Effect of cultivation conditions on the dimorphism of and heterologous protein production by *Arxula adeninivorans*

by

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Contents

Nomenclature

A	entropy constant
AOX	alcohol oxidase
°C	degree Celsius
cAMP	cyclic AMP
C_{crit}	critical dissolved oxygen concentration
cDNA	chromosomal DNA
DHAS	dihydroxyacetone synthase
DMSO	dimethylsulfoxide
DOT	dissolved oxygen tension
E_a	temperature coefficient
EDTA	ethylenediaminetetraacetic acid
FMD	formate dehydrogenase
g	gram or centrifugal force
GRAS	generally regarded as safe
h	hour
HOG	high osmolarity glycerol
HPLC	high performance liquid chromatography
HSA	human serum albumin
K	degrees Kelvin
kDA	kilodalton
l	litre
LB	Luria-Bertani
ln	natural logarithm
MAP	mitogen activated protein
mg	milligram (s)
min	minute

min	minute (s)
ml	millilitre (s)
ORF	open reading frame
PBS	phospate-buffered saline
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
PKA	protein kinase A
qO_2	specific rate of oxygen uptake
Q_s^{max}	maximum volumetric rate of substrate utilisation
q_s^{max}	maximum specific rate of substrate utilization
rDNA	ribosomal DNA
RNase	ribonuclease
rpm	revolutions per minute
s	seconds
$Y_{x/s}$	biomass yield coefficient
µg	microgram
µl	microlitre
µm	micrometer
μ_{max}	maximum specific growth rate

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Chapter 1

Introduction and Literature Review

1 Introduction

Heterologous protein production has recently become more important in science and industry. *Escherichia coli* was the first organism used to produce a commercial pharmaceutical (Domínguez *et al*. 1998; Swartz 2001). Since then, the production of more complex heterologous proteins became the focus of research. This initiated the search for hosts with the ability to produce these proteins.

One advantage of using yeasts in heterologous protein production is that they offer a eukaryotic system in a single celled organism. Furthermore, yeasts are able to adapt rapidly to changes in environmental conditions (Terentiev *et al.* 2003). The initial yeast system was based on baker's yeast, *Saccharomyces cerevisiae* (Gellissen *et al.* 2005). An extraordinary amount of research has been performed on this yeast species and a wealth of information is available on its molecular biology and physiology.

The rationale behind the production of heterologous proteins is to introduce a host organism that secretes active forms of a broad range of heterologous proteins more efficiently (Müller *et al.* 1998). *S. cerevisiae* has successfully been used as host for heterologous genes and a large number of genes have been cloned (Müller *et al.* 1998). Despite all the advantages associated with the use of *S. cerevisiae*, the limitations of this yeast have become apparent (Domíngues *et al.* 1998; Steinborn *et al.* 2006). These limitations include the tendency of *S. cerevisiae* to hyperglycosylate proteins, the relatively low yield of recombinant proteins compared to non-conventional yeasts, and the retention of proteins in the periplasmic space (Gellissen *et al*. 2005).

Alternative yeast systems have been defined that can potentially overcome the limitations of the traditional baker's yeast (Steinborn *et al.* 2006). Examples include the methylotrophic yeast *Pichia pastoris* and the dimorphic yeasts *Yarrowia lipolytica* and *Arxula adeninivorans.*

1.1 Objectives of this study

Arxula adeninivorans is a yeast strain with some interesting characteristics such as growth at high temperatures, the ability to utilise a wide range of substrates and an extreme halotolerance. These properties make this yeast an attractive host organism for heterologous gene expression. It is relatively new in the field of biotechnology and there is still much to learn about this yeast. The growth kinetics of this species and the effect of environmental conditions on its morphology under controlled cultivation conditions have not been well documented. Therefore, the aim of this study was a comparative investigation of the growth characteristics of *A. adeninivorans* strains LS3, G1211 (*LEU2*$^{+}$) and LS3/p*XynA*, focussing on the effect of temperature and dissolved oxygen tension (DOT) on the morphology and growth parameters of the strains. Strain G1211 (*LEU2*$^{+}$) is an auxotrophic mutant (*aleu2* mutant) of strain LS3 transformed with the pAL-ALEU2m plasmid and strain LS3/p*XynA* was derived from strain LS3 by transformation with the *Thermomyces lanuginosus* xylanase gene under the control of the *TEF1* promoter.

2 Literature Review

2.1 *Pichia pastoris* and *Yarrowia lipolytica* as non-conventional yeasts

Pichia pastoris, a methylotrophic yeast, was initially used for the production of single cell protein, but it was soon developed as a system for heterologous gene expression (Gellissen *et al.* 2005). It is able to grow on methanol as sole carbon and energy source and the key enzymes in the methylotropic pathway are alcohol oxidase (AOX), formate dehydrogenase (FMD) and dihydroxyacetone synthase (DHAS), the synthesis of which is regulated on transcriptional level (Domínguez *et al.* 1998).

Gene expression in *P. pastoris* is subject to a carbon source-dependent repression/derepression/induction regulation where the promoters are repressed by glucose, derepressed by glycerol and induced by methanol (Gellissen *et al*. 2005). Most foreign genes have been expressed under control of the *P. pastoris* promoter of the *AOX1* gene (Lee *et al.* 2003; Yu *et al.* 2007). The active status of the promoter is strictly dependent on the presence of methanol or methanol derivatives, but was also found to be dependent on the cellular environment of the specific host. Once transferred to

Hansenula polymorpha, the *P. pastoris*-derived *AOX1* promoter was active under conditions where glycerol served as carbon substitute (Gellissen *et al.* 2005).

Yarrowia lipolytica is widely used in the production of citric acid and peach flavour, and in the past was also used for single cell protein production (Beckerich *et al.* 1998). It's a dimorphic yeast and depending on the growth conditions, it is able to from either yeast cells or hyphae and pseudomycelia (Herrero *et al.* 1999; Spencer *et al.* 2002). *Y. lipolytica* metabolises only few sugars, but it can metabolise alcohols, acetate and hydrophobic substrates such as alkanes, fatty acids and oils. Many processes based on this yeast have been classified as GRAS (generally regarded as safe) by the American Food and Drug Administration (Madzak *et al.* 2005).

Expression of foreign proteins is achieved through the use of shuttle vectors (Gellissen *et al.* 2005). Integration of a linearised plasmid into the *Y. lipolytica* genome generally occurs by homologous recombination, which results in a high transformation efficiency and the precise targeting of the monocopy integration into the genome (Barth *et al.* 1997; Gellissen *et al.* 2005). Two strong constitutive promoters derived from the *Y. lipolytica TEF* and *RPS7* genes have been isolated, as well as a number of inducible promoters such as p*POX2,* p*ICL1* and p*POT1* derived from key enzymes in hydrophobic substrate utilisation (Juretzek *et al.* 2000; Müller *et al.* 1998).

2.2 *Arxula adeninivorans* as non-conventional yeast

A. adeninivorans, first described by Middelhoven *et al*. (1984), is an anamorphic, xerotolerant, ascomycetous, arthroconidial yeast. To date, nine strains of this species have been described, of which five were isolated in South Africa (Van der Walt *et al.* 1990). In recent years, strain LS3, isolated in Russia from wood hydrolysates (Samsonova *et al.* 1996), has gained considerable attention (Spencer *et al.* 2002).

A. adeninivorans is halotolerant and thermotolerant and is able to grow in NaCl solutions at concentrations as high as 20% and at temperatures of up to 48°C without previous adaptation, which is a very unusual property for a yeast species (Kunze *et al.* 1996; 1994; Yang *et al.* 2000). Furthermore, this yeast species is able to assimilate a wide range of carbon and nitrogen sources as well as phenols and hydroxybenzoates

(Middelhoven *et al.* 1991, 1992). The ability of *A. adeninivorans* to grow on many different substrates indicates the presence of a wide range of enzymes (Samsonova *et al.* 1996). Some of the enzymes produced by this yeast species are listed in Table1.1. The optimum temperature and pH values of these enzymes are in the range of 20 to 75°C and pH 4 to 7.5, respectively, and their molecular mass values are in the range of 60 to 600 kDa. This indicates that *A. adeninivorans* is able to secrete large protein molecules.

Table 1.1 Properties of enzymes produced by *Arxula adeninivorans* (adapted from Terentiev *et al*. 2003; Böer *et al.* 2005a; b; Kaur *et al.* 2007; Fiki *et al*. 2007)

Enzyme	Optimum values		Molecular mass (kDa)
	Temperature (°C)	pH	
Glucoamylase	60 - 70	4.0 - 5.0	225
Trehalase	45 - 55	4.5 - 4.9	250
Cellobiase	60 - 63	4.5	525 - 570
Invertase	55	4.5	600
β-D-xylosidase	60	5.0	60
Xylitol dehydrogenase	35	7.5	80
Acid phosphatase	60	4.8	350
Lipase	30	7.5	100
Transaldolase	20	5.5	140
3-Phytase	75	4.5	not determined

Like *Y. lipolytica*, *A. adeninivorans* is a dimorphic yeast and it was observed that dimorphism in strain LS3 is temperature dependent (Wartmann *et al.* 1995). LS3 grows as budding cells at 30°C; forms pseudomycelia at 42°C and at 45°C a mycelial culture can be observed (Fig. 1.1). Wartmann *et al.* (2000b) isolated mutants of strain LS3 with altered morphology after nitrosoguanidine mutagenesis. Mutant strain 135 formed mycelia when cultivated at 30°C (Fig. 1.2) and these mycelia secreted two-fold more protein than budding cells.

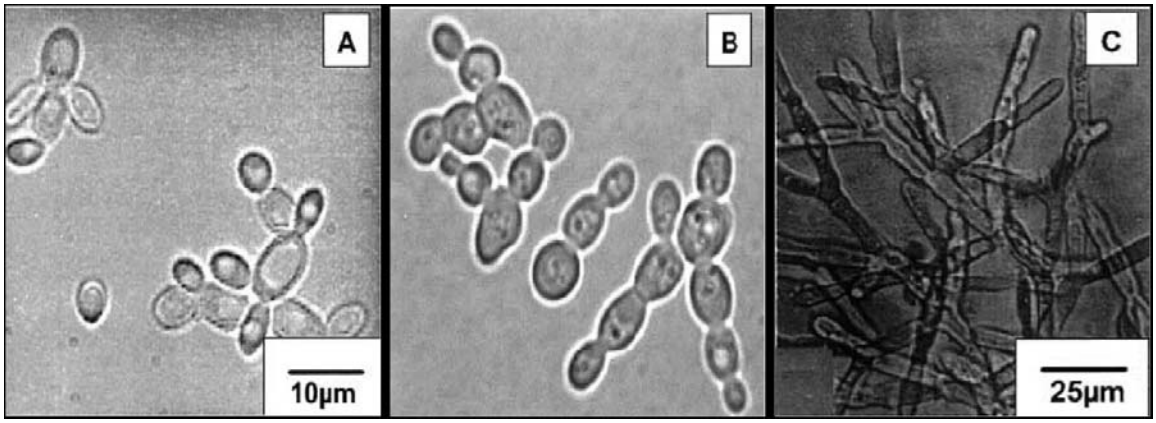


Figure 1.1 Micrographs of *Arxula adeninivorans* strain LS3 cultivated at 30°C (a), 42°C (b) and 45°C (c) (Wartmann *et al.* 2000b).

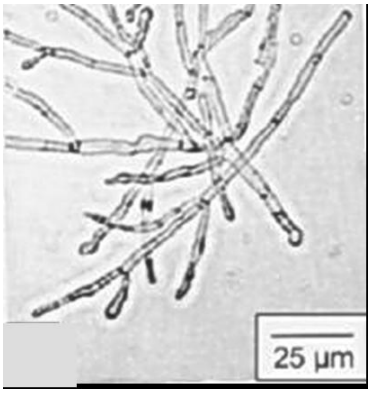


Figure 1.2 Micrograph of *A. adeninivorans* strain 135 grown at 30°C Wartmann *et al.* 2000b)

A. adeninivorans was identified as one of the most osmotolerant yeast species, being capable of growth in media containing NaCl at concentrations as high as 3.4 mol l^{-1} (Middelhoven *et al.* 1984; Yang *et al.* 2000). When exposed to high NaCl concentrations, *A. adeninivorans* cells react by producing and accumulating glycerol and erytrithol as compatible solutes (Yang *et al.* 2000). This tolerance is elicited by components of the high osmolarity glycerol (HOG) response pathway (Hayashi *et al.* 2006; Reynolds *et al.* 1998).

The HOG pathway is a signal transduction pathway linking osmo-sensing and gene expression for the production of compatible solutes. Böer *et al.* (2004a) found that the

AHOG1 gene was activated by phosphorylation when exposed to osmotic stress conditions. They found that the HOG pathway was regulated on a transcriptional level, something not described for any other yeast or filamentous fungus thus far. This property could account for the rapid adaptation and high osmotolerance of *A. adeninivorans*.

2.3 Molecular biology of *Arxula adeninivorans* strain LS3

A. adeninivorans is a haploid yeast and no sexual stage has yet been identified (Samsonova *et al.* 1989). Auxotrophic mutants with a broad spectrum of phenotypes have been isolated after nitrosoguanidine mutagenesis (Samsonova *et al.* 1989). By means of polyethylene glycol-induced fusion of spheroplasts, heterozygous diploids of auxotrophic mutants were obtained. The genome was analysed after segregation of diploids using benomyl-indused haploidization of parasexual hybrids (Kunze *et al.* 1996). In this way 32 genes could be assigned to four linkage groups, thus meeting the chromosome number of the *A. adeninivorans* genome and this was confirmed by relating the 32 auxotrophic mutants to particular chromosomes using pulsed field gel electrophoresis (PFGE) and subsequent DNA hybridization with specific probes (Samsonova *et al.* 1996). The mutant *A. adeninivorans* strain G1211 is a leucine negative strain and has been used in a wide range of heterologous gene expression studies (Samsonova *et al.* 1996; Böer *et al.* 2005; Steinborn *et al.* 2007); this strain was also used in this study.

A. adeninivorans has been developed as a host for heterologous gene expression. For assessment of the system, two model genes were selected; the *GFP* gene encoding intracellular green fluorescent protein, and the *HSA* gene encoding secreted human serum albumin (Wartmann *et al.* 2002a). The transformation/expression vector pAL-HPH1 (Fig. 1.3A) was used for the transformation (Terentiev *et al.* 2004b). In the case of *GFP* expression, the recombinant protein was localised in the cytoplasm and rendered the cells fluorescent. In the case of *HSA* expression based on an ORF including the native signal sequence at the 5'-end, more than 95% of the recombinant HSA was secreted into the culture medium. Budding cells as well as mycelia secreted similar levels of recombinant proteins, demonstrating a morphology-independent productivity (Wartmann *et al.* 2002a). This was in contrast to the secretion of native proteins where a

two-fold increase in protein excretion by mycelial cultures was observed (Wartmann *et al.* 2000b). Other genes expressed in *A. adeninivorans* include genes from the polyhydroxyalkoate biosynthetic pathway of *Ralstonia eutropha* and the *lacZ* gene from *E. coli* encoding β-galactosidase (Terentiev *et al.* 2004a; Wartmann *et al.* 2000a).

To date, three *A. adeninivorans*-based plasmids have been generated. The first plasmid constructed contains the *E. coli*-derived hygromycin B resistance gene (*hph*) as selection marker linked to the *A. adeninivorans TEF1*-promotor and the S. *cerevisiae PHO5*-terminator. The vector also contained unique restriction sites for the insertion of expression cassettes for heterologous genes and a 25S rDNA sequence for chromosomal targeting (Fig. 1.3A) (Rösel *et al.* 1998; Wartmann *et al.* 2002a).

The second and third plasmids were developed to avoid the use of toxic compounds or antibiotics during strain development. These plasmids were based on complementation of respective genes in auxotrophic strains (Wartmann *et al.* 2003b; 1998). The *AILV1* and *ALEU2* genes were used to complement the *ailv1* and *aleu2* auxotrophic strains. Plasmid pAL-ALEU2m harbouring the *ALEU2* gene for complementation and 25S rDNA for targeting were used to transform an *aleu2 A. adeninivorans* host (Fig. 1.3B).
Various *A. adeninivorans* genes have been isolated by PCR amplification using specific consensus primer sequences (Terentiev *et al.* 2003). The *TEF1* gene was one of the first genes isolated from the genomic library containing chromosomal DNA (cDNA) from LS3 (Rösel *et al.* 1995). The *TEF1* promoter is a constitutive promoter and was found to be active in all yeast species analysed (Terentiev *et al.* 2004b). The *TEF1* promoter provides a strong and constitutive expression of a heterologous gene, even when present in low copy numbers (Wartmann *et al.* 2003b). All *A. adeninivorans* genes isolated thus far are listed in Table 1.2. The first heterologous gene expressed in *A. adeninivorans* was the *Pseudomonas putida XylE* gene encoding the catechol 2,3-dioxygenase (Kunze *et al*. 1996). Since then, several heterologous genes were assessed for expressibility in *A. adeninivorans* (Table 1.3). *A. adeninivorans* is an interesting host for the production of heterologous proteins because all components needed for heterologous gene expression are available.

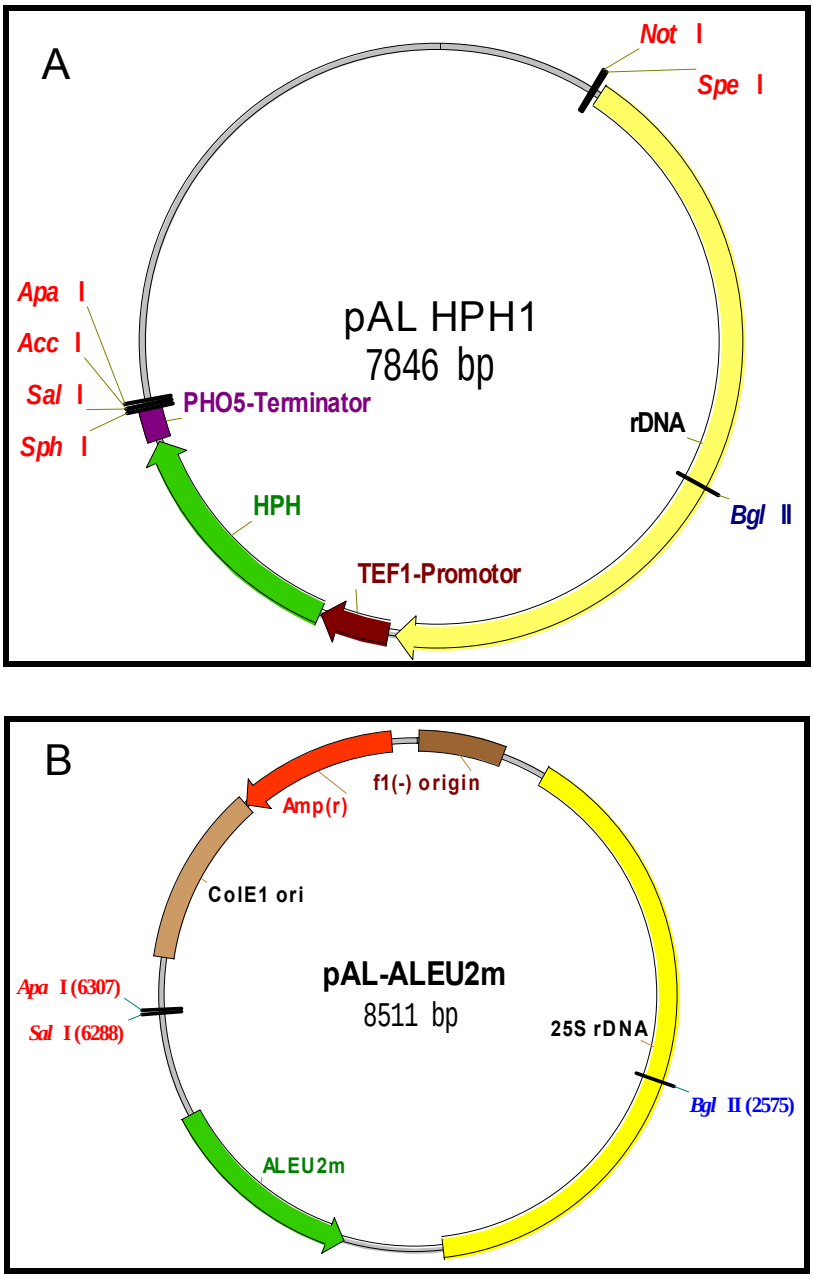


Figure 1.3 Physical maps of vectors for the *A. adeninivorans*-based expression platform. The vector pAL-HPH1 (A) contains the following elements: a 25S rDNA sequence (rDNA) chromosomal targeting, expression cassette for the *E. coli*-derived *hph* gene in the order *A. adeninivorans*-derived TEF1-promoter, the *hph*-coding sequence (HPH) and the *S. cerevisiae*-derived *PHO5*-terminator and vector pAL-ALEU2m (B) containing the selection marker *ALEU2m*. The vectors further contain unique *Apa*I and *Sal*I restriction sites for the insertion of the expression cassettes for heterologous genes and a unique *Bgl*II site within the rDNA sequence for linearization (Rösel *et al.* 1998; Wartmann *et al.* 2003b; Wartmann *et al.* 1998).

Table 1.2 Isolated and sequenced genes of the yeast *A. adeninivorans* strain LS3.

Gene	Gene Product	Accession no.	Reference
AXDH	Xilitol dehydrogenase	AJ748124	Böer *et al*. 2005
AILV1	Threonine deaminase	AJ222772	Wartmann *et al*. 1998
ALYS2	Amino-adipate reductase	Not sequenced	Kunze *et al.* 1996
ALEU2	β-Isopropylmalate dehydrogenase	AJ488496	Wartmann *et al.* 2003b
GAA	Glucoamylase	Z46901	Bui *et al.* 1996a
TEF1	Elongation factor 1α	Z47379	Rösel *et al*. 1995
ARFC3	Replication factor C component	AJ007712	Stoltenburg *et al*. 1999
AEFG1	Mitochondrial elongation factor G	AJ312230	Wartmann *et al.* 2001
AHSB4	Histone H4	AJ535732	Wartmann *et al*. 2003a
AFET3	Copper-dependent Fe(II) oxidase	AJ277833	Wartmann *et al*. 2002b
AINV	B-Fructofuranoside fructohydrolase	AJ580825	Böer *et al*. 2004b
APHO1	Acid phosphatase	AM231307	Kaur *et al.* 2007
ATRP1	Phosphoribosyl anthranilate isomerase	AM261500	Steinborn *et al.* 2007
ALIP1	Lipase	AJ879165	Böer *et al.* 2005a
ATAL	Transaldolase	AM400899	Fiki *et al.* 2007
25S rDNA	25S rRNA	Z50840	Rösel *et al.* 1996
AHOG1	Mitogen-activated protein (MAP) kinase	AJ626723	Böer *et al.* 2004a

Table 1.3 Heterologous genes expressed in *A. adeninivorans* LS3 (adapted from Böer *et al.* 2004a; b; 2005; Kunze *et al*. 1996; Terentiev *et al.* 2004a; Wartmann *et al*. 2000a; 2002a; 2003a; b)

Gene product	Promoter	Vector	Recombinant protein level
β-Galactosidase	*GAA*	pAL-HPH1	350 kU mg^{-1}
β-Galactosidase	*AHOG1*	pAL-HPH1	350 kU mg^{-1}
Green fluorescent protein	*TEF1*	pAL-HPH1	nd
Green fluorescent protein	*TEF1*	pAL-ALEU2m	nd
Green fluorescent protein	*AHSB4*	pAL-ALEU2m	nd
Human serum albumin	*TEF1*	pAL-HPH1	50 mg l^{-1}
Human serum albumin	*TEF1*	pAL-ALEU2m	50 mg l^{-1}
Human serum albumin	*AHSB4*	pAL-ALEU2m	50 mg l^{-1}
Catechol 2,3-dioxygenase	*AILV1*	I1-ALYS2	0.4 pkat mg^{-1}
Catechol 2,3-dioxygenase	*AINV*	pAL-ALEU2m	4.5 pkat mg^{-1}
Invertase	*TEF1*	pAL-ALEU2m	500 nkat ml^{-1}
β-Ketothiolase	*TEF1*	pAL-HPH1	2.2% PHA*
Acetoacetyl CoA reductase	*TEF1*	pAL-HPH1	2.2% PHA*
PHA synthase	*TEF1*	pAL-ALEU2m	2.2% PHA*
PHA synthase	*TEF1*	pAL-HPH1	2.2% PHA*

* per cent final product per dry weight.

