# THE GROWTH KINETIC CHARACTERISATION OF *SACCHAROMYCES CEREVISIAE* STRAINS TRANSFORMED WITH AMYLASE GENES

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# Chapter 1

# INTRODUCTION AND LITERATURE REVIEW

# 1 Introduction

The uses of ethanol include (a) potable ethanol, (b) fuel for vehicles, (c) a solvent for use in the pharmaceutical industry, (d) a chemical feedstock, (e) a disinfectant and (f) a co-surfactant in oil-water micro-emulsions (Lewis, 1996). Batch, fed-batch and continuous fermentation systems are used to produce ethanol (Keim, 1983).

Starch is an abundant renewable biopolymer (Chakrabarti and Storey, 1990) and, for a number of years, there has been considerable interest in using amylolytic yeasts for the direct fermentation of starch as an alternative to the conventional starch fermentation using commercial amylases for starch saccharification.

A microorganism suitable for commercial ethanol production must be able to ferment the substrate to ethanol without excessive formation of byproducts. It should also be tolerant to relatively high concentrations of ethanol without a significant loss of viability. Most yeasts, in particular *Saccharomyces cerevisiae*, have a relatively high ethanol tolerance. Yeasts can be classified into three groups based on their fermentation capacities. These include the non-fermentative, facultatively fermentative and obligate fermentative yeasts (Van Dijken and Scheffers, 1984). A total of 150 species of the approximately 400 recognised yeast species contain strains capable of utilising starch as carbon and energy source (Sá-Correia and Van Uden, 1981; Steyn and Pretorius, 1995). However, the naturally amylolytic yeasts are not suitable for commercial ethanol production from starch (see section 3.1).

Researchers at the Institute for Wine Biotechnology at the University of Stellenbosch, Stellenbosch, South Africa, constructed a number of recombinant strains of *S. cerevisiae* that contained amylase genes and thus had the potential to convert starch to ethanol. Fermentation trials were required to determine the efficacy of these strains for starch conversion.

## 2 Aim of study

The aim of this study was to evaluate *S. cerevisiae* yeast strains transformed with different amylase genes in terms of the relevant kinetic and stoichiometric parameters to assess their potential for commercial ethanol production by the direct fermentation of starch.

# 3 Literature review

## 3.1 Amylolytic yeasts

Most starch-converting yeasts hydrolyse only part of the starch and thus have low biomass yields in terms of the amount of carbon substrate supplied (Spencer-Martins and Van Uden, 1977; 1979). Table 1 indicates some yeast species known to produce the enzymes required for starch hydrolysis.

Although there are many amylolytic yeasts, as is evident from Table 1, certain species have been singled out for the purpose of this discussion: *Saccharomyces cerevisiae*, as it served as the host yeast for genetic manipulation in this investigation; *Lipomyces spencermartinsiae* (*kononenkoae*) and *Saccharomycopsis fibuligera*, as they were the donors of the amylase genes, and *Schwanniomyces occidentalis*, as it is a naturally amylolytic yeast. The latter three species are also known for their high amylase activities relative to the naturally amylolytic yeasts.

### 3.1.1 *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* is a lower eucaryote that is currently used as a model for genetic studies (Steyn and Pretorius, 1990). This intense interest in *S. cerevisiae* is due to a number of favourable factors, including (a) the vast amount of information available on its genetics and physiology (De Mot, 1990), (b) the advances made in molecular and cellular biology, especially in recombinant DNA techniques, and (c) the ease with which exogenous DNA can be introduced into its genome. These all facilitate easy genetic manipulation (Park *et al.*, 1993). *S. cerevisiae* can also easily be grown to a high cell density at a relatively low cost (Tokunaga *et al.*, 1997), which is an important consideration if the yeast transformant is destined for industrial applications. Its GRAS (generally regarded as safe) status, strong fermentative characteristics and high ethanol and acid tolerance make this yeast ideal for the fermentation of starch to ethanol (Ibragimova *et al.*, 1995), including potable ethanol. Due to its lack of amylolytic activity, with the exception of *S. cerevisiae* var. *diastaticus*, which has a limited dextrinase activity resulting from the production of an extracellular glucoamylase (Sills

