Evaluation of the effect of morphological control of dimorphic *Mucor circinelloides* on heterologous enzyme production

by

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Declaration

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

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Astrid Elizabeth Sindle Date

Summary

Filamentous fungi have been employed for production of heterologous proteins such as enzymes, antibiotics and vaccines due to their good secretion capacities and effective post-translational modifications of these proteins. With an improvent in recombinant DNA technologies it has become possible to express many useful proteins in species such as the *Aspergilli.* However the submerged cultivation of filamentous fungi is complicated by the difficulties in mixing and oxygen and nutrient transfer in the highly viscous culture fluids that result.

The purpose of the project was to investigate the potential of simultaneous control of morphology and production of enzymes in the dimorphic fungus, *Mucor circinelloides,* in order to overcome problems associated with the submerged cultivation of filamentous fungi. Dimorphic *M. circinelloides*, a zygomycete in the order *Mucorales,* occurs in a filamentous form or a yeast-like morphology in response to environmental conditions. Recently, advances were made in transformation of *Mucor*, and it has become possible to transform *M. circinelloides* to express heterologous proteins. The first example of a strong, regulated promoter from *M. circinelloides* being used for recombinant protein production was the expression of the glucose oxidase gene (from *Aspergillus niger*) under the control of the glyceradehyde-3-phosphate dehydrogenase (*gpd1*) promoter. Glucose oxidase (GOX) is an enzyme used to prevent oxidation of foods to extend shelf-life, to produce low-kilojoule beverages and to measure glucose levels in medical diagnostic applications.

The scope of this project was to establish the conditions for yeast and filamentous growth of *M. circinelloides* in order to allow control of morphology, and to evaluate enzyme production under these conditions. Enzyme production of the GOX producing mutant strain, that was recently constructed, was compared to that of a wild type *M.circinelloides* strain.

M. circinelloides was cultured in two-stage batch fermentations, firstly a yeast stage and then a filamentous stage. The yeast morphology was induced by anaerobic conditions while the filamentous morphology was achieved by exposure to air. The enzyme, biomass and metabolite production of the glucose-oxidase producing mutant strain and the wild type were monitored during the two-stage fermentations. GOX from the mutant and native amylase activity levels from the wild type were compared to each other and to other production systems for these enzymes.

The morphology could be maintained in a yeast form under N_2 with addition of ergosterol and Tween 80. The GOX activity levels in the culture fluid were comparable to some of the unoptimized GOX production systems in literature, but much lower than the optimized, recombinant GOX production systems that employ certain yeasts, or *Aspergilli* or *Penicillium*. The intracellular GOX levels were almost 6-fold higher than the extracellular levels which was unexpected as GOX is usually well-secreted. The morphological control improved the morphology for the initial yeast-stage of the fermentation but did not improve the morphology during the filamentous, enzyme-producing stage and it decreased the biomass yield and enzyme production by 50%.

The constraint of *Mucor* to its yeast-like form did not improve the broth homogeneity or enzyme production and increased the time required for enzyme production. In this study *M. circinelloides* did not perform that well against other species already used to produce these enzymes. However, *M. circinelloides* could be used to produce enzymes from zygomycetes that systems such as *A. niger* do not produce well.

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Opsomming

Filamentagtige fungi word gebruik vir produksie van heterloë proteïne soos ensieme, antibiotika en entstowwe omdat hulle proteïne effektief uitskei en na-translasie-modifisering van proteïne effektief uitvoer. Met die ontwikkeling van rekombinante DNA tegnologie het dit moontlik geword om nuttige proteïne in genera soos *Aspergillus* uit te druk. Die ondergedompelde kweek van filamentagtige fungi is moeilik omdat die menging, en suurstof- en voedsel oordrag in die hoë viskositeit kulture problematies is.

Die doel van die projek was om die potensiaal van gelyktydige beheer van morfologie en ensiem produksie in die dimorfiese fungus, *Mucor circinelloides*, te ondersoek ten einde probleme met ondergedompelde kulture op te los. Dimorfiese *M. circinelloides*, 'n sigomycete in die orde *Mucorales*, groei òf filamentagtig òf gis-agtig. Onlangs is vooruitgang gemaak met transformasie van *Mucor* vir heteroloë proteïn produksie. Die glukoseoksidase geen van *Aspergillus niger* is onder beheer van die gliseraldehied-3-fosfaat-dehidrogenasepromoter in *M. circinelloides* uitgedruk. Glukoseoksidase (GOX) is 'n ensiem wat oksidasie van voedsel voorkom om sodoende die raklewe verleng. Dit word ook gebruik vir die voorbereiding van lae-kilojoule drankies en die meting van glukose vlakke in mediese diagnostiese toepassings.

Die omvang van die projek was om die toestande van gis- en filamentagtige groei van *M. circinelloides* te bepaal en sodoende hulle morfologie te beheer, asook om ensiemproduksie onder hierdie omstandighede te evalueer. Die ensiemproduksie van die GOX-produsierende gemuteerde ras, wat onlangs saamgestel is, was met die van 'n "wilde" tipe *M. circinelloides* ras vergelyk.

M. circinelloides is in twee-fase lotfermentasies gekweek, eers in die gis-vorm, en toe in die filamentagtige vorm. Die gis-morfologiese vorm is onder anaerobiese toestande gestimuleer, terwyl die filamentagtige morfologiese vorm gestimuleer is deur blootstelling aan suurstof. Die ensiem-, biomassa- en metabolietproduksie van die GOX-produserende ras en 'n "wilde" tipe ras was bepaal in die twee-fase lotfermentasies. GOX aktiwiteit van die mutantras, en die amylase aktiwiteit van die "wilde" tipe ras is vergelyk, asook met dié van ander produksiesisteme.

Die morfologie kon in die gis-vorm behou word met behulp van N_2 asook die byvoeging van ergosterol en Tween 80. Die GOX aktiwiteit in die kwekingsvloeistof was vergelykbaar met dié van ongeoptimiseerde prosesse soos deurgegee in die literatuur. Die produksie was wel laer as in geoptimeerde rekombinante GOX produksie sisteme waar van sekere giste, *Aspergillus* of *Penicillium,* gebruik gemaak is. Die intrasellulêre GOX was ses maal hoër as die ekstrasellulêre vlakke. Dit was onverwags omdat die ensiem goed in die oorspronklike spesie uitgeskei is. Die morfologiese beheer het die morfologie van die gis-fase van die fermentasie verbeter, maar die morfologie van die filamentagtige, ensiem-produserende fase is nie verbeter nie. Die morfologiese beheer was nadelig vir biomassa opbrengs van ensiemproduksie.

Die beperking van *Mucor* in die gis-vorm het nòg die kwekingsvloeistof se eienskappe nòg die ensiemproduksie verbeter, en het ensiem produksie verleng. In hierdie studie was die ensiem produksie van *M. circinelloides* laer as dit vir ander spesies wat reeds vir ensiem produksie gebruik word. Nogtans kon *M. circinelloides* moontlik vir die produksie van sigomycete ensieme benut word, i.p.v. sisteme soos *A. niger* wat nie so effektief produseer nie.

Biographical Sketch

Astrid Sindle was born in Cape Town in 1979, she grew up in Table View, and attended Table View Primary and High schools. She graduated with a Bachelor of Science at the University of Cape Town in 2000, a Post-graduate Diploma in Engineering at Stellenbosch University in 2002 *cum laude*, and enrolled for her Masters in Engineering Science in 2004.

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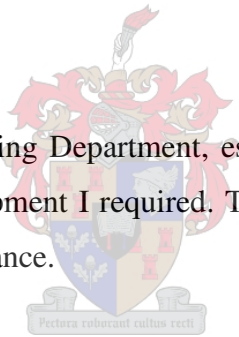

My family and friends for their encouragement and moral support. Kriek Britz for her encouragement over the last two years.

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My Father in heaven, for giving me this opportunity and providing for me.

Preface

This thesis is present as a compilation of six chapters. Chapters 3, 4 and 5 are written in a style similar to that expected for submission to a journal.

Chapter 1	General Introduction
Chapter 2	Literature Survey
Chapter 3	Materials and Methods
Chapter 4	Research Results Potential of a process including morphological control of dimorphic *M. circinelloides* for enzyme production
Chapter 5	Discussion and Conclusions
Chapter 6	Recommendations for Future Work

Table of Contents

Chapter 1. Introduction

Filamentous fungi are used commonly in industrial production of pharmaceuticals, bulk chemicals and commercial enzymes (Alexopoulos and Mims, 1979). Pharmaceuticals produced by fungi include the antibiotics, penicillin and cephalosporin, and virus–like particles for vaccines; and bulk chemicals include ethanol and citric acid (Li et al., 2000). Proteases and amylases are examples of enzymes produced on an industrial scale by fungi (Bhargava et al., 2003 and Li et al., 2000). In contrast with most bacteria and yeast, fungi are efficient protein secretors, and do not glycosylate proteins to the extent that other organisms do, and therefore are used for production of homologous and heterologous protein (Punt et al., 2002).

Proteins are usually secreted at the tip regions of the filamentous fungi (Wösten et al. 1991; Gordon et al. 2000 as quoted by McIntyre et al., 2002). Fungi are cultivated either on solid substrate or in submerged culture, but submerged culture is preferred in industry as the conditions of the culture broth can be well controlled and spores are not released into the air. However, the submerged culture of filamentous fungi presents engineering challenges due to their mycelial morphology. This mycelial morphology typically produces viscous fermentation broths and therefore presents problems in agitating, pumping and supplying O_2 to cultures, causing non-ideal mixing of the broth and poor O_2 and nutrient transfer to the fungi (Li et al., 2000). This translates into sub-optimal yields of biomass and other metabolites (Li et al., 2000).

In order to solve the problem, some approaches are to dilute the medium, increase agitation or encourage pelleted growth. In the case of dilution of the broth with water, this results in a large reactor size and more difficult downstream processing. An increase in agitation may break up hyphae and stunt growth, and release proteases, possibly degrading proteinaceous products (McIntyre et al., 2002). The approaches of dilution or increasing agitation have not been found to be to be satisfactory (Olsvick and Kristiansen, 1994 as quoted by Bhargava et al, 2003). Much research has gone into determining conditions that allow pelleted growth of *Aspergillus*, as pellets are more easily agitated than free filaments, however pellets often have nutrient limitation at the center (Li et al., 2000).

Besides problems caused by viscous fermentation media of filmentous fungi, there are also difficulties due to mycelial growth on the walls of the fermenter and above the surface of the medium. *Aspergillus niger* is one of the most commonly used filamentous fungi in industry, as it secretes large amounts of proteins, however the mycelia lead to problems in process control. It has been observed that in fermentation with *A. niger* the mycelia grow on walls of the fermenter as well as on the surface of the broth. The culture is non-homogeneous and the process is not controlled adequately for reproducible cultivations. Culturing of filmentous fungi has complications due to their mycelial morphology.

There are some dimorphic fungi, such as those of the genus *Mucor,* a zygomycete in the order *Mucorales,* that grow as yeast or filaments, in response to environmental variables (Orlowski, 1991). A process in which dimorphic *Mucor* can be cultivated in its yeast morphology would allow better control of O_2 and nutrient transfer than a filamentous culture. Biomass could first be grown in yeast form, and if protein production is found to be better in filamentous form, which is likely as proteins are secreted from hyphal tips, filamentous growth and concomitant protein production could be induced later in the fermentation. This method of growing *Mucor* may result in less viscous broth and reduce the time that filamentous broth must be controlled in a reactor. Morphology of *M. circinelloides* in submerged culture can be controlled by gas atmosphere and addition of ergosterol and Tween 80 to the medium. Under aerobic conditions *Mucor* is filamentous, whereas under anaerobic conditions it grows yeast-like. The gas mix of 30% CO_2 and 70% N_2 has been used for yeast cultures (Bartnicki-Garcia and Nickerson, 1962b, Lübbehüsen et al., 2003b). When Tween 80 and ergosterol are added to the medium, *Mucor* can also grow yeast-like under N_2 (Lübbehüsen et al., 2004).

Although the control of morphology of *Mucor* has been studied and conditions for yeast-like growth are known, the precise mechanism of morphological change is not yet known. The overarching requirement is for anaerobiosis for yeast; other than that there are nutritional requirements that need to be met: a fermentable hexose and complex nitrogen source. Also, ergosterol and Tween 80 have to be supplemented under N_2 but not under CO_2 to achieve yeast-like growth (Lübbehüsen et al., 2003b). It is not clear whether different stimuli activate the same pathway of morphological control or whether the way in which they work is completely different.

Some dimorphic fungi are associated with pathogenic activity, where one morph is pathogenic and the other is harmless. For example, *Candida* (a human pathogen) becomes filamentous with exposure to serum, and *Ustilago,* which infects corn, becomes filamentous as a response to a putative molecular signal from the host plant (Madhani and Fink, 1998). *Histoplasma capsulatum* is pathogenic in its yeast form, at 37˚C, but is harmless in its filamentous form, at 25˚C (Maresca and Kobayashi, 1989). In some organisms, the change in morphology is a response to nutritional stress; both *Saccharomyces cerevisiae* and *Cryptococcus neoformans* (causes meningitis) occur as yeasts, but become filamentous under nitrogen starvation in order to scavenge for nutrients (Madhani and Fink, 1998). It is possible that under certain conditions, the dimorphic behaviour of *Mucor* is a stress-response of the organism to nutritional starvation. It is not the fermentative metabolism (associated with anaerobiosis in many organisms) which determines morphology, as both yeast and filamentous cells are fermentative (Orlowski, 1991).

Until recently there had been slow progress in transforming *Mucor* to produce heterologous proteins, and there has been little information on heterologous protein production in *M. circinelloides.* However, recently, systems for transformation of *M. circinelloides* have been developed (Appel et al. 2004), and *M. circinelloides* has been transformed to over-express glucose oxidase (Larsen et al., 2004). *M. circinelloides* has also been transformed to produce carotenoid compounds (Papp et al., 2006). With the availability of these heterologous strains, it is possible to investigate heterologous protein production in combination with morphological control.

The purpose of this study is to investigate the potential of *M. circinelloides* for native and heterologous protein production while controlling morphology, by comparing the enzyme production of a mutant and of a wild type *M. circinelloides.* One of the glucose oxidase (GOX)-producing strains resulting from work by Larsen et al. (2004) was further investigated. The GOX-strain had been cultured in shake flasks (Larsen et al., 2004), but in this study it was cultured under conditions of controlled morphology in fermenters. Different substrate types and concentrations were investigated in addition to those used by Larsen et al. (2004). Where Larsen et al. (2004) assayed only extracellular GOX, in this study extracellular and intracellular GOX production was measured. The enzyme production was compared with current industrial enzyme production systems, such as *Aspergillus niger* cultivations. This enabled us to see if morphological control and enzyme production can be achieved in *Mucor*,

and whether this approach of reducing problems associated with viscous filamentous cultures is commercially viable.

Two *M. circinelloides* strains were used, a wild type and a glucose-oxidase producing mutant, to be evaluated by their amylase and GOX production respectively. The optimal conditions for yeast-like and filamentous morphology were determined, various media were screened for optimal biomass and enzyme production, and two-stage cultivations with a yeast stage and a filamentous stage were performed. Batch cultures in shake flasks and fermenters were used throughout, where biomass, ethanol, and enzyme production was measured and morphology was monitored by photographs.

Chapter 2. Literature Review: Potential of dimorphic fungus *Mucor circinelloides* as a host for heterologous protein production

In this literature review the following topics will be discussed: problems associated with cultivating filamentous fungi in submerged culture, an introduction to *Mucor*, the first systematic work done on understanding morphology of *Mucor*, observation of metabolism of *Mucor* in general, and more recent work focusing on growth and morphology of *M. circinelloides,* as well as a comparison of *M. circinelloides* with other expression systems.

2.1. Introduction

Filamentous fungi are used commonly in industrial production of pharmaceuticals, bulk chemicals and commercial enzymes. Examples of pharmaceuticals include the antibiotics, penicillin and cephalosporin, and virus–like particles for vaccines. Bulk chemicals such as ethanol and citric acid are produced by fungi. Proteases and amylases are examples of enzymes produced on an industrial scale by fungi (Bhargava et al., 2003). Fungi are efficient protein secretors and therefore are used for production of homologous and heterologous protein. Another advantage of filamentous fungi is that they do not destroy the functionality of heterologous proteins by glycosylating (adding sugar groups to) them to the extent that bacteria and yeasts do.

Fungi are cultivated either on solid substrate or in submerged culture. Submerged culture is preferred in industry as the conditions of the culture broth can be well controlled and spores are not released into the air. However, the submerged culture of filamentous fungi presents engineering challenges due to their mycelial morphology. This mycelial morphology typically produces viscous fermentation broths and therefore presents problems in agitating, pumping and supplying O_2 to cultures, causing non-ideal mixing of the broth and poor O_2 and nutrient transfer to the fungi. This translates into low yields of biomass and other metabolites. One possible approach to solve this problem is to dilute the broth through addition of water, although this results in the need for larger reactors and causes problems in downstream processing. The other reported approach is to increase the agitation rate to break up the

mycelium and reduce viscosity. If the broth is agitated too vigorously, hyphae fragment and growth is stunted; at the same time proteases may be released, degrading proteinaceous products including desired heterologous proteins (McIntyre et al., 2002). The approaches of dilution and of increasing agitation have not been found to be to be satisfactory (Olsvick and Kristiansen, 1994 as quoted by Bhargava et al., 2003).

Aspergillus niger is one of the most commonly used filamentous fungi in industry. It has been observed that in fermentations with *A. niger* the mycelia grow on the walls of the fermenter as well as on the surface of the broth. This means that the culture is non-homogeneous and the process is not controlled adequately for reproducible cultivations. To reduce wall growth, the reactor wall above the broth is sometimes cooled allowing condensation to wash mycelia down the wall and back into the broth. There are many reports detailing conditions that allow pelleted growth of *Aspergillus*, as pellets are more easily agitated than free filaments, however pellets often have nutrient limitation at the center.

Therefore, an organism that is an efficient protein secretor but that has unicellular morphology would be ideal for protein production (Papp et al., 2006). Some fungal species of the genus *Mucor* are dimorphic, existing in a filamentous and yeast-like form. The different morphologies are normally a response to environmental variables (Orlowski, 1991). A process in which *Mucor* can be cultivated in its yeast morphology would allow better control of O_2 and nutrient transfer. Biomass could first be grown in a yeast form, and if protein production is found to be better in the filamentous form, filamentous growth and concomitant protein production could be induced later in the fermentation. This method of growing *Mucor* may result in less viscous broth and reduce the time that filamentous broth must be controlled in a reactor. Dimorphic *Mucor* may have potential for cultivation in the form of a low-viscosity broth, for the purpose of heterologous protein production.

2.2. Effect of fungal morphology on protein secretion

The effect of a dimorphic shift in fungal morphology on protein production is not yet well documented in literature. However, in purely filamentous fungi, there have been some studies on the effect of fungal morphology on protein production. For example, in a study on the effect of fungal morphology on production of glucoamylase by recombinant *Aspergillus niger*, immobilised mycelial cultures had 10-fold more enzyme secretion than free-cell

pelleted cultures (Talabardon and Yang, 2005). This recombinant *A. niger* strain contained the gene for the glucoamylase-green fluorescence protein (GLA-GFP) fusion protein. Different morphologies were grown in three different culturing systems, namely stirred-tank bioreactors, a rotating fibrous bed and a static fibrous bed. Pelleted free-cell cultures of various sizes were grown in the stirred-tank bioreactors, and immobilized mycelial cultures were grown on cotton cloth in the rotating fibrous bed and the static fibrous bed. The expression of GLA-GFP was dependant on fungal morphology and was growth associated. The free-cell pellets produced 10 times less GFP and glucoamylase than the immobilized cultures. In free-cell cultures, release of the fusion protein occurred mainly as a result of cell autolysis, but in immobilised cultures protein secretion occurred from the beginning of the fermentation. Morphology strongly influenced protein secretion. Small 1 mm pellets secreted 82% of GFP produced, whereas larger pellets of 5 mm only secreted 57% of GFP. Immobilised cells secreted all the GFP produced. The greater amount of heterologous protein secretion in immobilized cells is attributed to the filamentous mycelial morphology since protein secretion occurred mostly at the tips of the hyphae. Protease secretion occurred during stationary phase or autolysis and was not dependant on morphology, although the immobilized cells had less protease activity. The rotating fibrous bed reactor yielded the best protein production as it provided conditions for the kind of cell morphology that was the most conducive to oxygen transfer and protein secretion (Talabardon and Yang, 2005). Therefore, the morphology does impact protein secretion, and when controlling the morphology of fungi, it is necessary to investigate the effect of morphological control on enzyme production.

2.3. Biomass concentration related to non-Newtonian behaviour and nutrient limitation in liquid culture

At high biomass concentrations culture fluid is very viscous, and fluid flow in a stirred-tank bioreactor can exhibit non-Newtonian behaviour (Goudar et al., 1999). To justify an attempt at the improvement of the broth rheology, by manipulation of morphology, the mycelial biomass concentrations encountered should be such that non-Newtonian flow and likely nutrient limitation would occur. Newtonian fluids have a constant viscosity, μ, across all shear rates. The shear rate is the velocity gradient perpendicular to the plane of shear. Examples of Newtonian fluids are water, most aqueous solutions, oils, corn syrup, glycerine and air.

Non-Newtonian fluids do not have a constant viscosity across all shear rates. They can for example, as in pseudoplastic fluids, have a lower apparent viscosity at higher shear rates. Non-Newtonian fermentation fluids typically use a power-law model in which the apparent viscosity, μ, of the fermentation broth can be related to the shear rate as

$$\mu = K(\gamma)^{n-1}$$

where K is the consistency index, n is the flow-behaviour index, and γ is the shear rate. A Newtonian fluid can be described by this equation when n is equal to 1. Empirical correlations have been proposed to relate the power-law parameters with fungal biomass concentration (Goudar et al., 1999). K tends to increase with biomass concentration thus the following equation has been used to describe the relationship between K and biomass concentration, X, where

$$K=aX^b$$

where a and b are empirical parameters (Olsvick and Kristiansen, 1994 as quoted by Goudar et al., 1999). This is inaccurate in batch fermentation systems where K and X exhibit a sigmoidal relationship, and not a power relationship. Thus a logistic equation was proposed to describe the dependence of K on X

$$K = \frac{K_0 e^{cX}}{1 - \frac{K_0}{K_f}\left(1 - e^{cX}\right)}$$

where K_0 and K_f are the initial and final values of K and c is a constant (Goudar et al, 1999). An equation was proposed to relate the flow-behaviour index, n, to the biomass concentration, X

$$n = \frac{1 - n_f}{1 + (dX)^e}$$

where d and e are constants and n_f is representative of the final value of n. This was found to accurately predict dependence of n on biomass concentration (Goudar et al., 1999). These

relationships do not account for the impact of microbial morphology which may change during the course of a fermentation, or be very different in different bioreactors, for example, a bubble column (Goudar et al., 1999). If n = 1, the fluid is considered Newtonian, but culture broths typically show n values between 1 and 0.2. To give an idea of the extent to which culture broths deviate from Newtonian flow, some values of biomass concentration at a value of n = 0.8 are given (Goudar et al., 1999). With *Penicillium chrysogenum*, n of 0.8 occurred at biomass concentrations of 2 g/l dry cell weight, in *A. awamori n* of 0.8 occurred at biomass concentrations of 3.5 g/l, and with *A. niger* at 9 g/l. Thus in a dispersed filamentous fungus such as *M. circinelloides n* less than 0.8 could be expected from biomass concentrations of 2 g/l upwards, thus any concentrations above this would show non-Newtonian flow characteristics and may be subject to nutrient limitation. The expected biomass concentrations for *M. circinelloides* in this project were up to 10 g/l cell dry weight. The value of n at 10 g/l biomass for *P. chrysogenum* was 0.2, for *A. awamori* it was 0.37 and for *A niger* it was 0.75 (Goudar et al., 1999). Thus the fermentation broth for *M. circinelloides* would be expected to exhibit definite non-Newtonian flow and high viscosity.