Apart from being used for heterologous gene expression, *A. adeninivorans* has also served as gene donor. The *GAA* gene encoding the biotechnologically important enzyme glucoamylase was identified from a cDNA library using an anti-glucoamylase antibody as probe for product detection and was expressed in *Kluyveromyces lactis* and *S. cerevisiae* (Bui *et al.* 1996a; b). The level of enzyme secretion was 20-fold higher in *K. lactis* than in *S. cerevisiae* transformants when using an identical construct for transformation (Bui *et al.* 1996a; b).

2.4 Dimorphism in fungi

Dimorphism is the ability of fungi to switch between a budding cell and a mycelial morphology. This is the reaction in response to an environmental stimulus (Cruz *et al.* 2000).

Dimorphism is usually associated with pathogenic fungi because the ability to switch between the morphological forms is considered necessary for virulence (Sudbury *et al.* 2004). It is generally suggested that the mycelial form promotes tissue penetration during early stages of infection, whereas the yeast form is more suited for spreading in the bloodstream (da Silva *et al.* 1999).

It was found that most fungi exhibit this morphological plasticity as an integral part of their biology. Even *S. cerevisiae* is able to grow filamentous as a means of foraging in response to nitrogen limitation (Ceccato-Antonini *et al.* 2004; Herrero *et al.* 1999; Saporito-Irwin *et al.* 1995). Generally, the morphological switch can be induced by a variety of environmental conditions such as temperature, pH shifts as well as changes in oxygen availability (Buffo *et al.* 1984; Cruz *et al.* 2000; da Silva *et al.* 1999). These conditions frequently include the imposition of a stress, for example, heat shock (Swoboda *et al.* 1995).

Ceccato-Antonini *et al.* (2004) reported on signalling pathways that transduce environmental signals into morphological switching. The first pathway is based on cAMP and protein kinase A (PKA) and the second on a mitogen activated protein (MAP) kinase-based module, which in *S. cerevisiae* is also used to transduce the mating pheromone response. Another pathway that has been reported to be involved in the morphological switch is the heat shock response pathway (da Silva *et al.* 1999). All living organisms respond to temperature elevation by producing heat shock proteins that seemingly protect cells against the damaging effects of the stress agent. These proteins could play a role in cellular development by stabilising the protein products of genes that are switched on during cellular differentiation and may be important in the morphological changes observed (Swoboda *et al.* 1995). da Silva *et al.* (1999) found that the *hsp70* gene encoding heat shock proteins were found to be expressed both during morphological switching and during heat shock in the yeast *Paracoccidiodes brasiliensis*. It seems that morphological switching might be an adaptational effect to increase the

resistance of the organism to environmental stress. In some species of the genus *Mucor* the culture conditions that promote fermentation also promote a yeast-like morphology, whereas oxidative conditions promote mycelial development (Rogers *et al.* 1975). During continuous cultivation in a chemostat the mycelial phase of *Mucor genevensis* was observed under conditions of a glucose limitation with dissolved oxygen concentrations of 2 to 12.5 μmol l^{-1}, but on the addition of glucose to the culture a complete reversion to the yeast phase was observed (Rogers *et al.* 1975).

Apart from *S. cerevisiae*, other non-pathogenic yeasts such as *A. adeninivorans* and *Y. lipolytica* are also classified as dimorphic. *Candida albicans* is a major fungal human pathogen and has the ability to grow in a variety of morphological forms. It is the model organism for the study of morphological switching (Swoboda *et al.* 1995).

These morphological forms range from unicellular budding yeast to true hyphae with parallel-sided walls and, in between these two extremes, the fungus can exhibit a variety of growth forms that are collectively referred to as pseudohyphae (Buffo *et al.* 1984).

Earlier literature ignored the pseudohyphal state or used the terms pseudohyphae and hyphae interchangeably, so it is necessary to distinguish between these two forms. Sudbery *et al.* (2004) reviewed this topic and described the difference between the two morphological forms in *C. albicans*. They stated that pseudohyphae are basically yeast cells modified by polarised growth and not fully separate after completion of each cell cycle; the superficial similarity to hyphae might just be illusory (Gow 1997; Merson-Davies *et al* 1989). Sudbery *et al.* (2004) described pseudohyphal cells as having a constriction at the neck of the mother cell and the bud and at every subsequent septal junction (Fig. 1.4A), whereas hyphae that develop from an unbudded yeast cell (also termed a blastopore) have no constriction at the neck of the mother cell (Fig. 1.4B). Furthermore, they found another difference between the two cell types, namely that in pseudohyphae both the width and length of the cells can vary enormously, so that at one extreme they resemble hyphae and at the other they can resemble yeast cells with elongated buds (Fig. 1.5). The width of the compartments that make up the filament is not constant, being wider at the centre than at the two ends (Fig. 1.5A). Hyphae, on the other hand have parallel sides along their entire length (Fig. 1.5B). Pseudohyphae tend to exhibit a considerable degree of branching compared to hyphae.

Despite the apparent differences between hyphae and pseudohyphae, it is striking that similar environmental conditions induce both morphologies, with the balance being tipped towards hyphae as the conditions become more extreme (higher temperature and pH) (Herrero *et al.* 1999; Sacco *et al*. 1981). Whether this difference between these cell morphologies is applicable to all dimorphic fungi still needs to be investigated.

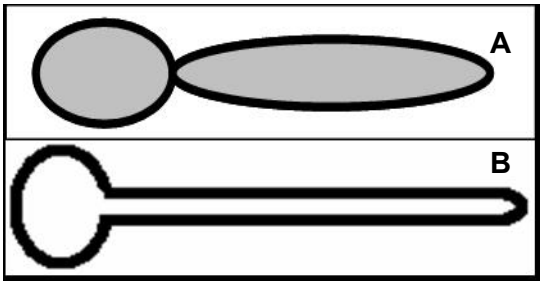


Figure 1.4 Differences between the development of pseudohyphae (A) and hyphae (B). There is a constriction between the neck of mother cell during pseudohyphal development (A) and no constriction between the mother cell and the emerging hyphae in B (adapted from Sudbery *et al.* 2004)

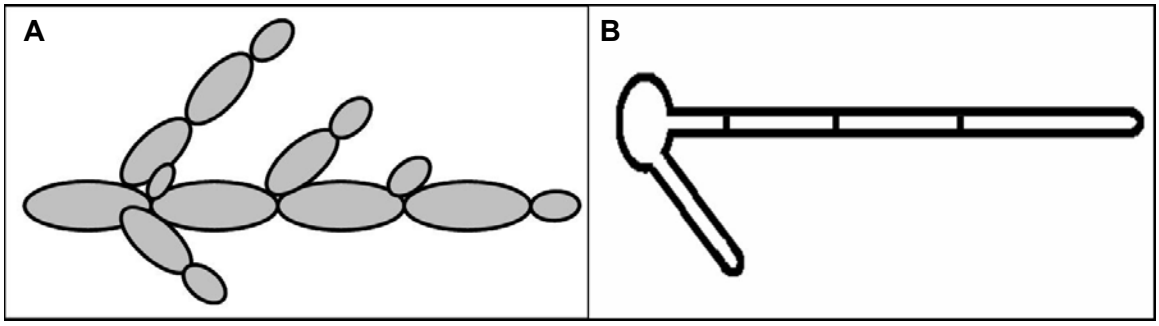


Figure 1.5 Difference between pseudohyphae (A), indicating the constrictions at branch site, non-parallel sides and extreme branching, and hyphae (B), indicating no constrictions at the branch sites and parallel sides. The pseudohyphae are more branched than hyphae (adapted from Sudbery *et al.* 2004)

In conclusion, a great amount of scientific data has been collected in a relatively short period on *A. adeninivorans*, mainly in respect of strain LS3. However, there are relatively few reports on the growth characteristics of this species. Because of its potential importance for biotechnological applications, particularly as eukaryotic host for the expression of heterologous proteins, a comprehensive study of the growth characteristics of this yeast species is desirable.

3 References

Barth G. and Gaillardin C. (1996) *Yarrowia lipolytica* In: Wolf K. (ed) Nonconventional yeasts in biotechnology: A handbook. Springer-Verlag, Berlin, Germany pp. 313-388.

Beckerich J., Boisramé-Baudevin A. and Gaillardin C. (1998) *Yarrowia lipolytica*: a model organism for protein secretion studies. Int. Microbiol. **1**: 123-130.

Böer E., Gellissen G. and Kunze K. (2005a) *Arxula adeninivorans* In: Gellissen G. (ed) Production of recombinant proteins. Novel microbial and eucaryotic expression systems. Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim pp. 89-110.

Böer E., Mock H. P., Bode R., Gellissen G. and Kunze G. (2005b) An extracellular lipase from the dimorphic yeast *Arxula adeninivorans*: molecular cloning of the *ALIP1* gene and characterization of the purified recombinant enzyme. Yeast **22**: 523-535.

Böer E., Wartmann T., Dlubatz K., Gellissen G. and Kunze G. (2004a) Characterization of the *Arxula adeninivorans AHOG1* gene and the encoded mitogen-activated protein kinase. Curr. Genet. **46**: 269–276.

Böer E., Wartmann T., Luther B., Manteuffel R., Bode R., Gellissen G. and Kunze G. (2004b) Characterization of the *AINV* gene and the encoded invertase from the dimorphic yeast *Arxula adeninivorans*. Antonie van Leeuwenhoek **86**: 121–134.

Böer E., Wartmann T., Schmidt S., Bode R., Gellissen G. and Kunze G. (2005c) Characterization of the *AXDH* gene and the encoded xylitol dehydrogenase from the dimorphic yeast *Arxula adeninivorans*. Antonie van Leeuwenhoek **87**: 233–243.

Buffo J., Herman M. A. and Soll D. R. (1984) A characterization of pH-regulated dimorphism in *Candida albicans*. Mycopath. **85**: 21-30.

Bui D. M., Kunze I., Förster S., Wartmann T., Horstmann C., Manteuffel R. and Kunze G. (1996a) Cloning and expression of an *Arxula adeninivorans* glucoamylase gene in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **44**: 610–619.

Bui D. M., Kunze I., Horstmann C., Schmidt T., Breunig K. D. and Kunze G. (1996b) Expression of the *Arxula adeninivorans* glucoamylase gene in *Kluyveromyces lactis*. Appl. Microbiol. Biotechnol. **45**: 102-106.

Ceccato-Antonini S. and Sudbery P. E. (2004) Filamentous growth in *Saccharomyces cerevisiae*. Brazil. J. Microbiol. **35**: 173–181.

Cruz J. M., Domínguez J. M., Domínguez H. and Parajó J. C. (2000) Dimorphic behaviour of *Debaryomyces hansenii* grown on barley bran acid hydrolyzates. Biotechnol. Lett. **22**: 605–610.

da Silva S. P., Borges-Walmsley M. I., Pereira I. S., de Almeida Soares C. M., Walmsley A. R. and Filipe M. S. S. (1999) Differential expression of an *hsp70* gene during transition from the mycelial to the infective yeast form of the human pathogenic fungus *Paracoccidiodes brasiliensis*. Mol. Microbiol. **31**: 1039-1050.

Domínguez Á., Fermiñán E., Sánchez M., González F. J., Pérez-Campo F. M., García S., Herrero A.B., San Vicente A., Cabello J., Prado M., Iglesias F.J., Choupina A., Burguillo F.J., Fernández-Lago L. and López M.C. (1998) Non-conventional yeasts as hosts for heterologous protein production. Int. Microbiol. **1**: 131–142.

Fiki A. E., Metabteb G. E., Bellebna C., Wartmann T., Bode R., Gellissen G. and Kunze G. (2007) The *Arxula adeninivorans ATAL* gene encoding transaldolase - gene characterization and biotechnological exploitation. Appl. Microbiol. biotechnol. **74**: 1292-1299.

Gellissen G., Kunze G., Gaillardin C., Cregg J. M., Berardi E., Veenhuis M. and van der Klei I. (2005) New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica* – A comparison. FEMS Yeast Res. **5**: 1079-1096.

Gow N.A.R. (1997) Germ tube growth of *Candida albicans*. Curr. Top. Med. Mycol. **8**: 43–55

Hayashi M. and Maeda T. (2006) Activation of the HOG pathway upon cold stress in *Saccharomyces cerevisiae*. J. Biochem. **139**: 797-803.

Herrero A. B., López M. C., Fernández-Lago L. and Domínguez A. (1999) *Candida albicans* and *Yarrowia lipolytica* as alternative models for analyzing budding patterns and germ tube formation in dimorphic fungi. Microbiol. **145**: 2727–2737.

Juretzek T., Wang H. J., Nicaud J. M. Mauersberger S. and Barth G. (2000) Comparison of promoters suitable for regulated overexpression of β-galactosidase in the alkaline-utilizing yeast *Yarrowia lipolytica*. Biotechnol. Bioprocess. Eng. **5**: 320-326.

Kaur P., Lingner A., Singh B., Böer E., Polajeva J., Steinborn G., Bode R., Gellissen G., Satyanarayana T. and Kunze G. (2007) *APHO1* from the yeast *Arxula adeninivorans* encodes an acid phosphatase of broad substrate specificity. Antonie van Leeuwenhoek **91**: 45-55.

Kunze G. and Kunze I. (1994) Characterization of *Arxula adeninivorans* strains from different habitats. Antonie van Leeuwenhoek **65**: 29–34.

Kunze G. and Kunze I. (1996) *Arxula adeninivorans* In: Wolf K. (ed) Nonconventional yeasts in biotechnology: A handbook. Springer-Verlag, Berlin, Germany, pp. 389 –395.

Lee C. Y., Lee S. J., Jung K. H., Katoh S. and Lee E. K. (2003) High dissolved oxygen tension enhances heterologous protein expression by recombinant *Pichia pastoris*. Process Biochem. **38**: 1147-1154.

Madzak C., Otterbein L., Chamkha M., Moukha S., Asther M., Gaillardin C. and Beckerich J. (2005) Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. FEMS Yeast Res. **5**: 635–646.

Merson-Davies L. A. and Odds F. C. (1989) A morphology index for cell shape in *Candida albicans*. J. Gen. Microbiol. **135**: 3143-3152.

Middelhoven W. J., Coenen A., Kraakman B. and Sollewijn Gelpke M. D. (1992) Degradation of some phenols and hydroxybenzoates by the imperfect ascomycetous yeasts *Candida parapsilosis* and *Arxula adeninivorans*: evidence for an operative gentisate pathway. Antonie van Leeuwenhoek **62**: 181–187.

Middelhoven W. J., de Jong I. M. and de Winter M. (1991) *Arxula adeninivorans*, a yeast assimilating many nitrogenous and aromatic compounds. Antonie van Leeuwenhoek **59**: 129–137.

Middelhoven W. J., Hoogkamer-Te Niet M. C. and Kreger-Van Rij N. J. W. (1984) *Trichosporon adeninovorans* sp. nov., a yeast species utilizing adenine, xanthine, uric acid, putrescine and primary *n*-alkylamines as the sole source of carbon, nitrogen and energy. Antonie van Leeuwenhoek **50**: 369–378.

Müller S., Sandal T., Kamp-hansen P. and Dalbøge H. (1998). Comparison of expression systems in the yeasts *Saccharomyces cerevisiae, Hansenula polymorpha, Kluyveromyces lactis, Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. Yeast **14**: 1267-1283.

Reynolds T. B., Hopkins B. D., Lyons M. R. and Graham T. R. (1998) The high osmolarity glycerol response (HOG) MAP kinase pathway controls localization of a yeast golgi glycosyltransferase. J. Cell Biol. **143**: 935-946.

Rogers P. J., Clark-Walker G. D. and Stewart P. R. (1975). Effects of Oxygen and glucose on energy metabolism and dimorphism of *Mucor genevensis* grown in continious culture: Reversibilitiy of yeasts-mycelium conversion. J. Bacteriol. **119**; 282-293.

Rösel H. and Kunze G. (1995) Cloning and characterization of a *TEF* gene for elongation factor 1α from the yeast *Arxula adeninivorans*. Curr. Gen. **28**: 360–366.

Rösel H. and Kunze G. (1996) Identification of a group-I intron within the 25S rDNA from the yeast *Arxula adeninivorans*. Yeast **12**: 1201–1208.

Rösel H. and Kunze G. (1998) Integrative transformation of the dimorphic yeast *Arxula adeninivorans* LS3 based on hygromycin B resistance. Curr. Gen. **33**: 157–163.

Sacco M., Maresca B., Kumar B. V., Kobayashi G. S. and Medoff G. (1981) Temperature- and cyclic nucleotide–induced phase transitions of *Histoplasma capsulatum*. J. Bacteriol. **146**: 117–120.

Samsonova I. A., Böttcher F., Werner C. and Bode R. (1989) Auxotrophic mutants of the yeast *Trichosporon adeninivorans*. J. Basic Microbiol. **29**: 675-683.

Samsonova I. A., Kunze G., Bode R and Böttcher F. (1996) A set of genetic markers for the chromosomes of the imperfect yeast *Arxula adeninivorans*. Yeast **12**: 1209–1017.

Saporito-Irwin S. M., Birse C. E., Sypherd P. S. and Fonzi W. A. (1995) *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. Mol. Cell. Biol. **15**: 601–613.

Spencer J. F. T., Ragout de Spencer A. L. and Laluce C. (2002) Non-conventional yeasts. Appl. Microbiol Biotechnol. **58**: 147–156.

Steinborn G., Böer E., Scholz A., Tag K., Kunze G. and Gellissen G. (2006) Application of a wide-range yeast vector (CoMed™) system to recombinant protein production in dimorphic *Arxula adeninivorans*, methylotrophic *Hansenula polymorpha* and other yeasts. Microb. Cell Factories **5**: 1475-2859.

Steinborn G., Wartmann T., Gellissen G. and Kunze G. (2007) Construction of an *Arxula adeninivorans* host-vector system based on *trp1* complementation. J. Biotechnol. **127**: 392-401.

Stoltenburg R., Lösche O., Klappach G. and Kunze G. (1999) Molecular cloning and expression of the *ARFC3* gene, a component of the replication factor C from the salt-tolerant, dimorphic yeast *Arxula adeninivorans* LS3. Curr. Gen. **35**: 8–13.

Sudbery P., Gow N. and Berman J. (2004) The distinct morphogenic states of *Candida albicans*. Trends Microbiol. **12**: 317-324.

Swartz J. R. (2001) Advances in *Escherichia coli* production of therapeutic proteins. Curr. Opin. Biotechnol. **12**: 195-201.

Swoboda R. K., Bertram G., Budge S., Gooday G. W., Gow N. A. R. and Brown A. J. P. (1995) Structure and regulation of the *HSP90* gene from the pathogenic fungus *Candida albicans*. Infect. Immun. **63**: 4506-4514.

Terentiev Y., Breuer U., Babel W. and Kunze G. (2004a) Non-conventional yeasts as producers of polyhydroxyalkanoates - genetic engineering of *Arxula adeninivorans*. Appl. Microbiol. Biotechnol. **64**: 376-681.

Terentiev Y., Gellissen G. and Kunze G. (2003) *Arxula adeninivorans* – a non-conventional dimorphic yeast of great biotechnological potential. Recent Research Development. Appl. Microbiol. Biotechnol. **1**: 135–145.

Terentiev Y., Huarto Pico A., Böer E., Wartmann T., Klabunde J., Breuer U., Babel W., Suckow M., Gellissen G. and Kunze G. (2004b) A wide-range integrative yeast expression vector system based on *Arxula adeninivorans*-derived elements. J. Ind. Microbiol. Biotechnol. **31**: 223–228.

Van der Walt J. P., Smith M. Th. and Yamada Y. (1990) *Arxula* gen. nov. (Candidaceae), a new anamorphic, arthroconidial yeast genus. Antonie van Leeuwenhoek **57**: 59–61.

Wartmann T. and Kunze G. (2000a) Genetic transformation and biotechnological application of the yeast *Arxula adeninivorans*. Appl. Microbiol. Biotechnol. **54**: 619–624.

Wartmann T., Bellebna C., Böer E., Bartelsen O., Gellissen G. and Kunze G. (2003a) The constitutive *AHSB4* promoter – a novel component of the *Arxula adeninivorans*-based expression platform. Appl. Microbiol. Biotechnol. **62**: 528–535.

Wartmann T., Böer E., Huarto Pico A., Sieber H., Bartelsen O., Gellissen G. and Kunze G. (2002a) High-level production and secretion of recombinant proteins by the dimorphic yeast *Arxula adeninivorans*. FEMS Yeast Res. **2**: 363–369.

Wartmann T., Erdmann J., Kunze I and Kunze G. (2000b) Morphology-related effects on gene expression and protein accumulation of the yeast *Arxula adeninivorans* LS3. Arch. Microbiol. **173**: 253–261.

Wartmann T., Gellissen G. and Kunze G. (2001) Regulation of the *AEFG1* gene, a mitochondrial elongation factor G from the dimorphic yeast *Arxula adeninivorans* LS3. Curr. Gen. **40**: 172–178.

Wartmann T., Krüger A., Adler K., Duc B. M., Kunze I. and Kunze G. (1995) Temperature-dependant dimorphism of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek **68**: 215–223.

Wartmann T., Rösel H., Kunze I., Bode R. and Kunze G. (1998) *AILV1* gene from the yeast *Arxula adeninivorans* LS3 - a new selective transformation marker. Yeast **14**: 1017–1025.

Wartmann T., Stephan U. W., Bube I., Böer E., Melzer M., Manteuffel R., Stoltenburg R., Guengerich L., Gellissen G. and Kunze G. (2002b) Post-translational modifications of the

AFET3 gene product – a component of the iron transport system in budding cells and mycelia of the yeast *Arxula adeninivorans*. Yeast **19**: 849–862.

Wartmann t., Stoltenburg R., Böer E., Sieber H., Bartelsen O., Gellissen G. and Kunze G. (2003b) The *ALEU2* gene – a new component for an *Arxula adeninivorans*-based expression platform. FEMS Yeast Res. **3**: 223-232.

Yang X., Wartmann T., Stoltenburg R. and Kunze G. (2000) Halotolerance of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek **77**: 303–311.

Yu M., Lange S., Richter S., Tan T. and Schmid R. D. (2007) High-level expression of extracellular lipase Lip2 from *Yarrowia lipolytica* in *Pichia pastoris* and its purification and characterization. Prot. Express. Purific. **53**: 255-263.

Chapter 2

Cloning of the *XynA* gene from *Thermomyces lanuginosus* and expression in *Arxula adeninivorans*

Abstract

Xylanases from thermophilic fungi have attracted considerable attention because of potential industrial applications. The xylanase gene from *Thermomyces lanuginosus* strain SSBP was used to transform *Arxula adeninivorans* strain LS3. This *XynA* gene was expressed under control of the strong, constitutive *Arxula*-derived *TEF1* promoter and integrated in the 25S rDNA locus. The plasmid copy number integrated into the rDNA locus was unknown. Little to no activity was found with these *A. adeninivorans* LS3 transformants, however, namely only 5.86 nkat ml^{-1} (0.35 U ml^{-1}) compared to the 4 418 nkat ml^{-1} (265 U ml^{-1}) obtained with the *T. lanuginosus* strain SSBP positive control. The protein itself might have been defective and since it was not secreted but accumulated intracellularly, this could also have resulted in the diminished activity observed.

1 Introduction

The main polysaccharide-containing renewable resources in nature are plant cell walls, which are composed of three major polymeric constituents: cellulose, hemicellulose and lignin (Biely 1993). Xylan, an abundant type of hemicellulose, consists of a backbone of β-D-1,4-linked xylopyranoside residues that can be substituted with acetyl, glucoronosyl and arabinosyl side chains (La Grange *et al*. 2001). It is second only to cellulose in natural abundance and represents a major carbon reserve in the environment (La Grange *et al.* 1996).

Xylan degradation is a process involving various enzymatic activities (Biely 1985). The most important enzyme is endo-β-1,4-xylanase (EC 3.2.1.8) that initiates the hydrolysis of xylan into xylo-oligosaccharides and β-D-xylosidase (EC 3.2.1.3.7) that hydrolyses xylo-oligosaccharides, although other debranching enzymes are thought to play a synergistic role in the hydrolysis of the side chain groups of xylan (Biely 1993; Ximenes *et al*. 1996). In recent years, interest in thermostable enzymes has increased

dramatically as resistance to thermal inactivation has become a desirable property of the enzymes used in many industrial applications such as use in animal feed, the pulp and paper and baking industries (Christopher *et al.* 2005; Damaso *et al*. 2003; Singh *et al*. 2000b).

