Crabtree-negative yeast (Figure 11). The acidification power of *L. thermotolerans* and *S. cerevisiae* increased as the growth rate increased, confirming the findings of van Hoek et al. [9,28] who have observed a direct relationship between growth rate and the fermentative capacity of *S. cerevisiae*. For both yeasts the change in acidification power was not as drastic as seen by van Hoek et al [9]. Similar to the findings of Gobbi et al. [66], *L. thermotolerans* showed a high ethanol tolerance and was not metabolically inhibited by the high ethanol concentration observed (13.9 g L^{-1}) during high growth rate production, hence the increased acidification power at high growth rates. The opposite was found for Crabtree-positive yeast *I. orientalis* and *M. pulcherrima* where higher acidification power was seen at lower growth rates.

The seemingly higher acidification power at lower growth rates may be due to high levels of reserve carbohydrates that are metabolised during the acidification power test before the external carbon source was added. By being produced at low growth rates the yeast could have been able to produce the necessary metabolites, for example glycogen and trehalose, which could aid with the process of adapting to a new environment [43]. Furthermore, the condition in which the AP test was conducted is not fully aerobic, which can limit the ability of the Crabtree-negative yeast to utilise the added glucose. This might be why the AP was lower at increased growth rates.

3.5.7 *Synthetic wine fermentations*

In an effort to evaluate the fermentation performance of *Lachancea thermotolerans*, *Issatchenkia orientalis* and *Metschnikowia pulcherrima* in industry-relevant conditions, synthetic wine fermentations were carried out by the Institute of Wine Biotechnology (IWBT), Stellenbosch, South Africa. By analysing the fermentation kinetics and metabolic contribution of each synthetic wine medium fermentation, the fermentative performance of each yeast product could be determined as a function of different production growth rates. Yeast biomass obtained from aerobic fed-batch cultures, designed to maximise biomass yields under yeast production conditions, were used for these wine fermentations.

The fermentation performance during synthetic wine medium fermentation of each yeast product was evaluated based on four parameters, namely: fermentation vigour, fermentation rate, fermentation purity and metabolite production. In synthetic wine medium fermentations the Crabtree-negative yeast showed a higher overall fermentation performance at low growth rates during yeast production in aerobic fed-batch, confirming the acidification power findings (Chapter 3). This may again be explained by higher reserve carbohydrates that assisted the organisms to adapt to a new environment [43]. While the fermentation purity remained

relatively constant, the fermentation vigour and fermentation rates of *L. thermotolerans* and *S. cerevisiae* increased with growth rates and reached maximum values at growth rates 0.20 h^{-1} and 0.22 h^{-1} respectively, also in agreement with AP results.

The contribution to wine quality is not only defined by the fermentation performance of a yeast. Total wine acidity and molecules that enhance the character or body of a wine are also important [66]. Glycerol is one of the most important by-products of alcoholic fermentation, with average concentrations of 7 $g.L^{-1}$. Its significance is only exceeded by ethanol and carbon dioxide [70,94]. Though its non-volatile nature excludes it from the aromatic profile of the wine, it enhances the wine by contributing to the residual sweetness and smoothness [70,94]. *I. orientalis* and *M. pulcherrima* produced high amounts of glycerol during synthetic wine medium fermentations, reaching 16.71 $g.L^{-1}$ and 16.75 $g.L^{-1}$ respectively. These concentrations were much higher than what was produced by the Crabtree-positive yeasts *L. thermotolerans* and *S. cerevisiae*, i.e., 9.32 $g.L^{-1}$ and 5.13 $g.L^{-1}$ respectively. The high amounts of glycerol was produced even though *I. orientalis* and *M. pulcherrima* consumed an average of only 37.6 g L^{-1} and 35.1 g L^{-1} sugar during the synthetic wine fermentations, respectively. This can be due to the high levels of stress these yeast encounter during fermentative conditions where reserve carbohydrates may serve as an additional carbon source to produce osmoregulation molecules like glycerol [43,70]. The glycerol yield on sugar of these yeast may also be higher than that of *S. cerevisiae* and *L. thermotolerans*. The yeast *Candida glycerinogenes* can produce 0.64 g g^{-1} of glycerol per gram of sugar which would have resulted in an average glycerol production of 24.1 g L^{-1} and 22.5 g L^{-1} if a total amount of 37.6 g L^{-1} and 35.1 g L^{-1} sugar was consumed, respectively [95]. Some experimental error may also exist however, one would expect the same error to be reflected in *L. thermotolerans* and *S. cerevisiae* fermentations as well.