2.4. *Mucor* dimorphism

Mucor is a zygomycete in the order *Mucorales*. Some *Mucor* species such as *M. rouxii, M. genevensis, M. bacilliformus* and *M. circinelloides (syn. racemosus)* exhibit dimorphic behaviour, while others are constrained to the filamentous form. Morphology changes in response to its environment, although the responses vary from species to species (Orlowski, 1991).

During the 1960's Bartnicki-Garcia and Nickerson. published a number of articles on the factors affecting the morphology of *M. rouxii*. This forms a good introduction to the subject, thus some of their findings are summarised below.

2.4.1. Mucor rouxii as an example of *Mucor* dimorphism

2.4.2. Gas Atmosphere

Batch experiments in submerged culture were performed on *M. rouxii* by Bartnicki-Garcia and Nickerson. (1962b) to elucidate morphogenesis in this species in different gas atmospheres. When cultivated in air or pure N_2 it grew filamentous, but when grown in

presence of pure CO_2 it grew yeast-like. Different ratios of CO_2:N_2 were compared. At a CO_2 level of less than 30% the morphology was a mixture of filamentous and yeast-like, while above 30% CO_2, cells were all yeast-like but growth was stunted. Figure 2-1 shows the proportions of filamentous to fragmentary (yeast) fractions at various CO_2 partial pressures. It was concluded that a ratio of 30% CO_2: 70% N_2 was the optimal gas mixture to induce yeast-morphology under their experimental conditions, while not compromising the growth of the fungus. In addition, it was found that CO_2 is assimilated by *Mucor*, and the amount of CO_2 fixation followed a similar trend to the growth, peaking at 0.3 atmospheres of CO_2 (Figure 2-2).

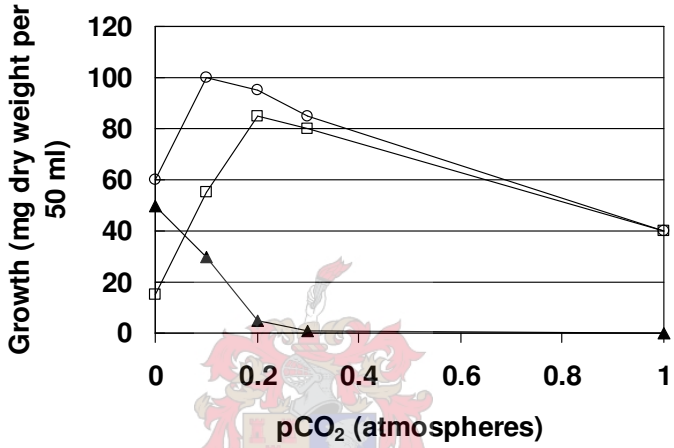

Figure 2-1. Effect of pCO_2 on growth and morphogenesis of *M. rouxii* incubated (24 hours) under mixtures of CO_2 and N_2 at a total pressure of 1.0 atm. O total growth, □ yeast-like fraction, ▲ filamentous fraction (Bartnicki-Garcia, 1963).

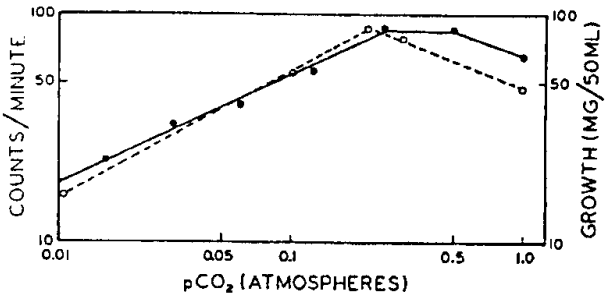

Figure 2-2. Comparative effect of pCO_2 on yeastlike growth (broken line) and $C^{14}O_2$ assimilation (solid line). (Bartnicki-Garcia and Nickerson, 1962a) Note that the x-axis is on logarithmic scale.

The biomass concentration was usually higher for aerobic growth, regardless of the pH, inoculum size and glucose concentration. Cell dry weight per litre of 48-hour shake flask

cultures with 20 g/l glucose were: under N_2 (mostly filamentous) 1.6 g/l, under CO_2 (yeast) 1.8 g/l and under air (filamentous) 6.6 g/l (Bartnicki-Garcia and Nickerson, 1962b).

2.4.3. pH

Only under N_2 was the effect of pH appreciable. Under an N_2 atmosphere, below pH 3 the growth was stunted in the form of ellipsoid cells. As the initial pH was increased above 3, the cells grew more filaments. The cells cultured under CO_2 developed yeast-like, and those incubated in air were typically filamentous regardless of pH.

2.4.4. Inoculum size

Inoculum size did not influence the morphology of cultures grown under air or CO_2. However, under N_2 a large inoculum ($3x\ 10^5$ spores per ml) resulted in spherical cells, while a small inoculum ($3x\ 10^3$ spores per ml) led to filamentous growth. Two factors are responsible: firstly, with a heavy inoculum, CO_2 is produced rapidly and would account for yeast-like growth. Secondly, the spores swelled greatly in initial stage of spore germination but did not reach the stage of filament formation (Bartnicki-Garcia and Nickerson, 1962b).

2.4.5. Glucose concentration

Under N_2, glucose concentration affected morphology, where a high glucose concentration apparently led to yeast-like growth, however these cells were most likely arthrospores. Arthrospores are rounded, undifferentiated hyphal cells often in rows resulting from fragmentation of a hypha, and serve to disseminate the fungus (Snell and Dick, 1957). Yeast cells and arthrospores can be confused, but they can be identified by the fact that yeast cells tend to have buds, whereas arthrospores do not bud. Bartnicki-Garcia (1963) state that the spherical cells observed at low pH, high inoculum size and low glucose concentration were arthrospores and not yeast cells. Glucose concentration also affected the biomass production; 48-hour shake-flask cultures had a maximum biomass concentration with 5% glucose under air, at 10% glucose under N_2 and 2% glucose under CO_2. Under all three atmospheres, biomass production was directly proportional to glucose concentration from 0 to 2% glucose (Bartnicki-Garcia and Nickerson, 1962b).

2.4.6. Cell wall composition

The morphology of the cell is defined by the cell walls. Knowledge of the difference between hyphal and yeast cell walls may assist in understanding and controlling morphology. Yeast cell walls have a slightly different chemical composition, and a different fine structure of the cell wall compared to hyphal cell walls. The yeast cell wall is typically thicker and multi-layered, comprising up to 40 % of the cell volume. A hyphal cell wall is single-layered. The composition of yeast and filamentous cell walls can be seen in Figure 2-3. Most substances were more abundant in the filamentous form; however mannose and protein levels were higher in yeast cell walls. How does this relate to gas environment? CO_2 fixation corresponded strongly with yeast-like development. There is fixation of CO_2 especially into aspartic acid, which may be incorporated into mannan and protein macromolecules in the cell–wall. Yeasts, in general, have mannan-protein complexes in the cell wall that are rich in aspartic acid (Bartnicki-Garcia, 1963).

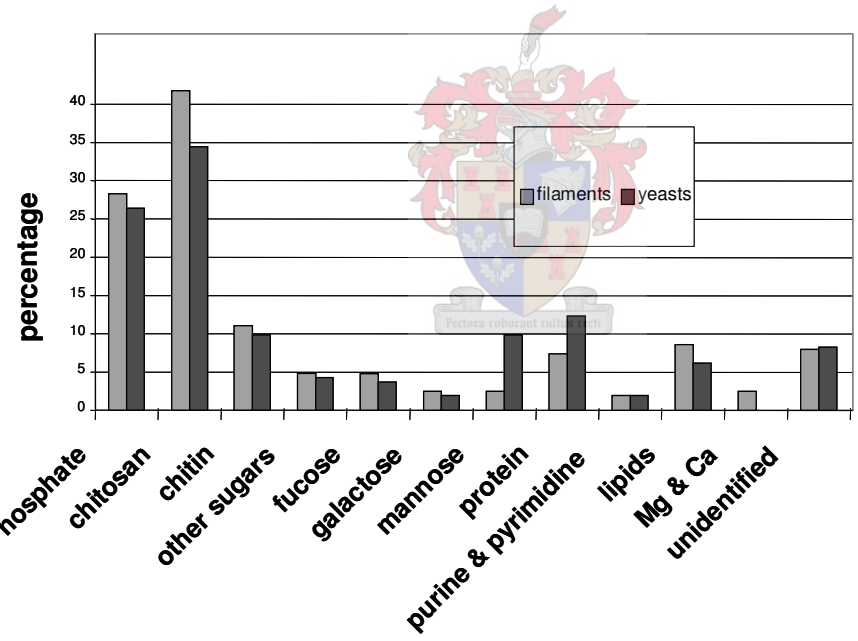

Figure 2-3. Cell wall composition of filamentous and yeast-like cells of *M. rouxii*. (Data from Bartnicki-Garcia, 1963).

The biochemical basis or morphological change is unclear, but the cell wall of yeast has 4 times more mannose and 40% more protein than the filamentous cell wall. Amongst typical culture variables such as the gas atmosphere, pH, inoculum size and glucose concentration, the gas atmosphere is the single most important factor in determining cell morphology.

2.5. Metabolism

The relationship between *Mucor*'s metabolism and morphology is discussed below. When morphology is controlled by manipulating culture conditions, the metabolism is also affected and this is important in commercial production of proteins or metabolites.

2.5.1. Carbon and energy metabolism

As mentioned previously, most observations suggest that aerobic growth leads to hyphal development, whereas anaerobiosis and alcoholic fermentation of a hexose are necessary for yeast development. However the relationship is not always so simple and different species react differently to environmental stimuli. *M. rouxii* requires anaerobiosis, a hexose as well as CO_2 (or high hexose concentration above 8%) for yeast growth. In the absence of a hexose and CO_2 it will grow mycelial even under anaerobic conditions (Orlowski, 1991).

M. genevensis, even in air atmosphere, will grow yeast-like at high hexose concentrations. In contrast, *M. racemosus* requires anaerobiosis but does not require CO_2 or glucose concentration greater that 0.1 % to grow in yeast form (Orlowski, 1991).

The effects of inhibitors of electron transport, oxidative phosphorylation or protein synthesis in mitochondria are discussed below. *Mucor* is constrained to yeast morphology when grown in presence of inhibitors of mitochondrial electron transport, oxidative phosphorylation or protein synthesis. There could be a link between aerobic respiration and Mucor morphology; however there are arguments against this (Orlowski, 1991).

Phenyl ethyl alcohol (PEA) constrains *M. rouxii* to yeast morphology. PEA was reported to stimulate alcoholic fermentation and inhibit oxidative phosphorylation. However if the carbon source is not a hexose *M. rouxii* grows as hyphae in presence of PEA. Oxidative phosphorylation should be depressed no matter what the carbon source (other sources were xylose, maltose, sucrose); therefore oxidative phosphorylation is not a critical controlling factor in morphology. As discussed by Orlowski, respiratory capacity is also not a critical morphological determinant (Orlowski, 1991).

Mitochondrial development and oxidative metabolism have been shown to be irrelevant in *M. genevensis* dimorphism. Anaerobically grown (under N_2) *M. racemosus* yeasts have fully developed mitochondria, and respiratory capacity of these cells is nearly as high as in

aerobically grown hyphae. It has been suggested that there is a nonrespiratory function of the mitochondrion necessary for hyphal development.

Both aerobic hyphae that have developed from yeasts, as well as anaerobic hyphae have a fermentative metabolism. Aerobic yeasts, that can be induced by adenosine 3´,5´-cyclic monophosphate (cyclic-AMP), have a fermentative metabolism. "Fermentation consistently accompanies yeast morphology but cannot be said to determine it" (Orlowski, 1991). *Mucor* yeasts produce ethanol, but ethanol produced in fermentation does not determine *Mucor* morphology as fermentative hyphae that produce large amounts of ethanol do exist.

A mutant of *M. racemosus* that behaves like a wild type with regard to morphology and energy-yielding catabolic pathways, except in the presence of glucose, has been isolated. With glucose the mutant is highly fermentative and grows as yeast whether O_2 is present or not. In this condition respiration is not inhibited, suggesting it is not a critical determinant of morphology. O_2 seems to be the primary regulator that independently controls expression of genes specifying catabolic and morphogenetic functions (Orlowski, 1991).

Why do *Mucor* yeasts not catabolize disaccharides? Production of the required enzymes may be repressed by glucose or cyclic AMP, or enzymes are not inserted into their functional site in the periplasmic space. It is not clear whether the inability of *Mucor* yeasts to catabolize disaccharides is directly related to morphogenesis (Orlowski, 1991).

2.5.2. Nitrogen metabolism

The nutritional requirements of *Mucor* yeasts are more specific than those of *Mucor* hyphae. *Mucor* yeasts require an aminated compound as a nitrogen source. A minimal medium for *M. racemosus* must contain glutamate for yeast growth, whereas an ammonium salt is sufficient to support hyphal growth. Nitrogen assimilation may proceed via different pathways in the two forms of *Mucor* spp. It was thought that perhaps the yeast form has a deficiency of biosynthetic (NADP-linked) glutamate dehydrogenase (GDH). NAD (nicotinamide adenine dinucleotide) and NADP (NAD phosphate) are important co-enzymes that function as hydrogen carriers in catabolic and anabolic redox reactions.

Biosynthetic and catabolic (NAD-linked) forms of GDH were found in *M. racemosus*. The NAD-dependant enzyme displays depressed activity in yeasts (10–fold lower). Exogenous cyclic AMP, which induces yeast morphology in aerobic atmosphere, represses activity of NAD-linked GDH to levels found in anaerobic yeasts, indicating a possible relationship of GDH to morphology. The activities of GDH in cells grown on complex nitrogen sources are normally higher than cells grown on inorganic nitrogen salts. However *M. racemosus* mutants have been found that, with cyclic AMP in air, grow hyphal while having depressed GDH activity. This raises doubts about the relationship between NAD-dependant GDH and morphology (Orlowski, 1991). Nonetheless, it is beneficial to add glutamate to the medium for the cultivation of *Mucor* yeast cells.

2.5.3. Endogenous small molecules

The "second messenger hypothesis" describes how an environmental variable stimulus may be changed to a physiological or morphogenetic response via a "messenger" molecule. Cyclic AMP was put forward as the "second messenger" although other substances have also been suggested. There is a consistent correlation between cell morphology and internal cyclic-AMP levels in *M. racemosus*. For example, when yeasts were converted to hyphae by change of atmosphere from CO_2 to air, cyclic AMP levels decreased 3-fold (Larsen and Sypherd, 1974, as quoted by Orlowski, 1991).

When a mutant *coy-1* was shifted from anaerobic to aerobic conditions the morphology remained yeast-like and internal cyclic-AMP levels remained high. This indicates that a change in cyclic–AMP levels is not simply due to change in gas atmosphere, but is correlated with morphology. Intracellular cyclic-AMP levels are considered the strongest correlate of *Mucor* dimorphism (Inderlied et al., 1985 as quoted by Orlowski, 1991).

2.5.4. Lipids

2.5.4.1. Lipids in *Mucor*; relating to morphology.

The composition of lipids was compared in yeasts and hyphae of *M. genevensis* and *M. rouxii*. Hyphae were found to have much higher levels of sterols and fatty acids than yeasts. Fatty acids in hyphae were predominantly unsaturated, however in yeasts they were mostly

saturated. The high level of sterols is not considered important in morphology (Weete et al., 1987 as quoted by Orlowski, 1991).

Cells induced to grow yeast-like aerobically in phenyl ethyl alcohol (PEA) had a typical hyphal pattern of lipid composition in the cytoplasmic membrane. But in the cell wall the lipid composition was more like that of anaerobic yeasts.

An antibiotic, cerulenin, blocks lipid synthesis by inhibiting fatty acid synthetases. In a yeast-to-hypha experiment, administering cerulenin prevented conversion to hyphae. It also blocked an increase in the rates of protein and RNA synthesis, and orthinine decarboxylase (ODC) activity. When Tween 80 (a complex mixture of fatty acids) was added it reversed the effects of cerulenin. Cerulenin inhibits the increased phospholipid synthesis associated with yeast-to-hypha transitions and also decreases phospholipid turnover. It seems that increases in the rate of lipid synthesis and phospholipid turnover are essential for *Mucor* yeast-to-hypha transitions. The presence of Tween 80 and ergosterol in culture medium can cause *M. circinelloides* to grow yeast-like (Lübbehüsen et al., 2003b).

2.5.4.2. Lipid production in industry

The fatty acid, gamma-linolenic acid (GLA), is a precursor of prostaglandins in the body, and dietary supplements of GLA have health benefits. GLA in supplements is usually oil extracted from evening primrose seeds. Some *Mucor* species contain high levels of GLA and have been used to produce GLA on an industrial scale (Jackson et al., 1998).

2.5.5. Enzymes

2.5.5.1. Enzymes and Morphology

Some enzymes involved in intermediary metabolism have been correlated with changes in the morphology of *Mucor*, for example, in *M. rouxii* pyruvate kinase occurs only in hyphae. There is a consistent correlation of ODC activity and *Mucor* morphogenesis. ODC plays a role in putrescine synthesis. SAM (S-adenosylmethionine) synthetase is closely linked to concentration of SAM which is a universal methyl donor and is correlated strongly to *Mucor* dimorphism (Orlowski, 1991).

Some carboxypeptidases are specific to a particular morphology of *Mucor racemosus*. Peptidase activity increases during yeast-to-hypha transitions and emergence of germ tubes sporangiospores (Orlowski, 1991).

Also, enzymes active in disaccharide catabolism have been investigated. *Mucor* is normally unable to utilize disaccharides, such as maltose, cellobiose and trehalose anaerobically. Maltose is cleaved by the enzyme α-glucosidase. Cytoplasmic α-glucosidase is induced in *M. rouxii* by exposure to maltose regardless of morphology or aerobic/anaerobic conditions. However the wall-associated α-glucosidase is only found in hyphae, not in yeasts, regardless of gas atmosphere thus yeasts cannot catabolize maltose. This enzyme does not appear in yeasts due to repression by cyclic-AMP (Orlowski, 1991).

β-glucosidase hydrolyses cellobiose, and is expressed in soluble and wall fractions of *M. racemosus*. It is expressed when *M. racemosus* is grown on all carbon sources besides hexoses. β-glucosidase is not found in yeasts because they require hexose for growth and have high intracellular cyclic-AMP, both of which inhibit β-glucosidase production. The presence of β-glucosidase is not a cause of morphogenesis as it can be fully repressed in hyphae (Orlowski, 1991).

Trehalase cleaves the trehalose of aerobically and anaerobically germinating sporangiospores of *M. rouxii*. Interestingly, although trehalase is present in anaerobic *Mucor* yeasts, trehalose does not support their growth (Orlowski, 1991).

Thus, *Mucor* yeasts do not use disaccharides for growth, in some cases they do not have the correct enzymes, or the enzymes are not in contact with the substrate.

2.5.5.2. Mucor Enzymes for Industrial Applications

Mucor species have been found to produce amylase, lipase, pectinase and proteases in plate assays (Alves et al., 2002). According to Bogar et al. (2003) the production of phytase, lipase and α-amylase by *Mucor racemosus* NRRL 1994 was more efficient in solid-state fermentation than in shake-flasks. For example, the amylase activity of shake flask cultures reached 0.5 Units per gram dry material (i.e. medium minus water) whereas amylase activity in solid-state fermentation was as high as 35 U/g dry material. In a study using the enzyme,

linamarase (β-D-glucosidase), to detoxify cassava of cyanide, *M. circinelloides* LU M40 produced 12.2 U linamarase activity per ml of culture supernatant (Petruccioli et al., 1999).

2.5.6. Macromolecular synthesis during morphological shift

Often when agents are added to cultivation broth to control morphology and prevent yeast-to-filaments morphogenesis, they stop not only the morphogenesis but the growth as well. For example, cycloheximide arrests morphogenesis to hyphae while inhibiting protein synthesis and RNA synthesis. Cell changes in the morphological shift from yeast to hyphae are discussed below.

The instantaneous rate of protein production increases during the morphological shift from yeast to filamentous cells. This was measured during CO_2-to-air and CO_2-to-N_2 shifts causing yeast-to-hyphae conversions of *M racemosus*. Protein synthesis initially increased with emergence of germ tubes and subsequently declined. Overall rate of protein synthesis was found to be a balance between the cellular ribosome concentration, the percentage of ribosomes recruited into the translation process and the rate of polypeptide chain elongation.

Different protein production rates would mean a change not only in quantity but also in the type of protein made. An adjustable rate of polypeptide growth offers potential for differential gene expression.

A basic protein designated S6 from the small ribosomal subunit correlates with the rate of protein synthesis. S6 displays various degrees of phosphorylation. The degree of phosphorylation of S6 correlates with the rate of protein synthesis, cell morphology and intra-cellular ATP level during CO_2-to-air morphological shifts (Orlowski, 1991).

Based on an overall view of correlates of morphogenesis a model is proposed by Orlowski (1991). Cyclic AMP and cyclic-AMP modulated protein kinase controls phosphorylation of ribosomal protein S6. S6, at appropriate polyamine concentration and in the presence of variably methylated EF-1α (a highly methylated protein, elongation factor - 1 α) could adjust rate of translation. The extent of EF-1α methlyation could be influenced by extracellular SAM concentration, determined by SAM synthetase activity.

During yeast-to-hypha shifts induced by a CO_2-to-air change there is an increase in DNA synthesis, RNA synthesis, chitin, chitosan and lipids. During germination and growth of *M. racemosus* in air or N_2 most proteins produced were the same, however a small number of proteins were different, but it is not known whether this is particularly due to metabolism or morphology.

2.5.7. Normal Growth and ageing

Hyphal growth on solid medium generally leads to sporangiospore production. In a liquid medium batch cultivation hyphae septate and form arthrospores. Orlowski states that continuous culture of *Mucor* hyphae is not possible but if attempted results in septation of hyphae and arthrospore formation in the mycelial mass. Batch cultivations result in yeast cells with so many bud scars that they are no longer capable of reproducing. In yeast continuous culture there are less old scarred cells (Orlowski, 1991).

The growth of coenocytic *Mucor* seems more 'primitive' than the strictly controlled growth of septate organisms like *Aspergillus*, or yeasts such as *Saccharomyces cerevisiae*. In *M. circinelloides* the yeast cells contain many nuclei (between 20 – 60), and buds on the cell also contain two to ten nuclei each. In a flow-through cell experiment, buds did not detach from the mother cell. In filaments large numbers of nuclei were clustered in random locations while arthrospores contained five to twelve nuclei (Lübbehüsen et al., 2003a).

2.5.8. Dimorphism as a stress response

In some organisms, change in morphology is a response to nutritional stress; both *S. cerevisiae* and *Cryptococcus neoformans* (causes meningitis) occur as yeasts, but become filamentous under nitrogen starvation (Madhani and Fink, 1998). In *Mucor*, it is possible that under anaerobiosis the yeast form predominates if all the nutritional requirements are met, but if a complex nitrogen source is not supplied, the filamentous morph of *Mucor* occurs as a stress-response to nitrogen starvation. The filamentous form may allow the organism to synthesise or forage for the compounds it requires (Gimeno et al., 1992). The mycelium is able to consume a much larger variety of substrates than the yeast form, especially under aerobic conditions. It is not the fermentative metabolism (associated with anaerobiosis in many organisms) which determines morphology, as both yeast and filamentous cells are fermentative (Orlowski, 1991).