A variety of bacteria, yeasts and filamentous fungi have the ability to degrade xylan by producing a range of enzymes (Sunna *et al.* 1997). The thermophilic deuteromycete fungus *Thermomyces lanuginosus* is one of the best xylanase producers yet reported (Singh *et al*. 2000a). Strains of *T. lanuginosus* thrive at temperatures of up to 60°C and are capable of producing a high activity of cellulase-free xylanase. In addition to its thermostability, the xylanase from this fungus is also active over a wide pH range (Singh *et al*. 2000c). To facilitate industrial thermophilic xylanase production, heterologous expression systems could be used in the production of large scale protein (Damaso *et al.* 2003).

The yeast expression systems offer a broader range of potential applications than bacterial expression systems. As a unicellular microorganism, yeast retains the advantages of bacterial systems of ease of manipulation and large scale, high density cultivation, while its eukaryotic sub-cellular organization is capable of post-translational processing and the modification of many heterologous proteins (Romanos *et al*. 1992*)*.

Arxula adeninivorans has been investigated as a host for the expression of heterologous proteins of biotechnological interest. The transformation system was developed by Rösel *et al.* (1998) is based on the integration of heterologous DNA into ribosomal DNA (rDNA). In this transformation system, selection is based on hygromycin B resistance conferred by the *Escherichia coli*-derived *hph* gene under control of the strong constitutive *Arxula TEF1* promotor. The vector also contains a 25S rDNA sequence for rDNA targeting. Linearization is required for high transformation frequencies. This vector has been successfully transformed into wild-type *A. adeninivorans* strains as well as mutant strains (Rösel *et al.* 1998; Wartmann *et al.* 2002). This chapter describes the cloning and expression of the *XynA* gene from *T. lanuginosus* strain SSBP in *A. adeninivorans* strain LS3.

2 Materials and Methods

2.1 Strains

Thermomyces lanuginosus (SSBP) was kindly supplied by Prof. S. Singh (Durban University of Technology, Durban) and *Arxula adeninivorans* strain LS3 was obtained from Prof. G. Kunze (Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany). *Eschericia coli* (XL-10 Gold) cells were obtained from Strategene.

2.2 Plasmid cloning vector

Plasmid pAL HPH1-TEF-XynA-PHO5 was kindly supplied by Prof. G. Kunze and the modified plasmid pAL HPH1-TEF-XynA-PHO5 by Prof. J. Albertyn (University of the Free State). The plasmid shown in Fig. 2.1 was linearised with the restriction enzyme *Bgl*II and integrated into the 25S rDNA locus of *A. adeninivorans* LS3.

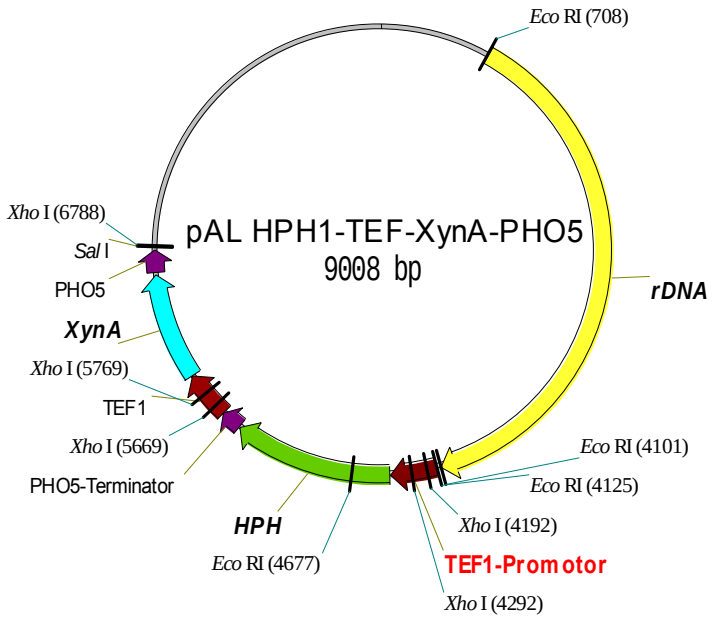


Figure 2.1 Physical map of pAL HPH1-TEF-XynA-PHO5 vector for the *A. adeninivorans*-based expression platform. The vector pAL-HPH1 contained the following elements: a 25S rDNA sequence (rDNA) chromosomal targeting, expression cassette for the *E. coli*-derived *hph* gene in the order *A. adeninivorans*-derived TEF1-promoter, the *hph*-coding sequence (HPH) and the *S. cerevisiae*-derived *PHO5*-terminator. The vector further contain unique *Sal*I restriction site for the insertion of the expression cassette containing the xylanase gene, *XYNA*.

2.3 Primers and restriction enzymes

Primers and modifying enzymes were purchased from Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) and used according to the conditions recommended by the suppliers, unless otherwise stated.

2.4 Growth conditions

Escherichia coli (XL-10 Gold) cells were grown in Luria-Bertani (LB) broth containing (per litre) 5 g yeast extract, 10 g tryptone, 10 g NaCl and subsequently incubated at 200 rpm on a rotary shaker at 37°C in the presence of an ampicilin concentration of 10 mg ml^{-1}.

Yeast cultures were maintained on YPD agar plates containing (per litre) 10 g yeast extract, 20 g peptone, 20 g glucose and 20 g agar at pH 5. To select for positive transformants, YPD agar was supplemented with hygromycin B to the concentration of 250 µg ml^{-1}. The inoculum was prepared by inoculating each of 250 ml erlynmeyer flasks containing 50 ml of YPD broth with positive transformed *A. adeninivorans* strain LS3 cells. These flasks were incubated at 200 rpm on a rotary shaker at 45°C for 16 h. Shake flasks cultures were prepared by inoculating each of 250 ml Erlenmeyer flasks containing 100 ml of the appropriate medium to approximately 0.5 absorbance units at 690 nm and incubated as above.

T. lanuginosus strain SSBP were grown at 50°C on potato dextrose agar (Merck Biolab diagnostics (Pty) Ltd, South Africa). The culture medium contained (per litre) 15 g oat spelts xylan (Sigma Chemical Co., St. Louis, MO, USA), 15 g yeast extract and 5 g KH_2PO_4 at pH 6.5. An agar block (1 cm^2) of an actively growing 5 day old culture was used to inoculate 100 ml growth medium in 250 ml Erlynmeyer flasks. These flasks were incubated at 200 rpm on a rotary shaker at 50°C for 5 days and the culture supernatant assayed for enzyme activity.

2.5 Recombinant DNA techniques

2.5.1 Propagation of plasmid DNA in *E. coli*

DNA was transformed according to the method described by Sambrook *et al*. (1989). *E. coli* (XL-10 Gold) competent cells were thawed on ice. A 5 µl volume of plasmid DNA

was added to 80 µl of competent cells and incubated on ice for 20 min. This mixture was subjected to heat shock at 42°C for 35 s. An 800 µl volume of LB broth (5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 10 g l^{-1} NaCl) containing 50 µl $MgCl_2$ (2 g l^{-1}) and 100 µl glucose (1 g l^{-1}) were added, the mixture incubated for 45 min at 37°C on a rotary shaker and subsequently centrifuged for 2 min at 16 100 x *g* and the supernatant removed. The pellet was dissolved in 100 µl of LB broth and plated on LB plates supplemented with ampicillin (60 mg l^{-1}), X-gal [5-bromo-4-chloro-3- indolyl-β-D-galactoside (40 mg l^{-1})] and IPTG [isopropylthio-β-D-galactoside (10 mg l^{-1})] and incubated overnight at 37°C.

2.5.2 Small scale plasmid isolation

Positive *E. coli* transformants, growing as single white colonies, were inoculated into 5 ml LB broth supplemented with ampicillin (10 mg ml^{-1}) and grown overnight at 37°C on a rotary shaker. Cells were harvested from LB broth by centrifugation at 16 100 x *g* for 30 s and the supernatant carefully aspirated. The pellet was resuspended in 400 µl sterile STET-buffer [0.1 mg l^{-1} NaCl, 5% Triton X-100, 10 mg l^{-1} Tris-HCl (pH 8), 1 mg l^{-1} EDTA (pH 8)]. A 25 µl volume of lysozyme (10 mg ml^{-1}; Roche) was added, the samples vortexed for 3 s and transferred to a boiling water bath for 40 s. The samples were centrifuged for 10 min at 16 100 x *g* and the cellular debris removed with a sterile toothpick. A 40 µl volume of 2.5 g l^{-1} sodium acetate (pH 5.2) and 420 µl of isopropanol were added to the supernatant, vortexed and left at room temperature for 5 min to precipitate. The samples were then centrifuged at 16 100 x *g* at 4°C for 2 min, the pellet washed with 70% cold ethanol and again centrifuged for 2 min as before. The supernatant was removed by aspiration and dried under vacuum. The pellet was resuspended in 50 µl of TE buffer (10 mg l^{-1} Tris-HCl, 1 mg l^{-1} EDTA, pH 8.0) supplemented with RNase (50 µg ml^{-1}).

2.5.3 Genomic DNA isolation from yeast cultures

The yeast cells were harvested from YPD medium by centrifugation for 1 min at 16 100 x *g*. The cells were resuspended in 500 µl cell lysis buffer (100 mg l^{-1} Tris-HCl (pH 8), 50 mg l^{-1} EDTA, 1% SDS). A 200 µl volume of glass beads (425 – 600 µm in diameter) was added to the cell suspension and vortexed for 4 min followed by immediate cooling on ice. The liquid phase was removed and 275 µl of ammonium acetate solution (7 g l^{-1};

pH 7) was added to the mixture, vortexed and incubated for 5 min at 65°C followed by 5 min incubation of ice. A 500 µl volume of chloroform was added, the sample vortexed and then centrifuged for 2 min at 16 100 x *g* at 4°C. The DNA was precipitated at -20°C for 30 min by the addition of 750 µl isopropanol. The sample was again centrifuged as above, the pellet washed with 70% cold ethanol and re-centrifuged. The supernatant was aspirated, the pellet dried under vacuum and subsequently resuspended in 100 µl of TE buffer supplemented with RNase (50 µg ml^{-1}).

2.5.4 Amplification of the XynA gene

Amplification of the double-strand DNA was done in a 50 µl reaction volume that consisted of 5 µl PCR buffer (containing 15 mg l^{-1} $MgCl_2$), 0.2 mg l^{-1} each of dCTP, dATP, dGTP, dTTP, 2 pmol each of *XynA*-1F (5'-AAG GAT CCA TGG TCG GCT TTA CCC CCG TTG-3') and *XynA*-1R (5'-AGA GTC GAC TTA GCC CAC GTC AGC AAC GGT C-3') primers (with the respective restriction enzyme sites for *Bam*HI and *Sal*I underlined), 5 µl of genomic DNA isolated from yeast cultures, 5 U Taq polymerase (Roche) and 37 µl nuclease free water. These reaction mixtures were subjected to denaturation, annealing and elongation for 30 s at 94°C, 1 min at 60°C and 2 min at 68°C, respectively, for 40 cycles. The reaction mixture was then maintained at 68°C for another 7 min to complete elongation. The amplified fragment was subsequently subjected to agarose gel electrophoresis.

2.6 Preparation of competent cells and DNA transformation

A. adeninivorans LS3 competent cells were prepared according to Rösel *et al.* (1998). The inoculum was prepared as mentioned in section 2.4. Shake flasks were inoculated to approximately 0.5 absorbance units at 690 nm in YPD medium and incubated at 30°C on a rotary shaker at 200 rpm overnight (under these conditions the wild type strain LS3 forms budding cells) until a cell concentration of about 10^8 cells ml^{-1} was reached, harvested by centrifugation and washed with water. The pellet was subsequently resuspended in 25 ml BICINE buffer I (1 g l^{-1} sorbitol, 10 mg l^{-1} BICINE-NaOH, pH 8.35, 3% PEG 1000, 5% DMSO), centrifuged and suspended in a 1 ml of the same buffer. Aliquots of 200 µl each of the competent cell suspension were transferred into sterile tubes and frozen at -80°C.

2.7 Yeast transformation

A. adeninivorans was transformed according to Rösel *et al.* (1998). A 1-3 µg amount of linearized plasmid DNA (pAL-HPH-TEF-XynA-PHO5-*Bgl*II) was pipetted onto frozen competent cells and mixed on a vortex mixer for 5 min at 37°C, 1 ml BICINE buffer II (40% PEG 1000, 0.2 g l^{-1} BICINE-NaOH, pH 8.35) added and the cells incubated at 37°C for 60 min. Thereafter, cells were centrifuged at 16 100 x *g* at 20°C for 5 min and washed with 1.5 ml BICINE buffer III (0.15 mg l^{-1} NaCl, 10 mg l^{-1} BICINE-NaOH, pH 8.35) and resuspended in 100 µl of the same buffer. Cells were plated on YEPD agar supplemented with hygromycin B (250 µg ml^{-1}) and incubated at 30°C for 2-4 days. The colonies obtained were then grown in 3 ml YPD medium at 30°C for 1 day.

2.8 Extraction of enzyme

The *XynA* gene contained no signal peptide and thus was expressed intracellularly. To extract the enzyme, the cells were incubated for 16 h at 45°C in 100 ml YPD medium, centrifuged at 14 000 x *g* and washed in phosphate buffer (pH 7.5) containing protease inhibitor (complete, EDTA-free protease inhibitor cocktail tablets, Roche) and Y-PER® yeast protein extraction reagent (PIERCE) added according to the manufacturer's recommendations. Following a 20 min incubation period at room temperature, 200 µl glass beads were added to the cell suspension and vortexed for 10 min in 30 s periods with cooling on ice in between. The suspension was then centrifuged for 20 min at 16 100 x *g* to pellet all the cell debris and glass beads and the supernatant subjected to the xylanase assay.

2.9 β-Xylanase assay

The endo-1,4-β-xylanase activity was assayed according to Bailey *et al*. (1992). Activity was determined by incubating 200 µl of enzyme solution at pH 6.5 and 70°C for 5 min in 1.8 ml of substrate solution comprising 0.1 g l^{-1} birchwood xylan (Sigma) in 0.05 g l^{-1} phosphate buffer, pH 6.5. Reducing sugars were assayed by the addition of 3 ml dinitrosalicylic acid (DNS) reagent (containing per litre: 16 g NaOH, 10 g dinitrosalycylic acid and 300 g potassium sodium tartrate tetrahydrate crystals), boiling for 5 min,

cooling, and measuring the absorbance at 540 nm against the reagent blank. Reagent blanks were prepared as above but using 200 µl of phosphate buffer instead of enzyme solution. Enzyme blanks for the correction of absorbance by background colour were prepared as above but with the 200 µl enzyme solution as the last addition. Absorbance values at 540 nm of enzyme blanks were subtracted from the absorbance values of samples. As control, the untransformed *A. adeninivorans* strain LS3 was subjected to the same conditions as transformed clones. A standard curve was constructed with D-xylose (Sigma) as standard. One nkat ml^{-1} of enzyme activity is defined as the formation of 1 nmol product (xylose) produced per second per millilitre of enzyme used.

3 Results

3.1 Transformation of *A. adeninivorans* LS3

Plasmid pAL HPH1-TEF-XynA-PHO5, linearised with the restriction enzyme *Bgl*II, was used to transform *A. adeninivorans* strain LS3 by integration in the 25S rDNA locus. Genomic DNA was then isolated from positive clones obtained from YPD agar plates supplemented with hygromycin B, using the technique described in section 2.8. Following genomic DNA isolation, vector integration was confirmed with an 850 bp band obtained after PCR (Fig. 2.2).

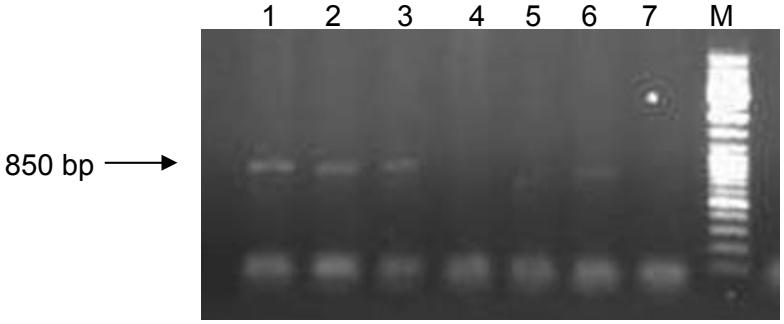


Figure 2.2 Agarose gel electrophoresis of the amplified fragment (850 bp) containing the *XynA* gene of *T. lanuginosus* SSBP. Lane M: λ phage DNA digested with *Eco*R and *Hind*III. Lanes 1, 2, 3, 5 and 6: fragments from positive *A. adeninivorans* clones containing the 850 bp PCR product. Lane 7: untransformed *A. adeninivorans* strain LS3 as negative control.

3.2 Screening for β-xylanase activity

Xylanase activity was assayed according to the method described by Bailey *et al.* (1992). The gene was only expressed intracellularly because no secretion signal was attached to the gene on the plasmid that was available at that time for the use in this study. Initially the transformants exhibited no activity thus the transformation procedure was repeated, doubling the quantity of plasmid DNA used during transformation. To determine activity; cells were grown until exponential phase, harvested and the enzyme extracted with Y-PER and glass beads. Breaking of the cells was more effective when done on exponentially growing cells than on cells already in the stationary growth phase. This could be due to the fact that the cell wall thickens when the cell enters the stationary growth phase (Werner-Washburne *et al.* 1993; Herman 2002). Cell breakage was monitored microscopically and the Y-PER/glass bead method proved to be the most effective method for the breaking of cells.

The *XynA* gene in the plasmid was under the control of the *A. adeninivorans TEF1* constitutive promoter, allowing constitutive expression without the use of an inducer. As control for the production of functional enzyme, the endo-β-xylanase activity of *T. lanuginosus* strain SSBP was also determined. The xylanase activity obtained with this *Thermomyces* strain was very low; therefore, the assay was modified by increasing the incubation temperature to 70°C instead of the 50°C stated in the method of Bailey *et al.* (1992) and the reaction pH increased from pH 5 to pH 6.5. These where the optimal temperature and pH values for activity of the *T. lanuginosus* xylanase (Singh *et al.,* 2000a). *T. lanuginosus* gave an activity of up to 4 418 nkat ml^{-1}, whereas the two positive yeast clones selected only reached an activity of 5.86 nkat ml^{-1} (Table 2.1). Clones were cultivated at 39°C as well as at 45°C and similar activities were obtained at both temperatures.

4 Discussion

The *XynA* gene was expressed in *Arxula adeninivorans* LS3 under control of the strong constitutive *Arxula* derived *TEF1* promoter and integrated in the 25S rDNA locus. The rDNA locus is an attractive target for multiple integration since it can contain up to 100 –

Table 2.1 Xylanase activity of *A. adeninivorans* transformants and *T. lanuginosus* shake flask cultures at 45°C after 16 h and 5 days, respectively.

Strains	Activity (nkat ml^{-1})	Activity (Units ml^{-1})
T. lanuginosus (SSBP)	4 418	265
A. adeninivorans LS3 (1)[a]	5.86	0.35
A. adeninivorans LS3 (2)[b]	5.43	0.33
A. adeninivorans LS3 (WT)	1.24	0.07

[a] Fig. 2.2 lane 1.
[b] Fig. 2.2 lane 2.

200 tandemly repeated units (Lopes *et al.* 1989; Kondo *et al.* 1995). Integration in the rDNA locus has been successfully employed in a wide range of species including *A. adeninivorans* strain LS3 (Klabunde *et al*. 2003; Terentiev *et al*. 2003; Wartmann *et al*. 2002). This allows for stable integration and the *TEF1* promoter allows for expression even under a low copy number integration (Terentiev *et al.* 2003). However, little to no activity was found with the *A. adeninivorans* strain LS3 transformants investigated here.

Heterologous xylanase genes have been successfully expressed in a number of microorganisms, including the *XynA* gene from *Thermomyces lanuginosus* in *Pichia pastoris* under the control of the *AOX1* promoter (Damaso *et al.* 2003). A xylanase activity of up to 5 000 nkat ml^{-1} was achieved in this host using methanol induction (Damaso *et al.* 2003). Furthermore, *T. lanuginosus* strain SSBP has been found to produce a β-xylanase activity of up to 59 600 nkat ml^{-1} when cultivated in a medium containing ground corn cobs as inducer (Singh *et al.* 2000b).

There are a number of factors that might have contributed to the low xylanase activity in *A. adeninivorans* strain LS3. Because the plasmid was integrated into the 25S rDNA, it is uncertain how many copies were integrated in this locus. The protein itself might have been defective. The low activity observed could also possibly have been due to the fact that the protein was intracellularly produced due to the absence of a secretion signal. This problem needs to be further investigated to determine whether the plasmid was

defective and also to optimize protein production in *A. adeninivorans* strain LS3. Further investigation should also be done to determine whether enzyme production is increased during bioreactor cultivation under controlled conditions.

5 References

Bailey M. J., Biely P. and Poutanen K. (1992) Interlaboratory testing of methods for assay of xylanase activity. J. Biochem. **23**: 257-270.

Biely P. (1985) Microbial xylanolytic systems. Trends Biotechnol. **3**: 286-290.

Biely P. (1993) Enzymological aspects of the production of microbial hemicellulases *In*: Hemicellulose and hemicellulases, M. P. Coughlan and G. P. Hazlewood (Eds). London, Portland Press. pp. 29-51.

Christopher L., Bissoon S., Singh S., Szendefy J. and Szakacs G. (2005) Bleach-enhancing abilities of *Thermomyces lanuginosus* xylanases produced by solid state fermentation. Process Biochem. **40**: 3230-3235.

Damaso M. C. T., Almeida M. S., Kurtenbach E., Martins O. B., Pereira N., Andrade C. M. M. C. and Albano R. M. (2003). Optimized expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*. Appl. Environ. Microbiol. **69**: 60 64-6072.

Herman P. K. (2002) Stationary phase in yeast. Curr. Opp. Microbiol. **5**: 602-607.

Klabunde J., Kunze G., Gellissen G. and Hollenberg C. P. (2003) Integration of heterologous genes in several yeast species using vectors containing a *Hansenula polymopha*-derived rDNA-targeting element. FEMS Yeast Res. **4**: 185-193.

Kondo K., Saito T., Kajiwara S., Takagi M. and Misawa N. (1995) A transformation system for the yeast *Candida utilis*: use of a modified endogenous ribosomal protein gene as a drug-resistant marker and ribosomal DNA as an integration target for vector DNA. J. Bacteriol. **177**: 7171-7177.

La Grange D. C., Pretorius I. S. and van Zyl W. H. (1996) Expression of a *Trichoderma reesei* β-xylosidase gene (*XYN2*) in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **62**: 1036-1044.

La Grange D. C., Pretorius I. S., Claeyssens M. and van Zyl W. H. (2001) Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* β-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. Appl. Environ. Microbiol. **67**: 5512-5519.

Lopes T. S., Klootwijk J., Veenstra A. E. van der Aar P. C., van Heerikhuizen H., Raue H. A. and Planta R. J. (1989) High-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*: a new vector for high-level expression. Gene **79**: 199-206.

Romanos M. A., Scorer C. A. and Clare J. J. (1992) Foreign gene expression in yeast: a review. Yeast **8**: 423-488.

Rösel H. and Kunze G. (1998) Integrative transformation of the dimorphic yeast *Arxula adeninivorans* LS3 based on hygromycin B resistance. Curr. Genet. **33**: 157-163.

Sambrook J., Fritsch E. F. and Maniatis T. (1989) Molecular cloning. A laboratory manual Vol. 1 (2nd ed.). Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York. pp. 1.80-1.81.

Singh S., Pillay B. and Prior B. A. (2000a) Thermal stability of β-xylanase produced by different *Thermomyces lanuginosus* strains. Enzyme Microbial Technol. **26**: 502-508.

Singh S., Pillay B., Dilsook V. and Prior B. A. (2000b) Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. J. Appl. Microbiol. **88**: 975-982.

Singh S., Reddy P., Haarhoff J., Biely P., Janse B., Pillay B., Pillay D. and Prior B. A. (2000c) Relatedness of *Thermomyces lanuginosus* strains producing a thermostable xylanase. J. Biotechnol. **81**: 119-128.