**Table 1.** Yeasts and their associated amylolytic enzymes

| Species (current name) | Synonyms | Amylolytic enzymes |
|---|---|---|
| *Ambrosiozyma monospora* | *Endomyces bispora* | Extracellular glucoamylase, no α-amylase |
| *Candida albicans* | *Candida claussenii, Candida langeronii* | Extra-and intracellular amylase |
| *Candida catenulata* | *Candida bruptii* | Extracellular amylase |
| *Candida diddensiae* | *Torulopsis saccharini* | Intracellular amylase |
| *Candida ernobii* | *Torulopsis ernobii* | Extra-and intracellular amylase |
| *Candida fennisa* | *Trichoderma fennicum* | α-Amylase, glucoamylase |
| *Candida silvanorum* | | α-Amylase, glucoamylase |
| *Candida tropicalis* | | Glucoamylase |
| *Debaryomyces polymorphus* | *Debaryomyces phaffii, Pichia polmorpha* | Extra-and intracellular amylase, no glucoamylase |
| *Filobasidium capsuligenum* | *Candida japonica* (asexual state) | Extracellular glucoamylase |
| *Lipomyces spencermartinsiae* | *Lipomyces kononenkoae* | Extracellular α-amylase, glucoamylase and isoamylase |
| *Lipomyces starkeyi* | | Extracellular α-amylase and glucoamylase |
| *Pichia anomala* | *Candida pelliculosa* | Intracellular amylase |
| *Saccharomyces cerevisiae* var. *diastaticus* | *Saccharomyces diastaticus* | Extracellular glucoamylase |
| *Saccharomycopsis fibuligera* | *Endomycopsis fibuliger, Endomyces fibuliger* | Extracellular glucoamylase and extracellular α-amylase |
| *Schwanniomyces occidentalis* | *Schwanniomyces alluvius* | Extracellular α-amylase and glucoamylase |
| *Trichosporon pullulans* | | Extracellular α-amylase and glucoamylase |
| *Zygosaccharomyces rouxii* | *Saccharomyces rouxii* | Intracellular α-amylase and glucoamylase |

Adapted from De Mot (1990); Gogoi *et al.* (1987); McCann and Barnett (1986).

and Stewart, 1982; Kim *et al.*, 1988), *S. cerevisiae* cannot use starch as its sole carbon and energy source (De Mot, 1990). In addition, *S. cerevisiae* is capable of utilising only a restricted range of carbon substrates (Park *et al.*, 1993).

### 3.1.2 *Schwanniomyces*

In contrast to *Saccharomyces,* this genus contains naturally amylolytic yeasts (Simões-Mendes, 1984). Prior to 1978, it was thought that only species of *Saccharomycopsis* and *Lipomyces* where able to produce both an α-amylase and a glucoamylase (Wilson *et al.*, 1982). Subsequently, the production and excretion of both an α-amylase and a glucoamylase by *Schwanniomyces occidentalis* have been described (Oteng-Gyang *et al.*, 1980; McCann and Barnett, 1986; Wilson and Ingledew, 1982). The genus *Schwanniomyces* was thought to be very promising, as strains within this genus hydrolysed a 2.5 % solution of soluble starch almost completely (De Mot, 1990). *Schwanniomyces* species, including *S. alluvius, S. castellii, S. persoonii* and *S. occidentalis,* are able to ferment and assimilate a large number of carbon compounds (Ingledew, 1987). *Schw. occidentalis* hydrolyses starch completely into glucose using α-amylase and glucoamylase (Strasser *et al.*, 1991). However, *Schwanniomyces* species are poor fermenters (De Mot *et al.*, 1985; Wilson et *al.*, 1982) and the activity of the α-amylase of *Schwanniomyces occidentalis* was greatly impaired, more so than its glucoamylase, by high ethanol concentrations (De Mot, 1990). Culture conditions may also affect the activity of amylase production, as a 10 % air saturation level is required by *Schw. occidentalis* for amylase production (De Mot, 1990). These yeasts also have a low ethanol tolerance and are not suitable for ethanol production on an industrial scale (Panchal *et al.*, 1982).

### 3.1.3 *Lipomyces spencermartinsiae (kononenkoae)*

This yeast secretes a group of highly efficient amylases, allowing for complete starch hydrolysis (Spencer-Martins and Van Uden, 1979). These include highly active extracellular α-amylases, glucoamylases, isoamylases, α-glucosidases (Kelly *et al.*, 1985), glucanohydrolases (Spencer-Martins, 1984) and dextranases (Koenig and Day,

1989). The α-amylase produced by this yeast does not require calcium ions for activity or stability and is not inhibited by EDTA (Aunstrup, 1983). Due to their non-fermentative capacity and low ethanol tolerance, these yeasts are not suitable for starch fermentation (Steyn and Pretorius, 1995). Additionally, the genetics of *L. spencermartinsiae* are poorly characterised. It has a slow growth rate and lacks GRAS status (Steyn and Pretorius, 1995).