2.6. Mucor circinelloides

This thesis deals specifically with *M. circinelloides*, and so the morphology, growth and genetic transformation of *M. circinelloides* are discussed in the following sections. *M. circinelloides* was named *M. racemosus* in the past. During the last five years there has been some literature published on growth and dimorphism of *M. circinelloides* (Lübbehüsen et al. 2003a, 2003b, 2004 and McIntyre et al., 2002). From this we see that when *Mucor circinelloides* is cultivated under aerobic conditions it grows filamentous, and under anaerobic conditions it grows yeast-like as shown in Figure 2-4. Besides gas atmosphere, the morphology can be influenced by addition of morphogens. In the lifecycle of *M. circinelloides* in submerged cultivation, after germination of the sporangiospore development can either be yeast-like or filamentous (Figure 2-5). As with *M rouxii,* anaerobic cultivation of *M. circinelloides* requires supplementation of a fermentable hexose and a complex nitrogen source.

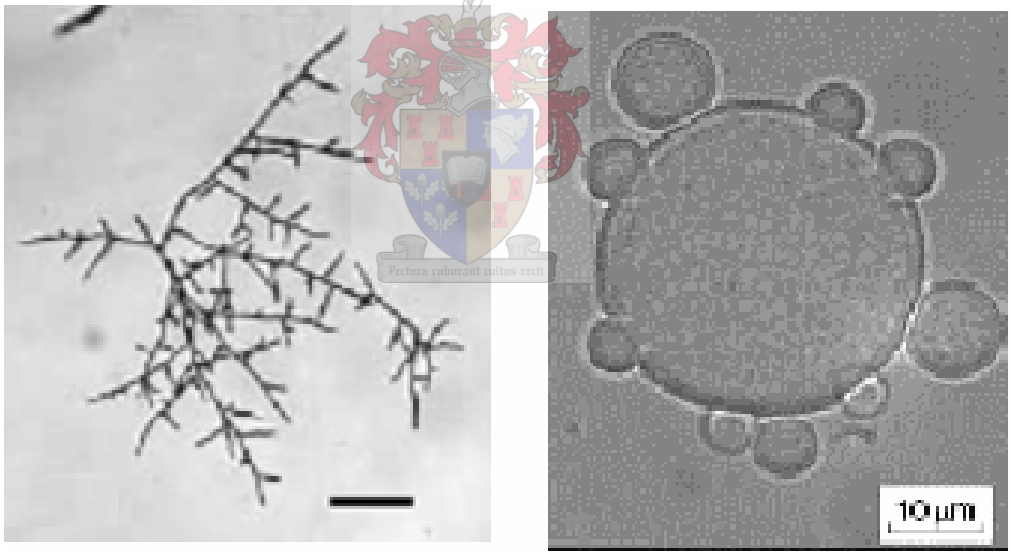

Figure 2-4. Filamentous (left) and yeast (right) morphology of Mucor circinelloides. The bar on left is 300 µm. Photographs were taken of fungi grown in a flow-through cell (Lübbehüsen et al., 2003a).

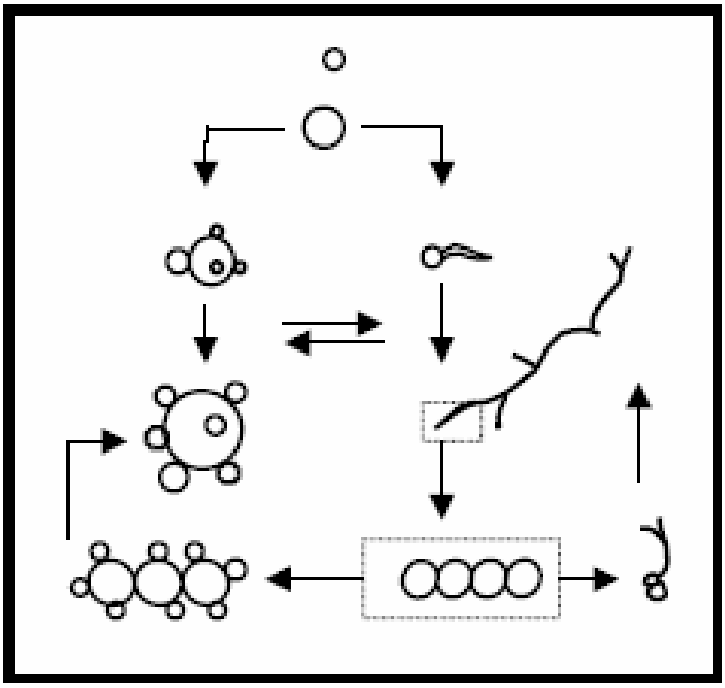

Figure 2-5. Life cycle of *Mucor circinelloides* during submerged growth. After swelling of the sporangiospores growth continues in either a polarized (hyphal) or isotropic (yeast-like) growth depending on the environment. Changing cultivation conditions triggers a dimorphic shift from one from to the other. After exponential growth septa are produced in the usually aseptate hyphae that differentiate into chains of arthrospores (box). These eventually fragment and again can develop into yeasts or hyphae, governed by the environmental conditions (Lübbehüsen et al., 2003a).

2.6.1. Gas Atmosphere

A ratio of 30% CO_2: 70% N_2 has been used successfully to induce yeast-like growth in *M. circinelloides* based on work by Bartnicki-Garcia and Nickerson (1962a) mentioned in previous sections about *M. rouxii*. In Table 2.1 it can be seen that in Vogel's medium (a synthetic medium) with 100% N_2 growth was mixed (filaments and yeast). However, when it is grown in the complex medium, YPG, (containing yeast extract, peptone and glucose) it grows yeast-like even under 100% N_2 (McIntyre et al., 2002). As with *M. rouxii,* it seems that the gas atmosphere is the most important factor in morphological changes, however there is an interaction with other factors such the medium and addition of morphogens which will be discussed below.

Table 2.1. Overview of effects of media and gas atmosphere on morphology in batch cultivation experiments with *M. circinelloides* (McIntyre et a.l, 2002)

Medium	Gas Atmosphere	Morphology	Comments
Vogel's	Air	Arthrospores	Higher spore inoculum
YPG	Air	Filamentous	
Vogel's	Air	Filamentous	
YPG	70% N_2 / 30% CO_2	Yeast	
Vogel's	70% N_2 / 30% CO_2	Yeast	
YPG	100% N_2	Yeast	
Vogel's	100% N_2	Filamentous and yeast	
Vogel's	No aeration	Yeast	Sparge with CO_2/N_2 when sampling
Vogel's	Shift	Yeast to filamentous	

The changes in morphology during a shift cultivation in which *M. circinelloides* was grown first anaerobically and thereafter aerobically are shown in Figure 2-6 and biomass, ethanol and glucose levels in the same fermentation are tracked in Figure 2-7.

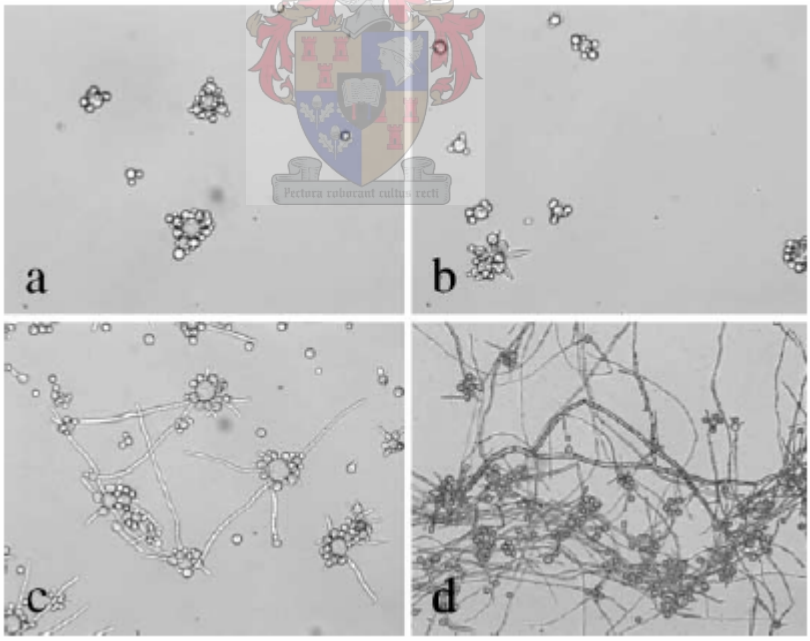

Figure 2-6. Dimorphic shift of *M. circinelloides* from the yeast form under anaerobic conditions (a), showing the emergence of germ tubes within 1 hour of exposure to air (b), subsequent extension of hyphae (c) and filamentous growth (d), (McIntyre et al., 2002).

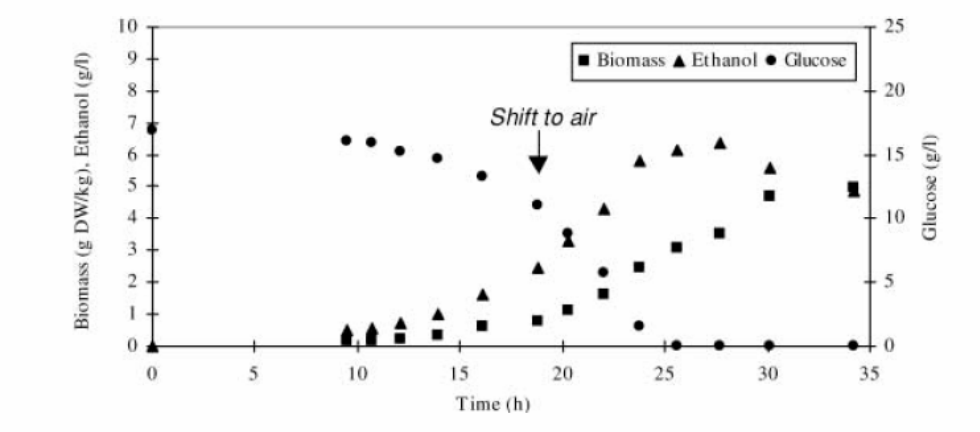

Figure 2-7. Shift cultivation where the sparge gas was switched from 30% CO2 / 70%N2 to air after 19h of cultivation. Values for biomass (g dry weight (DW)/kg) residual glucose (g/l) and ethanol formation (g/l) over time course of the process are shown (McIntyre et al., 2002).

2.6.2. Carbon sources

M. circinelloides can assimilate a large variety of carbon sources under aerobic conditions (Table 2.2). Only certain carbon sources can be fermented. Under anaerobic conditions *Mucor* requires a fermentable hexose. For example, *M. circinelloides f. circinelloides* CBS 108.16 can ferment galactose, glucose, and maltose (Botha et.al., 1997). A more quantitative analysis showing biomass yield and ethanol production on various carbon sources (aerobic growth) can be seen in Table 2.3.

Table 2.2: Carbon sources tested on *Mucor circinelloides f. circinelloides* CBS 108.16. + indicates the carbon source was assimilated, - indicates it was not assimilated (Botha et al. , 1997).

Assimilation of carbon sources			
Pentoses		Polysaccharides	
D-arabinose	-	Inulin	+
L-arabinose	+	Soluble starch	+
D-ribose	+	Salicin	+
D-xylose	+	Alcohols	
Hexoses		Erythritol	-
D-galactose	+	ethanol	+
D-glucose	+	Galactitol	-
L-rhamnose	-	Glycerol	-
L-sorbose	-	Inositol	-
D-fructose	+	D-mannitol	+
Disaccharides		Methanol	-
Cellubiose	+	Ribitol	+

Lactose	-	Sorbitol	+
Maltose	+	Organic acids	
Melibiose	-	Acetic acid	+
Sucrose	-	Butanoic acid	+
Trehalose	+	Citric acid	-
Trisaccharides		Formic acid	-
Melezitose	+	Gluconic acid	+
Raffinose	-	Lactic acid	+
		Succinic acid	+
		Propionic acid	-

Table 2.2: (continued) Below, + indicates the carbon source was fermented, - indicates it was not fermented.

Fermentation of carbohydrates by *Mucor*			
Pentoses		Disaccharides	
D-arabinose	-	Maltose	+
L-arabinose	-	Sucrose	-
D-ribose	-	Trisaccharides	
D-xylose	-	Raffinose	-
Hexoses			
D-galactose	+		
D-glucose	+		

Table 2.3: **Biomass level (g dry weight (DW)/l) , yield of biomass on substrate (Ysx, g/g) and presence of ethanol in shake flask cultivation of *Mucor circinelloides* with a range of carbon sources in Vogel's medium. Results are from 72 hours of cultivation (McIntyre et al., 2002). (ND= not determined)**

Carbon source	Biomass (gDW/l)	Ysx (g/g)	Ethanol production
D-glucose	7.0 ±0.1	0.38	+
D-mannose	6.8 ± 0.2	0.37	+
D-xylose	5.8 ± 0.1	0.32	+
D-galactose	5.5 ± 0.3	0.30	+
L-arabinose	4.0 ± 0.2	0.32	+
Maltose	4.0 ± 0.2	0.60	-
Starch	3.1 ± 0.3	0.31	+
Glycerol	2.2 ± 0.2	0.60	-
Lactose	1.6 ± 0.1	ND	-
Sucrose	1.6 ± 0.1	ND	-
Ethanol	1.5 ± 0.1	ND	ND
No addition	1.5 ± 0.1	ND	-

M. circinelloides was also grown on mixed sugars, glucose and xylose (each 10g/l) and the organism first consumed all the glucose, whereafter it rapidly switched to consuming xylose. On consumption of xylose biomass production was high and ethanol production was much less (McIntyre et al., 2002). Thus, many different carbon sources can be used in medium for filamentous growth but not all can be fermented. For yeast-like growth, carbon sources must be fermentable.

2.6.3. Addition of Morphogens: ergosterol and Tween 80

Ergosterol and Tween 80, fatty acid supplements routinely included in medium for anaerobic cultivation of *S. cerevisiae*, were both added to the medium, resulting in yeast-like growth in the presence of N_2 or an N_2/CO_2 mixture. However, filamentous growth occurred with air or when only ergosterol or Tween 80 were present on their own (Table 2.4). The addition of ergosterol and Tween 80 enabled yeast cells to be cultivated under pure N_2, without the need for CO_2, and also resulted in a larger specific growth rate (Lübbehüsen et al., 2003b). A concentration of 0.02 g/l ergosterol and 0.42 g/l Tween 80 gave the largest specific growth rate and biomass yield (Lübbehüsen et al., 2003b).

Table 2.4: Overview of batch cultivation experiments in Vogel's medium with different sterol and lipid supplements (Lübbehüsen et al., 2003b)

Gas Atmosphere	Supplement	Morphology
Air	-	Filamentous
100% N_2	-	Filamentous
70% N_2 / 30% CO_2	-	Yeast
Air	Ergosterol and Tween 80	Filamentous
100% N_2	Ergosterol and Tween 80	Yeast
70% N_2 / 30% CO_2	Ergosterol and Tween 80	Yeast
100% N_2	Cholesterol and Tween 80	Filamentous
100% N_2	Ergosterol	Filamentous
100% N_2	Cholesterol	Filamentous
100% N_2	Tween 80	Filamentous

2.6.4. Phenyl ethyl alcohol (PEA)

Addition of PEA to fermentation broth causes *M. circinelloides* to grow yeast-like in air. The effects of this morphogen were discussed in the metabolism section above. A concentration of 0.23 % v/v was sufficient to induce yeast under air (Lübbehüsen et al., 2004a).

2.6.5. Effect of gas atmosphere on Metabolism

Figure 2-8 illustrates glucose use and biomass and ethanol production in fermentation experiments. Biomass produced under aerobic conditions is significantly more than that produced under anaerobic conditions. In Figure 2-8, amongst the anaerobic yeast cultures the specific growth rates and biomass yields were highest with pure N_2. In aerobic cultivation it can again be seen that the biomass concentration is 3-fold higher than in anaerobic cultivations. The aerobic ethanol production was more rapid than anaerobic ethanol production. However, due to subsequent ethanol utilization under aerobic conditions, the final ethanol concentration under aerobic conditions was half that under anaerobiosis, as shown in Figure 2-8 (Lübbehüsen et al., 2004).

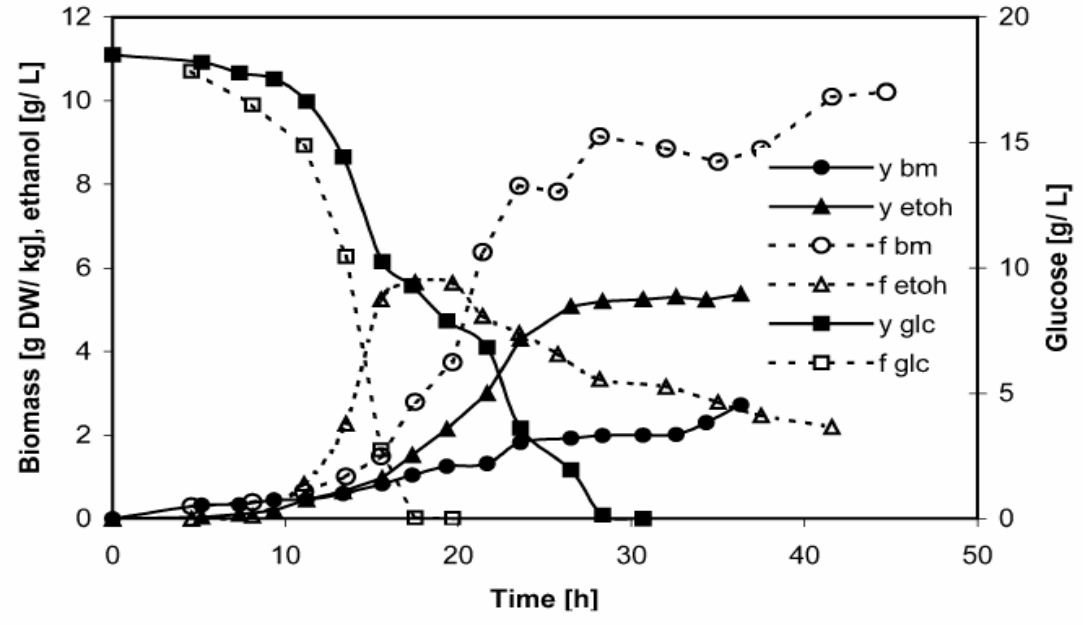

Figure 2-8. Cultivation of *M. circinelloides* ATCC 1216B in Vogel's medium sparged with air (filamentous growth) or 30% CO_2/70% N_2 (yeast-like growth) respectively. Values for biomass (grams of dry weight per kilogram), ethanol (grams per litre) and residual glucose (grams per litre) over the time course of the fermentation are shown. *Y* Yeast growth, *f* filamentous growth, *bm* biomass, *etoh* ethanol, *glc* glucose (Lübbehüsen et al., 2004).

2.6.6. Ethanol production

As discussed in previous sections *M. circinelloides* readily produces ethanol. Because ethanol is also produced aerobically, *M. circinelloides* is classified as Crabtree positive. This represents a challenge for fermentation as a high ethanol yield results in less biomass yield per gram of carbon source. When ethanol is added to the medium, concentrations of ethanol above 10 g/l noticeably decrease biomass yield of the organism. When ethanol was increased from 5 to 10 g/l the final biomass concentration decreased by 10% (Lübbehüsen et al., 2004).

M. circinelloides is relatively intolerant of ethanol. A decrease in biomass and delay in germination occurred as more ethanol was added to the medium. In aerobic conditions below 10 g/l ethanol, morphology was filamentous, between 10 – 30 g/l morphology was mixed, and above 50 g/l no germination occurred (Lübbehüsen et al., 2004). In *M. fragilis* morphology is yeast-like above a certain ethanol concentration. With increased ethanol concentration, increasing lipid unsaturation was demonstrated to maintain an effective plasma membrane (Serrano et al., 2001 , quoted by Lübbehüsen et al., 2004). If *M. circinelloides* responds

similarly to high ethanol levels it would be difficult to change the morphology to filamentous in order to secrete proteins (Lübbehüsen et al., 2004).

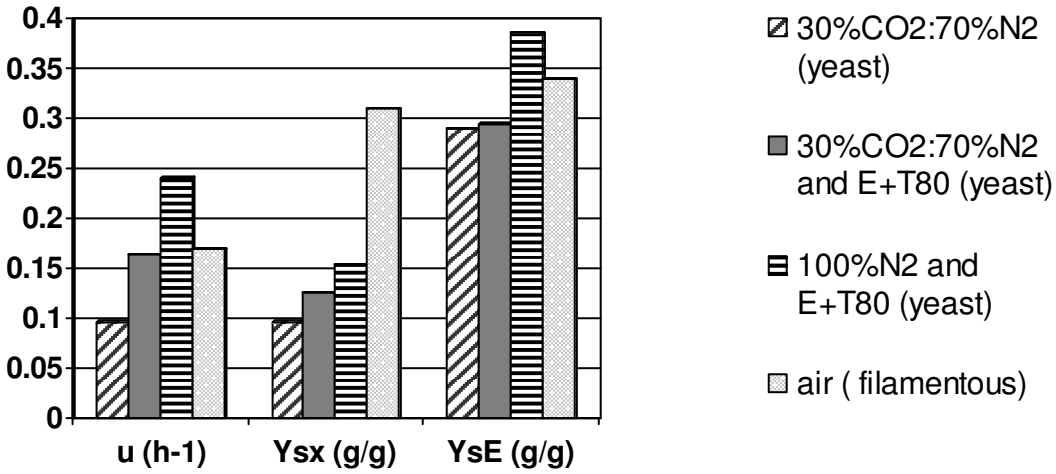

Figure 2-9. A comparison specific growth rates,μ, yield of biomass, Ysx and yield of ethanol Y_{SE} for batch fermentations. (air (filamentous) data were taken from a different set of experiments but they were run under mostly similar conditions). E + T80 is ergosterol and Tween 80 (Data from tables in Lübbehüsen et al., 2003b and McIntyre et al., 2002)

2.6.7. Process parameters and protein production

The effects of the gas atmosphere, carbon source, and nitrogen source, amongst others, on biomass and morphology have been well studied, however there is little data on effects of process parameters on protein production, or how to quantify protein production in *M. circinelloides* effectively. Total protein levels would be a good benchmark for comparison with other heterologous protein production systems.

2.7. Transformation of *Mucor circinelloides*

In the past 20 years *M. circinelloides* has been transformed using episomal plasmids, however the plasmids are segregationally unstable, resulting in a few of the cells carrying the plasmid. A new multicopy vector was developed for *M. circinelloides* using geneticin resistance for selection of transformants (Appel et al., 2004).

Expression and secretion of active glucose oxidase 1 (GOX) was demonstrated using a glyceraldehyde-3-phosphate dehydrogenase gene gpd1P-expression cassette on an episomal plasmid (Wolff and Arnau, 2002). Expression of the gpd1 gene was stronger when growing cells in glucose as opposed to glycerol or ethanol.

Subsequently, the glyceraldehyde-3-phosphate dehydrogenase gene (gpd1P) was characterised using glucose oxidase strains containing integrated expression cassettes by Wolff and Arnau (2002), in one of the first integrated expression systems in zymgomycetes (Larsen et al., 2004).

Recently, astaxanthin biosynthesis genes have been expressed in *M. circinelloides* for the production of carotenoids which represents another step forward in heterologous expression in this species. "Autoreplicative expression vectors containing bacterial astaxanthin biosynthesis genes were constructed, and a beta carotene–producing strain was transformed with them" (Papp et al., 2006).

2.8. A comparison of commonly used expression systems

In order to assess the potential of *M. circinelloides* as a host for heterologous protein production, it should be compared to currently used expression systems (Table 2.5). Although *M. circinelloides* has been transformed using episomal vectors it does not have a popular or commonly used expression vector. It was found that transformants were unstable and many lost their plasmids. Some work was done on creation of an integrative expression system (Wolff and Arnau, 2002). A new vector has been developed with more stable transformants (Appel et al., 2004) (Papp et al., 2006).

There is little information in literature about the protein secretion of *M. circinelloides* and post-translational modification of proteins. It was anticipated that *M circinelloides* would have similar protein secretion capabilities as *Aspergillus niger* which is well known for being a good secretor. If so, *M. circinelloides* would be an attractive host for protein production. One of the project objectives was that the research in this study would enable the missing sections of Table 2.5 to be filled in.