Sunna A. and Antranikian G. (1997) Xylanolytic enzymes from fungi and bacteria. Crit. Rev. Biotechnol. 17**: 39-67.**

Terentiev Y., Gellissen G. and Kunze G. (2003) *Arxula adeninivorans* – A non-conventional dimorphic yeast of great biotechnological potential. Recent Res. Devel. Applied Microbiol. Biotechnol. **1**: 135-145.

Wartmann T., Böer E., Pico A. H., Sieber H., Bartelsen O., Gellissen G. and Kunze G. (2002) High-level production and secretion of recombinant proteins by the dimorphic yeast *Arxula adeninivorans*. FEMS Yeast Res. **2**: 363-369.

Werner-Washburn., Braun E., Johnston G. C. and Singer R. A. (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*. Bicrobiol. Rev. **57**: 383-401.

Ximenes F. A., Silveira F. Q. P. and Filho E. X F. (1996) Production of β-xylosidase activity by *Trichoderma harzianum* strains. Curr. Microbiol. **33**: 71-77.

Chapter 3

Effect of temperature on the specific growth rate and morphology of *Arxula adeninivorans* strains

Abstract

Dimorphism in *Arxula adeninivorans* strain LS3 is known to be temperature dependent, a trait apparently not shared with the other strains of this species. In accordance with the literature, in shake flask cultures the morphology of LS3 changed with an increase in temperature from predominantly budding cells at 30°C to pseudomycelia at 42°C and resembled a true mycelial culture at 45°C. By contrast, the morphology of strain G1211 (*LEU2*$^+$), which is the auxotroph derived from strain LS3 by transformation with the plasmid pAL-ALEU2m, remained pseudomycelial at 45°C.

Cultivation in a temperature gradient incubator revealed a small but significant difference in the maximum specific growth rates (μ_{max}) of strains LS3 and G1211 (*LEU2*$^+$), namely 0.48 and 0.52 h^{-1}, respectively. The minimum and maximum temperatures were 18 and 46°C, respectively, with the optimum temperature in the range of 37 to 39°C. Thus, genetic transformation exerted a small effect on the specific growth rate and morphology of *A. adeninivorans* LS3.

1 Introduction

The dimorphic yeast *Arxula adeninivorans* is able to grow at cultivation temperatures of up to 48°C (Wartmann *et al.* 1995). However, no detailed report on the cardinal temperatures of *A. adeninivorans* is available in the literature. Dimorphism in *A. adeninivorans* strain LS3 is known to be temperature dependent (Wartmann *et al.* 1995). At a cultivation temperature of 30°C it grows as budding yeast cells, whereas at 42°C pseudomycelia are formed and true mycelia appeared at a cultivation temperature of 45°C (Wartmann *et al.* 1995). This trait is more pronounced in strain LS3 than in the other strains of *A. adeninivorans* (Böer *et al.* 1994).

Because genetic transformation might have an affect on the yeast physiology, a comparative investigation of the growth characteristics of strains LS3 and

G1211 (*LEU2*$^+$) was conducted. *A. adeninivorans* strain G1211 (*LEU2*$^-$) is an auxotrophic mutant strain (*aleu2* mutant) obtained by mutagenesis of strain LS3 with nitrosoguanidine. Strain G1211 (*LEU2*$^+$), obtained from strain G1211 (*LEU2*$^-$) by transformation with an empty plasmid pAL-ALEU2m, was used to determine whether the transformation resulted in apparent physiological changes. Stain G1211 (*LEU2*$^+$) was used as it has been used for a wide range of heterologous gene expressions and was the only strain available for this study. It was kindly provided by Prof. G. Kunze of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany.

In this chapter, the temperature profiles of the above two strains of *A. adeninivorans* were determined to confirm the temperature requirements. Furthermore, since *A. adeninivorans* strain Y1224T required the addition of thiamine and biotin in the culture medium for growth (Kurtzman *et al.* 1998), the growth of strain LS3 in a culture medium devoid of growth factors was compared to its growth in a medium containing yeast extract to determine whether this strain had a similar growth factor requirement.

2 Materials and Methods

2.1 Yeasts strains

Arxula adeninivorans strain LS3 was obtained from the MIRCEN yeast culture collection of the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, and strain G1211 (*LEU2*$^+$) was kindly provided by Prof. G. Kunze of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. The cultures were routinely maintained on YPD slants, stored at 4°C and sub-cultured every 6 months.

2.2 Inoculum and culture medium

A. adeninivorans was routinely grown at 30°C on YPD agar plates containing (per litre) 10 g yeast extract, 20 g peptone, 20 g glucose and 20 g agar at pH 5. The minimal medium contained (per litre) 0.25 g citric acid, 5.5 g $(NH_4)_2SO_4$, 10 g KH_2PO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 20 g glucose and was supplemented with 0.1 ml of a trace element stock solution containing (per litre) 0.035 g $FeSO_4.7H_2O$; 0.007g

$MnSO_4.H_2O$ or 0.01 g $MnSO_4.5H_2O$; 0.011 g $ZnSO_4.7H_2O$; 0.001 $CuSO_4.5H_2O$; 0.002 g $CoCl.6H_2O$; 0.0013 g $Na_2MoO_4.2H_2O$; 0.00035 g KI; 0.002 g H_3BO_3; 0.0016 g $Al_2(SO_4)_3.18H_2O$ and 1 ml of a vitamin stock solution containing (per litre) 0.5 g biotin and 1 g thiamine HCl. The biotin was first dissolved in 20 ml of a solution of 0.1 mol NaOH l^{-1} and made up to 1 l with distilled water. Before adding thiamine, the pH was adjusted to 6.5 and subsequently re-adjusted to pH 6.5 (Schulze 1995).

The inoculum was prepared by inoculating each of 250 ml Erlenmeyer flasks containing 100 ml of the appropriate medium with cells from 24 h YPD agar plates. These flasks were incubated at 200 rpm on a rotary shaker at 30°C for 16 h.

2.3 Shake flask cultivation

The growth of *Arxula adeninivorans* strain LS3 was compared using two different media, namely YPD broth and a minimal medium without yeast extract. Shake flasks were inoculated to approximately 0.5 absorbance units at 690 nm and incubated at 30°C on a rotary shaker at 200 rpm. The inoculum was grown in the corresponding medium.

The vitamin dependence of strain LS3 was tested by comparing its growth in minimal medium devoid of vitamins to that in minimal medium containing the vitamins biotin and thiamine. The inoculum was prepared as above and used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of the appropriate medium that were subsequently incubated at 30°C on a rotary shaker at 200 rpm.

2.4 Determination of the cardinal temperatures

The influence of temperature on the growth rate of *A. adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) was determined by growing the yeasts in a temperature gradient incubator (Scientific Instruments, Inc., New York, USA) consisting of an aluminium bar that was cooled at one end and heated at the other to obtain a stable temperature gradient. The bar contained thirty equidistant sample wells on both sides into which L-shaped tubes of optically selected glass (40 ml total volume and 17 mm in diameter) were inserted and capped with loose-fitting metal caps. Whereas one side of the bar was loaded with tubes containing yeast cultures, the corresponding tubes on the other

side contained only water to allow frequent monitoring of the temperature. A built-in shaker, rocking the bar through a 30° arc at 44 oscillations min^{-1}, provided mixing of the culture as well as aeration. Sterile culture tubes, each containing 10 ml YPD broth, were equilibrated in the sample wells for 24 h. Each tube was subsequently inoculated with 0.1 ml from a 16 h shake flask culture of strain LS3 or G1211 (*LEU2*$^{+}$) grown in YPD at 30°C. Growth was monitored turbidimetrically using a WPA CO8000 Cell Density Meter (WPA, Cambridge, UK) at 600 nm by removing tubes sequentially at 2 min intervals without stopping the shaker. The maximum specific growth rate (μ_{max}) was calculated from the exponential growth curve where ln values of the absorbance readings were plotted as a function of time. The Arrhenius equation

$$\mu_{\max} = Ae^{-\left(\frac{E_a}{RT}\right)} \quad (1)$$

was used to obtain a linear relationship between growth rate and temperature, where A is an entropy constant, E_a the temperature coefficient (also known as the activation energy), R the universal gas constant (8.314 J $mol^{-1}K^{-1}$) and T the absolute temperature in K. On taking natural logarithms, equation 1 becomes

$$\ln \mu = \ln A - \left(\frac{E_a}{R}\right)\frac{1}{T} \quad (2)$$

The value of E_a was thus derived from the slope of the plot of ln μ versus 1/T.

Statistical analysis was done using an unpaired t-test with a 95% confidence interval that was calculated using GraphPad InStat version 3.05 (GraphPad Software Inc., San Diego, CA, USA).

The influence of temperature on the morphology was investigated by examining the growth at 30, 42 and 45°C in shake flasks using a light microscope (Zeiss Axioplan, Colorview soft imaging system, Germany).

3 Results

3.1 Growth factor requirements of *Arxula adeninivorans* LS3

The influence of the culture medium composition on the maximum specific growth rate was determined in duplicate by comparing the growth of *A. adeninivorans* strain LS3 in YPD broth and in minimal medium which contained biotin and thiamine (Fig. 3.1). The highest μ_{max} value of 0.49 h^{-1} (±0.02 h^{-1}) was obtained in YPD broth, whereas the μ_{max} value in the minimal medium was 0.46 h^{-1} (±0.05 h^{-1}). These values, representing the mean values of duplicate experiments, indicated only a slight difference between the two culture media; therefore, all subsequent shake flask and bioreactor experiments were performed using the minimal medium.

The dependence of *A. adeninivorans* strain LS3 on vitamins was determined by comparing growth in minimal medium with and without vitamins (Fig. 3.2). Strain LS3 grew slower on agar plates containing minimal medium devoid of vitamins than on the YPD agar plates. When these cells where used to inoculate shake flasks containing minimal medium without the two vitamins, no growth was observed. Therefore, the inoculum was subsequently prepared by using cells grown on YPD agar plates. The inoculum prepared in this manner was capable of growth in the minimal medium devoid of vitamins. The mean μ_{max} value of 0.43 h^{-1} (±0.005 h^{-1}) recorded in triplicate experiments, using minimal medium supplemented with vitamins was by far greater than the value of 0.11 h^{-1} (±0.06) in minimal medium without vitamins. Furthermore, strain LS3 stopped growing after only 6 h of cultivation. The initial growth could have been due to the carry over of vitamins from the agar plates used for preparation of the inoculum. When investigating growth in a medium containing only one of the above two vitamins, μ_{max} values of 0.40 h^{-1}(±0.005 h^{-1}) and 0.43 h^{-1} (±0.008 h^{-1}) were obtained for growth with biotin and thiamine, respectively. This suggested that biotin could substitute for thiamine. There was no significant difference in the specific growth rate of strain LS3 grown in a medium containing both biotin and thiamine and in a medium containing only one of the two vitamins. This is a phenomenon that can not presently be explained.

3.2 Effect of temperature on the specific growth rate

The temperature profiles of *A. adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) are shown in Figs. 3.3A and B. With an increase in temperature up to 39°C the growth rate increased, but on further increase the growth rate rapidly declined. The optimum temperature of both strains was in the range of 37 to 40°C, where the highest specific growth rate recorded for strain G1211 (*LEU2*$^+$) ranged from 0.46 to 0.52 h^{-1} with a mean value of 0.49 h^{-1} (±0.027 h^{-1}) and for strain LS3, ranged from 0.4 to 0.47 h^{-1} with a mean value of 0.43 h^{-1} (±0.025 h^{-1}) (Figs. 3.3A and B). Statistical analyses were performed to see whether the difference between the growth rates was significant. The P value of 0.0036 was very significant. The highest temperature where growth was recorded was 46°C in the case of both strains, whereas both strains failed to grow at or above 47°C, and the lowest temperature where growth was recorded was 18°C.

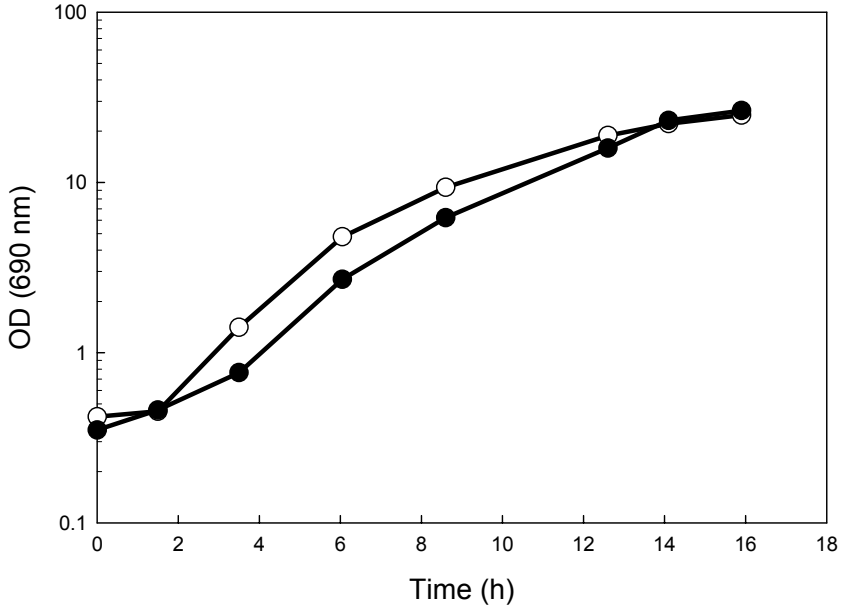


Figure 3.1 Typical Growth curves of *A. adeninivorans* strain LS3 in shake flasks at 30°C using YPD broth (○) and minimal medium (●). The μ_{max} values were calculated over the intervals 2-6 h and 4-8 h for the YPD broth and minimal medium, respectively.

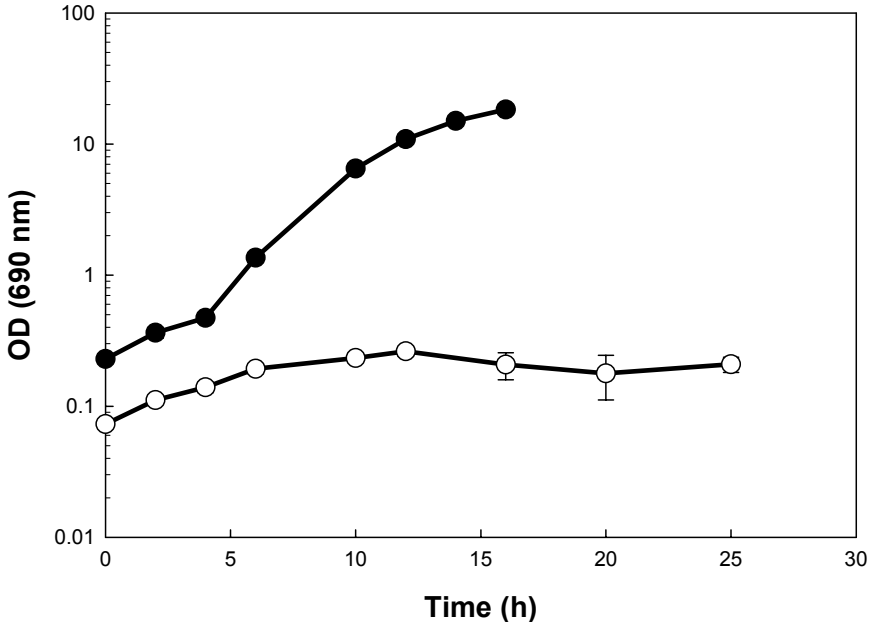


Figure 3.2 Typical growth curves of *A. adeninivorans* strain LS3 in shake flasks at 30°C using minimal medium (●) and minimal medium without vitamins (○). The mean values of three experiments are shown with error bars indicating the standard deviation of the mean. The μ_{max} values were calculated over the intervals 5-10 h and 0-6 h for the minimal medium and minimal medium without vitamins, respectively.

A high degree of scatter of the growth rate values was observed at temperatures higher than 20°C, which increased with an increase in incubation temperature, even though the incubation temperature in this range fluctuated by less than 0.3°C during the experiment. The Arrhenius model describes the relationship between the rate of a chemical reaction and the reaction temperature. This model is also used to describe the effect of temperature on the specific growth rate of an organism (Pirt 1975). Arrhenius transformations of the temperature profiles are shown in Figs 3.4A and B. The temperature coefficients (E_a), determined by linear regression of the slopes of the regions denoted as a - b, c – d and e – f, are shown in Table 3.1. Two distinct inflection points were observed with both strains at point's c and d. From these temperature coefficients it was clear that both strains were more sensitive to a change in temperature in the lower temperature range than within the range of about 22 to 39°C. The entropy constant (A) was also determined (Table 3.1), as this value together with the E_a value facilitated modelling of the maximum specific growth rate as a function of the incubation temperature.

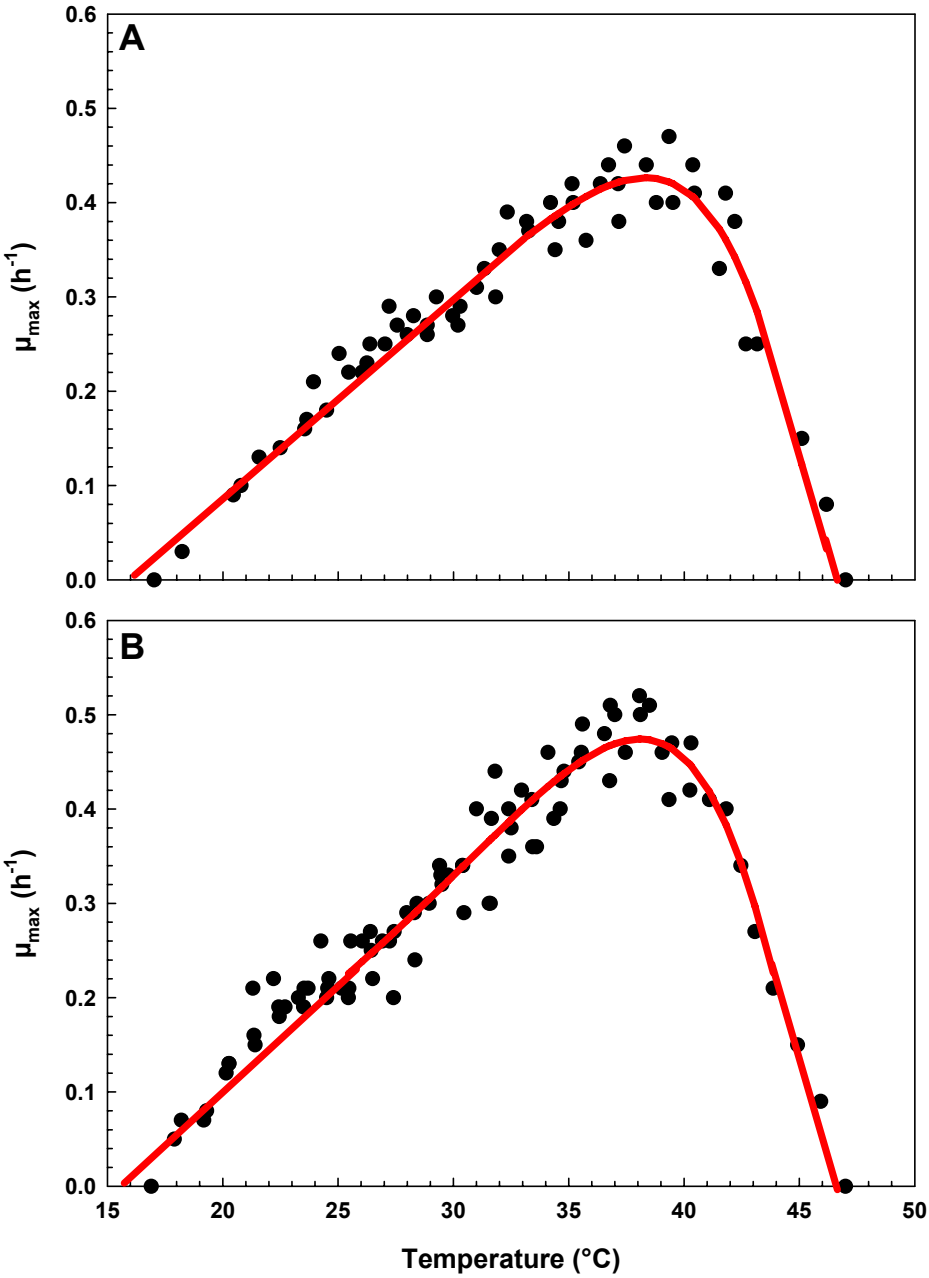


Figure 3.3 Temperature profiles of *A. adeninivorans* strains LS3 (A) and G1211 (*LEU2*$^+$) (B) grown in YPD broth in a temperature gradient incubator. The data points represent four experiments with each strain.

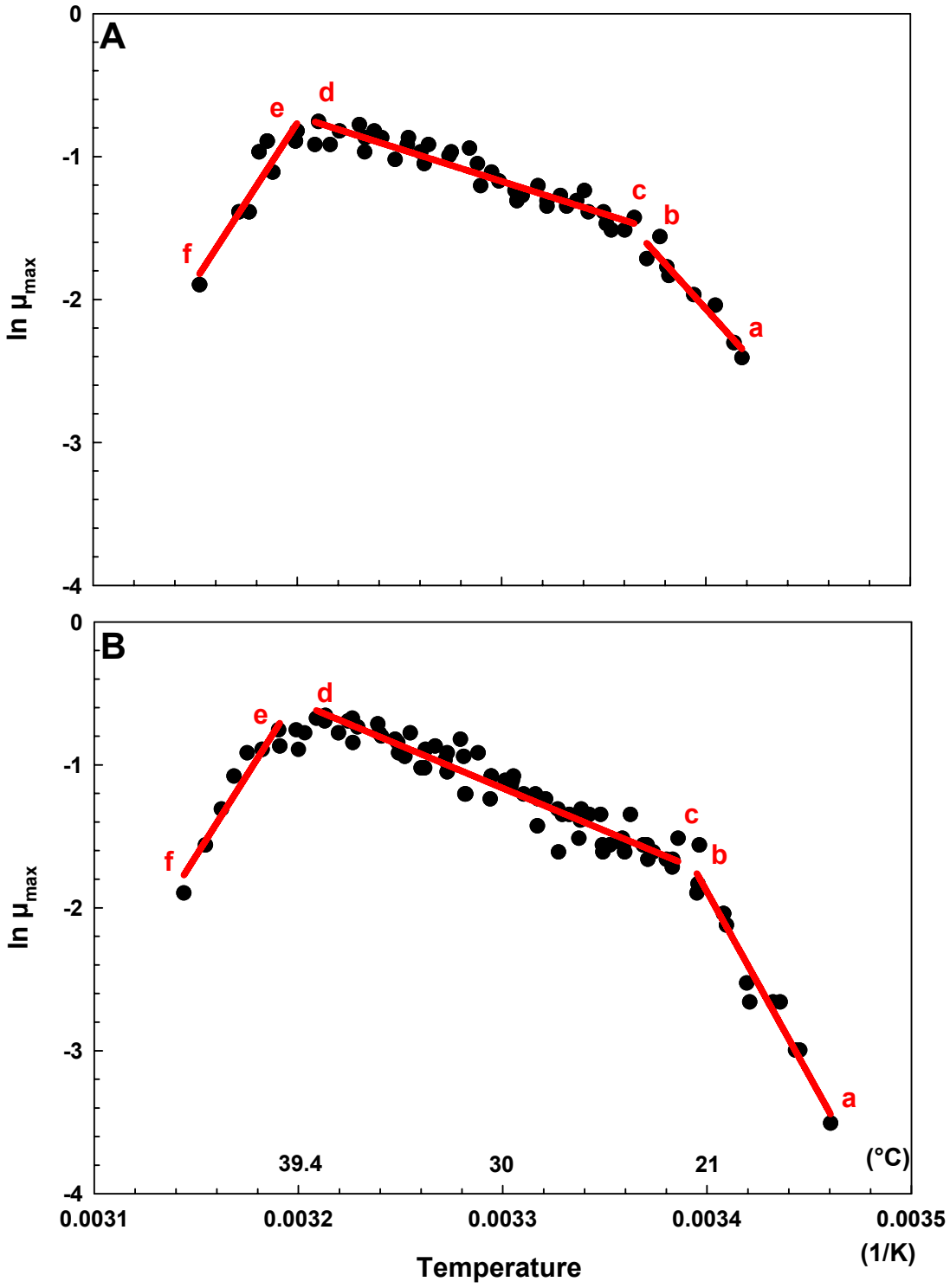


Figure 3.4 Arrhenius plots of *A. adeninivorans* strains LS3 (A) and G1211 (*LEU2*$^+$) (B) grown in YPD broth in a temperature gradient incubator. The gradients of slopes a-b, c-d, and e-f were used to calculate the temperature coefficient E_a.