In terms of acid production, the growth rate had no apparent effect on total acid production for any of the yeasts. Even though *L. thermotolerans* is also targeted to improve wine acidity [66], no significant advantage over any of the other yeast was seen (Figure 13).

Producing *L. thermotolerans* and *S. cerevisiae* at increasing growth rates up to 0.20 h^{-1} and 0.22 h^{-1} respectively, resulted in an increase in the overall fermentation performance. The fermentation performance of *L. thermotolerans* compared well to that of *S. cerevisiae*. However, the higher glycerol production, similar fermentation rates and a lower fermentation vigour seen with *L. thermotolerans* might prove this organism to be more desirable over *S. cerevisiae* as a primary fermenter, especially in countries where a higher than optimal alcohol level poses problems [65,66]. Although the Crabtree-negative yeast *I. orientalis* and *M. pulcherrima* did not ferment to the same extent as the Crabtree-positive yeast, high amounts of glycerol was produced, which can contribute greatly to the overall quality of a wine. Producing *I. orientalis* and *M. pulcherrima* at lower growth rates increased their ability to survive during synthetic wine fermentations, possibly lending them the time to contribute to the wine quality.

3.5.8 *Production of non-Saccharomyces yeast for commercial application*

It is important to avoid the loss of carbon to unwanted by-products, such as ethanol in the case of glucose-sensitive yeast [30], to successfully produce yeast at high cell densities. Losses in biomass yield can be circumvented by controlling the substrate feed rate in fed-batch and continuous cultures, thereby controlling the specific growth rate according to the model described by Monod [30,96]. Even though the productivity of all the fed-batch cultures were higher at faster growth rates, maximum biomass yields were achieved at slower growth rates, accompanied by minimal residual substrate (Figure 9 and Figure 10). From an industrial point a view, higher volumetric productivity will increase the utilisation of capital equipment, while

higher biomass yields will reduce the operational cost of medium components for biomass production. Both may therefore incur economic benefits in yeast biomass production, although in the present study, higher biomass yields were preferred.

The metabolic state or vitality of the Crabtree-negative yeast was, as with the biomass yield, higher at slower growth rates, which is in accordance with the biomass production results (Figure 11). The vitality of the Crabtree-positive yeast increased only slightly as the growth rate was increased. Therefore, producing these yeasts at slower growth rates will not be detrimental to the cultures' vitality.

Even though carbon metabolism in yeast has received much attention, it is still far from being fully understood, especially for yeasts other than *S. cerevisiae*. In fact, in most cases *S. cerevisiae* was not as sensitive to changes in fed-batch feed rates as any of the *non-Saccharomyces* strains. This again suggests that successful production of yeast species other than *S. cerevisiae* cannot be defined exclusively from current knowledge resolving around baker's yeast production and metabolism.

3.5.9 *Pilot scale cultivation*

When the k_La was determined at pilot scale to confirm whether the oxygen transfer capacity of the 150 L bioreactor would be sufficient, it was possible to achieve a k_La of 121.32 h^{-1} at an agitation rate of 450 RPM while maintaining a constant volumetric air flow rate of 0.75 vvm. The k_La of 130.68 h^{-1} achieved at bench scale could not be reached at pilot scale. Constructing transfer Equation 40 from the pilot scale k_La data instead of the bench scale data, as in Equation 39, the relationship between k_La, agitation and volumetric air flow could be expressed as

$$k_La = 12.28\left(\frac{P_g}{V_L}\right)^{0.26}(v_s)^{0.014} \quad (40)$$

which predicts a more realistic agitation of 505.2 RPM to achieve a k_La of 130.68 h^{-1} during pilot scale cultivations. The pilot scale reactor therefore imposed a physical constraint due to a maximum agitation of 450 RPM. However, the k_La of 121.32 h^{-1} compared well to the k_La determined by Anane et al. [38], the k_La range proposed by Doran et al. [52] and the 130.68 h^{-1} achieved in the bench scale bioreactors. Therefore, it was concluded that the pilot scale bioreactor could indeed maintain aerobic conditions. A dissolved oxygen (DO) percentage higher than 20 % of saturation could be maintained throughout all pilot scale cultivations.