Table 2.5. Comparison between *Saccharomyces cerevisiae*, *Pichia pastoris*, *Aspergillus niger* and *Mucor circinelloides* as yeast or fungal expression systems.

Feature	***S.cerevisiae***	***P. pastoris***	***A. niger***	***M. circinelloides***
Expression vectors	Episomal and integrated vectors. Moderate expression promoters.	Episomal and integrated vectors. High expression promoters.	Episomal and integrated vectors. Moderate to high expression promoters.	Relatively stable transformants.
Biomass production	Crabtree positive; low biomass yield in batch 10-50 g/l	Crabtree negative; high biomass yield in batch 150 g/l	Low	Crabtree positive, about 10 g/l in batch – not optimized
Secretion of protein	Limited	Efficient	Very efficient	Not in literature
Protein yield	100-1000 mg/l	10 g/l (30% total protein)	Good	Not in literature
Post-translational modification	Tends to hyperglycosylate	Moderate glycosylation	Moderate glycosylation	Not in literature
Applications	CO_2 and ethanol production	Heterologous production of pharmaceutical products	Heterologous proteins	

2.9. Production of Glucose Oxidase

Glucose oxidase (glucose:1-oxido-reductase) is an enzyme that oxidises glucose to glucono-δ-lactone and hydrogen peroxide. Glucose oxidase in used in determination of glucose levels in medical diagnostic tests, for example blood and urine glucose measurements. It is also added to food to prevent oxidation and discolouration, and to remove excess glucose to produce low-alcohol wine (Malherbe et al., 2003). The chemical reaction for the enzyme-catalysed oxidation of glucose is as follows:

$$\text{Glucose} + O_2 + H_2O \rightarrow \text{glucono-}\delta\text{-lactone} + H_2O_2$$

The main species of fungus used in glucose oxidase production are *Aspergillus niger* and *Penicillium amagasakiense*. *Saccharomyces cerevisiae* has also been used to express the *A. niger* GOX gene. Some examples of glucose oxidase activity achieved are shown in Table 2.6. Two methods of glucose oxidase measurements are commonly employed: a coupled oxidative reaction of o-dianisidine with hydrogen peroxide (Fiedurek et al., 1986) and use of a dissolved oxygen probe to measure O_2 utilization rate (Mischak et al., 1985). In a survey by Hatzinikolou and Macris (1995) maximum GOX activity by GOX overproducing organisms was reported for *Penicillium notatum* (1.8 U/ml), *A. alternata* (0.4 U/ml), and four different strains of *A. niger* (4-5 U/ml); these are not in Table 2.6.

Table 2.6. Glucose oxidase production systems in literature

Species	Extra-cellular	Intracellular	Assay method	Unit definition	Reference:
A. niger		cell-free extracts: shake flask: 0-0.01 U/mg protein	DO probe, volume = 3 ml, 0.1 M Citrate/phosphate buffer. pH 5.6, catalase 400 µg, extract (sample), glucose 100 mmol	1U= 0.5 µmol 0_2 consumed per minute	Van Dijken and Weenhuis, 1980
A. niger		cell-free extracts: fermenter: 2.55-4.70 U/mg protein		1 U = 0.5 µmol O_2 consumed/minute	Van Dijken and Weenhuis, 1980
A.niger	culture fluid 0.27 U/ml or 4.58 U/mg protein, 73.7% of total GOX	Cell-free extracts: 0.059 U/ml or 0.48 U/mg protein, 16.2%; Cell debris (cell wall plus membrane): 0.037 U/ml, 10.1 % of total GOX	DO probe: 0.1M phosphate buffer, pH 6.0, measure decrease in DO after adding glucose (70mM),	1 U not defined	Mischak et al., 1985
A.niger	0.8 U/ml '	1.5 U/ml	DO probe, pH 5.1, T=35˚C, added 0.1M acetate buffer + 65mM glucose	1U/ml broth = oxidation of 1µmol/min glucose at pH 5.1, T 35˚C (Sigma units)	Traeger et al., 1991
A.niger	2.7-3.2 U/ml (filtrate)		Assay : o-dianisidine	1 U = production of µmol of H_2O_2 per minute = 1µmol glucose/min, µmol O_2/min	Fiedurek et al., 1986
A.niger; Shake flask culture:	9 U/ml specific activity:1.55 U/mg cell dry weight	8 U/ml specific activity: 1.38 U/mg cell dry weight	Assay : o-dianisidine		Fiedurek and Gromada, 1997
A.niger; (shake flask culture)	total GOX(cell-bound and supernatant):1.74 U/ml (glucose) and 0.25 U/mg cell dry weight, optimized 7.5U/ml		Assay: o-dianisidine, pH 5		Hatzinikolau and Macris, 1995

Table 2.8 (continued) Glucose oxidase production systems in literature

Species	Extra-cellular	Intracellular	Assay method	Unit definition	Reference:
P. variable	supernatant: 19 U/ml		substrate: benzoquinone (1glucose + 1benzoquinone →D-gluconic acid + hydroquinone)	1U = reduced 1µmol substrate per ml per minute	Petruccioli et al., 1995
S. cerevisiae (heterologous GOX); Shake flask; 2% glucose + 1.5% EtOH	460U/ml = 8.85 g/l GOX		100ul sample with 0.05 M sodium acetate buffer (pH 5.1), 2.4 ml 0.21M o-dianisidine, 0.5ml 10% glucose, 0.1 ml 60u/ml peroxidase, incubated 10 min. Stopped by addition of 4N H_2SO_4, OD at 500nm.	*A. niger* GOX (Sigma, type 5) as standard	Park et al., 2000
Hansenula polymorpha, fed-batch fermentation	445 IU/ml (2.25 g/l or 2.2% dry weight). Specific activity: 4.4 U/mg cell dry weight	76 IU/ml (0.38 g/l or 0.4% dry weight)			Hodgkins et al., 1993
S.cerevisiae	100 U/ml extra cellular	5 U/ml intracellular	o-dianisidine , pH 4.5, 37˚C, stopped with 0.3 ml 4 M H_2SO_4 , abs. 500 nm (ref to Park , 2000)	standard curve with commercial enzyme	Malherbe et al., 2003
A. niger, grown in fermenter:		intracellular and cell wall: 850 +/- 45 U/ml; equivalent to *0.157 µM glucose/ml/min*	Incubation 30 min at 30˚C, pH 5.6 followed by DNS assay to measure glucose remaining	One unit of glucose oxidase activity converts 1.0 µg of glucose per 30 min at 30°C.	Kona et al., 2001

Note: 1 Unit of glucose oxidase activity is generally equal to 1 µmol glucose consumed per minute per ml sample. The author's definitions of 1U have been included, and those not considered to be equivalent to 1 µmol glucose/min/ml have been converted in italics. In the oxygen uptake rate method (DO probe) 1 unit = 0.5 µmol O_2/ml/min =1µmol glucose/ml/min therefore no conversion is necessary. Since the assays used were not identical the values are not strictly comparable, however they serve to give an idea of GOX production.

2.10. Conclusion

The objective of this study was to evaluate the potential of *M. circinelloides* for heterologous protein production. Two model systems were chosen, a wild type and a mutant, and the morphology and enzyme production of both were compared. The criteria for a good heterologous protein production system were that it would have successful expression vectors, efficient biomass production, good protein secretion, high protein yield, and little post-translational modification in the form of glycosylation.

The morphology of *M. circinelloides* can be controlled by manipulation of growth conditions. The key parameters are gas environment and carbon source. *Mucor* will grow as a yeast under anaerobiosis subject to the conditions that its requirements for a fermentable hexose, a complex nitrogen source and in some cases lipid or fatty acid supplements are met. Recently, methods for transformation of *Mucor* have been developed, enabling the expression of heterologous proteins in this species. Challenges anticpated in morphological control of *Mucor* are the unwanted metabolic channelling of carbon source to ethanol instead of biomass, and the very specific requirements of *Mucor* yeast cells for growth. In assessing Mucor by heterologous GOX production, it is interesting to note that activity up to 400 U/ml has been measured for heterologous GOX of other species.

Based on information in the literature on recent advances in heterologous enzyme expression, it appears that *M. circinelloides* could have potential for heterologous protein production. This study is an evaluation of dimorphic *M. circinelloides* for heterologous enzyme production. The feasibility of morphological control of *M. circinelloides* for improved broth rheology was to be investigated. Thus, the effect of morphological control on enzyme production in *M. circinelloides* was analysed as there was no information in literature about this. This was done by comparing the physiology and enzyme production of a recombinant and a wild type *M. circinelloides* strain under morphological control. The substrate uptake and biomass, ethanol and enzyme production were measured. The production levels of enzymes were compared to other systems. It was expected that the control of morphology in the yeast form would improve broth rheology and lead to better bioreactor control, and that perhaps enzymes would be produced more efficiently overall than in fully filamentous cultures.

Chapter 3. Materials and Methods

3.1. Strains

M. circinelloides. f janssenii CBS 232.29 (from Stellenbosch University culture collection) and *M. circinelloides* KFA199 (received from José Arnau, Denmark) were used in the study. The glucose oxidase (E.C 1.1.3.4) gene (*gox1*) from *A. niger* with the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd1*P) was inserted into KFA199 as follows: *M. circinelloides* strain ATCC 90680 was transformed with a *gpd1*P-*gox1* gene integrated to construct strain UPO1171 (Larsen et al., 2004). Strain UPO1171 was subjected to mutagenesis and screening steps and KFA199 was selected (Arnau, personal communication). In previous publications *M circinelloides* ATCC 1216b has been referred to as *M. circinelloides, M. racemosus*, and *M. circinelloides* syn. *racemosus* and is referred to as *M. circinelloides* in this study.

3.2. Preparation of spores

Spores from the above cultures were stored in 30% glycerol at –80˚C for long-term storage. Strain CBS 232.29 was grown on Malt Extract Agar plates containing 2% malt extract, 1% Agar, 0.05% streptomycin and 0.025 % chloramphenicol, then transferred to rice flasks to increase the number spores. Strain KFA199 was first grown on YNB plates (YNB 6.7 g/l, ammonium sulphate 1.5 g/l, glucose 20 g/l, agar 20%) to select for leu+ strains, to ensure only cells with the *gpd1*P-gox gene were used to inoculate rice flasks. Rice flasks contained 30g rice autoclaved with 6 ml YPG (glucose 20 g/l, peptone 10 g/l, yeast extract 3 g/l) (McIntyre et al., 2002), which were inoculated with about 1 ml spore solution and 6 ml sterile water. After 5-7 days of incubation at 26˚C spores were harvested by washing with 0.9% NaCl solution and stored in 20 ml aliquots at 4˚C. This spore solution was used to inoculate cultures. Cultures from the plates were used to inoculate rice flasks and prepare spore solution as above.

3.3. Media

Several media were used in shake flask experiments: YPG (McIntyre et al., 2002), YNB without amino acids (Difco) (YNB 6.7 g/l, ammonium sulphate 1.5 g/l), Hansson medium (Hansson and Dostalek, 1988), and Vogel's medium. Hansson medium contained per litre: yeast extract 5 g, KH_2PO_4 2.4 g, $FeCl_3.6H_2O$ 15 mg, $ZnSO_4.7H_2O$ 7.5 mg, $CuSO_4.5H_2O$ 0.5 mg, $MnSO_4.H_2O$ 10 mg, $MgSO_4.7H_2O$ 0.5 mg, $CaCl_2$ 0.1 g, NH_4Cl 1 g, and various concentrations of carbon source.

Vogel's medium was used for fermentations. Vogel's medium contained per litre: 20 ml Vogel's solution, 5 g casamino acids, 1.5 g glutamate, 1 ml biotin solution, 1 ml vitamin solution (nicotinic acid 1 g/l, thiamine chloride 1 g/l), and a carbon source. The Vogel's salts stock solution (50X) contained per litre Na_3-citrate 125 g, KH_2PO_4 250 g, NH_4NO_3 100 g, $MgSO_4.7H_2O$ 10 g, $CaCl_2.2H_2O$ 5 g, and trace element solution 5 ml. The trace element solution contained per 100 ml; citric acid 5 g, $ZnSO_4.7H2O$ 5 g, $Fe(NH_4)(SO_4)_2.6H_2O$ 1 g, $CuSO_4.5H_2O$ 0.25 g, $MnSO_4.H_2O$ 0.05 g, H_3BO_3 0.05 g, $NaMoO_4.2H_2O$ 0.05 g, H_2O 95 ml (Vogel, 1956 quoted on Fungal Genetics Stock Centre website, 2005) and (McIntyre et al., 2002). The biotin solution contained 0.5 mg per ml (a few drops in 10 M NaOH were added to dissolve biotin). For anaerobic growth Vogel's medium was supplemented with ergosterol and Tween 80 stock solution to final concentration of 0.02 g/l ergosterol and 0.42 g/l Tween 80. The ergosterol/Tween stock (500X) was made by dissolving 2 g ergosterol in 100 ml boiling ethanol. 42 g Tween 80 was added and the solution made up to 200 ml by adding ethanol. This was stored at -20˚C.

3.4. Shake flask conditions

In the case of YPG and YNB, 60 ml medium was autoclaved in 250 ml baffle Erlenmeyer flasks at 121ºC for 15 min. For Vogel's medium, carbon source (usually 20 g/l), casamino acids and glutamate were autoclaved, and Vogel's salts, vitamin and biotin solutions were filter sterilized and added afterwards to make up a final volume of 60 ml. The media were inoculated with 10^6 spores per ml medium. Shake flasks were incubated at 26˚ C on an orbital shaker at approximately 100 rpm for up to 140 hours.

3.5. Fermenter medium

500 ml glucose solution was autoclaved in the fermenter vessel at 121˚C for 15 minutes. Other medium components (for Vogel's medium) were filter sterilized through a 0.22 μm filter and added. For anaerobic growth, ergosterol and Tween 80 was injected into the fermenter. Fermenters were inoculated with spore solution to a concentration of $1x10^6$ spores/ml. In 'shift fermentations' after cultures had reached stationary phase they were shifted from anaerobic to aerobic conditions. At the shift 200 ml of 4.5X medium (with either 18 g glucose or 9 g starch) was added, to make up a total volume of 900 ml. Immediately after the addition of medium, the concentration of glucose was 20 g/l (for KFA199) and the starch concentration was 10 g/l (for CBS 232.29). The concentration of other medium components was at least that of the starting medium.

3.6. Cultivation conditions

Fermentations were performed in 1.3 litre fermenters (Bioflow 110 Non-Jacketed Vessels, New Brunswick Scientific Co.) with a working volume of 900 ml. The impellor arrangement consisted of one 6-blade rushton turbine fixed above a pitched blade impellor. Some anaerobic fermentations were done using N_2 only (99.999% N_2, Instrument grade, Afrox), others were done with a gas mix of 30% CO_2 and 70% N_2. Where specified, a Model 1000 CRS (Chromatography Research Supplies) oxygen trap was used to remove trace amounts of O_2 from the sparge gas. In anaerobic fermentations, N_2 or N_2/CO_2 was sparged at 0.3 vvm and the agitation rate was 200 rpm. During shift fermentations, in the anaerobic phase 0.3 vvm N_2 was sparged and agitation was 200 rpm. In the aerobic phase air was sparged at 1 vvm and after the shift agitation was increased in steps from 200 to 500 rpm over 3 hours. Gas flow rate was controlled by rotameters. Temperature was maintained at 28°C ± 0.1 °C. pH was controlled at 5 ± 0.02 by addition of 2 M KOH or 2 M HCl. Dissolved oxygen levels were measured by a Mettler-Toledo oxygen probe in the fermenter. Antifoam A (Sigma) at a concentration of 2 ml/l was pumped in to suppress foaming when deemed necessary. Exhaust gas from the fermenter was analysed by an Innova Airtech Instruments 1309 Gas Analyser connected to an Innova 1313 Fermentation Monitor where O_2 and CO_2 were monitored.

3.7. Fermentation Sampling

Samples of fermentation broth were taken approximately every 4 hours. A sample of about 25 ml was taken at each time-point, and 20 ml used for dry weight. In the case of yeast growth, the remainder was centrifuged at 6500 rpm for 3 minutes and the supernatant was stored at –20˚C for further analyses. In the case of mycelial growth the broth was filtered through Miracloth (Calbiochem, Cat no. 475855) for dry weight determination, and the filtrate was stored at –20˚C for other analyses.

3.8. Analyses: Dry weight determinations

Biomass was measured by cell dry weight. For yeast cells, 10 ml culture broth was filtered through 0.45 µm acetate filters and rinsed twice with RO (reverse osmosis) water. For mycelia approximately 10 ml culture broth was filtered through Miracloth filters and rinsed twice, and the filter was squeezed gently to remove excess liquid. The filters were dried at 65˚C until constant weight. Each dry weight determination was done in duplicate. Masses were recorded to four decimal places. Absorbency of yeast cultures was measured at 600 nm.

3.9. Metabolite and glucose concentrations

Glucose, glycerol, ethanol, acetate and gluconic acid concentrations in culture supernatant were measured by HPLC, with a Biorad Aminex HPX-87H column at a temperature of 45˚C, and a mobile phase of 0.005 M sulphuric acid pumped at 0.6 ml/min. A Waters refractive index detector and Waters PDA UV detector were used.

3.10. Starch concentration

Starch was measured by the phenol-sulphuric acid total sugar assay. 200 µl supernatant from fermentation broth was placed in a test-tube, 200 µl 10 % phenol solution, and then 800 µl concentrated sulphuric acid was added. Colour development was measured as absorbance at 490 nm. A standard curve of various starch concentrations was made up to quantify the amount of starch in the samples.

3.11. Cell extracts: Cytoplasmic and membrane protein extraction

Cytoplasmic and membrane protein fractions were extracted from mycelia. Mycelial cells from fermentation samples were washed twice with RO water, squeezed to remove excess water and stored at –20˚C. The cells were ground under liquid N_2 using a mortar and pestle.

0.3 g ground cell mass was placed in an eppendorf tube, 1 ml extraction buffer was added, it was vortexed, incubated on ice for 10 min, then centrifuged at 14 000 rpm for 15 minutes at 4˚C. The extraction buffer contained 50 mM sodium phosphate buffer (pH 7), 1 mM EDTA, 20 mM PMSF, and 0.1 % Triton X-100. The centrifuged supernatant (cell-free extract) was used for protein and enzyme assays.

3.12. Glucose oxidase and amylase assays

Glucose oxidase was measured using a coupled-enzyme assay with o-dianisidine (Worthington website and Celliers, personal communication). 0.1 M phosphate buffer of pH 7 was prepared by dissolving 0.53 g KH_2PO_4 and 1.06 g K_2HPO_4 in 100 ml water. A mixture of 50 ml 10% glucose and 240 ml phospate buffer was oxygenated at 25˚C for at least 10 minutes until saturated, 18 ml was withdrawn and to that 148 µl o-dianisidine (6 mg/ml) and 620 µl peroxidase (60 U/ml) were added to make up the reaction mixture. 100 µl sample was pipetted into a 4 ml cuvette, 3.0 ml reaction mix was added and absorbance read at 426 nm at 30 second intervals for 3 minutes. The temperature was maintained at 25˚C in the Beckman spectrophotometer. The initial rate (ΔA /min), was used to calculate activity according to the formula U/ml= (ΔA/min x 3.1)/(8.3 x 0.1). Use of this formula to calculate activity was compared to a standard curve constructed using commercial GOX and gave equivalent activities. One unit oxidises one µmol of o-dianisidine per minute at 25°C and pH 7.

Amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1961). A substrate solution of 0.1 % starch in 0.05 M citrate buffer (pH 6) was used. 450 µl substrate solution was equilibrated to 50˚ C, 50 µl enzyme sample was added, and incubated for 10 minutes at 50˚ C. 750 µl DNS was added, the mixture was boiled for 15 minutes and absorbance read at 540 nm (Bogar et al., 2003). Amylase activity was also evaluated by placing 10 µl sample on a 1% starch agar plate, incubating at 30˚C and rinsing with Gram's iodine. Activity was indicated by clear zones on the plate.

3.13. Protein Determination

Protein in culture supernatant was measured by the bicinchoninic acid (BCA) method with a kit from Pierce (Cat no. 23227) using a microplate assay. Protein in cell extracts was determined by the Bradford assay (Biorad Protein Assay kit, USA). 50 µl sample was vortexed with 2.5 ml Bradford reagent, left for 5 minutes, and absorbance read at 595 nm on a

Pharmacia LKB Ultrospec III spectrophotometer. A standard curve was made using bovine serum albumin.

3.14. Photographs: Morphology

Wet mounts of yeast and mycelia were prepared. Photographs of cell morphology were taken with a Nikon Coolpix digital camera mounted on a microscope.

Chapter 4. Results

Wild-type and recombinant *Mucor* strains were cultivated in batch culture to evaluate *Mucor* as a production system for heterologous proteins. Of specific interest was the control of morphology in the yeast-like and filamentous forms through the manipulation of cultivation conditions, the fungal physiology associated with morphological changes, as well as the production levels of native and heterologous enzymes. In Figure 4-1 the relationship of experiments to each other is illustrated. The results are organised into two sections, firstly control of morphology and secondly enzyme and biomass production.

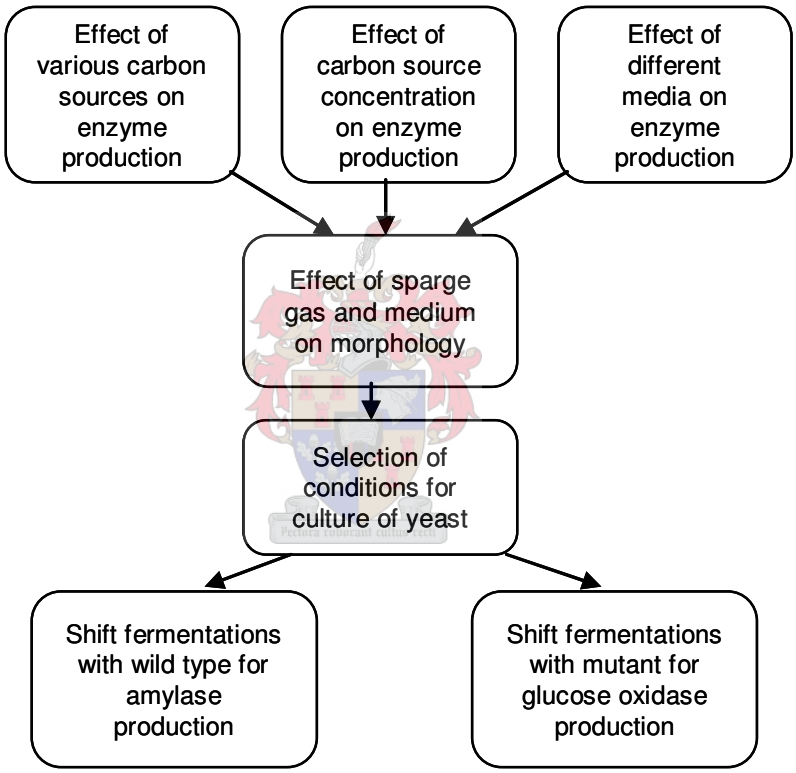

Figure 4-1. Flow-diagram of broad idea behind experiments.

4.1. Control of Morphology in Yeast Form

Morphology of *M. circinelloides* was studied in anaerobic cultivations to determine suitable conditions for control of the organism in a yeast morphology. Effect of gas atmosphere and medium components on cell appearance (Section 4.1.1) and specific growth rates (Section 4.1.2) were taken into consideration in selecting conditions for morphological control – the

aim being to achieve yeast morphology while getting an efficient biomass yield and minimal ethanol production. Cultivation was in fermenters with a working volume of 900 ml, with Vogel's medium or YPG both containing 20 g/l glucose. Spores were used as the inoculum and anaerobic conditions were maintained by sparging N_2 or CO_2/N_2. Cell dry weight, absorbency, glucose and ethanol concentrations were measured.