Table 3.1 Temperature coefficients and entropy constant of *Arxula adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) calculated from the Arrhenius plots shown in Fig. 2.4A and B, respectively.

Strain	Curve	Temperature range °C	Temperature coefficient (E_a) kJ.mol^{-1}	A	Correlation coefficient (r)
LS3	A-B	19 – 24	146	8.74×10^{24}	0.91
	C-D	24 – 39	38	9.96×10^{5}	0.89
	E-F	40 – 44	-221	5.77×10^{-38}	0.87
G1211 (*LEU2*$^+$)	A-B	15 – 21	211	5.28×10^{36}	0.95
	C-D	22 – 39	51	1.72×10^{8}	0.91
	E-F	40 – 45	-189	1.89×10^{-32}	0.92

3.3 Effect of temperature on the morphology

During the stationary phase of shake flask cultures at 30°C, both strains exhibited predominantly a budding cell morphology (Figs. 3.5A and B). Some pseudomycelial cells were observed during the cultivation of both strains. Cells of strain G1211 (*LEU2*$^+$) appeared smaller than those of strain LS3 and the μ_{max} value of strain G1211 (*LEU2*$^+$) at 30°C (0.39 h^{-1}) was less than that of strain LS3 (0.44 h^{-1}) (Table 3.2). One-way analysis of variance (ANOVA) was done using an unpaired t-test comparison on the μ_{max} values of strains grown at 30°C and was found to be significant.

At 42°C both strains formed pseudomycelia, characterised by cell constrictions at the septa, septa at branching sites and a short apical cell (Figs. 3.5C and D). At 45°C the morphology of strain LS3 seemed to change to that of a true mycelium (Fig. 3.5E), but on closer inspection, constrictions at the septa as well as septa at the branching sites were still evident. Strain G1211 (*LEU2*$^+$) retained the pseudomycelial form observed during growth at 42°C (Fig. 3.5F). On average the cell length of LS3 appeared greater than that of the mutant strain G1211 (*LEU2*$^+$). The growth curves of the two strains at the different temperatures are shown in Fig. 3.6 and the mean specific growth rates of triplicate experiments are summarised in Table 3.2. Strain G1211 (*LEU2*$^+$) exhibited significantly

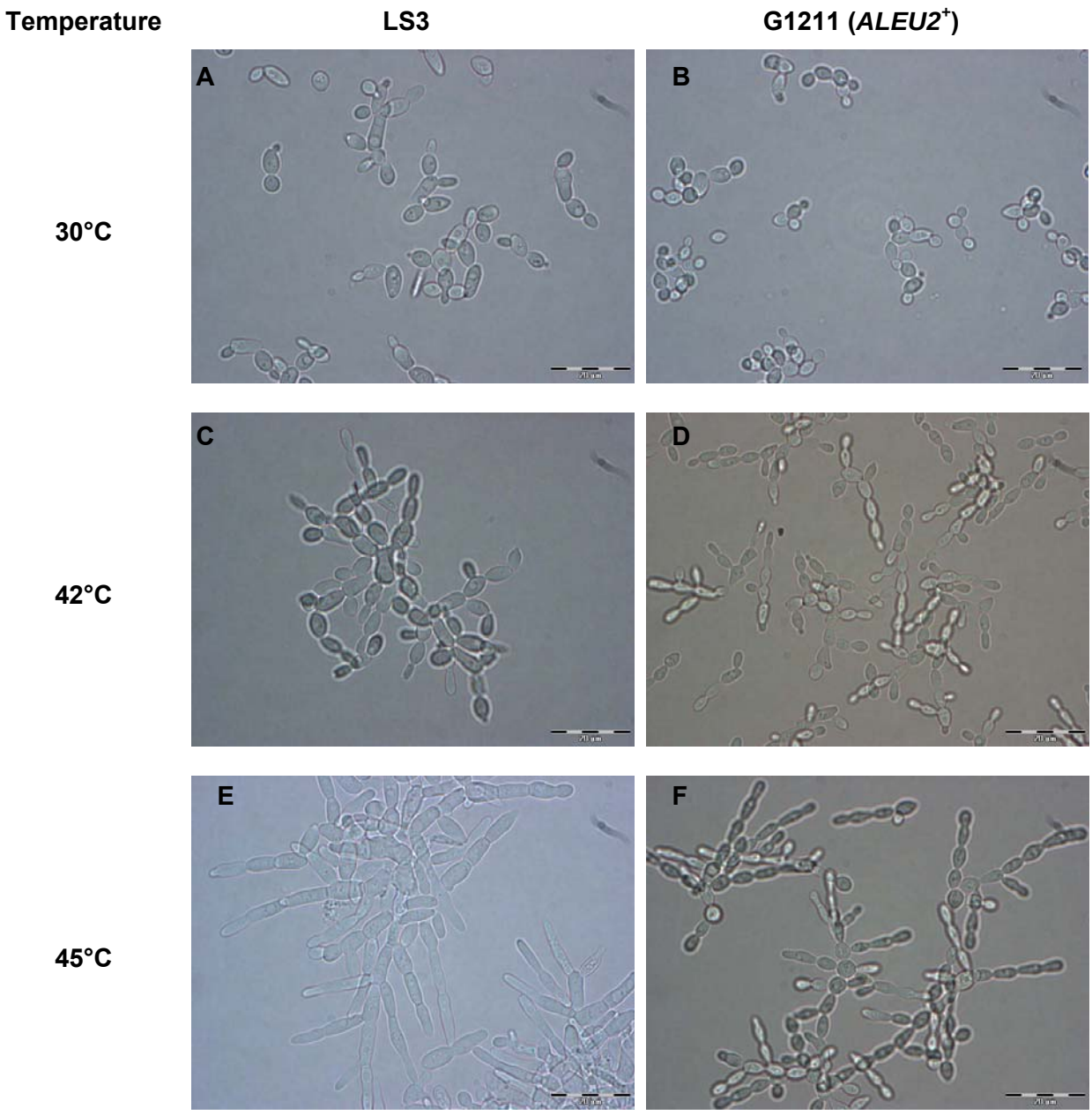


Figure 3.5 Light micrographs at 100× magnification of *A. adeninivorans* strains at the end of shake flask cultivation at different temperatures: LS3 (A) and G1211 (*LEU2*+) (B) at 30°C; LS3 (C) and G1211 (*LEU2*$^+$) (D) at 42°C; LS3 (E) and G1211 (*LEU2*$^+$) (F) 45°C. The bar in each micrograph represents 20 µm.

Table 3.2 Maximum specific growth rates of *A. adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) at different cultivation temperatures in shake flasks using minimal medium. Standard deviations of the mean values from triplicate experiments are indicated in brackets.

Cultivation temperature (°C)	μ_{max} (h^{-1})	
	LS3	**G1211 (*LEU2*$^+$)**
30	0.44 (±0.013)	0.39 (±0.017)
42	0.50 (±0.022)	0.55 (±0.02)
45	0.35 (±0.027)	0.47 (±0.013)

higher μ_{max} values at 42 and 45°C, respectively, compared to strain LS3 at the same temperatures. However, at 30°C strain G1211 (*LEU2*$^+$) had a lower μ_{max} value than strain LS3. In general the growth rate was higher during shake flask cultivation compared to the growth rates determined in the temperature gradient incubator. This could have been due to an oxygen limitation, especially at the higher temperatures, in the culture tubes of the temperature gradient incubator.

4 Discussion

There was a clear difference in the μ_{max} values of the strains obtained in shake flask cultures at 30, 42 and 45°C. The specific growth rate of strain G1211 (*LEU2*$^+$) during cultivation at 30°C was significantly lower than that of LS3, but significantly higher at 42 and 45°C. Both strains LS3 and G1211 (*LEU2*$^+$) displayed a yeast-like morphology at a cultivation temperature of 30°C and a pseudomycelial morphology at 42°C. Although the morphology of both strains remained pseudomycelial at 45°C, the appearance of the cells differed. The cells of LS3 closely resembled mycelia with longer cell segments, but restrictions at the septa could be observed. This was in contrast to literature where it is stated that strain LS3 forms true mycelia at 45°C (Wartmann *et al*. 1995). At 42 and 45°C the cells of strain G1211 (*LEU2*$^+$) formed pseudomycelia with short sausage-like cells.

A possible reason for this change in morphology could be a result of a heat shock response elicited by an increase in cultivation temperature. It has been postulated that heat shock

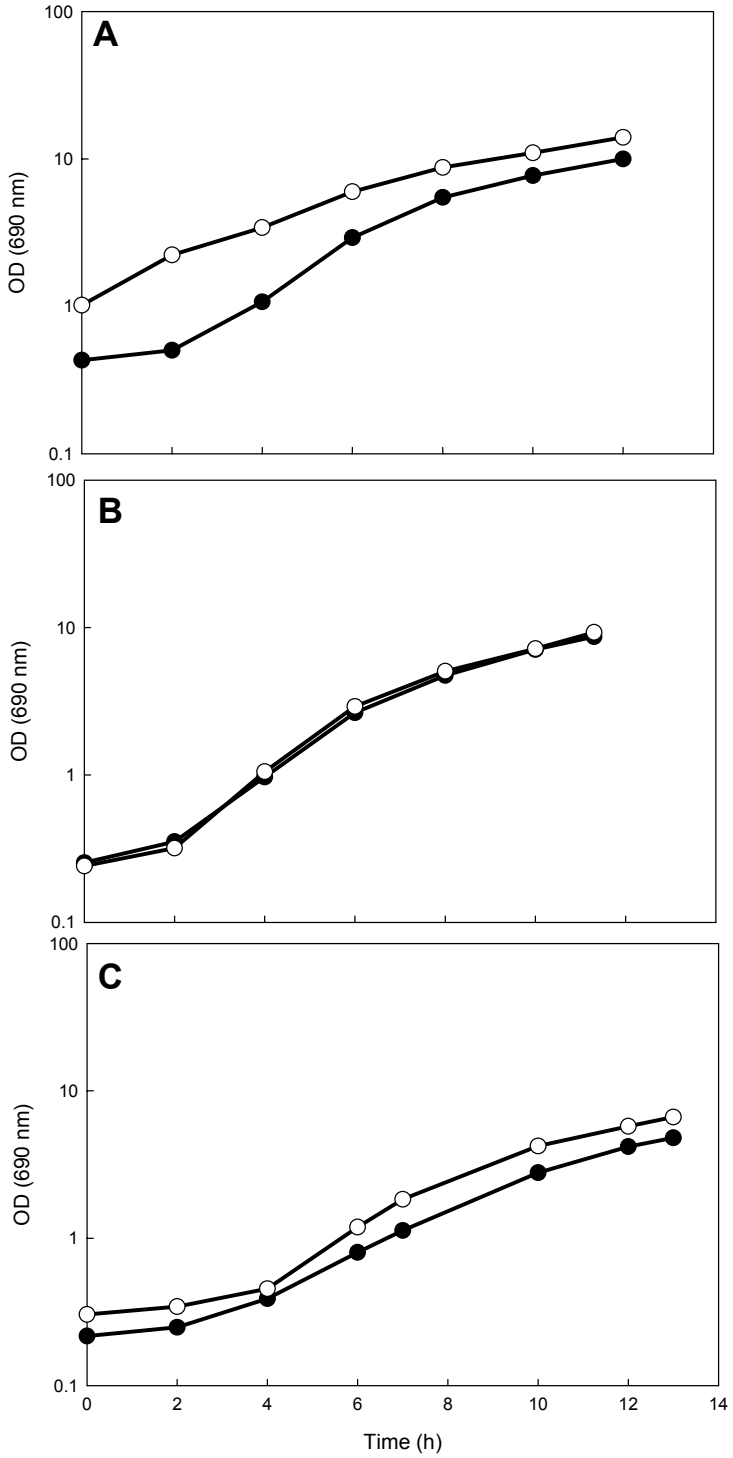


Figure 3.6 Typical growth curves of *A. adenivorans* strains LS3 (●) and G1211 (*LEU2*$^{+}$) (○) in shake flasks using minimal medium at different temperatures, namely 30°C (A), 42°C (B) and 45°C (C).

and the heat shock proteins could trigger a morphological response in the pathogenic dimorphic fungus *Candida albicans,* resulting in the switch from a yeast-like culture to a mycelial culture (Swoboda *et al.* 1995). This was also observed in another pathogenic dimorphic fungus, *Histoplasma capsulatum* (Minchiotti *et al*. 1991). Hayashi *et al.* (2006) suggested that changes in cultivation temperature could affect the membrane structure of cells, influencing the cellular metabolism and resulting in changes in the growth rate. This might also impact on the cell morphology. The effect of oxygen on the cell morphology has also been investigated in the bacterium *Helicobacter pylori* as well in the fungus *Candida albicans* as a possible trigger to morphological switching (Donelli *et al.* 1998; Herrero *et al.* 1999). However, this aspect has not yet been investigated in *A. adeninivorans*.

Cultivation in a temperature gradient incubator revealed a small but significant difference in the maximum specific growth rate of strains LS3 and G1211(*LEU2*$^+$) in the optimum temperature region of 37 to 39°C, which were 0.48 and 0.52 h^{-1}, respectively. The minimum and maximum temperatures were 18 and 46°C, respectively. The cell morphology of both strains changed upon increase in temperature but it was only at 45°C where there was a significant difference in the appearance of the cells between the two strains.

An Arrhenius plot of the temperature data provided a linear relationship between temperature and the growth rate of the culture over distinct different temperature regions (Figs. 3.4A and B). Changes in the temperature coefficient value suggest, as indicated by the two distinct inflection points in the Arrhenius plots of both strains, changes in the rate-controlling reactions or in the metabolic regulation of cells (Pirt 1975); this indicates sensitivity of the culture to changes in temperature in these regions.

The four-fold increase in the E_a value at point c indicated that at below 24 and 19°C, respectively, a change in temperature had a major influence on the physiology of the cells of strains LS3 and G1211 (*LEU2*$^+$) (Figs. 2.4A and B). The increase in the E_a value at point d suggested similar effects. The E_a value recorded over the range of 40 to 45°C for strain LS3 (221 $kJ.mol^{-1}$) was similar to that of strain G1211 (*LEU2*$^+$) (188 $kJ.mol^{-1}$), but in the range 15 to 24°C the E_a value of strain G1211 (*LEU2*$^+$) was higher at 211 $kJ.mol^{-1}$ than that of LS3 at 146 $kJ.mol^{-1}$. Thus, strain G1211 (*LEU2*$^+$) appeared more sensitive to a change in temperature in the lower temperature ranges than LS3.

5 References

Böer E., Terentiev Y., Wartmann T., Gellissen G. and Kunze G. (1994) The *Arxula adeninivorans*-based expression platform. *In* Satyanarayana T. and Johri B. N. (eds.), Microbial Diversity Current Perspectives and Potential Applications. I.K. International Pvt. Ltd., New Delhi, pp. 948 – 965.

Donelli G., Matarrese P., Fiorentini C., Dainelli B., Taraborelli T., Di Campli E., Di Bartolomeo S. and Cellini L. (1998) The effect of oxygen on the growth and cell morphology of *Helicobacter pylori*. FEMS Microbiol. Lett. **168**: 9 – 15.

Hayashi M. and Maeda T. (2006) Activation of the HOG pathway upon cold stress in *Saccharomyces cerevisiae*. J. Biochem. **139**: 797 – 803.

Herrero A. B., López M. C., Fernández-Lago L. and Domínguez A. (1999) *Candida albicans* and *Yarrowia lipolytica* as alternative models for analysing budding patterns and germ tube formation in dimorphic fungi. Microbiology **145**: 2727 – 2737.

Kurtzman C. P. and Fell J. W. (1998) The yeasts, a taxonomic study. 4th ed. Elsevier, New York. pp 441– 442.

Minchiotti G., Gargano S. and Maresca B. (1991) The intron-containing *hsp82* gene of the dimorphic pathogenic fungus *Histoplasma capsulatum* is properly spliced in severe heat shock conditions. Mol. Cell. Biol. **11**: 5624 – 5630.

Pirt S. J. (1975) Principles of microbe and cell cultivation. Blackwell Scientific Publications, Oxford, pp. 137 – 142.

Schulze U. (1995) Anaerobic physiology of *Saccharomyces cerevisiae*. PhD Thesis. Department of Biotechnology, Technical University of Denmark, Lyngby. pp. 147 – 148.

Swoboda R. K., Bertram G., Budge S., Gooday G. W., Gow N. A. R. and Brown J. P. (1995) Structure and regulation of the *HSP90* gene from the pathogenic fungus *Candida albicans*. Infect. Immun. **63**: 4506 – 4514.

Wartmann T., Krüger A., Adler K., Duc B. M., Kunze I. and Kunze G. (1995) Temperature-dependant dimorphism of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek **68**: 215 – 223.

Chapter 4

Influence of Oxygen on the growth rate and morphology of *Arxula adeninivorans* strains

Abstract

The effect of temperature and dissolved oxygen tension (DOT) on the cell morphology of three strains of *Arxula adeninivorans* was investigated in batch culture. The critical dissolved oxygen concentration (C_{crit}) value was 0.016 mmol O_2 l^{-1} (9.34% of saturation) and 0.013 mmol O_2 l^{-1} (7.39% of saturation) of *A. adeninivorans* strains LS3 and G1211 (*ALEU2*$^+$) respectively. This indicated that *A. adeninivorans* G1211 (*ALEU2*$^+$) had a slight but significantly lower C_{crit} value than strain LS3. Under oxygen-sufficient or oxygen-limited conditions, the cell morphology of strain LS3 shifted from yeast-like to pseudomycelial on increasing the temperature from 39 to 45°C, whereas strain G1211 (*LEU2*$^+$) retained a pseudomycelial morphology irrespective of the DOT and temperature. Strain LS3/p*XynA* was a pseudomycelial culture during oxygen-sufficient batch cultivation but changed to a yeast-like morphology during oxygen-limited conditions at 39°C. The specific growth rates of all the strains were lower at a DOT of 1% saturation compared to a DOT of 30% saturation. Thus, genetic transformation of *Arxula adeninivorans* LS3 exerted an effect on its response to temperature and DOT in terms of its morphology.

1 Introduction

Dimorphism in fungi refers to two morphological states of the same organism, namely budding cells and mycelia (Wartmann *et al.* 2000). Dimorphic transformation in fungi has been ascribed to environmental stimuli such as changes in the culture medium and substrate limitation (Cruz *et al.* 2000). During substrate limitation, induction of cell elongation and hyphae may occur as a means of increasing the cell surface area to facilitate uptake of nutrients and the combined effect of temperature and oxygen availability can also result in a change in cell morphology (Ceccato-Antonini *et al.* 2004; Cruz *et al.* 2000). Oxygen availability decreases with an increase in temperature and in Chapter 3 it was shown that in shake flasks an increase in cultivation temperature from

30°C to 45°C brought on a switch in morphology from a yeast-like to a pseudomycelial morphology of *Arxula adeninivorans* strains LS3 and G1211 (*ALEU2*$^+$).

Oxygen is required as a final electron acceptor during the oxidative phosphorylation step in aerobic respiration. At low concentrations, oxygen can limit the metabolism of organisms as the limiting substrate, thus leading to a decrease in the growth rate. The respiration rate, and thus the growth rate, becomes independent of the dissolved oxygen concentration above a certain value termed the critical dissolved oxygen tension (C_{crit}) (Pirt 1975).

In industrial aerobic microbial processes it is desirable to know the feasible limits of maximum biomass concentration that can be sustained by practicable oxygen transfer rates before growth becomes limited by oxygen (Mavituna *et al.* 1985). Thus far, no published report is available on the effect of oxygen availability on the morphology and growth rate of *A. adeninivorans.* In this chapter the effect of the dissolved oxygen level on the morphology of *A. adeninivorans* was investigated.

2 Materials and methods

2.1 Yeast strains

Arxula adeninivorans strains LS3, G1211 (*LEU2*$^+$) and LS3/p*XynA* were used in this study and were maintained as described in Chapter 3.

2.2 Determination of the effect of DOT on growth and morphology

Batch cultivation studies on the influence of different dissolved oxygen concentrations on *A. adeninivorans* were conducted in a 3 l stirred tank reactor (Chemap CF 3000, Volketswil, Switzerland) using a 2.5 l working volume and fitted with one disk turbine impeller, or a 15 l Biostat C reactor (B. Braun Biotech International, Melsungen, Germany) with a working volume of 8 l and fitted with three Rushton disc impellers and one propeller. Minimal medium was used to prepare the inoculum as described in Chapter 3, as well as for bioreactor cultivations. Dow Corning 1520 silicone antifoam (Dow Corning Corporation, USA, repackaged by BDH Laboratory Supplies, Poole, England) was also added at a concentration of 0.2 ml l^{-1}. The pH was controlled at

pH 5.0 by automatic titration with 5 N KOH and 5 N H_2SO_4. Bioreactor vessels were inoculated to 0.2 absorbance units at 690 nm.

The dissolved oxygen tension (DOT) in the culture was monitored with a polarographic pO_2 electrode (Mettler Toledo, Halstead, UK). The DOT was controlled at the determined set point by automatic cascade control of the stirrer speed between 400 and 700 rpm and the aeration rate between 0.4 and 4 slpm using a Braun bioreactor. Some experiments were conducted using a Chemap bioreactor and the DOT was controlled at the determined set point by automatic variation of the stirrer speed between 400 and 700 rpm and manual control of aeration rate between 1 and 2 l min^{-1}. Batch cultures at different dissolved oxygen concentrations of 30 and 1% DOT were investigated at 39 and 45°C.

The critical dissolved oxygen concentration was determined in the Chemap reactor by monitoring the rate of oxygen uptake using a batch culture aerated at 1 l min^{-1}. Determinations were performed during the exponential growth phase of the culture by briefly increasing the stirrer speed to obtain a DOT value of at least 70% of saturation. The aeration was then interrupted and the stirrer briefly stopped to allow air bubbles to escape from the culture broth. After 5 s agitation was resumed at 200 rpm to effect mixing but at the same time minimising surface aeration. The rate of decrease of the DOT was recorded at 2 s intervals using a Squirrel 1200 series data logger (Grant Instruments, Cambridge, England). The C_{crit} value was defined as the point above which the rate of oxygen uptake (and thus the growth rate, assuming a constant biomass yield on oxygen) was independent of the DOT as indicated by the point where the trace deviated from linearity.

Since oxygen can also serve as primary growth-limiting nutrient in a culture, a Michaelis-Menten type relationship between the oxygen concentration and the specific rate of oxygen uptake (qO_2) exists as described by the equation

$$qO_2 = qO_2^{\max}\left(\frac{c}{k_o + c}\right) \qquad (1)$$

where qO_2^{max} is the maximum specific rate of oxygen uptake, C the dissolved oxygen concentration and k_o the saturation constant for oxygen (Pirt 1975). An alternative procedure to determine C_{crit}, therefore, is to use a plot of qO_2 vs DOT, where C_{crit} is indicated by the point where the qO_2 becomes a function of the dissolved oxygen concentration. The solubility of oxygen in distilled water, correcting for temperature and ambient pressure, was used in the calculations.

2.3 Microscopy

Cell morphology was monitored during cultivation using a light microscope (Zeiss Axioplan, Colorview soft imaging system, West Germany). The morphology was also monitored using differential interference contrast microscopy (Zeiss Axioskop, Germany) or phase contrast microscopy (Nikon Eclipse E400).

2.4 Analytical procedures

Culture turbidity was measured with a Photolab S6 photometer (WTW, Weilheim, Germany) at 690 nm. Dry biomass concentration was determined gravimetrically by washing and drying duplicate samples overnight at 105°C to constant mass. Glucose was determined by HPLC (Waters Breeze equipped with a differential refractive index detector) at 84°C using a Waters sugarpack column (300 x 7.8 mm) and deionised water as mobile phase at a flow rate of 0.5 ml min^{-1}. The sample was filtered using a 0.45 µm acetate filter and 20 µl were injected into the system. Quantification was done using a standard curve of glucose.