The aim of the pilot scale cultivations was to produce *L. thermotolerans* at a growth rate as close to the critical growth rate as possible, ensuring the highest possible biomass yield [30]. Based on the bench scale fermentations, a growth rate of 0.13 h^{-1} would ensure the highest possible biomass yield (Figure 9). During the pilot scale cultures it was possible to maintain a constant specific growth rate of 0.117 h^{-1} and 0.133 h^{-1} throughout the respective fed-batch phases. At μ = 0.117 h^{-1} *L. thermotolerans* reached an $Y_{x/s}$ of 0.54 g g^{-1}, which correlated with the 0.56 g g^{-1} achieved at μ = 0.20 h^{-1} during bench scale cultures. However, unlike the bench scale culture, no ethanol was produced, which indicates that the low $Y_{x/s}$ might be due to the transient conditions found in large bioreactors [97]. At μ = 0.133 h^{-1}, ethanol production did occur, which reached a yield of 0.20 g g^{-1} on sugar, resulting in $Y_{x/s}$ = 0.42 g g^{-1}. This compared to the values achieved at a growth rate between 0.20 h^{-1} and 0.21 h^{-1} in bench scale cultivation.

The shift in the critical growth rate seen in the pilot scale fermentation may be due to ineffective mixing, which generates concentration gradients in the bioreactor, the so-called ‘scale-up effect’ [54,57,97], since no apparent oxygen or nitrogen limitation occurred. Feeding the carbon source at one zone may also have augmented the formation of such a concentration gradient. Each time a cell entered the high concentration zone, in this case high sugars, the short-term Crabtree effect came into effect and resulted in ethanol production and ultimately a loss in biomass yield [57,97]. At bench scale these effects were avoided by means of more

efficient mixing and the feed medium entering at two different points. Unlike the catabolite repression seen at high growth rates during bench scale fermentations, the ethanol production in pilot scale occurred at low growth rates and minimal sugar accumulation (1.6 g L^{-1} total sugar), indicating overflow metabolism instead.

The AP of *L. thermotolerans* following pilot scale cultures was higher at μ = 0.117 h^{-1} than at μ = 0.133 h^{-1} (Table 4). This may be due to augmented transient conditions generated at higher feed rates, increasing the level of gradient formation in the bioreactor, which can reduce the metabolic state of the yeast because of the ongoing change in metabolism [46,97].

3.6 Conclusions

The growth characteristics of *Metschnikowia pulcherrima*, *Issatchenkia orientalis* and *Lachancea thermotolerans* were unlike that of *Saccharomyces cerevisiae*. *M. pulcherrima* and *I. orientalis* did not resemble a Crabtree-positive metabolism, since no ethanol was produced during aerobic cultures. *L. thermotolerans* produced ethanol during aerobic fed-batch cultures when a high substrate feed rate was employed. However, the ethanol production of *L. thermotolerans* was not as vigorous as that of *S. cerevisiae.* This was possibly due to more efficient respiratory capacities, more efficient oxidative phosphorylation, and a lesser sensitivity towards glucose or the use of alternate carbon sources in the growth medium.

During fed-batch *L. thermotolerans* cultures ethanol production was avoided at growth rates below 0.17 h^{-1}, the critical growth rate. Even though no definite critical growth rate was observed for *M. pulcherrima* or *I. orientalis*, the biomass yields of both *M. pulcherrima* and *I. orientalis* were indirectly proportional to the specific growth rate in aerobic fed-batch cultures.

The fermentative performance following the aerobic fed-batch production process differed between Crabtree-positive and Crabtree-negative yeast. The fermentative performance of the Crabtree-negative yeast *M. pulcherrima* and *I. orientalis* was highest at slow growth rates,

whereas the fermentative capacity of the Crabtree-positive yeast *L. thermotolerans* and *S. cerevisiae* increased at increasing growth rates.

When compared to the same growth rate, a lower biomass yield for *L. thermotolerans* was achieved on scale-up than that achieved at bench scale. This is possibly due to the "scale-up effect" where substrate gradients could have resulted in overflow metabolism. This can be avoided by feeding that substrate at two different points or by feeding a more dilute medium.

Ultimately, all of the *non-Saccharomyces* yeasts should be produced at slower growth rates, compared to their respective μ_{max} values, where the biomass yield is at a maximum. Therefore *M. pulcherrima*, *I. orientalis* and *L. thermotolerans* should be produced at 0.10 h^{-1}, 0.11 h^{-1} and 0.12 h^{-1}, respectively. Producing these organisms at a growth rate that favours a high biomass yield will not be detrimental to the fermentative performance of the yeast product.

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Chapter 4

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