In order to inhibit filamentous growth, gas atmospheres of N_2 and 30% CO_2/ 70%N_2 were used and ergosterol and Tween 80 were added to the medium (Lübbehüsen et al., 2003b); the experimental conditions are summarized in Table 4.1. Since *M. circinelloides* becomes filamentous when exposed to trace amounts of O_2 (Orlowski, 1991) in some experiments N_2 was passed through an oxygen trap to remove trace amounts of O_2 and the purified N_2 was used to sparge fermentations. It has been shown that the addition of ergosterol and Tween 80 (E/T80) can support yeast-like growth in Vogel's medium under N_2 (Lübbehüsen et al., 2003b), therefore E/T80 was added to some fermentations to see the effect on morphology. A concentration of 0.02 g/l ergosterol and 0.42 g/l Tween 80 was used, as was found to give the largest specific growth rate (Lübbehüsen et al., 2003b).

4.1.1. Morphological appearance of yeast-like and filamentous cells

The yeast morphology was achieved in both the wild type (CBS 232.29) and mutant (KFA199) under the following conditions: N_2 passed through oxygen trap (pure N_2), CO_2/N_2 mixture, and N_2 when ergosterol and Tween 80 were added to the medium (Table 4.1).

Table 4.1. Conditions under which morphological response of *M. circinelloides* to the environment was tested and the morphological response.

strain	gas	medium	other	morphology
CBS 232.29	N_2	Vogel's		yeast/filamentous
CBS 232.29	CO_2/N_2	Vogel's		Yeast
CBS 232.29	N_2	YPG		Yeast
CBS 232.29	N_2	Vogel's	ergosterol/Tween 80	Yeast
CBS 232.29	N_2	Vogel's	oxygen trap	Yeast
KFA199	N_2	Vogel's		yeast/filamentous
KFA199	N_2	Vogel's	ergosterol/Tween 80	yeast
KFA199	N_2	Vogel's	oxygen trap	yeast

4.1.1.1. Morphology of cells under 70% N_2 and 30% CO_2:

Under 70 % N_2/ 30% CO_2, in Vogel's medium without E/T80, cells were all yeast-like in both CBS 232.29 (Figure 4-2) and KFA199 (photographs not shown). The yeast cells exhibited profuse budding (Figure 4-2 c) in comparison with the N_2-only atmosphere (Figure 4-4).

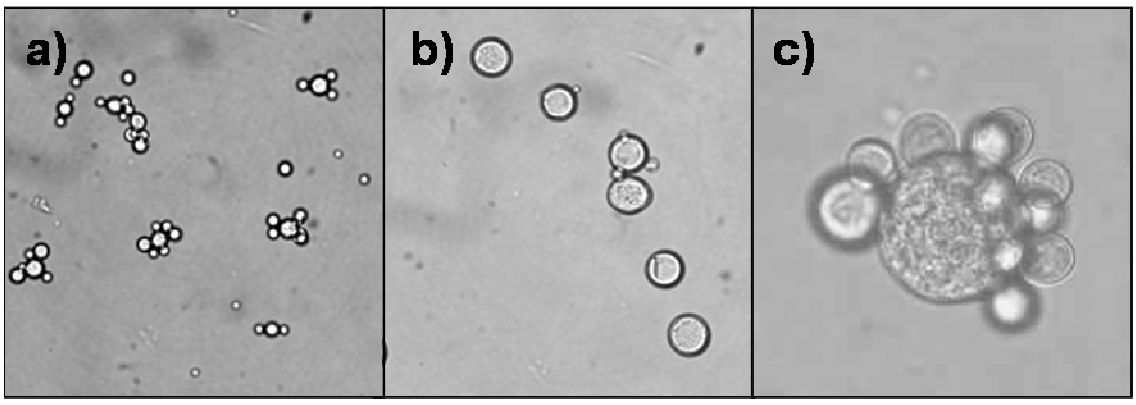

Figure 4-2. CBS 232.29 budding yeast cells from fermentation with CO_2/N_2, a) 17 hours (400X), b) 74.4 hours (400X) c) enlarged image of multipolar budding cell.

4.1.1.2. Morphology of Cells under N_2, no ergosterol and Tween 80 supplementation

As can be seen in Table 4.1, when strains were grown in Vogel's medium under N_2, without E/T80, mixed morphology resulted. In 'mixed morphology' on a microscopic scale, cells were yeast-like with short filaments extending, for both KFA199 and CBS 232.29 (Figure 4-3), however the culture appeared yeast-like on a macroscopic scale (Section 4.1.1.6). In YPG under N_2, budding yeast cells resulted (photographs not shown) similar to those in Vogel's medium under N_2/CO_2 (Figure 4-2).

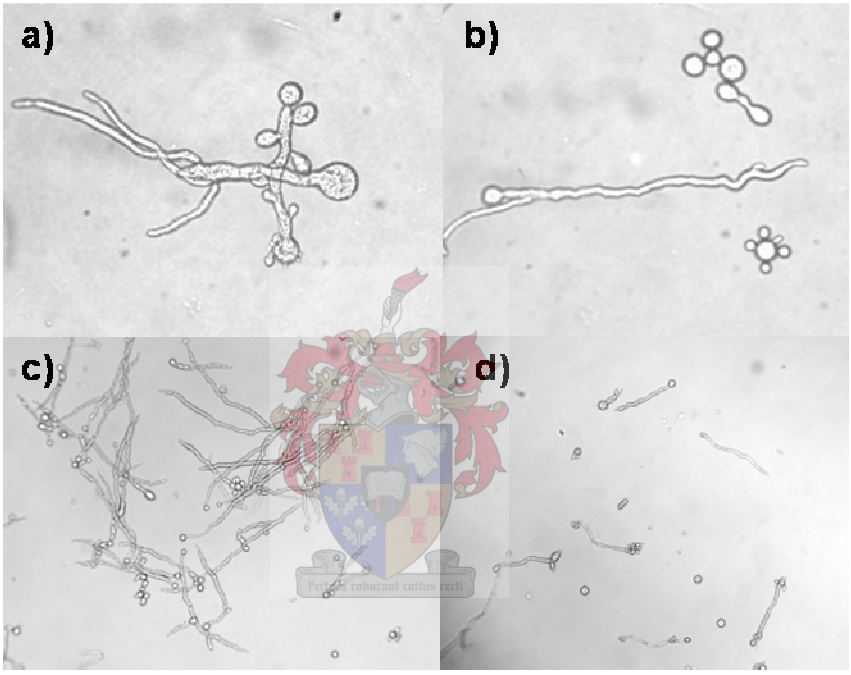

Figure 4-3. CBS 232.29, mixed morphology under N_2, without ergosterol and Tween 80: a) and b) yeast-like with filaments and buds (400X). c) CBS 232.29 and d) KFA199 hyphae grown from yeast cells exposed to air (100X).

4.1.1.3. Morphology of cells under N_2, when an oxygen trap was used

When trace amounts of O_2 in sparge gas were removed by means of an oxygen trap, and the cultivation medium was Vogel's medium without E/T80, morphology was yeast-like (Figure 4-4). CBS 232.29 cells appeared more spherical while KFA199 cells were oblong with much more buds, but in both cases morphology was clearly yeast-like (Figure 4-4). Other than this

small difference, the wild type, CBS 232.29, and mutant, KFA199, exhibited similar morphology under identical conditions.

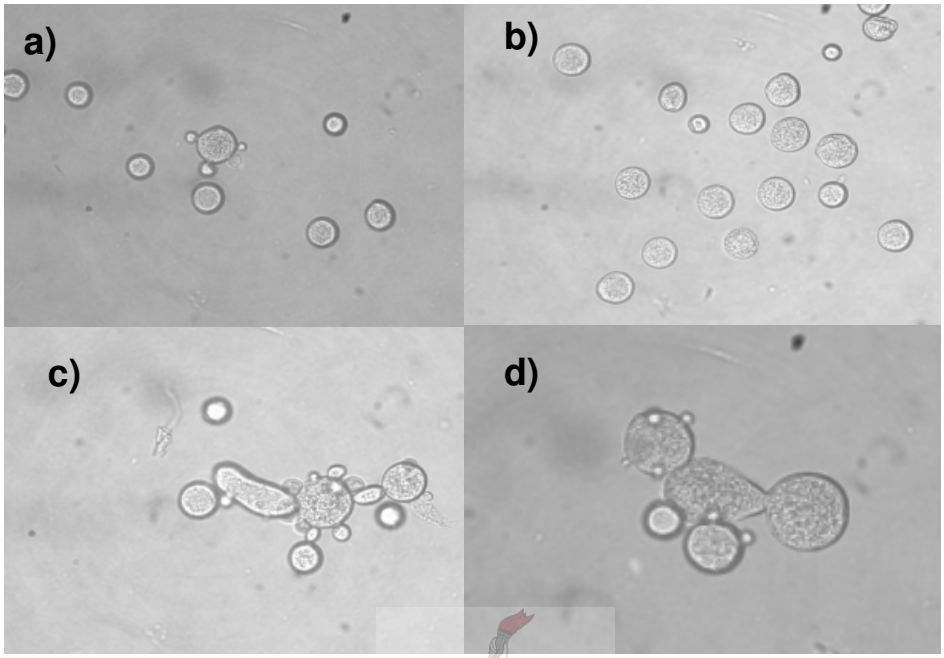

Figure 4-4. Yeast cells under purified N_2, with oxygen trap (400x): a) and b) CBS 232.29 (round cells), c) and d) KFA199 (cells more oblong).

4.1.1.4. Morphology of cells under N_2 with ergosterol and Tween 80

Ergosterol and Tween 80, sterol and fatty acid supplements often added for anaerobic cultivation of *S. cerevisiae,* were included in the Vogel's medium to allow yeast-like growth under N_2 (Lübbehüsen et al., 2003b). Under N_2 in Vogel's medium with addition of E/T80 morphology was completely yeast-like in both strains. KFA199 is shown in (Figure 4-5 a) and CBS 232.29 is shown in Figure 4-6 a) and b). In CBS 232.29 there was no budding and in KFA199 budding was less than observed under N_2/CO_2.

4.1.1.5. Morphology of cells during shift fermentations

Shift fermentations were performed by first growing *M. circinelloides* yeast cells under N_2 with E/T80, then when the culture reached stationary phase at about 30 hours the fermentation was shifted into an aerobic phase of filamentous growth for enzyme production. The shift comprised of changing sparge gas from N_2 to air, increasing agitation from 200 to 500 rpm and adding more medium to make up the same working volume as in the beginning. The medium that was added contained all media components so that growth was carbon-limited

and in the case of KFA199 glucose was added to make up a final concentration of 20 g/l; in the case of CBS 232.29 starch was added to make up a final concentration of 10 g/l.

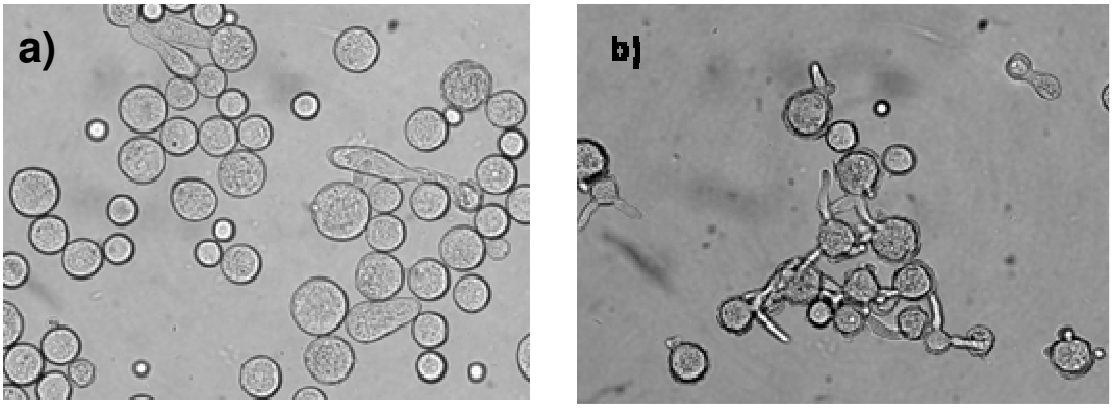

Figure 4-5. KFA199 cells (N_2, with ergosterol and Tween 80) grown on glucose: a) yeast cells before shift at 34 hours and b) yeast cells with polarized growth after shift at 37 hours.

In Figure 4-5 and Figure 4-6 the morphology of yeast cells before the shift and yeast/filamentous cells after the shift are shown. Figure 4-5 shows shift fermentations with KFA199: a) shows the yeast cells and b) shows cells four hours after the shift, where yeast cells have filaments beginning to extend. Figure 4-6 shows a shift fermentation with CBS 232.29 where a) and b) show the yeast phase and c) to f) show the change from yeast to filamentous under aerobiosis.

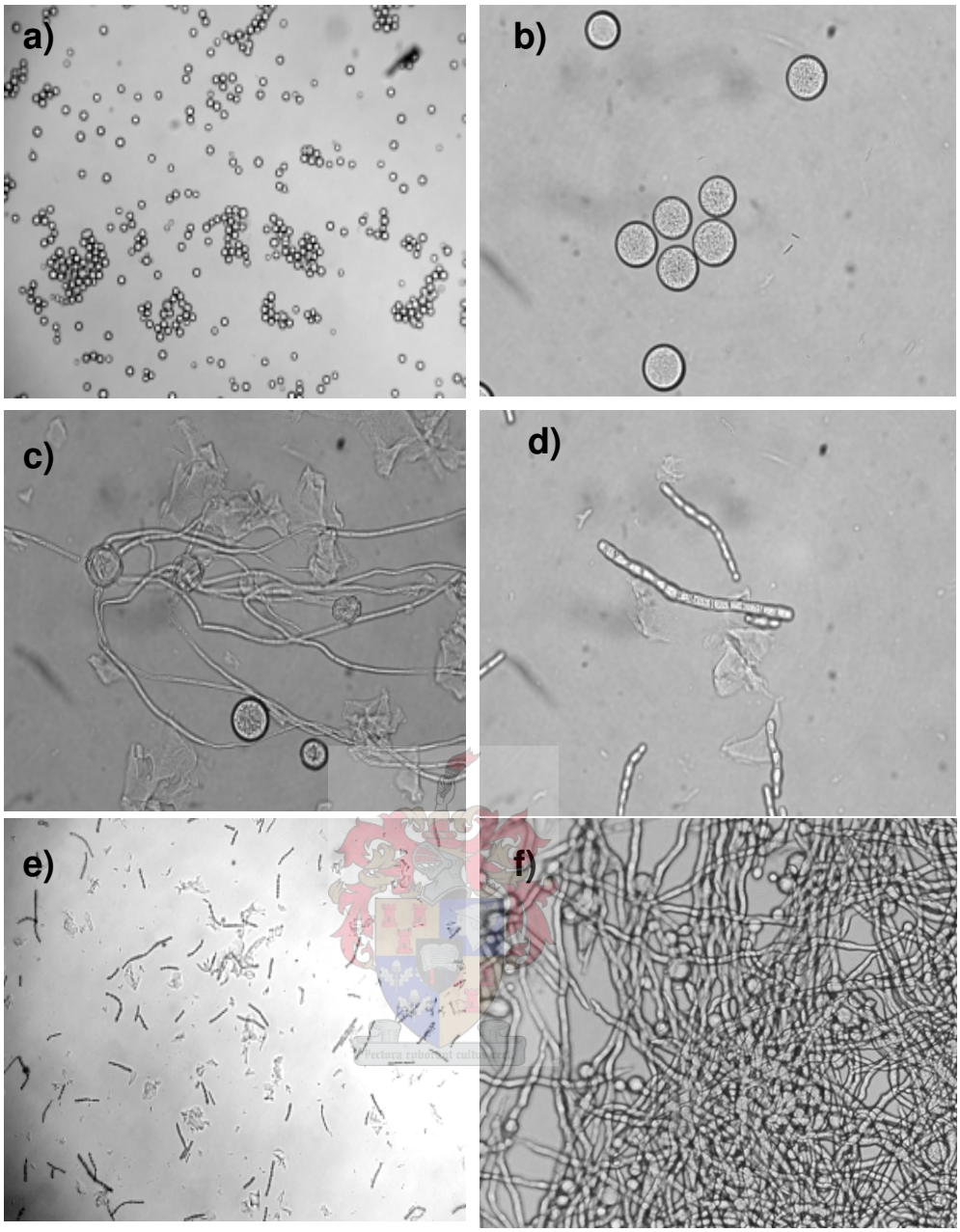

Figure 4-6. Shift fermentation with CBS 232.29, under N_2: a) yeast cells at 19 hours (100X), b) yeast cells at 22 hours (400X), c) after shift: yeast and filaments at 45 hours, d) filaments at 60 hours e) mostly filamentous growth at 60 hours (100X) f) fully filamentous growth at 80 hours.

4.1.1.6. Appearance of the culture in fermenters

Before embarking on shift fermentations a few aerobic fermentations were carried out to try out various impeller arrangements, for example, one pitched blade, one Rushton turbine, two Rushton turbines or one pitched blade and one Rushton turbine. In the fermentation with one pitched blade mounted below and one Rushton Turbine (Figure 4-7) the culture was well

mixed and there was minimal wall growth thus this was chosen for future fermentations. However, in the long fermentations the growth still occurred on the walls and probes in the latter part.

The change in morphology during a shift fermentation was not only visible under the microscope but also in the fermenter. In Figure 4-8 the large-scale view of a shift fermentation is shown. Figure 4-8 a shows the yeast phase in which the culture is easily mixed, Figure 4-8 b the filamentous phase at 60 hours where growth is completely filamentous and beginning to pack on fermenter walls. Figure 4-8 c shows the end of the fermentation with filaments settled out and the significant extent of growth on walls, impellors and probes (about 100 hours) showing difficulties of growing this filamentous fungus in submerged cultivation. From 50 hours onwards, the shifted culture (Figure 4-8) was as viscous as those cultures that were grown aerobically from the beginning (Figure 4-7).

4.1.2. Specific growth rates under various gas atmospheres

As part of morphological control was the requirement for efficient biomass production, since biomass was the desired product in the anaerobic phase. The specific growth rates of cultures under various conditions for morphological control comprising anaerobic growth on glucose (Table 4.1) and aerobic growth on starch were compared (Table 4.2).

In Table 4.2 specific growth rates and biomass yields of *M. circinelloides* yeast cultures are compared, values on the left are from this study and those on the right hand side are from literature. Morphology was yeast-like with the exception of the first row showing filamentous (aerobic) cultures for comparison to yeast cultures. Glucose (20 g/l) was used as a substrate for all anaerobic cultures. In all cases KFA199 grew about half the rate of the wild type, CBS 232.29. In both strains the difference in the specific growth rate between cells under CO_2/N_2 (yeast) and N_2 (mixed morphology) was not significantly differnent, however under CO_2/N_2 the final biomass concentration was much less (1.4 – 1.8 g/l) than that under N_2 (2.5 - 3 g/l). The specific biomass yield under CO_2 was also significantly less at 0.07 to 0.09 g/g and that under N_2 was 0.14 to 0.19 g/g. The specific growth rate under N_2 with addition of ergosterol and Tween 80 was the fastest in both strains, and yeast morphology was maintained, thus these conditions were chosen for culturing yeast during shift fermentations.

Figure 4-7. Fermenter showing appearance of filamentous fungi, impellor arrangement was one Rushton turbine and one pitched blade.

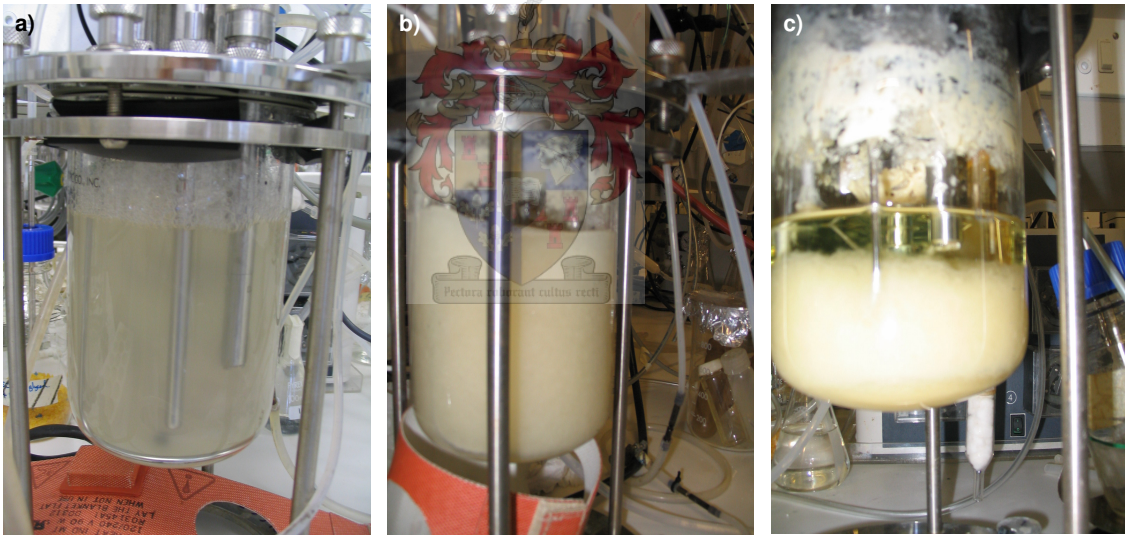

Figure 4-8. a) Yeast-like growth under N_2 with E/T80 (20 hours), b) filamentous growth after the shift (60 hours) and c) filaments packed on wall, probes and impellor at the end of the fermentation (100 hours).

Table 4.2. Specific growth rates and biomass yield, Ysx, of *M. circinelloides* under various gas atmospheres.

From this study					
Strain	**Gas**	**Comment**	**μ (h-1)**	**Ysx**	**Morphology**
CBS 232.29	Air	Starch	0.43 ± 0.08	0.46	Filamentous
CBS 232.29	N_2		0.075 ± 0.004	0.14	Yeast/ Filamentous
KFA199	N_2 (trap)		0.041	0.10	Yeast
CBS 232.29	N_2 (trap)		0.086	0.10	Yeast
KFA199	CO_2/N_2		0.034 ± 0.004	0.09	Yeast
CBS 232.29	CO_2/N_2		0.090 ± 0.016	0.07	Yeast
KFA199	N_2	E/T80	0.14 ± 0.02	0.16	Yeast
CBS 232.29	N_2	E/T80	0.226 ± 0.015	0.19	Yeast
From literature					**Reference**
ATCC 1216b	Air	Shakeflask, starch		0.60	McIntyre et al., 2002
ATCC 1216b	Air		0.17	0.31	Lübbehüsen et al., 2004
ATCC 1216b*	CO_2/N_2		0.097	0.097	Lübbehüsen at al., 2003b
ATCC 1216b*	CO_2/N_2	E/T80	0.164	0.126	Lübbehüsen et al., 2003b
ATCC 1216b*	N_2	E/T80	0.241	0.154	Lübbehüsen et al., 2003b

*** Unless otherwise stated values are for batch cultivation in a fermenter with 20 g/l glucose; ATCC1216b* strain was a leu- ATCC1216b transformed with pEUKA2::PG1 to create a stable strain containing wild type leuA. E/T80 refers to ergosterol and Tween 80.**

4.1.3. Measurement of biomass using absorbency compared to cell dry weight

Absorbency as a measure of biomass could be related to cell dry weight under certain conditions (Figure 4-9). Even though absorbency is commonly used to measure biomass, due to the marked changes in cell size and cell morphology, a simple relationship between

absorbency and cell dry weight (DW) did not exist. Under the N_2 atmosphere the absorbency showed a linear relationship to DW, and under the CO_2/N_2 atmosphere the absorbency also showed a relationship to DW. However, the N_2 and CO_2/N_2 absorbency-DW relationships were different to each other (Figure 4-9). The best fit regression for the CO_2/N_2 data was a straight line, while the best fit for N_2 data was a quadratic function. The mutant and wild-type had similar DW-absorbency plots under N_2, but at the beginning and end of the exponential phase there were larger deviations from a linear trend, as well as deviations between fermentations shown by more scatter at the high and low values (Figure 4-9).