The maximum specific growth rate of a batch culture was determined by linear regression analysis with Microsoft Excel of the exponential growth phase where the natural logarithm of the dry biomass concentration was plotted as a function of time. The maximum volumetric rate of substrate utilisation (Q_s^{max}) was calculated as

$$Q_s^{\max} = \frac{ds}{dt} = \frac{(s_t - s_0)}{(t_2 - t_1)} \quad (1)$$

from the maximum slope of the substrate concentration curve plotted as a function of time. The maximum specific rate of substrate utilisation (q_s^{max}) was calculated as

$$q_s^{\max} = \frac{ds}{dt} \cdot \frac{1}{x} \quad (2)$$

The biomass yield coefficient ($Y_{x/s}$) was calculated as

$$Y_{x/s} = \frac{(x_t - x_0)}{(s_0 - s_t)} \quad (3)$$

where x_0 and s_0 were the initial biomass and substrate concentrations, respectively, and x_t and s_t the corresponding concentrations when the highest biomass concentration was reached.

3 Results

3.1 Determination of the critical dissolved oxygen tension (C_{crit})

The C_{crit} values of *A. adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) were determined in batch culture. Representative profiles showing the decrease in DOT plotted as a function of time for both strains LS3 and G1211 (*LEU2*$^+$) are shown in Figs. 4.1A and 4.2A respectively. C_{crit} was read off the y-axis at the point where the trace deviated from linearity. These data allowed construction of a curve where the specific oxygen uptake rate (q_{O2}) was plotted as a function of the dissolved oxygen concentration (Figs. 4.1B and 4.2B). In the latter case, C_{crit} is the dissolved oxygen concentration where the respiration rate becomes independent of the dissolved oxygen concentration. This is indicated in Figs. 4.1B and 4.2B as the value on the x-axis corresponding where the tangent lines intersect, indicated by (a), according to Pirt (1975). This value indicated that strain LS3 would become oxygen-limited below a DOT value of 9% and strain G1211 (*LEU2*$^+$) below 7% (Table 4.1). Possibly a physiologically more meaningful value of C_{crit} is indicated by point (b) in Figs. 4.1B and 4.2B, below which qO_2 clearly was dependent on the DOT. These C_{crit} values of strains LS3 and G1211 (*LEU2*$^+$) were equivalent to 12% and at 10% of saturation, respectively.

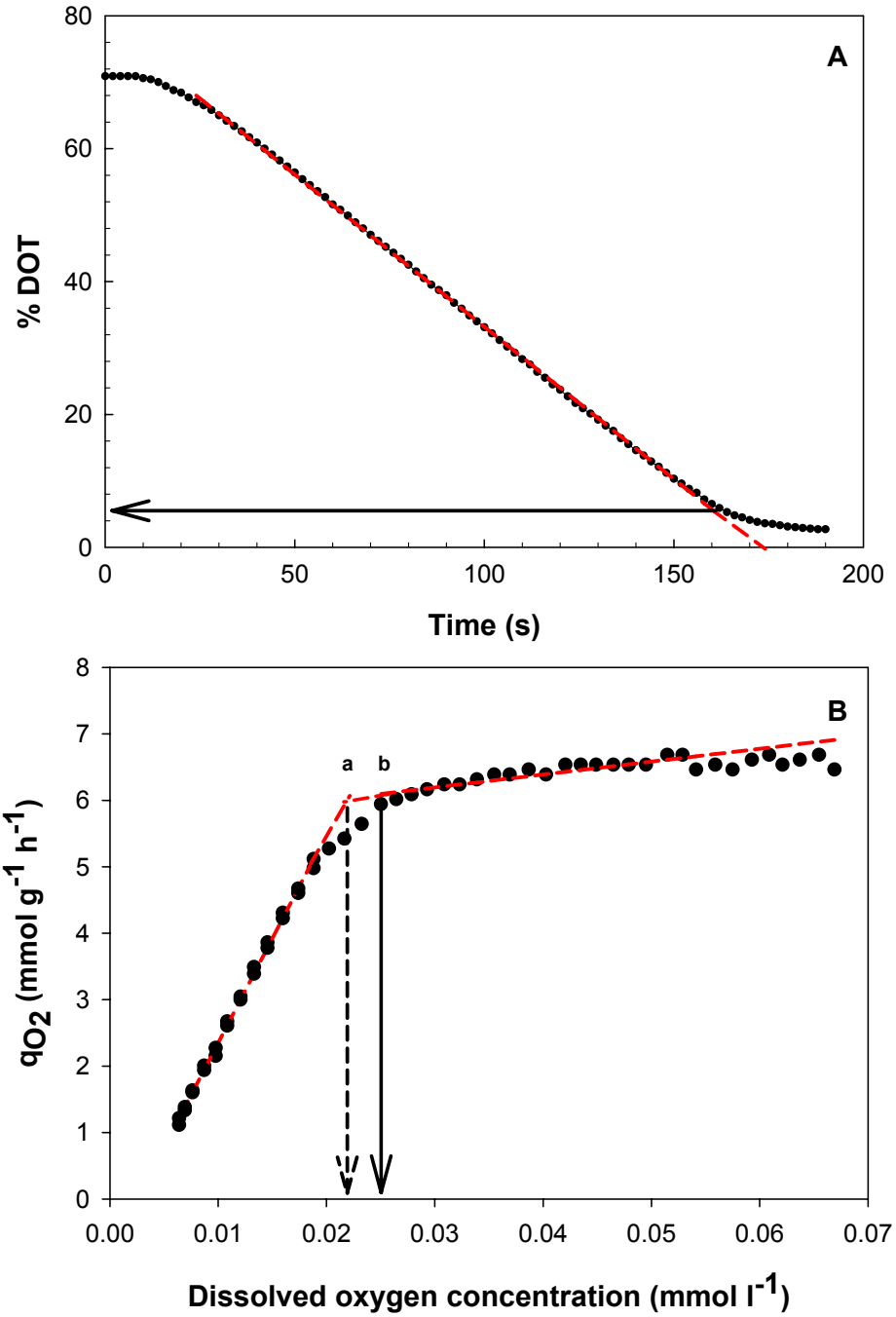


Figure 4.1 A: Representative profile of the decrease in the dissolved oxygen tension following interruption of the air supply to a culture of *A. adeninivorans* LS3 grown in chemically defined medium at 39°C and pH 5 in a Chemap bioreactor. The arrow indicates the C_{crit} value. **B:** The relationship between the specific rate of oxygen uptake (q_{O_2}) and the dissolved oxygen concentration. Point (a) indicates the C_{crit} value according to Pirt (1975) and point (b) indicates the C_{crit} value as determined where the q_{O_2} curve deviates from linearity. The corresponding C_{crit} values are summarised in Table 4.1.

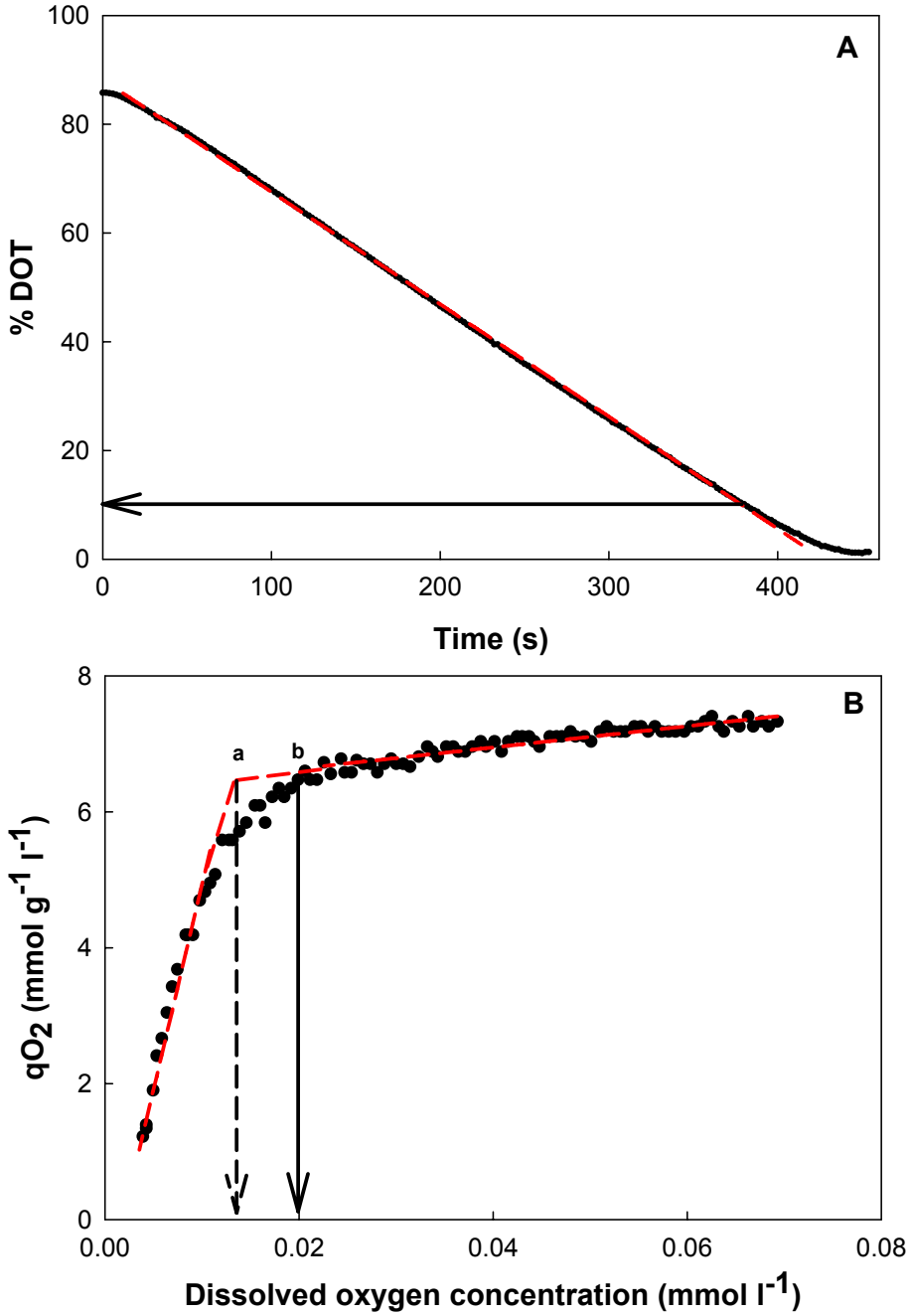


Figure 4.2 A: Representative profile of the decrease in the dissolved oxygen tension following interruption of the air supply to a culture of *A. adeninivorans* G1211 (*LEU2*$^+$) grown in chemically defined medium at 39°C and pH 5 in a Chemap bioreactor. The arrow indicates the C_{crit} value. **B:** The relationship between the specific rate of oxygen uptake (q_{O2}) and the dissolved oxygen concentration. Point (a) indicates the C_{crit} value according to Pirt (1975) and point (b) indicates the C_{crit} value as determined where the q_{O2} curve deviates from linearity. The corresponding C_{crit} values are summarised in Table 4.1.

Table 4.1 The critical dissolved oxygen concentration (C_{crit}), determined from two different plots

Strain	DOT vs time [a]		Q_{O2} vs O_2 concentration[b]		Q_{O2} vs O_2 concentration[c]	
	mmol O_2 l^{-1}	% of saturation	mmol O_2 l^{-1}	% of saturation	mmol O_2 l^{-1}	% of saturation
LS3	0.0199 (± 0.0039)	11 (± 2.22)	0.016 (± 0.0034)	9 (± 1.91)	0.022 (± 0.0035)	12 (± 1.98)
G1211 (*LEU2*$^+$)	0.016 (± 0.0023)	9 (± 1.24)	0.013 (± 0.0018)	7 (± 1.02)	0.018 (± 0.002)	10 (± 1.11)

[a] C_{crit} determined as the value on the y-axis corresponding to the point where the trace of DOT vs time deviated from linearity (Figs. 4.1A and 4.2A).
[b] C_{crit} determined according to Pirt (1975), Figs. 4.1B and 4.2B point (a).
[c] C_{crit} determined as the value on the x-axis corresponding to the point where the trace of q_{O2} vs O_2 concentration deviated from linearity, Figs. 4.1B and 4.2B point (b).

One-way analysis of variance (ANOVA) was done using an unpaired t-test comparison on the C_{crit} values of strains LS3 and G1211 (*LEU2*$^+$). When comparing the C_{crit} values of LS3 and G1211 (*LEU2*$^+$) obtained from the intersection (a) of tangent lines (Figs. 4.1B and 4.2B), the P-value of 0.0721 obtained from six experiments was considered not quite significant, but when comparing the C_{crit} values obtained from point (b), the P-value of 0.0442 was significant (Figs. 4.1B and 4.2B). Strain G1211 (*LEU2*$^+$), therefore, exhibited a slightly lower critical dissolved oxygen tension than strain LS3. No C_{crit} value for strain LS3/*XynA* was determined.

3.2 Effect of different DOT values on morphology and the growth rate

3.2.1 Growth at 39°C

The effect of DOT in the yeast morphology and growth rate was determined by growing *A. adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) in batch culture using minimal medium at 39°C and 45°C and DOT values of 1% and 30% at each of the respective temperatures. The transformed strain LS3/*XynA* was included in the study to determine whether the insertion of the plasmid had any effect on the morphology and growth rate of the yeast growing at 39°C, which was the optimal temperature for the strain LS3 as determined in Chapter3. The optimal growth conditions for the strain LS3/*XynA* were not

determined during this study. According to literature, the morphology of *A. adeninivorans* strain LS3 was yeast–like at 30°C, pseudohyphae at 42°C and a true mycelium at 45°C (Wartmann *et al*. 1995).

For aerobic cultivation in a 3 l bioreactor, the DOT value was maintained at 30% by cascade control of the pO_2 by means of the agitation rate from 400 to 500 rpm and the air flow rate from 1 to 2 l min^{-1}. The agitation rate was maintained within a narrow range to minimise the effects of shear stress on the cell. Oxygen limiting conditions were achieved by initially switching off the air flow and when the DOT reached 1% of saturation, the point when exponential growth normally started, switching the air flow back on and maintaining the DOT at 1% of saturation.

The cultivation profiles of strains LS3, G1211 (*LEU2*$^+$) and LS3/p*XynA* grown at 39°C are shown in Figs. 4.3, 4.4 and 4.5. The culture parameters are shown in Table 4.2. Glucose was completely utilised by strain LS3 grown at DOT values of 1 and 30% saturation (Figs. 4.3A and B), but with strain G1211 grown at a DOT of 1% saturation (Fig. 4.4A) residual glucose remained. This suggested that glucose was not the primary growth limiting substrate, since it was not completely utilised when the culture entered the stationary growth phase. During the growth of strain LS3/p*XynA* at a DOT of 30% saturation, glucose was completely utilised by the time the culture reached stationary phase (Fig. 4.5B), but when grown at a DOT of 1% saturation growth was slower and the glucose was not completely utilised after 22 h of cultivation (Fig. 4.5A). When strain G1211 (*LEU2*$^+$) was grown at a DOT of 30% saturation, the cultivation was stopped after 13 h due to excessive foaming and wall growth (Fig. 4.4B). This was found during repeat experiments and it hindered the monitoring of growth since the biomass separated between the culture broth and the foam. Under these conditions foaming usually occurred during the exponential growth phase and this could have been due to production of polysaccharides or due to cell lysis (Abdullah *et al*. 2000). Foaming also occurred during the exponential growth phase of strains LS3 and LS3/p*XynA*, but not to the extent found with strain G1211 (*LEU2*$^+$) and could be controlled by the addition of the antifoaming agent.

With all strains, the highest maximum specific growth rate (μ_{max}) and biomass concentration were obtained during growth at a DOT of 30% saturation, except in the case of strain G1211 (*LEU2*$^+$) where the highest biomass concentration was obtained at

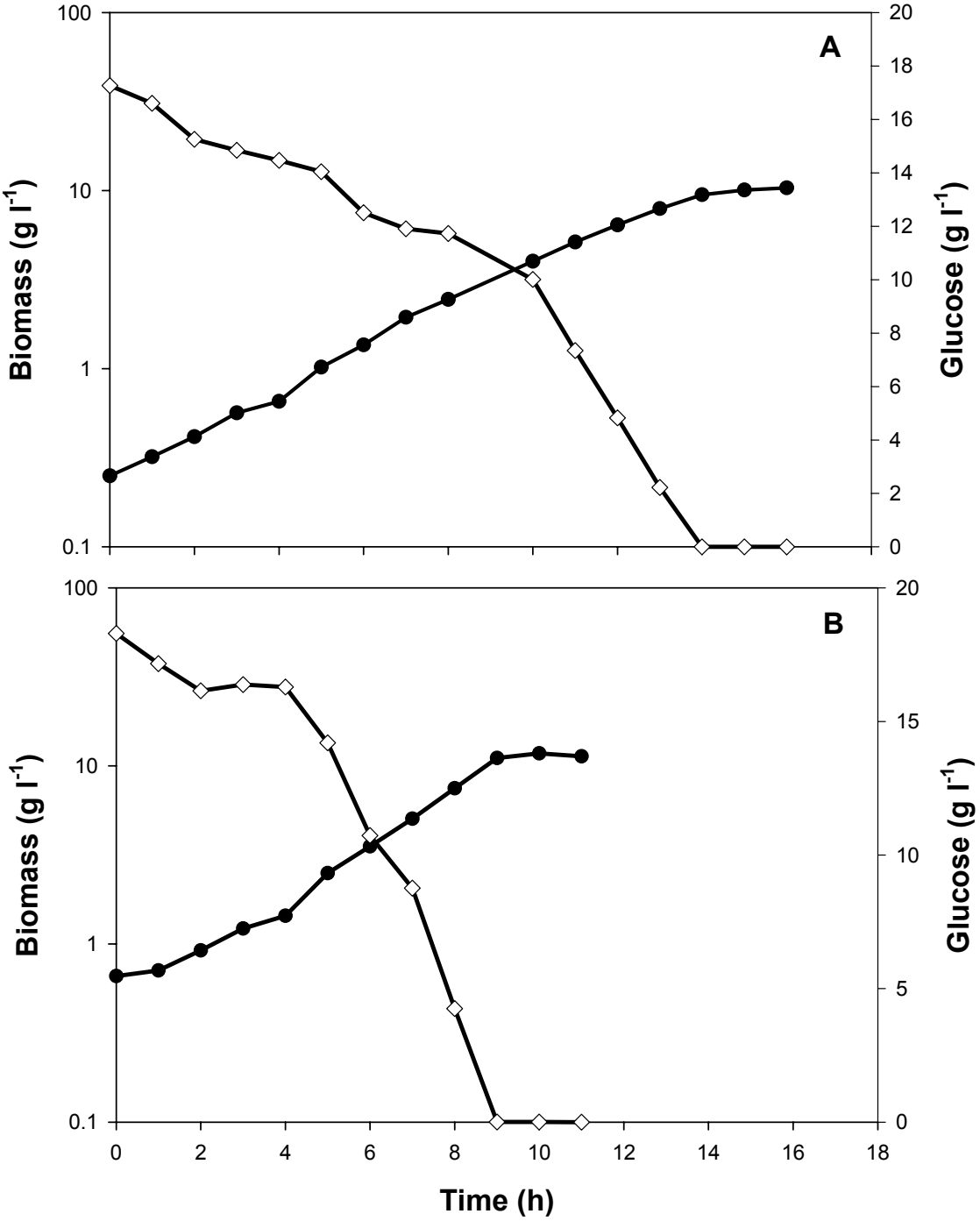


Figure 4.3 Typical cultivation profiles of *A. adeninivorans* LS3 grown at 39°C in batch cultures in a Braun bioreactor using minimal medium, showing concentrations of biomass (●) and glucose (◊). A: 1% DOT. B: 30% DOT.

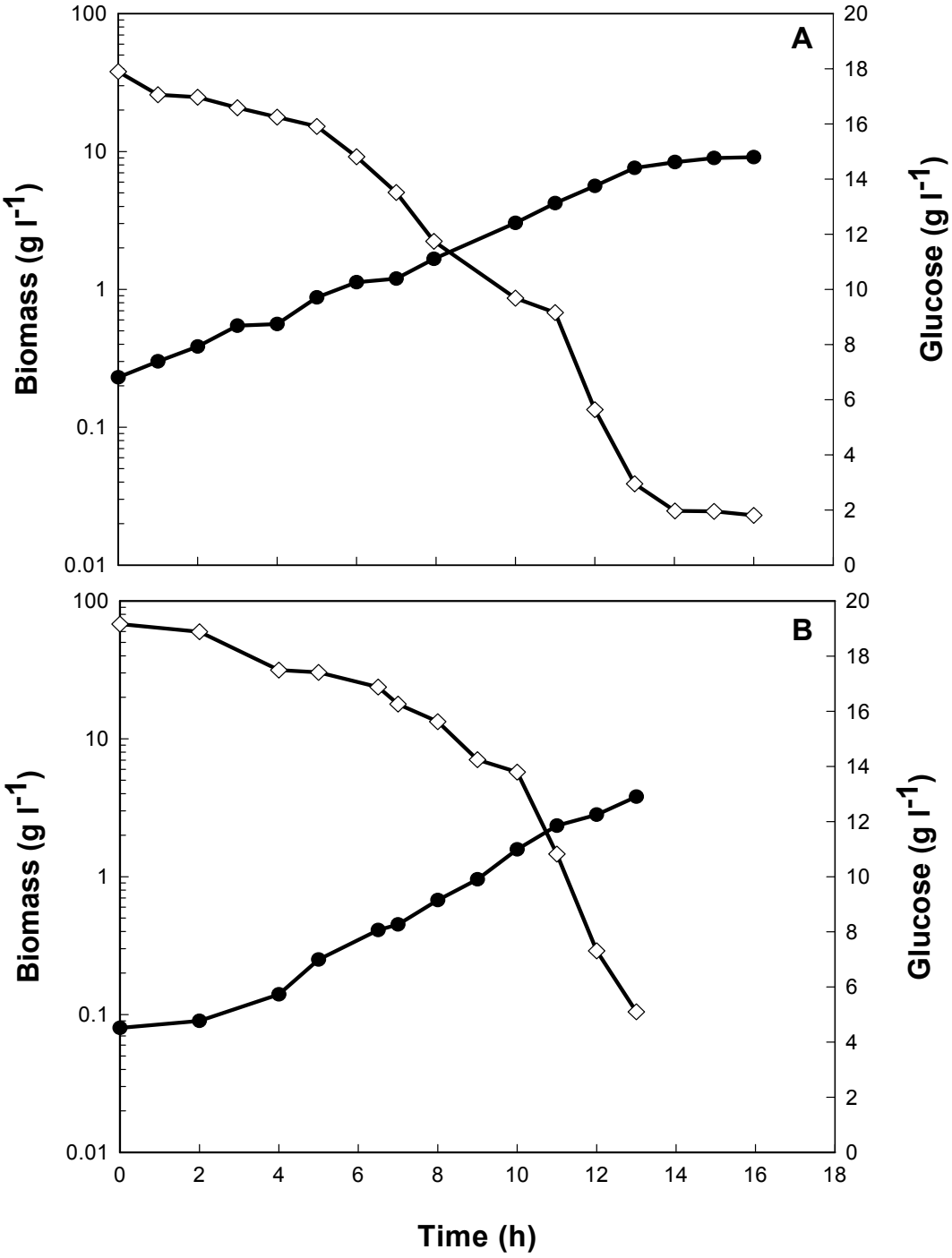


Figure 4.4 Typical cultivation profiles of *A. adeninivorans* G1211 (*LEU2*$^+$) grown at 39°C in batch cultures using minimal medium, showing concentrations of biomass (●) and glucose (◊). A: 1% DOT grown in a Braun bioreactor. B: 30% DOT grown in a Chemap bioreactor

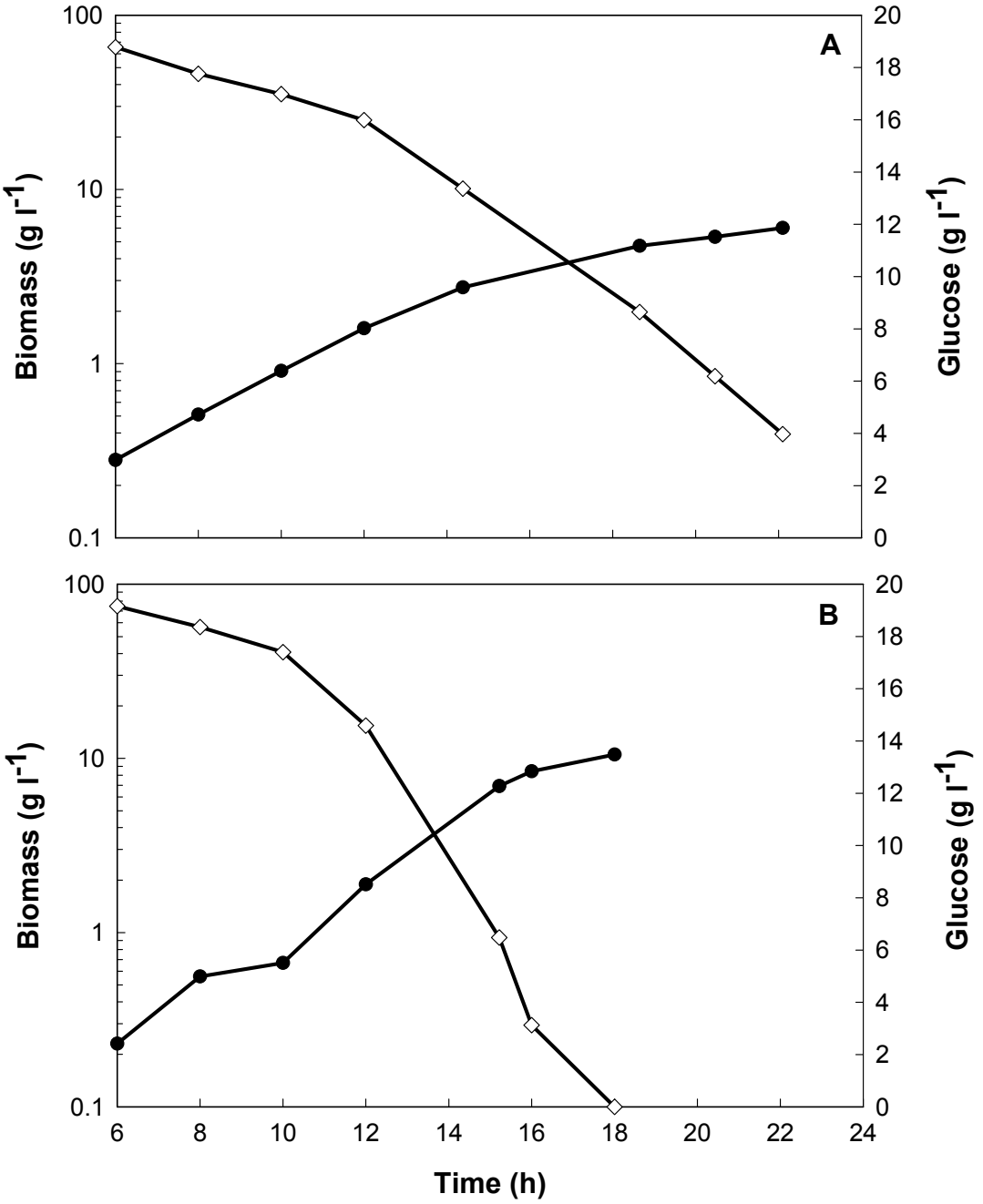


Figure 4.5 Typical cultivation profiles of *A. adeninivorans* LS3/p*XynA* grown at 39°C in batch cultures in a Chemap bioreactor using minimal medium, showing concentrations of biomass (●) and glucose (◊). A: 1% DOT. B: 30% DOT.