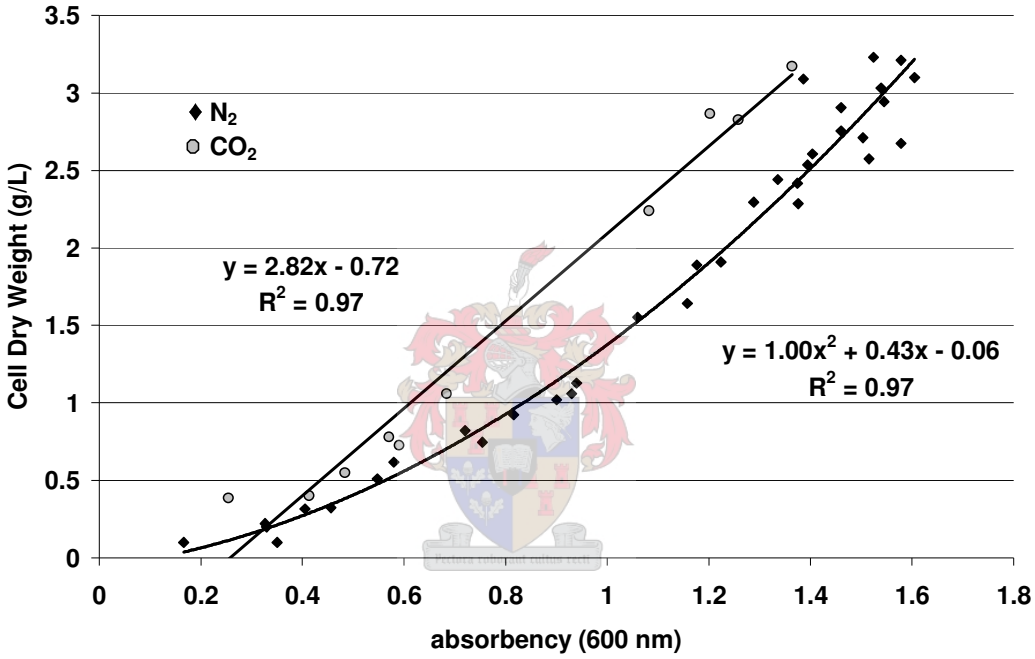

Figure 4-9. Comparison of relationships of cell dry weight with absorbency of culture fluid under N_2 and N_2/CO_2 atmospheres. The regression equations and corresponding R^2 values are shown alongside the regression curves.

The absorbency measurements proved difficult as the cells settled out very quickly and thus if absorbency was not measured immediately after shaking the sample, the absorbency values were inaccurate. Diluting the sample to measure absorbency also led to deviations compared to undiluted samples in the range of 0.050 (in absorbency) when both diluted and undiluted samples were supposedly in the linear range of the spectrophotometer. Therefore, dry weight measurements were used as a more accurate measure of biomass throughout the study, and were used in all biomass-related calculations e.g. specific growth rates and biomass yields.

4.2. Influence of media components on enzyme production by *M. circinelloides*

4.2.1. Enzyme production by wild-type strain in shakeflasks

The influence of media on the enzyme and biomass production by the wild-type and mutant was measured in shake flask cultures. Firstly, native protein and enzyme production of a few different wild-type strains were compared. *Mucor* readily grows on starch and some strains grow on cellulose and therefore amylase, cellulase and β-glucosidase activities, amongst others, were assayed. However, only amylase activity gave significant results. Extracellular protease activity was also assayed to determine if low levels of native enzymes were caused by proteolytic degradation, but no measureable levels of extracellular proteases could be detected. One wild-type strain, CBS 232.29, was selected and tested for amylase activity on various media, namely, YNB (yeast nitrogen base) with starch or maltose, YPG, and Vogel's medium, where the greatest amylase activity (0.60 U/ml) and dry weight (5.8 g/l) were found in Vogel's medium with starch (Table 4.3). The protein levels were lower than that measurable by the Bradford assay.

Table 4.3. Comparison of different media types on amylase and protein production of CBS 232.29 in culture supernant of shake flasks

Medium		DW (g/l)	Amylase (U/ml)	Protein (Bradford)
YNB	0.5% starch	2.8	ND	ND
YNB	0,5% maltose	3.2	0.48	ND
Yeast, Peptone	2% Glucose	3.6	ND	_
Vogel's	0.5% starch	5.8	0.60	ND

ND=not detected

In a subsequent experiment in Vogel's medium in which some shake flasks contained casamino acids and others did not, the addition of casamino acids gave marginally higher biomass, but slightly lower amylase activity. The biomass and amylase activity levels at 86

hours were 9.9 ± 1.4 g DW/l and 0.36 ± 0.10 U/ml activity with casamino acids, and 9.6 ± 2.1 g DW/l and 0.61 ± 0.09 U/ml without casamino acids, respectively.

4.2.2. Glucose oxidase production in shake flask cultures with KFA199

The production of an *Aspergillus niger* glucose oxidase (GOX) enzyme by a mutant, transformed strain of *Mucor*, KFA199, was used a model system to evaluate the production capacity of *Mucor* for heterologous fungal enzymes. The effect of four media, all of which could support both yeast-like and filamentous growth, on heterologous GOX production levels was assessed in shakeflask cultures cultured for 144 hours (data not shown). Out of Vogel's YPG, YNB and Hansson media, the highest levels of extracellular GOX activity were obtained during cultivation in Vogel's (0.37 ± 0.02 U/ml). Activity in Hansson medium (0.25 ± 0.02 U/ml) was 33% lower than in Vogel's medium, and GOX activity in YNB and YPG was 73% lower than Vogel's at about 0.1 ± 0.02 U/ml. GOX activity continued to increase with time, until the final timepoint of 144 hours.

The effect of different carbon sources in combination with Vogel's medium on GOX production levels was also determined in shakeflask cultures (Figure 4-10). While galactose and gluconic acid lactone gave high activities, it was decided to use glucose, which gave slightly lower activity, for further fermentations as it is more economical and also provides a good benchmark.

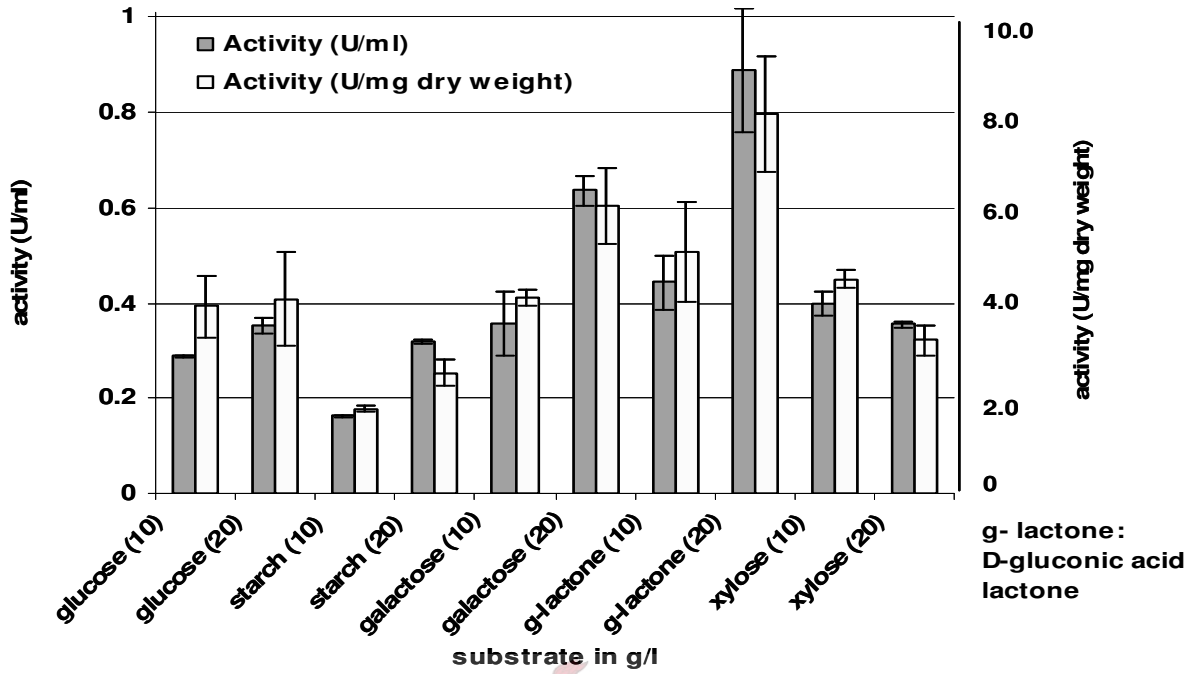

Figure 4-10. GOX activity of KFA199 (shake flask culture of 100 hours) on different carbon sources, in supernatant (U/ml) and activity per dry weight (U/mg).

The effect of the concentration of glucose in the medium on GOX production was determined by culturing shakeflasks with an initial concentration of 10, 15 and 20 g/l glucose. The highest glucose concentration (20 g/l) yielded the highest extracellular GOX activity (0.40 ± 0.8 U/ml culture), as shown in Table 4.4., however the GOX production per cell dry weight was approximately the same, 0.05 ± 0.01 U/mg, at all three glucose concentrations. Assuming all the glucose was utilized after 85 hours, the biomass yields for 20, 15, 10 g/l glucose were 0.37, 0.42 and 0.44 g DW per g glucose, respectively.

Table 4.4. GOX Activity and dry weight of shake flask cultures (85 hours) with different starting glucose concentrations

Glucose concentration	GOX (U/mg)		GOX: U/ml		DW (g/l)	
20 g/l	0.054	±0.012	0.40	±0.08	7.3	±0.3
15 g/l	0.048	±0.006	0.30	±0.02	6.3	±0.5
10 g/l	0.050	±0.004	0.22	±0.02	4.3	±0.1

4.3. Comparison of enzyme production by wild type and recombinant strain in batch fermentations with controlled morphology

Mucor was cultivated in shift fermentations (Section 4.1.1.5), starting with an anaerobic stage of yeast morphology followed by a stage of aerobic filamentous growth for enzyme production (Figure 4-11 to Figure 4-16). At least three shift fermentations were performed with CBS 232.29 and KFA199.

4.3.1. Substrate uptake and product formation during shift fermentations with KFA199

In Figure 4-11, the profiles of substrate utilization and product formation during a shift fermentation of KFA199 with glucose are shown. After a lag phase, glucose was consumed fairly rapidly in the yeast phase and fully exhausted, whereupon more glucose was added, resulting in rapid consumption during the filamentous phase of the fermentation. Ethanol was produced at high levels during anaerobic growth, as well as in aerobic growth, until glucose ran out at which point the organism started using ethanol as a substrate. The filamentous biomass yield was more than the yeast biomass yield, glycerol was produced only anaerobically and acetate production (not shown) was negligible (Figure 4-11). Acetate production was negligible during the course of the fermentation. Carbon balancing of the main products viz. biomass, ethanol and glycerol (only anaerobically was glycerol produced) accounted for about 90% of the products on a C-mole basis (data not shown).

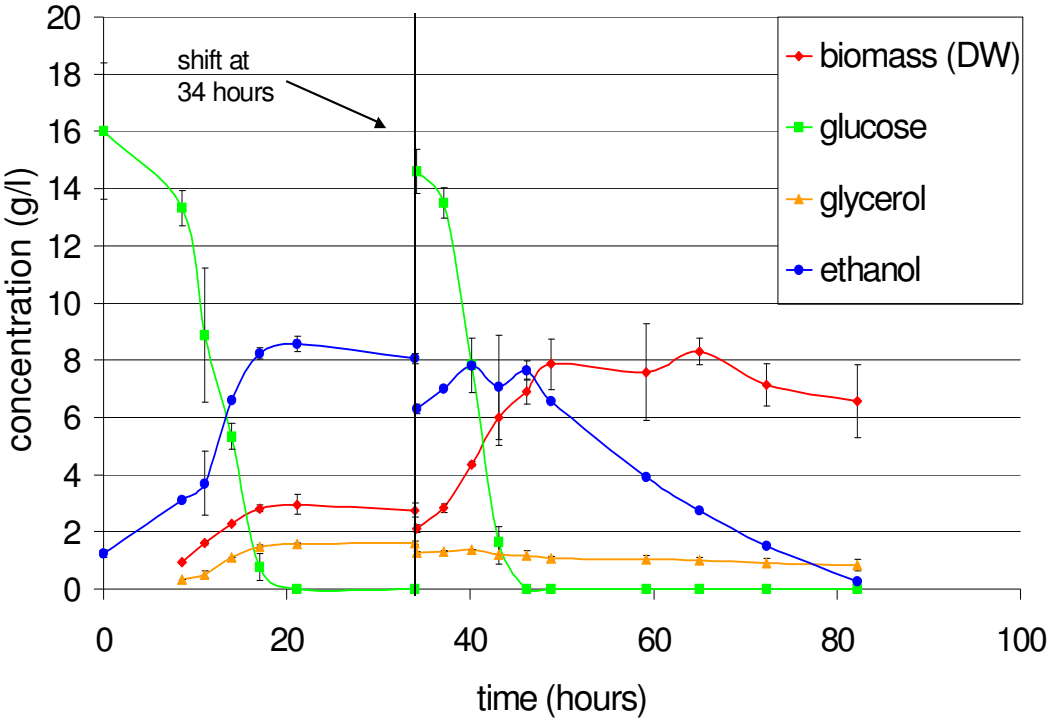

Figure 4-11. Graph of substrates and products averaged over two shift fermentations with KFA199 on glucose. The shift was at 34 hours as indicated by the vertical line. At the shift glucose was added and air was sparged from the shift onwards.

In Figure 4-12 it can be seen that GOX enzyme activity in supernatant was essentially zero in anaerobic conditions, increasing gradually after the shift, with a dramatic increase towards the end of the fermentation as biomass decreased, possibly indicating that the enzyme leaked out as cells began to lyse. The highest measured enzyme activity in KFA199 occurred after stationary phase at 80 hours, when the filaments started fragmenting due to autolysis and ageing, and cell dry weight had decreased by 2 g/l from its peak level of 8.5 g/l. Cytoplasmic and membrane bound (intracellular) GOX also increased up until the end of the fermentation (Figure 4-12). The highest measured extracellular GOX activity of KFA199 was 0.14 ± 0.03 $U/ml_{supernatant}$ and intracellular GOX activity was 0.81 ± 0.12 $U/ml_{culture\ broth}$. The highest GOX activities that were assayed occurred at the end of the fermentations.

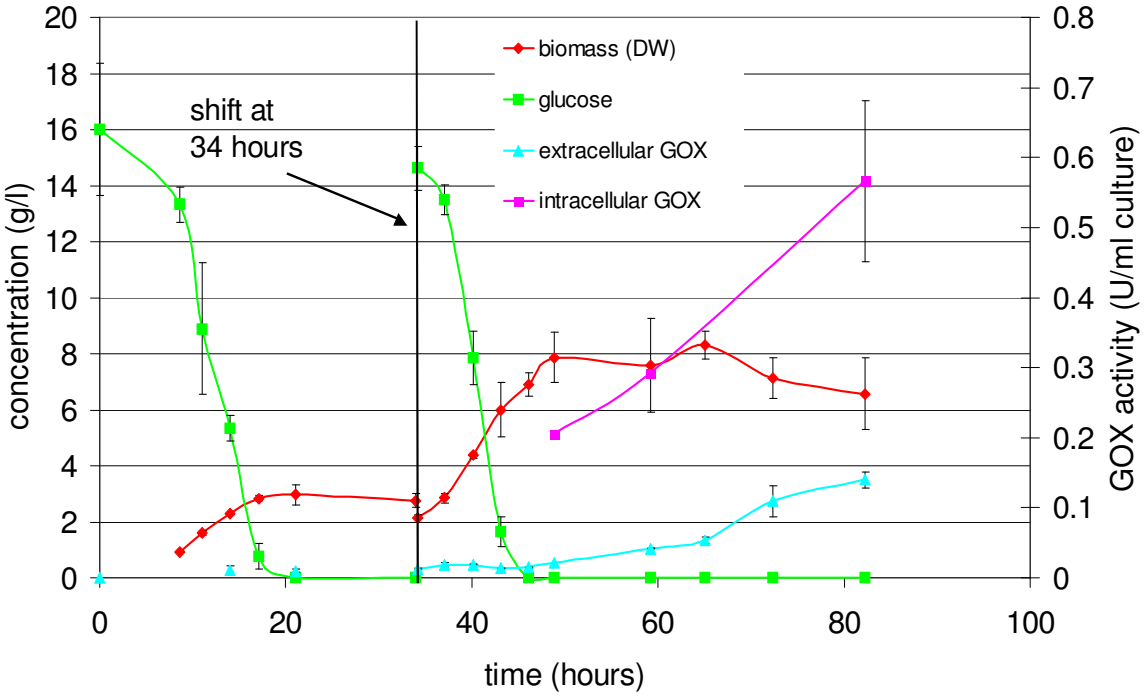

Figure 4-12. Enzyme activity averaged over two shift fermentations with KFA199 on glucose. The shift was at 34 hours as indicated by the vertical line.

In Figure 4-13 the specific glucose oxidase activity during a shift fermentation with KFA199 is shown. The increase in specific extracellular GOX from 60 hours to 84 hours indicates likely release due to cell lysis, as the biomass decreased while enzyme activity increased.

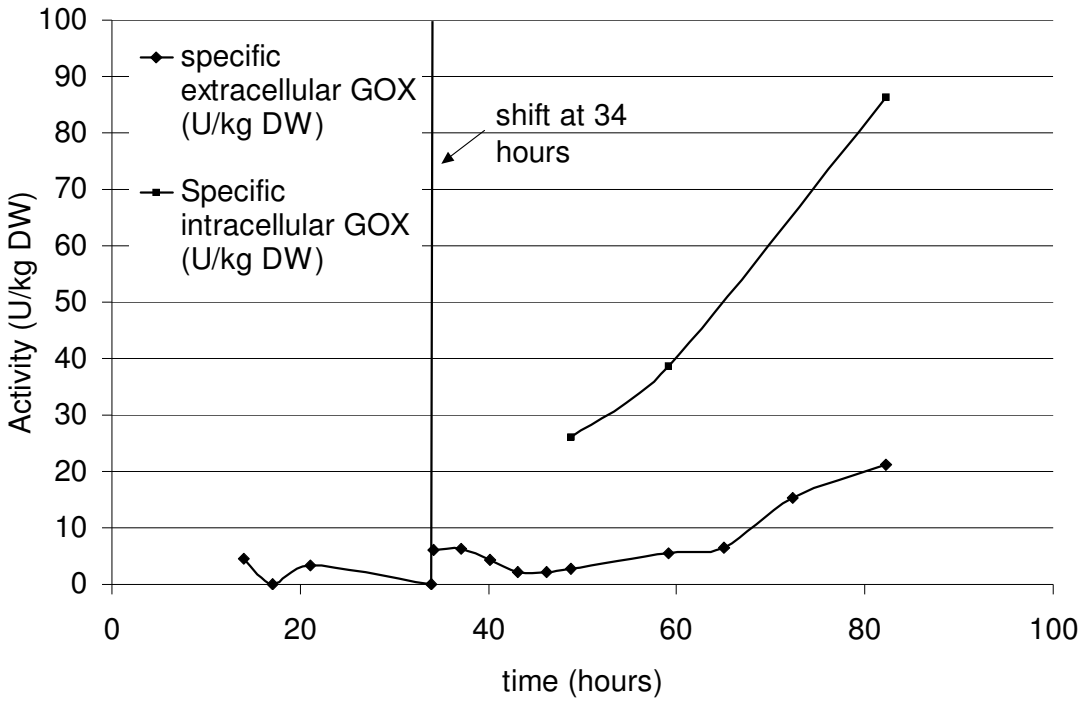

Figure 4-13. Specific glucose oxidase activity during a shift fermentation with KFA199. The shift from anaerobic to aerobic conditions occurred at 34 hours. DW is cell dry weight.

4.3.2. Substrate uptake and product formation during shift fermentations with CBS 232.29

In fermentations with the wild type, the anaerobic yeast phase was similar to that of the mutant with regard to biomass and ethanol production (Figure 4-14). The concentrations of ethanol, cell dry weight, and glycerol decreased at the shift because they were diluted by addition of more medium. At the shift, starch was supplied as a substrate, and biomass production was more (9.4 ± 1.6 g/l) than under glucose with KFA199 (8.5 ± 1.3 g/l). The biomass production continued for a longer period until 85 hours with starch, as opposed to biomass production stopping at 60 hours under glucose. The biomass production (accompanied by morphological shift from yeast to filamentous) for 32-40 hours after the shift was faster than that during the remainder of the growth phase of 40 – 60 hours (Figure 4-11 and Figure 4-14).

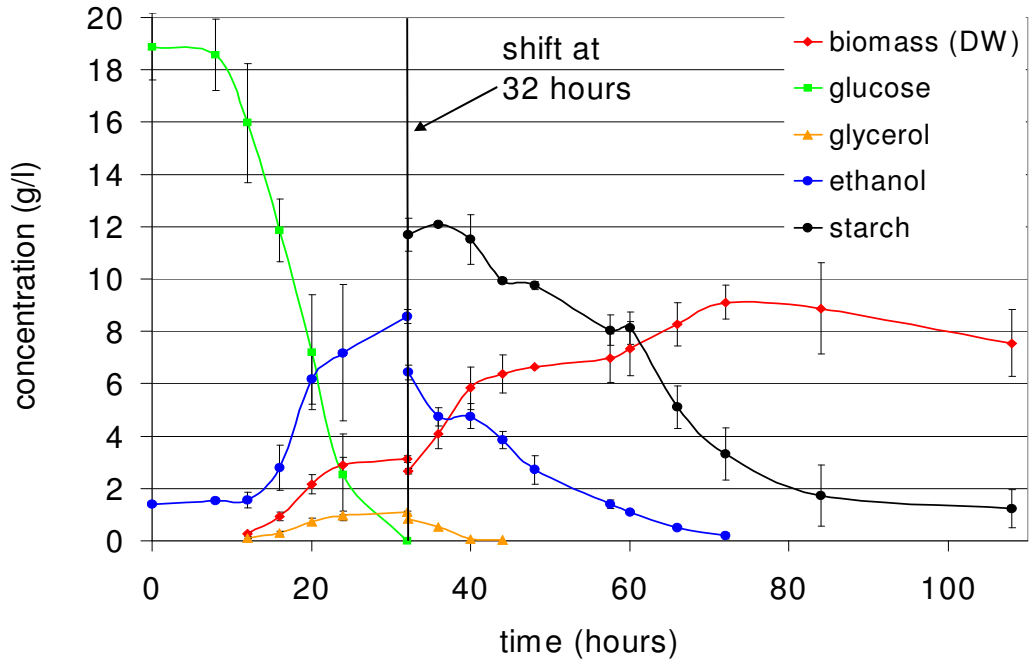

Figure 4-14. Shift fermentation with CBS 232.29, showing glucose and starch uptake (g/l) and dry weight, glycerol, ethanol levels (average of two fermentations).

The amylase activity in the supernatant increased with biomass increase, and therefore amylase secretion seems to be growth related. Amylase activity in the supernatant peaked at about 80 hours (Figure 4-15). The maximum amylase activity of CBS 232.29 was measured to be 0.31 U/ml in the culture supernatant and 0.20 ± 0.01 U/ml$_{\text{culture broth}}$ intracellularly. For CBS 232.29, the maximum enzyme activity (0.31 U/ml culture supernatant) coincided with maximum biomass levels at 65 hours, and activity subsequently decreased as filamentous biomass decreased due to fragmentation. There were noticeable oscillations in the extracellular amylase activity levels and this variation is attributed to inaccuracies arising from the amylase assay method. The assay method using DNS has been known to be inaccurate at times (Celliers, personal communication).