Table 4.2 The effect of different DOT values (% of saturation) and temperatures on the kinetic and stoichiometric parameters of *A. adeninivorans* strains LS3, G1211 (*LEU2*$^+$) and LS3/p*XynA* grown in batch cultures using minimal medium.

Parameter	Strain LS3				Strain G1211 (*LEU2*$^+$)				Strain LS3/p*XynA*	
	39°C		45°C		39°C		45°C		39°C	
	1%	30%	1%	30%	1%	30%	1%	30%	1%	30%
Cultivation Time (h)[a]	16	14	26	23	16	13	23	26	22	19
Biomass (g l^{-1})[b]	10.36	10.90	2.90	8.89	9.09	2.80	0.82	11.06	6.02	10.52
μ_{max} (h^{-1})	0.28 ±0.02	0.36 ±0.01	0.16 ±0.03	0.16 ±0.02	0.27 ±0.03	0.38 ±0.02	0.05 ±0.04	0.18 ±0.04	0.27 ±0.02	0.44 ±0.01
			0.27[c] ±0.03	0.47[c] ±0.02			0.34[c] ±0.02	0.30[c] ±0.01		
Q_s^{max} (g l^{-1} h^{-1})	0.39	0.93	3.01	1.75	0.72	0.38	1.43	1.02	0.825	0.44
			0.34[c]	0.39[c]			0.23[c]	0.30[c]		
q_s^{max} (g g^{-1} h^{-1})	0.20	0.67	1.38	0.31	0.82	0.38	2.43	0.43	0.52	0.79
			1.46[c]	0.84[c]			0.79[c]	0.36[c]		
$Y_{x/s}$ (g g^{-1})	0.51	0.54	0.13	0.44	0.55	0.18	0.047	0.46	0.39	0.51

[a] Time of cultivation until stationary phase or cultivation stopped due to excessive foaming

[b] Maximum biomass reached during cultivation

[c] Parameters calculated during growth at 42°C

Q_s^{max} Maximum volumetric rate of substrate uptake, calculated from the maximum slope of the curve of substrate concentration vs time

q_s^{max} Maximum specific rate of substrate uptake

$Y_{x/s}$ Biomass yield coefficient on glucose assimilated

a DOT of 1% of saturation. This could, however, have been due to the fact that cultivation had been prematurely terminated. With the exception of strain LS3, the maximum volumetric rate of substrate uptake (Q_s^{max}) of all strains was higher during cultivation at a DOT of 1% than at 30% saturation. This could have been due to the substrate being utilised for product formation. However, a maximum ethanol concentration of 0.04 g l^{-1} was recorded with strain G1211 (*LEU2*$^+$) growing at a DOT of 1% and even lower concentrations with strain LS3 (0.0016 g l^{-1}). No acetic acid production was observed.

The cell morphology during the stationary phase of batch cultivation at 39°C is shown in Fig 4.6. At a DOT of 30% strain LS3 grew as budding cells (Fig 4.6D), whereas strain G1211 (*LEU2*$^+$) grew as pseudohyphae (Fig 4.6E) and strain LS/p*XynA* was a mixture of pseudohyphae and budding cells (Fig 4.6F). A population that consisted solely of pseudohyphal cells was not obtained. This could have been due to shear stress that caused the cells to break off. At a DOT of 1% saturation both strains LS3 and LS3/p*XynA* had a budding cell morphology whereas strain G1211 (*LEU2*$^+$) was pseudohyphal (Fig. 4.6A, B and C). No cells containing parallel cell walls were observed with any of the strains, whereas a considerable number of constrictions were observed. The agitation speed had been maintained relatively constant during each experiment, therefore the effect of shear stress on the morphology of the yeasts would not have been a variable.

During growth at 39°C and a DOT of 30% saturation, strain LS3 underwent a transition from budding cells to pseudohyphae and back again, whereas the morphology of strain G1211 (*LEU2*$^+$) remained pseudohyphal and strain LS3/p*XynA* grew as a mixture of budding cells and pseudohyphae with long cell segments (Table 4.3). When the cultivation conditions were shifted to oxygen-limited conditions at a DOT of 1% saturation and 39°C, the morphology of both strains LS3 and G1211 (*LEU2*$^+$) did not change but the morphology of strain LS3/p*XynA* changed from a mixture of pseudohyphae and budding cells to a predominantly budding cell culture. The same trend regarding cell morphology was observed, irrespective of which bioreactor was used. This ruled out the possibility that the differences in morphology between the strains could have been due to differing shear forces as a consequence of the different bioreactor configurations.

3.2.2 Growth at 45°C

The cultivation profiles of strains LS3 and G1211 (*LEU2*$^+$) grown at 45°C are shown in Figs. 4.7 and 4.8. Cultivation at 45°C proved a bit tricky, however, as both strains were unable to grow when inoculated into the medium at 45°C. Therefore, the cultivation was started at a lower temperature of 42°C and increased to 45°C approximately after 5 h of inoculation when the cells had started to grow. Agitation was maintained at 500 rpm because at lower agitation rates growth on the vessel wall as well as clumping occurred,

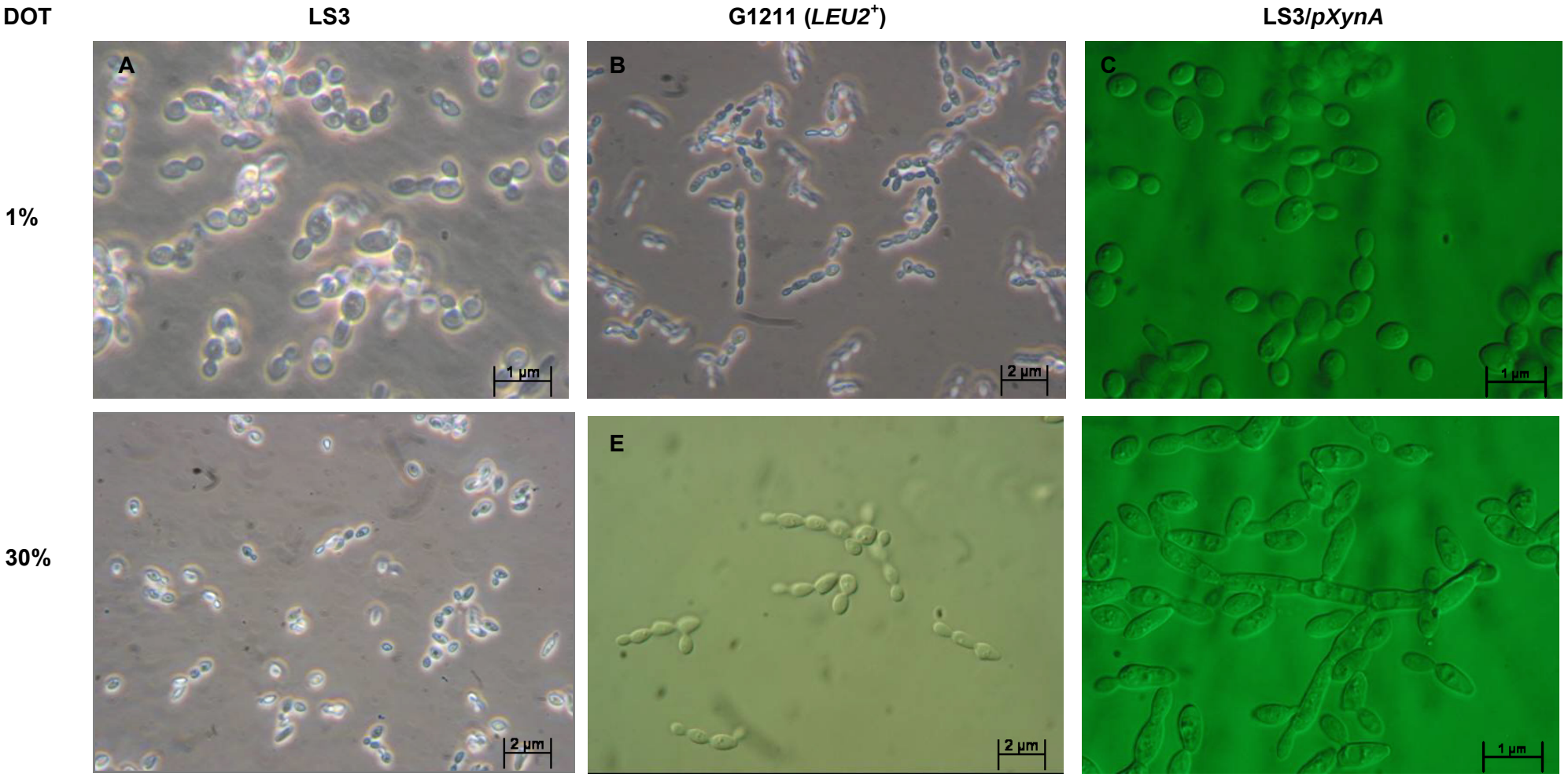
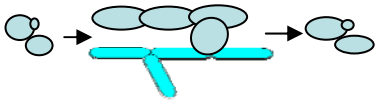
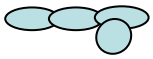
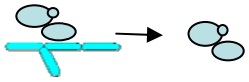
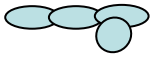
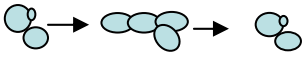
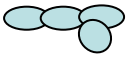
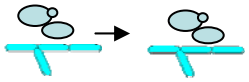
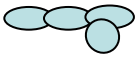
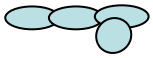


Figure 4.6 Light micrographs at the end of batch cultivation of *A. adeninivorans* strains grown at 39°C. A: strain LS3 grown at a DOT of 1% in a Braun bioreactor, B: strain G1211 (*LEU2*$^+$) grown at a DOT of 1% in a Braun bioreactor, C: strain LS3/p*XynA* grown at a DOT of 1% in a Chemap bioreactor, D: strain LS3 grown at a DOT of 30% in a Braun bioreactor, E: strain G1211 (*LEU2*$^+$) grown at a DOT of 30 % in a Chemap bioreactor and F: strain LS3/p*XynA* grown at a DOT of 30% in a Chemap bioreactor.

which made it impossible to accurately monitor the growth of the culture. The cultivation was stopped when excessive foaming occurred, which also hampered the cultivation process. At the higher temperature the oxygen limitation had a very noticeable effect on the growth of both the yeast strains. When the in temperature was increased from 42 to 45°C, the growth rate decreased, as evident from the noticeable decrease in the slope of

Table 4.3 Changes in morphology observed during batch cultivation of *A. adeninivorans* strains LS3, G1211 (*LEU2*$^+$) and LS3/p*XynA* at 39 and 45°C and at a DOT of 1 and 30% saturation, respectively, using either a Braun or a Chemap bioreactor.

DOT (%)	Temperature (°C)	Strain		
		LS3	G1211 (*LEU2*$^+$)	LS3/p*XynA*
1	39			
	45			ND
30	39			
	45			ND

ND Not determined

the exponential phase at this point. Although the temperature was increased to 45°C after 5 h of cultivation, the decrease in growth rate was consistently only apparent about 3 h later, as can be seen in Fig 4.7B. Generally a low growth rate was observed at a DOT of 1% saturation and 45°C. Both yeasts failed to completely utilise glucose under these conditions.

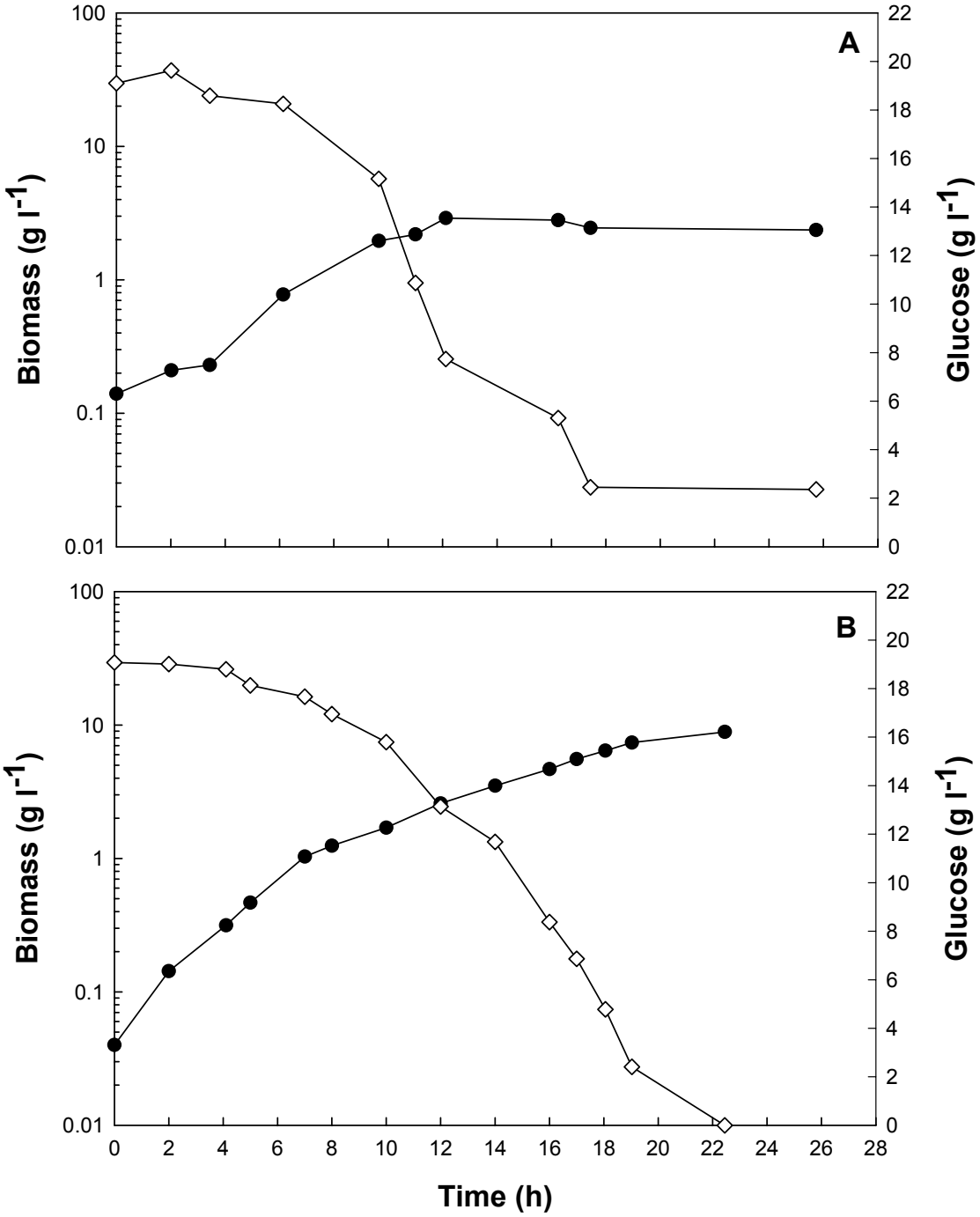


Figure 4.7 Cultivation profiles of *A. adeninivorans* strains LS3 grown at 45°C in batch cultures in a Chemap bioreactor using minimal medium, showing concentrations of biomass (●) and glucose (◊). A: 1% DOT. B: 30% DOT.

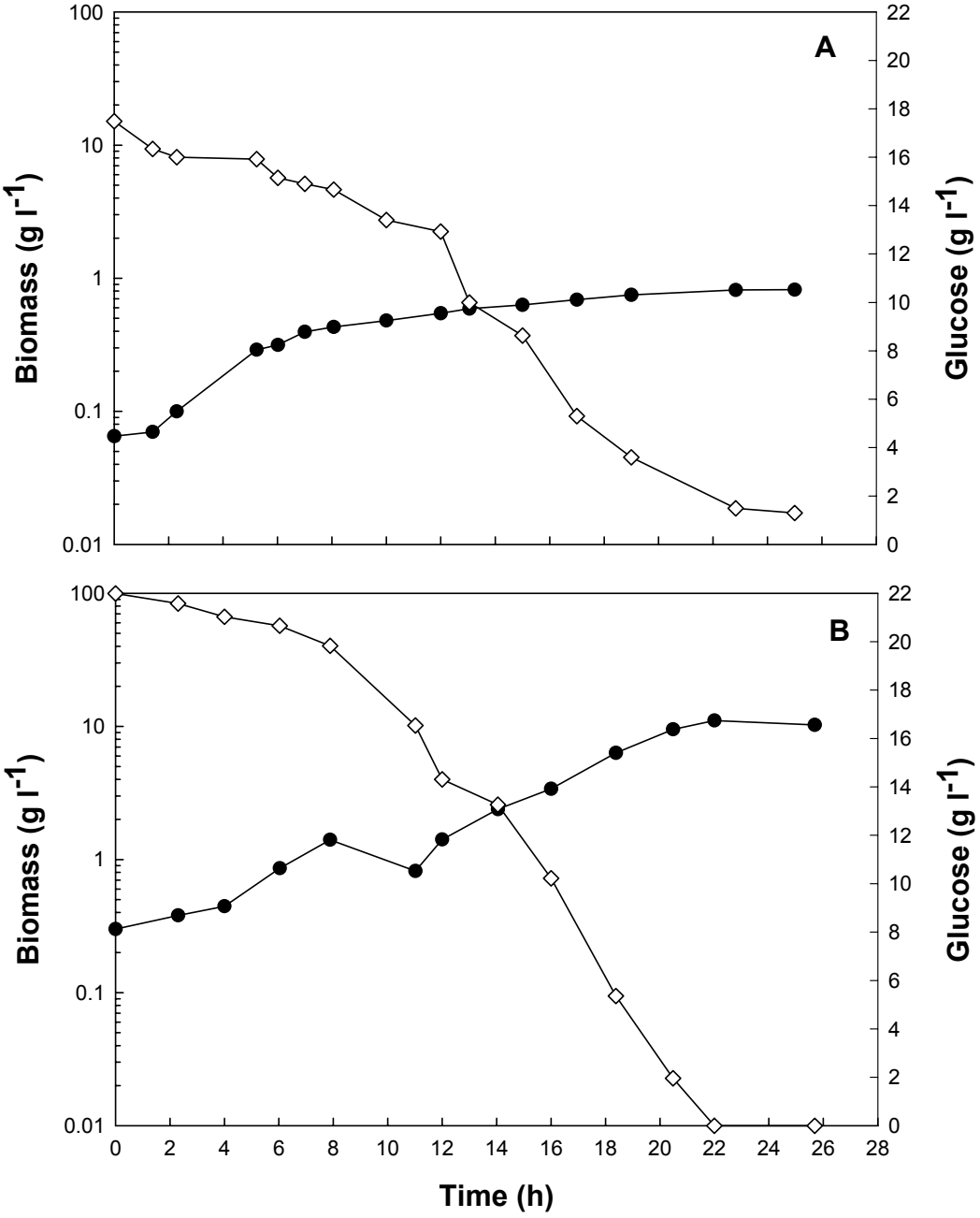


Figure 4.8 Cultivation profiles of *A. adeninivorans* strains G1211 (*LEU2*$^+$) grown at 45°C in batch cultures in a Chemap bioreactor using minimal medium, showing concentrations of biomass (●) and glucose (◊). A: 1% DOT. B: 30% DOT.

With the increase in cultivation temperature the growth rate of the strains decreased as well as the final biomass concentration. In general the biomass yield coefficient was higher during cultivation at 30% DOT than at 1% at both 45 and 39°C, and it was higher at 39°C than at 45°C.The volumetric rate of substrate uptake of all the strains was higher during cultivation at a DOT of 1% than at 30% saturation.

During the stationary phase of batch cultures at 45°C (Fig 4.9) the morphology of strains LS3 and G1211 (*LEU2*$^+$) was pseudohyphal during growth at both DOT values of 1 and 30%. The only apparent difference between the latter two strains was found at a DOT of 1% saturation at 45°C, where the morphology of strain LS3 was pseudohyphal with long cell segments resembling hyphae, whereas the morphology of strain G1211 (*LEU2*$^+$) was also pseudohyphal but with short cell segments (Figs. 4.9A and B). Although Wartmann *et al*. (1995) reported that at 45°C strain LS3 exhibited mycelial growth, no true mycelium were observed during the cultivation of strains LS3 and G1211 (*LEU2*$^+$) at 45°C.

5. Discussion

Morphological switching from yeast to filamentous forms can be induced by a variety of environmental and stress conditions such as a change in cultivation temperature, pH, nutritional limitation and a lack of oxygen (Sudbery *et al*. 2006). *Arxula adeninivorans* strain LS3 has a temperature dependent dimorphism where it grows as budding cells at 30°C and switches to hyphal growth at 45°C with an intermediate pseudohyphal stage at 42°C (Wartmann *et al.* 2000). The oxygen solubility in a culture medium is dependent on the cultivation temperature and could be a factor influencing the change in morphology by causing added stress of the cells in combination with the increased cultivation temperature. Cruz *et al*. (2000) found that a decrease in the dissolved oxygen concentration caused a transition from budding cells to hyphae in a culture of *Debaryomyces hansenii* and an increase in DOT reversed this morphological switch. By contrast, it was found that when *A. adeninivorans* LS3 was grown at 45°C and conditions were shifted from aerobic to anaerobic, a transition from hyphal growth to budding cells was observed (Wartmann *et al.* 2000).

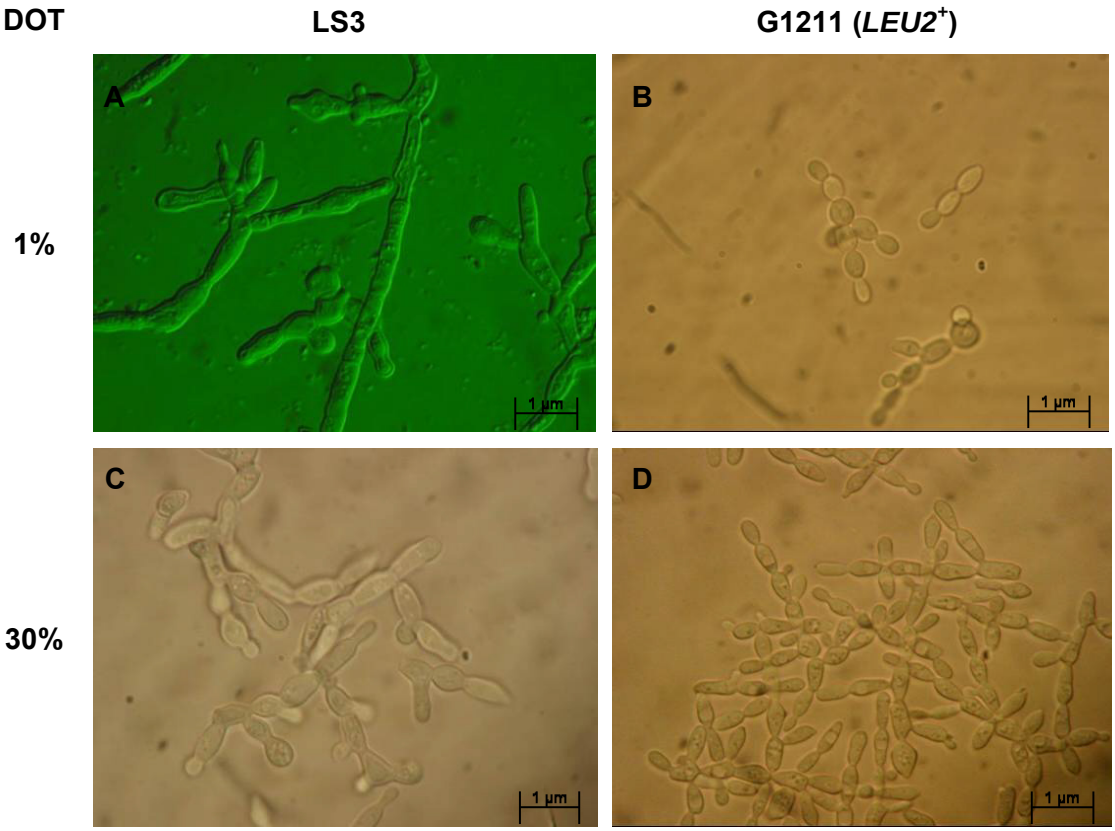


Figure 4.9 Light micrographs at the end of batch cultivation of *A. adeninivorans* strains grown in a Chemap bioreactor at 45°C. A: LS3 grown at a DOT of 1%, B: G1211 (*LEU2*$^+$) grown at a DOT of 1%, C: LS3 grown at a DOT of 30% and D: G1211 (*LEU2*$^+$) grown at a DOT of 30%.

Three methods to determine the critical oxygen concentration (C_{crit}) are demonstrated in Figs 4.1 and 4.2. Oxygen can serve as the growth-limiting substrate and a Michaelis-Menten type relationship between the oxygen concentration and specific rate of oxygen uptake exists. Determination of the C_{crit} values according to the method described by Pirt (1975) was probably the most accurate method because it was very difficult in some cases to determine the precise point of deviation of the DOT curve from linearity in Figs. 4.1B point b. The C_{crit} values of *A. adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) were 0.016 mmol l^{-1} (9% of saturation) and 0.013 mmol l^{-1} (7% of saturation), respectively. This suggested that strain G1211 (*ALEU2*$^+$) had a slightly but significantly lower critical dissolved oxygen concentration than strain LS3. The C_{crit} value of strain LS3/*XynA* was

not determined. The general trend observed for the biomass yield coefficient was that it was higher during cultivation at a DOT of 30% saturation than at 1% at both 45 and 39°C and that the biomass yield was higher at 39°C than at 45°C (Table 4.2). With the exception of strain LS3 at 39°C, the volumetric rate of substrate uptake by all the strains was higher during growth at a DOT of 1% than at a DOT of 30% at 39°C and 45°C. During conditions of external stress, such as low oxygen concentrations or high cultivation temperatures, maintenance energy requirements may increase considerably and could account for the lower biomass yield and growth rate observed during growth at 45°C and a DOT of 1% (Gustafsson *et al.* 1993; Pirt 1965). No appreciable levels of ethanol and no acetic acid production were found with any of the strains.

Cultivation of strain G1211 (*ALEU2*$^+$) at 39°C and 30% was hampered due to the production of excessive foam in the cultivation broth. Foaming may be aggravated towards the end of a batch culture due to extracellular polysaccharide production, an initial high sugar concentration during early stages of cultivation, and the presence of proteins, peptides and fatty acids released by lysed cells towards the end of cultivation (Abdullah *et al.* 2000). Cells entrapped in a stable foam may form a crust sticking to the reactor wall or around probes and sample ports, affecting the functioning of the probes and making it impossible to obtain a homogenous culture sample.

According to Sudbery *et al.* (2006), a pseudohyphal culture is formed when the daughter bud elongates to form a daughter cell remaining attached to the mother cell after septum formation. The elongation of buds in pseudohyphae can be so extreme that these filaments superficially resemble hyphae. This was seen during the cultivation of strain LS3 at 45°C and a DOT of 1% saturation (Fig 4.9A). This differs from a previous report stating that under anaerobic conditions strain LS3 grew as budding cells (Wartmann *et al.* 2000). At 39°C oxygen limitation had no apparent effect on the morphology of strains LS3 and G1211 (*LEU2*$^+$), whereas strain LS3/p*XynA* changed from a pseudohyphall to a budding cell morphology. This was unexpected as it would seem more likely that cells would change to a hyphal form during oxygen limitation, as was found in the case of *D. hansenii* (Cruz *et al*. 2000). Increasing the temperature to 45°C together with an oxygen limitation had an effect on the morphology of strain LS3 but not on strain G1211 (*LEU2*$^+$). Both strains exhibited a pseudomycelial morphology at 45°C.

The low growth rate observed in the bioreactor with both strains LS3 and G1211 (*LEU2*$^+$) correlated with the findings of growth studies using a temperature gradient incubator (Chapter 3). This, however, was not observed during shake flask cultivations at 45°C where growth rates of up to 0.35 and 0.47 h^{-1} were recorded with strains LS3 and G1211 (*LEU2*$^+$), respectively. A possible reason could have been that it was not always possible to maintain an exact cultivation temperature in shake incubators, whereas during bioreactor cultivations better control of the cultivation temperature was possible.

It is clear that the genetic transformation of *A. adeninivorans* strains had an effect on the morphology and the kinetic parameters of this yeast species. The morphology of strain G1211 (*ALEU2*$^+$) was apparently not sensitive to the temperature or oxygen levels used in this investigation. The morphology of strain LS3, on the other hand, was affected by the change in temperature as well as oxygen level. A change in DOT affected the morphology of strain LS3/p*XynA*.

6. References

Abdullah M. A., Ariff A. B., Marziah M., Ali A. M. and Lajis N. M. (2000) Strategies to overcome foaming and wall-growth during the cultivation of *Morinda elliptica* cell suspension culture in a stirred-tank bioreactor. Plant Cell Tiss. Org. Cult. **60**: 205-212.

Ceccato-Antonini S. R. and Sudbery P. E. (2004) Filamentous growth in *Saccharomyces cerevisiae*. Braz. J. Biol. **35**: 173–181.

Cruz J. M., Domínguez J. M., Domínguez H. and Parajó J. C. (2000) Dimorphic behaviour of *Debaryomyces hansenii* growing on barley bran hydrolyzates. Biotechnol. Lett. **22**: 605–610.

Gustafsson L., Ölz R., Larsson K., Larsson C. and Adler L. (1993) Energy balance calculations as a tool to determine maintenance energy requirements under stress conditions. Pure Appl. Chem. **65**: 1893-1898.

Mavituna F. and Sinclair C. G. (1985) A graphical method for the determination of critical biomass concentration for non-oxygen-limited growth. Biotechnol. Lett. **7**: 69-74.

Pirt S. J. (1965) The maintenance energy of bacteria in growing cultures. Proc. R. Soc. Lond. B. Biol. Sci. **163**: 224-231.

Pirt S. J. (1975) Principles of microbe and cell cultivation. Blackwell Scientific Publications, Oxford. pp. 137 – 142.

Sudbery P., Gow N. and Berman J. (2006) The distinct morphogenic states of *Candida albicans*. Trends Microbiol. **12**: 317-324.

Wartmann T., Erdmann J., Kunze I. and Kunze G. (2000) Morphology-related effects on gene expression and protein accumulation of the yeast *Arxula adeninivorans* LS3. Arch. Microbiol. **173**: 253-261.

Wartmann T., Krüger A., Adler K., Duc B. M., Kunze I. and Kunze G (1995) Temperature-dependant dimorphism of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek **68**: 215–23.

Chapter 5

General Discussion and Conclusions

Dimorphism is the ability of fungi to switch between budding cells and hyphae in response to an environmental stimulus (Cruz *et al.* 2000). *Arxula adeninivorans* is a dimorphic yeast and dimorphism in strain LS3 is temperature dependent (Wartmann *et al.* 1995). This yeast species is halotolerant, thermotolerant and can assimilate a wide range of carbon and nitrogen sources (Kunze *et al*. 1997; Yang *et al.* 2000). Strain LS3 has been developed as host for heterologous gene expression and the transformation system based on the integration of heterologous DNA into ribosomal DNA (rDNA) was developed by Rösel *et al*. (1998).

The aim of this study was a comparative investigation of the growth characteristics of *A. adeninivorans* strains LS3, G1211 (*LEU2*$^{+}$) and LS3/p*XynA*, focusing on the effect of temperature and dissolved oxygen tension (DOT) on the morphology and growth parameters of the strains. Strain G1211 (*LEU2*$^{+}$) is an auxotrophic mutant (*aleu2* mutant) of strain LS3 transformed with the pAL-ALEU2m plasmid and strain LS3/p*XynA* was derived from strain LS3 by transformation with the *Thermomyces lanuginosus* xylanase gene under the control of the *TEF1* promoter.

Little to no xylanase activity was found with strain LS3/p*XynA*, the highest activity recorded being 5.86 nkat ml^{-1} (0.35 U ml^{-1}). The copy number integrated into the rDNA locus was unknown and the protein was produced intracellularly. A low copy number should not have been a problem since the *TEF1* promoter provides strong constitutive expression of a heterologous gene, even when present in low copy numbers (Steinborn *et al.* 2007; Wartmann *et al.* 2003). This particular vector has also been tested on a range of alternative yeast species including *Saccharomyces cerevisiae, Pichia pastoris* and *Debaryomyces hansenii* and all strains exhibited heterologous gene expression at a similar high level (Terentiev *et al*. 2004). The xylanase protein itself might have been defective since it accumulated intracellularly and this might have resulted in the low activity observed. However, the reason for the low xylanase activity is not clear.

In this investigation, cultivation in a temperature gradient incubator revealed a small but significant difference in the maximum specific growth rates (μ_{max}) of strains LS3 and G1211 (*LEU2*$^+$), namely 0.45 and 0.52 h^{-1}, respectively. The minimum and maximum temperatures were 18 and 46°C, respectively, with the optimum temperature in the range of 37 to 39°C. This is the first time that the cardinal temperatures of *A. adeninivorans* strains LS3 and G1211 (*LEU2*$^+$) are reported and it would seem that the mutation or the transformation slightly enhanced the growth rate of this species.

In accordance with literature, in shake flask cultures the morphology of strain LS3 changed with an increase in temperature, from budding cells at 30°C to pseudohyphae at 42°C. Although the cells did not form true hyphae at 45°C, the length of the cell segments increased and the morphology resembled a mycelial culture. Similarly, the morphology of strain G1211 (*LEU2*$^+$) at 30°C was budding cells and pseudohyphae at both 42 and 45°C. A comparison of morphology using shake flasks might not be reliable because the cultivation conditions are not rigorously controlled.

Oxygen solubility in culture broth is dependent on the temperature and could be a factor influencing the change in morphology by causing additional stress on the cells in combination with the increased cultivation temperature. Three methods to determine the critical oxygen concentration (C_{crit}) are illustrated in Figs 4.1 and 4.2. The first method was based on a graphical determination from a plot of DOT as a function of time, where the C_{crit} value was the point where the DOT curve deviated from linearity (Figs 4.1A and 4.2A). Oxygen can serve as the growth-limiting substrate and a Michaelis-Menten type relationship between the oxygen concentration and specific rate of oxygen uptake exists, therefore, allowing for a physiologically more meaningful method of determining C_{crit} from a graph were the specific rate of oxygen uptake (q_{O2}) is plotted as a function of the dissolved oxygen concentration. The C_{crit} value was determined from the point were the trace of q_{O2} deviated from linearity. Because the point of deviation is difficult to determine, the method described by Pirt (1975) determined C_{crit} at the intersection of tangent lines (Figs 4.1B and 4.2B). Using the latter method the C_{crit} determined for strains LS3 and G1211 (*LEU2*$^+$) was 0.016 mmol l^{-1} (9% of saturation) and 0.013 mmol l^{-1} (7% of saturation) respectively. This is the first time the C_{crit} values are reported for strains LS3 and G1211 (*LEU2*$^+$). These values were higher than those reported for *S. cerevisiae*, namely 0.0037 – 0.0046 mmol l^{-1} (1.6 – 2% of saturation) (Bailey *et al.* 1977).

In this investigation, during batch cultivation the DOT had no effect on strain LS3 at 39°C, but at 45°C an oxygen limitation resulted in an increase in the length of the cell segments. By contrast, the morphology of strain G1211 (*LEU2*$^+$) was not influenced by either temperature or DOT. At a cultivation temperature of 39°C, an oxygen limitation caused a shift in morphology of strain LS3/p*XynA* from a mixture of pseudohyphae with long cell segments and budding cells to predominantly budding cells. This is in contrast to what was found in the case of *D. hansenii* where an oxygen limitation caused a transition from budding cells to hyphae (Cruz *et al*. 2000).

In conclusion, the morphology of *A. adeninivorans* strain LS3 was influenced by an increase in cultivation temperature, but the DOT only had an effect on the morphology at a high cultivation temperature of 45°C. Genetic transformation had an effect on the morphology of strain LS3, since an oxygen limitation at 39°C caused a shift in the morphology from a mixture of budding cells and pseusohyphae to predominantly budding cells in the transformed strain LS3/p*XynA*, whereas in strain G1211 (*LEU2*$^+$) only pseudohyphae were observed at both cultivation temperatures and DOT values. The C_{crit} value of strain G1211 (*LEU2*$^+$) was significantly lower than that of strain LS3 and its μ_{max} value at the optimum temperature was significantly higher, but with strain LS3/p*XynA* only the morphology was affected by its genetic transformation.

References

Bailey J. E. and Ollis D. F. (1977) Biochemical engineering fundamentals. McGraw-Hill, New York, pp 422.

Cruz J. M., Domínguez J. M., Domínguez H. and Parajó J. C. (2000) Dimorphic behaviour of *Debaryomyces hansenii* growing on barley bran hydrolyzates. Biotechnol. Lett. **22**: 605–610.

Kunze G. and Kunze I. (1997) *Arxula adeninivorans* In: Wolf K. (ed) Nonconventional yeasts in biotechnology: A handbook. Springer, Heidelberg, pp. 389 –395.

Pirt S. J. (1975) Principles of microbe and cell cultivation. Blackwell Scientific Publications, Oxford, pp 137 – 142.

Rösel H. and Kunze G. (1998) Integrative transformation of the dimorphic yeast *Arxula adeninivorans* LS3 based on hygromycin B resistance. Curr. Gen. **33**: 157–163.

Steinborn G., Wartmann T., Gellissen G. and Kunze G. (2007) Construction of an *Arxula adeninivorans* host-vector system based on *trp1* complementation. J. Biotechnol. **127**: 392-401.

Sudbery P., Gow N. and Berman J. (2006) The distinct morphogenic states of *Candida albicans*. Trends Microbiol. **12**: 317-324.

Terentiev Y., Breuer U., Babel W. and Kunze G. (2004) Non-conventional yeasts as producers of polyhydroxyalkanoates - genetic engineering of *Arxula adeninivorans*. Appl. Microbiol. Biotechnol. **64**: 376-681.

Wartmann T., Bellebna C., Böer E., Bartelsen O., Gellissen G. and Kunze G. (2003) The constitutive *AHSB4* promoter – a novel component of the *Arxula adeninivorans*-based expression platform. Appl. Microbiol. Biotechnol. **62**: 528–535.

Wartmann T., Krüger A., Adler K., Duc B. M., Kunze I. and Kunze G. (1995) Temperature-dependant dimorphism of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek **68**: 215–223.

Yang X., Wartmann T., Stoltenburg R. and Kunze G. (2000) Halotolerance of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek **77**: 303–311.

Summary

Arxula adeninivorans strain LS3, a dimorphic yeast with potential for biotechnological applications, has in recent years has been studied for the production of heterologous proteins. The growth kinetics of this species and the effect of environmental conditions on its morphology under controlled cultivation conditions have not been well documented. Because genetic transformation might affect the yeast physiology, a comparative investigation of the growth characteristics of strains LS3, G1211 (*LEU2*$^{+}$) (the auxotroph derived from strain LS3) and LS3/p*XynA,* derived from strain LS3 by transformation with the *Thermomyces lanuginosus* xylanase gene under the control of the *TEF1* promoter, was conducted, focusing on the effect of temperature and dissolved oxygen tension (DOT) on the morphology and growth parameters of the strains.

This xylanase gene (*XynA*) was integrated in the 25S rDNA locus and expressed under control of the strong constitutive *Arxula*-derived *TEF1* promoter. The plasmid copy number integrated into the rDNA locus was unknown. Little to no activity was found with the *A. adeninivorans* LS3 transformants, namely only 5.86 nkat ml^{-1} (0.35 U ml^{-1}) compared to the 4 418 nkat ml^{-1} (265 U ml^{-1}) obtained with the *T. lanuginosus* strain SSBP positive control. The protein itself might have been defective and since it accumulated intracellularly, this could also have resulted in the diminished activity observed.

Cultivation in a temperature gradient incubator over a wide temperature range revealed significant differences in the maximum specific growth rates of the strains LS3 and G1211(*LEU2*$^{+}$), namely 0.48 and 0.52 h^{-1}, respectively. The minimum and maximum temperatures were 18 and 46°C, respectively, with the optimum temperature in the range of 37 to 39°C. In accordance with the literature, in shake flask cultures the morphology of LS3 changed with an increase in temperature from predominantly budding cells at 30°C to pseudohyphae at 42°C and resembled a mycelial culture at 45°C. By contrast, the morphology of strain G1211 (*LEU2*$^{+}$) remained pseudohyphal at 45°C.

During batch cultivations no true hyphae were observed, but a change in morphology, from budding cells to pseudohyphae, was observed during cultivation at different

dissolved oxygen concentrations at different temperatures for LS3 and LS3/p*XynA*. G1211 (*LEU2*$^{+}$) remained a pseudohyphal culture. The critical dissolved oxygen concentration (C_{crit}) value, determined from the intersection of tangent lines of a plot of specific rate of oxygen uptake as function of dissolved oxygen concentration, of strains LS3 and G1211 (*LEU2*$^{+}$) was 0.016 mmol l^{-1} (9% of saturation) and 0.013 mmol l^{-1} (7% of saturation), respectively. This revealed that *A. adeninivorans* strain G1211 (*LEU2*$^{+}$) had a slight but significantly lower C_{crit} than strain LS3.

This study showed that insertion of plasmid pAL-ALEU2m has a significant effect on the specific growth rate and on the morphology of *Arxula adeninivorans* LS3. Insertion of plasmid p*XynA* had only a slight effect on the morphology but no effect on the specific growth rate.

Keywords: *Arxula adeninivorans*, critical dissolved oxygen tension, dimorphic, morphology, pseudohyphae, yeast

Opsomming

Arxula adeninivorans stam LS3, ‘n dimorfiese gis met potentiaal vir biotegnologiese toepassings, is die afgelope jare vir die produksie van heteroloë proteïene bestudeer. Die groeikinetika en die effek van omgewingsfaktore op die morfologie van hierdie spesies is nie goed gedokumenteer nie. Omdat genetiese transformasie dalk die morfologie van die gis kon affekteer, is ‘n vergelykende studie tussen die drie *A. adeninivorans* stamme LS3, G1211 (*LEU2*$^{+}$) (die ouksotrofiese mutant van stam LS3) en LS3/p*XynA*, afgelei van stam LS3 deur transformasie met die *Thermomyces lanuginosus* xilanase-geen onder die beheer van die *TEF1* promoter, onderneem met die fokus op die invloed van temperatuur en die opgeloste suurstofspanning (DOT) op die morfologie en groeiparameters van die stamme.

Die xilanase geen (*XynA*) is in die 25S rDNA lokus geïntegreer en onder die beheer van die sterk konstitiewe *A. adeninivorans* promoter *TEF1* uitgedruk. Die plasmied kopie-getal wat in die rDNA lokus geïntegreer is, was onbekend. Baie min tot geen aktiwiteit is met die *A. adeninivorans* transformante gevind, naamlik slegs 5.86 nkat ml^{-1} (0.35 U ml^{-1}) in vergelyking met dìe 4 418 nkat ml^{-1} wat in die *T. lanuginosus* stam SSBP positiewe kontrole gevind is. Die proteïen self kon defektief gewees het en aangesien dit intrasellulêr opgehoop het, kon dit dalk ook tot die verlaagde aktiwiteit tot gevolg gehad het.

Kweking in ‘n temperatuurgradiënt inkubator oor ‘n wye temperatuurreeks het betekenisvolle verskille in die maksimum spesifieke groeisnelhede van die twee stamme LS3 en G1211 (*LEU2*$^{+}$), naamlik 0.48 en 0.52 h^{-1} onderskeidelik, aangetoon. Die minimum en maksimum temperature was 18 en 46°C, onderskeidelik, met die optimum temperatuur in die gebied van 37 tot 39°C. In ooreenstemming met die literatuur het die morfologie van skudfleskulture van stam LS3 met ‘n verhoging in temperatuur vanaf oorwegend botselvormende selle by 30°C na pseudohifes by 42°C verander en by 45°C het die selle soos `n miselium-agtige kultuur gelyk. In teenstelling hiermee, het die morfologie van stam G1211 (*LEU2*$^{+}$) by 45°C pseudohife-agtig gebly.

Tydens lotkultuur kwekings is geen ware hifes gevind nie, maar ‘n verandering in morfologie vanaf botselvormende selle na pseudohifes is wel tydens kweking van

stamme LS3 en LS3/p*XynA* by verskillende DOT-waardes en temperature opgemerk. Stam G1211 (*LEU2*$^+$) het `n pseudohife-kultuur gebly. Die kritiese opgeloste suurstofkonsentrasie (C_{crit}) van stamme LS3 en G1211 (*LEU2*$^+$) wat vanaf die kruispunt van die raaklyne van die grafiek van spesifieke snelheid van suurstof-opname (q_{O2}) as ‘n funksie van die opgeloste suurstofkonsentrasie bepaal is, was 0.016 mmol l^{-1} (9% versadiging) and 0.013 mmol l^{-1} (7% versadiging), onderskeidelik. Dit beteken dat die C_{crit} waarde van *A. adeninivorans* stam G1211 (*LEU2*$^+$) beduidend laer as dié van stam LS3 was.

Hierdie studie bewys dat die invoeging van die plasmied pAL-ALEU2m ‘n betekenisvolle effek op die spesifieke groeisnelheid en die morfologie van *Arxula adeninivorans* LS3 gehad het. Die invoeging van die plasmied p*XynA* het slegs ‘n geringe effek op die morfologie maar geen effek op die spesifieke groeisnelheid gehad nie.