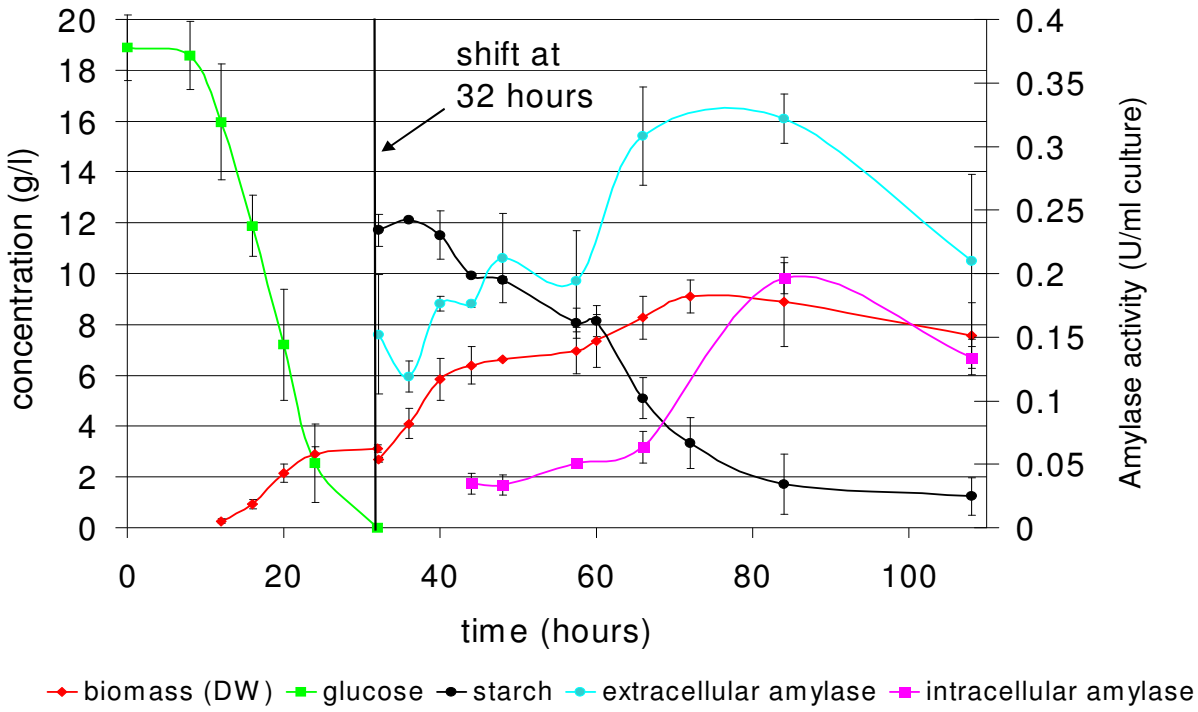

Figure 4-15. Shift fermentation with CBS 232.29, showing glucose and starch uptake (g/l) and dry weight, glycerol, ethanol levels (average of two fermentations).

In Figure 4-16 the specific amylase activity is shown during shift fermentations with CBS 232.29. The specific extracellular amylase activity remained at a relatively constant level of 28 to 38 U/kg cell dry weight, therefore the amylase activity was growth related i.e. the more biomass, the more amylase activity.

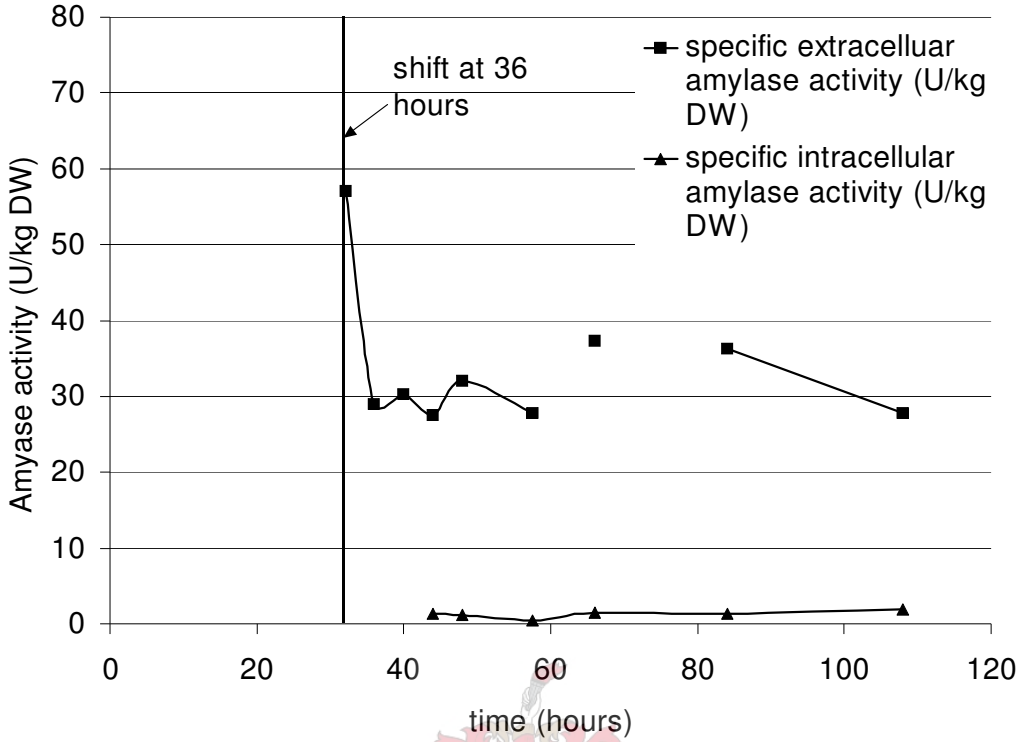

Figure 4-16. Specific amylase activity during shift fermentations with CBS 232.29. The shift from anaerobic to aerobic conditions occurred at 36 hours.

4.3.3. Specific growth rates and biomass and ethanol yields of shift fermentations

In Table 4.5 the specific growth rate, biomass yield and ethanol yield are compared before and after the shift. The values in Table 4.5 were calculated by taking the mean of the specific growth rates and yields of at least three fermentations. In the case of KFA199, the mean specific growth rate was slightly higher for anaerobic (0.14 ± 0.02 h^{-1}) than aerobic growth (0.12 ± 0.01 h^{-1}) (but not significantly different according to Student's t-test), the biomass yield of anaerobic (0.16 ± 0.02 g/g) was half that of aerobic (0.31 ± 0.01 g/g) and the ethanol yield was almost three times higher in anaerobic growth (0.42 ± 0.02 g/g) compared to aerobic (0.15 ± 0.04 g/g). For CBS 232.29, the specific growth rate anaerobically was 0.24 ± 0.02 h^{-1}, double the aerobic growth rate of 0.11 ± 0.03 h^{-1} (significantly different according to Student's t-test). In CBS232.29 the biomass yield, *expressed on C-mole basis*, of yeast was

much lower (0.22 ± 0.07 C-mole biomass formed. per C-mole glucose consumed) than filamentous growth (0.4 ± 0.1 C-mole biomass formed. per C-mole glucose consumed) and the ethanol produced anaerobically was used as a substrate aerobically.

Table 4.5. Biomass yield, ethanol yield, and specific growth rates of shift fermentations 1) average of at least 3 fermentations with CBS 232.29 2) average of 3 fermentations with KFA199

strain	before shift (yeast: anaerobic)			after shift (filamentous: aerobic)		
	specific growth rate μ (h-1)	Biomass Yield Ysx	Ethanol Yield YsE	specific growth rate μ (h-1)	Biomass Yield Ysx	Ethanol Yield YsE
KFA199 (glucose)	0.14 ±0.02	0.16 ±0.02 g/g 0.19 ±0.02 (Cmol)	0.42 ±0.02 g/g 0.93 ±0.04 (Cmol)	0.12 ±0.01	0.31 ±0.01 (g/g) 0.33 ± 0.05 (Cmol basis)	0.15 ±0.04 (g/g) 0.33 ± 0.18 (Cmol)
CBS 232.29 (starch)	0.24 ±0.02	0.19 ±0.06 g/g 0.22 ± 0.07 (Cmol)	0.48 ±0.19 1.06 ± 0.42 (Cmol)	0.11 ±0.03	0.4 ± 0.1 (Cmol basis)*	not produced

* Cmol yield for CBS 232.29 calculated as cell dry weight produced (in Cmol) over substrate consumed, where substrate was the sum of starch, ethanol and glycerol (in Cmol) used.

In comparing the two strains to each other, the yeast phase in CBS 232.29 had a larger specific growth rate (0.24 vs. 0.14), biomass yield (0.19 vs. 0.16) and ethanol yield (0.48 vs. 0.42) than KFA199. Despite CBS 232.29 using starch and KFA199 using glucose in the aerobic phase, the specific growth rates were very similar, 0.11 ± 0.03 and 0.12 ±0.01 respectively. The biomass yield of KFA199 on glucose (0.33 ± 0.05 C-mole biomass/C-mole glucose) was higher than that of CBS 232.29 on starch (0.4 ± 0.1 C-mole biomass/C-mole substrate). The final cell dry weight for CBS 232.29 after the exhaustion of substrate was 9.4 ± 1.6 g/l in the shift fermentations with 20 g/l glucose and 10 g/l starch. The final cell dry weight of KFA199 was 7.5 ± 1.3 g/l on a total of 40 g/l glucose, where 20 g/l glucose was present initially and 20 g/l was added at the shift (data not shown).

Chapter 5. Discussion

The objective of this study was to evaluate the potential of *M. circinelloides* for heterologous protein production. Two model systems were chosen, a wild type and a mutant, and the morphology and enzyme production of both were compared. The criteria for a good heterologous protein production system were that it would have successful expression vectors, efficient biomass production, good protein secretion, high protein yield, and little post-translational modification in the form of glycosylation (Table 2.5). The criteria examined in this study were biomass production, enzyme secretion and enzyme yield. Good protein secretion and high protein yields are often found in filamentous fungi, but the use of such an organism necessitates the control of morphology, as morphology can have a significant impact on protein secretion and bioreactor control (Li et al., 2000). Bioreactor control involves the control of rheology and mixing of fermentation liquid to optimize nutrient and O_2 transfer to the organism. The morphology of filamentous fungi changes depending on the conditions under which it is placed and optimization of conditions to control morphology is not trivial (Li et al., 2000). The morphology of the filamentous fungus *A. niger* has been manipulated to obtain a pelleted form, or in other cases, a dispersed form to optimize production of various compounds. Unlike most filamentous fungi used in industry, *M. circinelloides* can grow as a yeast, which is beneficial for bioreactor control, therefore *M. circinelloides* was grown as a yeast in this study. Once the conditions for yeast-like growth were established, the organism was cultured in a yeast form and subsequently in a filamentous form in shift fermentations, the first phase to grow biomass and the second phase to produce the reporter enzymes. Other substances such as glucose and ethanol were measured to calculate biomass yield and ethanol yield in order to determine the efficiency of biomass production.

5.1. Heterologous protein production with morphological control – conditions for yeast-like growth

5.1.1. Anaerobic growth

M. circinelloides was cultivated under various anaerobic conditions in order to determine the most satisfactory conditions for morphological control. The cells were grown under 30 % CO_2 and 70% N_2, under purified N_2 and under N_2 with and without E/T80 in the medium.

The first condition tested was growth under N_2 without E/T80 as anaerobiosis had been reported to yield yeast-like growth (Lübbehüsen et al., 2003b). The culture seemed to contain yeast-like cells, where mixing and homogeneity in the fermenter were good, but under the microscope, it was apparent that the cells had filaments extending from them. The filamentation was dramatically less than under air, but the morphology was definitely not purely yeast-like, as one could see yeast-like cells with filaments growing out of them. From the perspective of their rheological properties these cells were acceptable, but as is discussed later the specific growth rate was less than the cells grown with E/T80 and so these conditions were not chosen for further work. In the literature, under anaerobic conditions without gas sparging, yeast cells were grown, and one would think that, if O_2 is the critical factor, with N_2 being sparged that yeast cells would also result (Lübbehüsen et al., 2003a), but the situation was more complex.

Filamentous growth under N_2 may have occurred due to trace amounts of O_2 in the sparge gas and although the O_2 was very low (3 ppm) it was obviously enough to stimulate formation of filaments. Lübbehüsen et al. (2003a) were able to cultivate pure yeast in their anaerobic fermentations with no continuous sparging. This suggests that complete anaerobiosis is sufficient for yeast-like growth. However, they sparged with CO_2/N_2 for an hour after sampling to eliminate air that entered during sampling, and it seems that this addition of CO_2/N_2 at regular intervals (about every 4 hours according to their data) may have been sufficient to prevent filamentous growth and restrict *M. circinelloides* to yeast. They reported that sparging with N_2 alone was not enough to prevent filamentous growth (Lübbehüsen et al., 2003a). This begs the question, was the critical factor simply complete absence of O_2, or was the CO_2 necessary for yeast-like growth? In this case the CO_2 probably helped to maintain yeast morphology, as there was no ergosterol and Tween 80 supplementation (refer to first paragraph of this section).

In the next set of experiments trace O_2 was removed from the sparge gas by a Gas Chromatography oxygen trap and yeast-like cells resulted (Section 4.1.1.3). The sparge gas was analytical grade N_2 (99.999% pure) as used in all experiments, and this was passed through the trap, whereafter O_2 would be below 70 ppb. Under these conditions of complete absence of O_2 *M. circinelloides* grew yeast-like. This correlates with results from Phillips and Borgia (1985) and with a review paper by Orlowski (1991), however the requirement of strict anaerobiosis would be difficult to maintain cost-effectively under industrial conditions.

M. circinelloides was grown under 30% CO_2 and 70% N_2 and budding yeasts resulted (Section 4.1.1.1). This was despite the fact that both the N_2 and CO_2 contained trace amounts of O_2, the CO_2 containing more than the N_2. If CO_2 is present, the trace O_2 does not cause filamentous growth. This agrees with results from Lübbehüsen et al. (2003a). The cells appeared smaller than those under N_2 and it was interesting to see that there was much more budding with CO_2/N_2 than under N_2 alone. Even though there was more budding, the specific growth rate was similar to that under N_2 alone, without E/T80. The biomass yield under CO_2/N_2 was 30% less in the wild type strain, and 10% less in the mutant, than under N_2 with the oxygen trap. CO_2 can be assimilated by *Mucor* (Barnicki-Garcia and Nickerson, 1962a), but it appears to have a negative effect on biomass yield (this study, and Lübbehüsen et al., 2003b).

The defined Vogel's medium was compared with the complex medium, YPG (yeast, peptone, glucose) under N_2. In Vogel's medium (without E/T80) mixed yeast/filamentous morphology was observed while in YPG only yeast was observed under the same gas atmosphere. This further substantiates the interaction between gas atmosphere and nutrients in the medium on the morphology – *M. circinelloides* can grow yeast-like under N_2 as long as certain nutrients are supplied, either a rich medium or ergosterol and Tween 80. It could be that the yeast extract in YPG provides the sterols and fatty acids required for yeast-like cell development. The disadvantage of YPG was that enzyme expression was only 30% of that in Vogel's medium and so while it gave the desired morphology it did not give much enzyme activity (Section 4.2.1), thus YPG was not used for subsequent fermentations.

The conclusions of the investigation on morphology were that yeast could be attained with a few different approaches, i.e. complete absence of O_2, or with CO_2/N_2, or with addition of

E/T80 and sparging with N_2. The most satisfactory conditions were found to be growth under N_2 with addition of ergosterol and Tween 80 (E/T80) to the medium. Under these conditions the cells remained yeast-like and the specific growth rate was more than 150% greater than under other conditions (growth rates to be discussed in more detail later). The morphology of cells grown under N_2 with E/T80 was yeast-like (Section 4.1.1.4), and there was budding in KFA199, but very little budding in CBS 232.29. The budding was suppressed in the wild type by the E/T80, and growth occurred through the enlargement of existing cells.

5.2. Growth rates: Effect of morphological control on physiology

As the objective of morphological control was to produce biomass (in an easily mixed form), it was not only the morphology that was important but also the cellular growth rate and biomass yield as an indication of efficient biomass production. The growth rate and biomass yield were compared under CO_2/N_2, N_2, and N_2 with addition of E/T80 to the medium, and also aerobic conditions.

Growth rates were determined by plotting cell dry weight vs. time of fermentation and also the natural logarithm of cell dry weight vs. time (graphs not shown). The growth did not follow the typical exponential model as predicted by the Monod equation (Shuler and Kargi, 2002, p176) or the logistic equation (Shuler and Kargi, 2002, p180). Especially in the anaerobic phase growth appeared linear, where a plot of cell dry weight vs. time yielded a straight line. This leads one to believe that growth is limited in some way, since unlimited growth of unicellular fungi by budding in a batch culture is usually exponential. Limited growth could result from nutrient limitation or cell stress under anaerobic conditions. Because exponential growth rates are conventionally used in literature, the exponential growth rate, otherwise known as the specific growth rate, was used to compare growth with values in previous publications.

5.2.1. Anaerobic growth and ethanol production

Out of the anaerobic conditions, the growth under N_2 with E/T80 in Vogel's medium had the largest specific growth rate (Table 4.2). The specific growth rate was 2.5 times greater for the wild-type and 3.5 times greater in the mutant strain compared to the same conditions without

E/T80, and the biomass yield was also greater; thus it was the most favourable system overall (Table 4.2). These were the optimal conditions for both wild type and mutant strains. Under these conditions the wild type strain had a specific growth rate of 0.24 ± 0.02 h^{-1} and biomass yield of 0.19 g/g, and the mutant strain had a specific growth rate of 0.14 ± 0.02 h^{-1} and a biomass yield of 0.16 g/g (Table 4.2). This is less than the theoretically possible biomass yield of 0.51 g/g and this is explained by the large amount of ethanol that was produced. Under anaerobiosis and in the presence of relatively high glucose concentrations *M. circinelloides* converts a large proportion of glucose to ethanol (Lübbehüsen et al., 2004). More details of ethanol yields are given later.

The specific growth rate of the wild type strain was slowest under N_2 (no E/T80) at 0.075 h^{-1}, with a biomass yield of 0.14 g/g and growth was faster under CO_2/N_2 at 0.090 h^{-1} but with a low biomass yield of 0.07 g/g (Table 4.2). In the mutant strain, growth under N_2 with E/T80 (0.14 h^{-1}) was faster than that under CO_2/N_2 (0.034 h^{-1}) and the biomass yield under N_2 with E/T80 (0.16 h^{-1}) was greater than under CO_2/N_2 (0.09 h^{-1}) (Table 4.2). The addition of E/T80 definitely increased anaerobic specific growth rates as seen in this study and in experiments by Lübbehüsen et al. (2003b). However the increase in specific growth rate upon addition of E/T80 was much greater in this study than in the one by Lübbehüsen et al.(2003b).

In general, the wild type strain, CBS 232.29 grew faster and had a higher biomass yield than the mutant strain, KFA199 (Table 4.2). With E/T80, CBS 232.29 grew 60% faster than KFA199 and had a biomass yield that was 18% greater. It could be that the mutant does not produce biomass as well due to the mutagenesis it has undergone. The difference could also be due to the different strains used i.e. the mutant strain was not constructed from the wild type strain used in the investigation and that one strain has a tendency to produce more biomass or more ethanol.

In Table 4.2 the specific growth rates and biomass yields of this study, with *M. circinelloides* strain CBS 232.29, and those in literature, *M. circinelloides* strain ATCC1216b, are compared. While there are differences, the numbers are of the same order of magnitude (Table 4.2). The specific growth rate under CO_2/N_2 in this study, 0.09 h^{-1}, was very similar to that reported in the literature, 0.097 h^{-1}. Under N_2 with E/T80, the specific growth rate in this study, 0.226 h^{-1} (Table 4.2), was also similar to the value in literature, 0.241 h^{-1}. The biomass yields from this study, 0.07 g/g, were a little lower than in the literature, 0.097 g/g,

under N_2/CO_2 (Table 4.2). The biomass yields under N_2 with E/T80 in this study were 27% higher, 0.19 g/g, than those reported in the literature, 0.15 g/g (Lübbehüsen et al., 2003b).

In anaerobic growth, ethanol was produced at the expense of biomass. For heterologous protein production, it is desirable to have more biomass to make the process economically feasible. Methods of decreasing ethanol production such as using fed-batch fermentations could be investigated, as ethanol yield coefficients decrease when less sugar is supplied (Lübbehüsen et al., 2004, and Rangel-Porras et al., 2005).

The cells were grown on Vogel's medium, a defined medium. However, faster growth could be expected on a complex medium as more nutrients are supplied. It may also be cheaper to use a complex medium than to use Vogel's medium. Complex media such as those containing yeast extract, peptone or corn steep liquor may allow fast growth while maintaining yeast morphology as we have seen YPG (a complex medium) allows yeast growth at a similar rate to Vogel's medium (defined medium). If Vogel's medium with E/T80 is to be used routinely, an alternative source of fatty acids and sterols should be investigated e.g. coconut oil cake (Bogar et al., 2003) because E/T80 is expensive.

5.2.2. Aerobic growth and ethanol production

The growth aerobically in shift fermentations was 3 times slower than in ordinary aerobic fermentations. The growth after the shift, which included morphological change from yeast to filaments, occurred in two phases: initial faster growth for about 12 hours followed by slower growth for the next 20 hours. Both segments of the growth curve were linear but with different slopes. The growth was not as fast as expected compared to aerobic growth directly from spores (no yeast phase) it seems to 'get stuck' after a while – perhaps due to filaments experiencing shear stress and also switch to ethanol utilization or nutritional limitation. The specific growth rates after the shift (0.11 – 0.12 h^{-1}) were slower than specific growth rate of a non-shift fermentation (about 0.43 h^{-1}), this represents quite a large difference and the post-shift growth may be slower as the cells have to adjust to the new environment and change morphology (Table 4.5). The ethanol levels are relatively high at 6-8 g/l which could retard growth (Lübbehüsen et al., 2004), whereas much less ethanol is present in an aerobic non-shift fermentation.

The behaviour of cells after the shift i.e. in the aerobic phase showed certain trends.: In the glucose fermentations the growth was diauxic (Figure 4-11). Growth was fast until glucose was mostly used up and then the organism switched to ethanol consumption with slower growth as ethanol is more difficult to metabolise than glucose. In starch fermentations there was rapid growth and rapid ethanol usage and then as starch began to be utilized growth slowed down (Figure 4-14). Perhaps the enzymes necessary for starch break-down were only sufficient to break down starch at a limited rate. It is possible that the initial fast growth after the shift was due to energy stored within the cell (such as glycogen), which could then be used quickly under aerobic conditions (Lin et al., 2001). It has been reported that *S. cerevisiae* yeast cells store energy in the form of glycogen when they are under stress, or when they are ageing (Lin et al., 2001). The *Mucor* yeast cells could have been under stress due to anaerobiosis, and ageing as they were in stationary phase before the shift, and thus may have been stimlated to store glycogen.

Biomass yields in *Mucor* are lower than in some other filamentous fungi, as a large amount of glucose is used to produce ethanol. We can see that, as reported by Lübbehüsen et al., *Mucor* is Crabtree-positive, capable of fermentative metabolism under aerobic conditions such that it produces ethanol aerobically. This is shown by ethanol production under aerobic conditions, after the shift in shift fermentations (Section 4.3). Ethanol yields in aerobic cultures (0.15 g/g) were lower than in anaerobic cultures (0.42 g/g) grown on glucose. NAD-dependent alcohol dehydrogenase (ADH), an enzyme related to alcohol production in *M. circinelloides*, has activity levels about 2.5-fold higher in yeast cells than in the mycelium (Rangel-Porras et al., 2005). Higher ADH activity in yeast cells explains higher ethanol yields anaerobically. In contrast, Lübbehüsen et al. (2004) reported higher ethanol yields aerobically (0.34 g/g), than anaerobically (0.29 g/g). The maximum ethanol level of anaerobic cultures by Lübbehüsen et al. (2004) was approximately 5.5 g/l and in this study it was 8 g/l. High ethanol yields are undesirable for biomass production, as substrate is diverted to ethanol production instead of biomass production, and ethanol present in fermentation broth may inhibit growth. The ethanol is not wasted though, as *Mucor* exhibits diauxic growth, using ethanol after the glucose in the medium is exhausted thus the ethanol is still used to make biomass eventually. ADH is believed to play a role not only in ethanol production but also in utilization as levels rise during ethanol utilization (Rangel-Porras et al., 2005). From another point of view, high ethanol yields can be seen as an advantage because *M. circinelloides* has attractive prospects for industrial ethanol production due to its ability to consume a large

variety of substrates such as wood hydrozylates originating from the pulping industry (Millati et al., 2004 and Sues et al., 2005).

5.3. Enzyme production levels of *M. circinelloides*

Enzyme activity of both the wild type and mutant was measured in shake flask cultures and fermenters to evaluate the enzyme yield of *M. circinelloides.* In the wild type strain, grown on starch, amylase activity was measured to get an idea of the amount of enzyme that could be produced by fermentation of the wild type. In the mutant strain, grown on glucose, glucose oxidase activity was measured to determine the heterologous enzyme production. These enzyme activity levels gave an indication of the potential of *M. circinelloides* to be used as an enzyme/protein producer which, along with morphological control, was one of the main criteria for evaluating the potential of the *Mucor* species for protein production.

5.3.1. Enzyme activity in shake flasks

Enzyme activity was measured in shake flask cultures to select a suitable medium and get an indication of how the enzyme activity varied with substrate concentration and over the course of the cultivation. Various media were tested in shake flasks and the biomass yield and enzyme activity measured. The requirements of media were two-fold, firstly to support yeast-like growth and secondly to encourage enzyme production. Out of the media tested, Vogel's was superior for enzyme production in both the wild type and mutant strain.

Vogel's medium was formulated for cultivation of *Neurospora crassa (*Fungal Genetics Stock Centre website, 2005*)*, a filamentous fungus, with a morphology similar to *Mucor* (but not dimorphic) and thus it is understandable that this medium is suitable for cultivation of *Mucor.* In an analysis of *M. circinelloides* growth on five different defined media, namely Yeast, Vogel's, SIV, and Fungal medium 1 and 2, McIntyre et al. (2002) found that growth on Vogel's medium resulted in the highest specific growth rate (0.19 h^{-1}), final biomass concentration (4.2 g/l dry weight) and yield on glucose (0.26, c-mole basis). The inclusion of biotin, casamino acids, glutamate, niacinamide and thiamine chloride in Vogel's medium may result in faster growth of *M. circinelloides* in this medium compared to the other media which did not contain these nutrients (McIntyre et al., 2002).

The effect of carbon source quantity in the medium on enzyme production was checked. This was of especially of interest in the fermentations with KFA199 with regard to glucose (Larsen et al., 2004), but for completeness the starch concentration is discussed as well. The amount of starch that could be added to the medium was limited by the low solubility of starch (even after boiling) and thus 10g/l of starch was used for the fermentations based on the solubility of starch. It was not considered important to investigate this further as high starch concentrations increase the viscosity of fermentation fluid and diminish O_2 transfer (this was observed in dissolved oxygen levels of starch vs. glucose medium) and low starch concentrations would not provide enough substrate to continue growth for a sufficient time in a batch cultivation.

The effect of glucose concentration on GOX expression was important as the GOX gene was inserted with the *gpd1* promoter which is stimulated by glucose (Larsen et al., 2004). The greater the glucose concentration, the greater the glucose oxidase production (Table 4.4). In experiments with 10, 15 and 20 g/l glucose, the glucose oxidase production was greatest in the 20g/l glucose cultures (0.40 U/ml). The increase in enzyme activity with increase in glucose concentration was due to the larger amount of biomass that resulted, as the yield of GOX per g biomass was not significantly different at the different glucose levels, at 0.051 ± 0.003 U GOX/ g DW (Table 4.4).

The GOX levels in shake flasks (with 20 g/l glucose) with a maximum of 0.40 U/ml (about 1.96 mg/l) were comparable to that measured by Larsen *et al*. (2004) of 0.2 – 1.8 mg/l of GOX. The cultures by Larsen et al. (2004) were in a similar medium (but with 50 g/l glucose instead of 20 g/l) with a strain of *M. circinelloides* (UPO1171) that is parental to the KFA199 strain used in this study. Considering that Larsen et al. (2004) used a larger glucose concentration, and that large glucose concentrations induce *gpd1*P-regulated expression, more GOX activity could be expected from KFA199 at larger glucose concentrations. It must be added that the GOX activity between this study and that of Larsen et al. (2004) can be compared legitimately. In both cases, the commercial GOX enzyme from *A.niger* was used as a standard for GOX assays and because the same benchmark was used the activities can be compared with confidence. In addition, both strains used came from the same parental strain and were grown in the same medium.

In this study *gpd1*P was stimulated by high glucose concentrations (Larsen et al., 2004), so this creates a dilemma between low glucose concentration for biomass production (to reduce ethanol production) or high glucose concentration for enzyme production. A way to overcome this issue, would be to use a fed-batch feeding strategy to produce biomass and then in the filamentous phase feed larger concentrations of glucose for enzyme production under *gdp1*P. This is likely to work for filamentous growth but not for yeast-like growth. If anaerobiosis causes fully fermentative metabolism in the yeast-like cells then a fed-batch strategy is unlikely to reduce the ethanol yield. The specific ethanol yield for KFA199 was 0.42 and for CBS 232.29 it was 0.48 g/g. This indicates a predominantly fermentative metabolism and so it would seem that reducing ethanol yield in the yeast morphology may not be improved by a different feeding strategy.

5.3.2. Enzyme production in *Mucor* yeasts

Once the effect of media on enzyme activity was analysed in shake flask cultures, fermentations were performed in Vogel's medium. In fermentations, enzyme activity was measured in yeast phase of growth but found to be very low, a maximum of 0.010 ± 0.007U/ml or 3.37 U/g DW, and during aerobic growth activity was higher (21.8 U/g DW) and increased with time. Why are enzymes hardly secreted in yeast-like growth? In the mutant, GOX production should have been stimulated with the presence of glucose as the *gpd1* promoter switches on with glucose. Yeast cells contain three times more cAMP than hyphae (Orlowski, 1991). cAMP has been found to repress wall-associated α-glucosidase in *Mucor* and cAMP also inhibits β-glucosidase production (Orlowski, 1991). The presence of high levels of cAMP in *Mucor* yeasts could repress the production of glucose oxidase. Alternatively, cAMP could allow GOX production but repress secretion of the enzyme, as it has been found that cAMP allows intracellular α-glucosidase production but not wall associated α-glucosidase in *Mucor* yeasts. Another reason could be that the enzymes are present but not functional. Enzymes have been found in yeast cells (e.g. β-glucosidase) but were not inserted correctly in the periplasmic space thus were not functional (Orlowski, 1991).

It could be that the cells are under stress and therefore do not secrete enzymes. Yeast growth rates may be limited by the unfavourable environment of anaerobiosis as indicated by linear growth curves of yeast, suggesting cellular stress. The yeast cell wall is five to ten times

thicker than the filament cell wall, and the yeast cell wall is multi-layered, whereas the hyphal cell wall is single-layered (Bartnicki-Garcia, 1963) and therefore enzyme secretion will be more difficult as the enzyme must pass through a thicker cell wall. It is difficult to stimulate native enzyme production in yeast morphology, as in order to attain yeast, glucose (or a fermentable hexose) must be used as a substrate and this suppresses enzyme (amylase) production. For example, amylases or cellulases cannot be stimulated by provision of a suitable substrate such as starch or cellulose as the yeast cell simply cannot utilize these substrates. For the wild type, production of amylase was not possible in yeast as yeast could not grow on starch (Orlowski, 1991). In the mutant, even though glucose stimulates GOX production, it did not do so in the yeast phase but only in the filamentous phase.

5.3.3. Enzyme activity in shift fermentations

Since the yeast phase could not yield measurable quantitites of the reporter enzymes, it was necessary to shift the cultures to filamentous morphology to produce enzymes. In the wild type strain, the secreted amylase increased as biomass increased (Figure 4-16). The levels of amylase in the culture supernatant were generally low (a maximum of 0.31 U/ml in fermentations and 0.6 U/ml in shake flasks). Bogar et al. (2003) report a similar level of α-amylase activity of 0.72 U/ml from shake flask cultures of *M. circinelloides*. Despite low enzyme secretion, the growth of the wild type on starch was good and the cell dry weight was about 10 g/l similar to that achieved in *Aspergillus*, indicating that if the enzymes are not readily secreted, they may be cell-associated. However intracellular enzyme activity was relatively low (maximum of 0.10 ± 0.01 U/ml broth). *Mucor* seems to be conservative in secreting hydrolytic enzymes and does not secrete enzymes to the same extent that *A. niger* does. In another study, *M. circinelloides* produced 13 $U/g_{substrate}$ (0.72 U/ml) while *A. ficuum* produced 22 $U/g_{substrate}$ (1.23 U/ml) amylase activity in submerged fermentation (Bogar et al., 2003). In solid state fermentation, *M. circinelloides* produced 35 U/g substrate, while *A. ficuum* produced 130 U/g substrate (Bogar et al., 2003). In shake flask optimization of extracellular α-amylase production by an *Aspergillus* wild type strain on rice noodle waste-water, the unoptimized or baseline amylase concentration was 4 U/ml and optimized amylase production was 36.5 U/ml (Pimpa, 2004). The amylase assays by Bogar et al. (2003) and Pimpa (2004) were done in a similar manner to those in this study – starch degradation and assay of reducing sugars by DNS with glucose as a standard. (The pH, temperature and buffer used in the assay were the same as in this study (Bogar et al., 2003 and Pimpa, 2004).)

In the mutant strain, the GOX levels in supernatant from fermentations were 0.14 U/ml (equivalent to about 0.79 mg/l). Larsen et al. reported a similar amount of 0.2 – 1.8 mg/ml GOX activity in shake flask cultures. The conversion from U/ml to mg/l is done according to the units per mg of the GOX enzyme used as a standard. The same batch of enzyme was used as a standard throughout. The maximum shake flask levels were 0.40 U/ml, more than that from fermenters (0.14 U/ml), perhaps due to fermenter enzyme levels not reaching a maximum as fermentations were stopped while GOX was still increasing, or conditions being less favourable for enzyme production. The initial yeast phase may decrease enzyme levels because the cells have experienced stress, and in addition, inhibition of enzyme production may occur due to ethanol secreted into the medium. The filamentous morphology is also different in the shake flasks compared to the fermenter, in the shake flasks filaments were longer and intertwined with one another, and in the fermenter the filaments were shorter and more easily separable, as a result of shear stress from the impellors, and this could influence enzyme production. Enzyme production has been shown to vary with fungal morphology and branching and the length of hyphal branches (te Biesebeke et al., 2005).

In some examples from literature of extracellular GOX activity (Table 2.6), fermentations with *A. niger* yielded 2.7-3.2 U/ml GOX activity (Fiedurek at al., 1986) and 9 U/ml (Fiedurek et al., 1997), and shake flask cultures yielded 0.27 U/ml (Mischak et al., 1985). These were for native protein. The GOX levels in this study were 44% higher than those of Mischak et al. (1985) but lower than the others. Recombinant GOX was produced in *S. cerevisiae* at 100 U/ml (Malherbe et al., 2003) and in *H. polymorpha* at 445 IU/ml or 4.42 IU/mg cell dry weight, where biomass was 100.6 g/l (Hodgkins et al., 1993). These recombinant levels (in U/ml) reported in literature were approximately 100 times higher than those from *A. niger* but it should be noted that the biomass concentration of the yeasts was much higher than that of *A.niger,* thus the specific GOX yield of the yeasts is estimated to be only ten times higher than those of *A. niger*.

GOX levels in cytoplasmic and membrane fraction were 0.81 ± 0.12 U/ml compared to 0.14 ± 0.03 U/ml in the culture supernatant, indicating that the enzyme is not being secreted efficiently (at the end of the fermentation, about 80 hours). Expressed as units GOX activity per dry weight, intracellular GOX was 123 U/g and extracellular was 21.8 U/g DW. It is likely that the GOX is leaking out of the cells, evidenced by 1) the intracellular concentration is higher than the extracellular concentration and 2) the GOX only increases as biomass

decreases after stationary phase (Figure 4-11), indicating release of enzyme upon cell lysis. This was unexpected as the GOX gene has a secretion signal (Larsen et al., 2004, NCBI website).. The secretion signal may not be as effective in *M. circinelloides* as in *A. niger* and perhaps the use of a homologous secretion signal would improve GOX secretion.

Research on the distribution of GOX in *A. niger* shows that GOX can be found both intra- and extracellularly. In one study, 38% of the GOX was found to be extracellular and 62% intracellular (Clarke et al., 2006). The greater extracellular GOX activities in this study can be partly explained by the fact that the native enzyme is predominantly intracellular in *A. niger*. However, the ratio of intra- to extracellular GOX in this study was much greater than in *A. niger* (Clarke, et al, 2006).

Larsen et al. (2004) suggest that the late release of GOX is due to developmental regulation of *gpd1*P, supported by presence of regulatory sequences like ATGAAAT which are also in the *M. circinelloides ga1* promoter. In literature GOX increase is reported in stationary phase although it is not clear if cells had begun to lyse by then (Larsen et al., 2004). In cultures of the parental strain, UPO1171, the GOX levels also increased after a long time, from about 72 hours onwards, and increased up until 96 hours when the culture was stopped (Larsen et al., 2004).

The GOX levels produced by the recombinant strain KFA199 are lower than those obtained by most other species reported in the literature, although in the same range as some, for example, Mischak et al. (1985). However, there is still a lot optimization that can be done on the *M. circinelloides* cultivations. Considering that this is the first application of a strong, regulated promoter for heterologous protein production in *M. circinelloides* (Larsen et al., 2004) it shows potential for use of *M. circinelloides* for heterologous gene expression and supports and expands on work done by Larsen et al. (2004).

5.4. Protein measurements

In general, total secreted protein levels were low and information on secreted *Mucor* proteins in other literature is scarce. (The results of Bradford assays on culture supernatant indicated very low protein levels.) Using a more sensitive assay, the BCA protein assay, gave

unreliable results due to interference of substances in the culture supernatant. Low levels of protein in culture supernatant are beneficial from the point of view that when a recombinant protein is secreted, there will be only small amounts of native proteins and therefore purification of the desired protein is easier in the downstream processing stage.

Chapter 6. Conclusions: The Potential of *Mucor* as an expression system

The study dealt with controlling the morphology of *M. circinelloides* in yeast-like form with the eventual aim of improving the broth rheology. It was found that reactor conditions were more homogenous in the case of yeast-like morphology. However no significant levels of enzymes were measured during yeast-like growth and thus shifting to filamentous morphology was necessary to allow enzyme production. Enzymes were then produced in the filamentous phase of growth. GOX release into the culture supernatant occurred mainly on cell lysis. The filamentous broth at this stage was very viscous. Extracellular GOX production in fermentors was half that in shake flasks. The control of morphology thus led to less overall enzyme production and less biomass yield than shake flask cultures. Therefore the hypothesis of improvement in biomass and or enzyme production by morphological control was disproved.

The biomass levels and biomass yields in the yeast-like phase were low. The biomass yield of KFA199 was 0.16 g/g and of CBS 232.29 was 0.19 g/g, and these were both half that of the respective biomass yields of the filamentous phase. There is no clear way to increase the biomass yield of the yeast-like phase because of its inherent fermentative metabolism under anaerobiosis. The low biomass yield is an obstacle in the cultivation strategy which aims to achieve high biomass concentration in the yeast-like phase.

Along with low biomass yields is the fact that the ethanol production levels in the yeast-like phase were high, at between 6-8 g/l. Also, there was an average ethanol yield of 0.45 g/g for the KFA199 and CBS 232.29 (Table 4.5). If anaerobic conditions are essential for yeast-like morphology, and they are the most established method, then the reduction of ethanol yield is difficult.

When the enzyme production achieved in this study is compared with other enzyme expression systems, *Mucor* does not compare favourably with the well-established systems for GOX and amylase production. Yes, *Mucor* is dimorphic and will grow in yeast form and is thus convenient to cultivate and easily mixed. With yeast-like cells the broth is homogenous, samples are representative and there is no wall growth. The specific growth

rate of the yeast was significantly faster than that of the mycelia under the conditions tested; this is an interesting result and could possibly be exploited. The disadvantages of yeast are that the conditions for yeast growth yield low biomass and high ethanol concentrations and do not allow enzyme production. Futhermore, the medium requirements for yeast growth are very specific; only a fermentable hexose can be used (Orlowski, 1991) and amino acids, sterols and fatty acids must be supplemented. The fermentable hexose, in this case glucose, depresses production of native substrate hydrolases (e.g. amylase).

Enzyme production occurs only in the filamentous phase, after a long period of filamentous growth, by which stage the broth consistency appeared just as cumbersome as if one had grown the *Mucor* aerobically from the beginning. Even the highest enzyme levels that were measured were low in comparison with reported production systems. The total fermentation time to achieve enzyme production with a shift is longer than without a shift without achieving a less filamentous morphology or increased enzyme activity. Thus morphological control and simultaneous secretion of enzymes is not advantageous. If *Mucor* yeasts can be engineered to produce valuable substances then morphological control would be an advantage. Even though optimized industrial fermentations for GOX are better than *Mucor*, when compared to some other GOX productions systems such as *A.niger* before they were refined and improved, *Mucor* is on a par with them (Table 2.6). If enzymes can be secreted in earlier stages of the fermentation, perhaps under control of a different promoter or with a better enzyme secretion signal, and the growth conditions optimized then the enzyme production could be increased.

GOX production in the *M. circinelloides* strain, KFA199, was predominantly intracellular, at 82 hours 15% was extracellular and 85% was intracellular. It is preferable to have most of the enzyme secreted, as it is easier to separate out of the culture fluid than the cells in the downstream processing. If the enzyme is present inside the cells, the cells must first be disrupted before the enzyme can be extracted. This makes the downstream processing more complicated and costly. Thus, the criteria of good biomass production and enzyme secretion for a promising host for heterologous enzyme production were therefore not evident in this study.

M. circinelloides could perhaps be exploited successfully for other applications besides glucose oxidase production. Rather than competing with the *Aspergilli* for production of common enzymes, *Mucor* could find an application for production of niche enzymes that are not easily produced in other fungal systems. Since *Mucor* is a zygomycete, it has potential for production of zygomycete enzymes that are not successfully produced in the *Aspergilli* and, for example, production of phytase and lipase (Bogar et al., 2004). *Mucor* has been used successfully to synthesize carotenoid compounds (Papp et al., 2006 and Navarro et al., 2000) and gamma linolenic acid (Dabee, 1996). It also seems to perform better in solid state fermentations for enzyme production than in liquid medium (Bogar et al., 2003).

Chapter 7. Recommendations for Future Work

Aspects that could be looked at to improve enzyme production in *M. circinelloides* such as the fermenter size, feeding strategy, GOX secretion, medium optimization, and agitation are discussed below.

For filamentous cultures of *M. circinelloides* a fermenter with a working volume of about 1.5 litres or more is recommended, as in small fermenters the proportion of filaments on probes and the fermenter wall is large, and use of a larger fermenter will decrease the proportion of growth on probes and walls. In addition, a larger volume of culture will be disturbed less by sampling, as each sample removed is represents only a small portion of the broth. An observation from fermentations was that it is better to use one impellor instead of two in a 1 litre fermenter, because the level of liquid in the fermenter dropped due to sampling and the upper impellor was no longer submerged at the end of the fermentation.

With the batch fermentations the biomass yield of yeast cultures was quite low, but a fed-batch fermentation could give higher biomass yields. At higher glucose concentrations *M. circinelloides* produces more ethanol (Lübbehüsen et al., 2004), therefore in a glucose-feeding fed-batch scenario the glucose concentration would be kept low and favour the production of biomass instead of ethanol. By decreasing the glucose concentration, the specific growth rate and biomass yield were increased and the ethanol yield was decreased (Lübbehüsen et al., 2004). For 10 and 20 g/l glucose, the specific growth rates were 0.35 h^{-1} and 0.17 h^{-1}, biomass yields 0.41 g/g and 0.31 g/g, and ethanol yields 0.14 g/g and 0.34 g/g, respectively (Lübbehüsen et al., 2004). This was for filamentous growth thus yeast growth should still be investigated. A fed-batch fermentation for filamentous growth would give better biomass yields and specific growth rates (based on above comments) and this may also result in more enzyme production in the culture.

A matter of concern was the secretion of GOX; the levels of GOX in the supernatant increased as biomass decreased in the latter stages of the fermentation, probably as cells lysed (Discussion - Section 5.3.3). Therefore the culture had to be maintained for a long period of time, in shake flasks about 144 hours and in the fermenter in excess of 80 hours, well after onset of stationary phase. This long culture time increased problems associated with

filamentous fungi as in the latter stage of fermentation, mycelia gathered extensively on the walls and probes and the broth was non-homogenous. One could sequence the heterologous gene in KFA199 to confirm that the secretion signal is present. The secretion signal is present in the *A. niger* gox1 gene that was inserted into KFA199 (NCBI website, 2006 and Larsen et al., 2004) and it is possible that the secretion signal is not as effective in the new host, *M. circinelloides*, as in *A. niger*.

The fermentation time for KFA199 shift fermentations could be increased above 82 hours. The enzyme levels were still increasing at the time that the cultivation was stopped and higher GOX activities could result if the culture is maintained for a longer period.

Two other strains would be of interest to test in shake flasks or fermenters: a parental of KFA199, namely UPO 1171, and a derivative strain which has constitutive expression, GG103A, in other words GOX expression is independent of glucose concentration. It would be an advantage to have expression independent of glucose concentration, allowing low glucose concentrations to be used while accomplishing good GOX expression and thereby reducing ethanol production by *M. circinelloides* which occurs even aerobically as *M. circinelloides* is Crab-tree positive (Larsen et al., 2004). The other strain, UPO1171, may secrete GOX earlier on in the fermentation before the broth becomes very filamentous (Larsen et al., 2004).

The Vogel's medium used in this study was satisfactory for cultivation of *Mucor*, however it would be expensive to use this defined medium in industrial scale fermentations. The use of corn steep liquor or yeast extract and peptone may reduce costs. The ergosterol and Tween 80 added for yeast growth under N_2 is expensive and since YPG also supported yeast-like growth the addition of yeast and/or peptone to Vogel's medium could be studied to dispense with the need for E/T80 addition. The complex medium may also increase the specific growth rate which is a benefit for industrial production as fermentation time would be reduced. Another medium with potential is Hansson medium (Section 4.2.2). The GOX expression and biomass production in Hansson medium was similar to that in Vogel's, and Hansson medium is a simpler and cheaper medium.

One could use alternative strategies for dealing with filamentous growth besides changing the cell morphology. The fermenter set-up could be different to that used in this study. For example, high agitation rates could be used to keep filaments short and improve broth rheology. The advantages of high agitation are large O_2 transfer rates and avoiding O_2 limitation, minimizing wall and probe growth, possibly increased branching of filaments with more tips (smaller hyphal growth units) and more enzyme secretion from the hyphal tips (te Biesebeke et al., 2005). Another approach would be to use an air-lift fermenter, as shake flask cultures had higher activity, and see if less disturbance of mycelia from agitation improves enzyme production.

Chapter 8. References

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