### 3.1.4 *Saccharomycopsis fibuligera*

*Saccharomycopsis fibuligera,* formerly known as *Endomycopsis fibuliger,* can be found in bread, macaroni, ragi and cattle fodder. This yeast produces an extracellular α-amylase (Steverson *et al.*, 1984). It was determined that the dominant enzyme was α-amylase (Steverson *et al.*, 1984). The presence of saccharifying and dextrinising enzymes, which exhibited a strong debranching activity, was also reported (Steverson *et al.*, 1984). A number of papers reported that *S. fibuligera* gave higher amylase activity values than *L. spencermartinsiae* (Abouzied and Reddy, 1987).

## 3.2 Starch

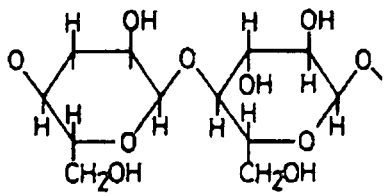
Starch is one of the most abundant plant polysaccharides, second only to cellulose (Guzmán-Maldonado and Paredes-López, 1995; Stewart, 1987). It serves both as a long and short-term storage molecule. On a long-term basis, it is stored in seeds, tubers and rhizomes and used only when energy is required for germination (Goodwin and Mercer, 1983). Natural starch is insoluble and is present in plants as microscopic granules (Bentley and Williams, 1996). Starch contains up to 20 % water, of which 10 % is chemically bound to the starch in concentric layers arranged around the hilum (Goodwin and Mercer, 1983).

A large percentage of agricultural biomass, including cereals, corn, potatoes, cassava and rice, can be used as potential sources of starch (Nigam and Singh, 1995). On average the above-mentioned substrates consist of 60 to 75 % starch on a dry weight basis. These substrates could thus be used as a carbon feedstock for ethanol production,

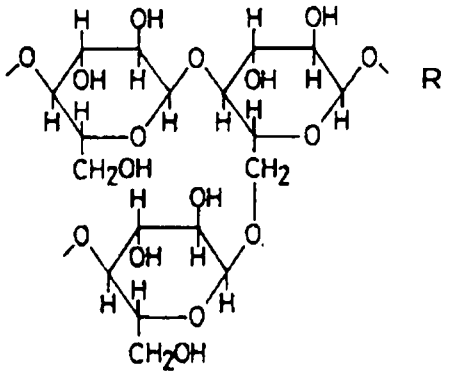
with little waste (Nigam and Singh, 1995). Prolonged storage is facilitated due to the chemical composition and high density of starch in comparison to other forms of biomass, thus decreasing transport and pre-treatment costs (Abouzied and Reddy, 1986).

### 3.2.1 Structure and composition

In the raw state, starch molecules are round or of irregular shape and vary in length from 1 to 100 μm. Internal H-bonds bind the starch granules, thus very little water is absorbed (Nigam and Singh, 1995). Starch, a bipolymer, consists of alpha-D-glucose residues linked to form large macromolecules (Nigam and Singh, 1995). This carbohydrate comprises two high molecular weight polysaccharides, amylose and amylopectin (Figure 1), which are both glucose polymers, but which differ in physical properties (Table 2). Amylose and amylopectin are usually present in the ratio of 1:3 to 1:4 (Bentley and Williams, 1996). However, these ratios may vary depending on the source of the starches, as indicated in Table 3.



*amylose*



*amylopectin*

**Figure 1.** The chemical structure of amylose and amylopectin. Adapted from Finn (1987). The reducing end is denoted by R.

The amylose fraction, constituting *ca.* 30 % of the starch, has a linear structure consisting of long unbranched chains of D-glucose units linked by α-1,4 bonds (Finn, 1987). It has a double helix crystalline structure containing six D-glucose molecules per turn (Nigam and Singh, 1995). Amylose is soluble in hot water, but the resulting solution is unstable and precipitates spontaneously. This process, known as retrogradation, an irreversible process that arises due to the tendency of the amylose molecules to align themselves side by side to form insoluble aggregates by hydrogen bonding (Goodwin and Mercer, 1983). A helical coil forms when amylose is suspended in water; this produces a blue colour when reacted with iodine, because the iodine halide occupies a position at the interior of the coil.

**Table 2.** A comparison of the physical properties of amylose and amylopectin.

| Property | Amylose | Amylopectin |
|---|---|---|
| Structure | Essentially linear | Branched |
| Bonds | Alpha-1,4 | Alpha-1,6 and alpha-1,4 |
| Stability in aqueous solutions | Retrogrades | Stable |
| Degree of polymerisation | $10^2$-$10^4$ | $10^4$-$10^5$ |
| Average chain length (glucose units) | $10^2$-$10^4$ | 20-30 |
| Portion of starch | 20-25 % | 75-80 % |
| Reaction with iodine | Blue colour | Purple to red colour |
| Iodine complex $\lambda_{max}$ (nm) | 650 | 550 |

Adapted from Fogarty and Kelly (1979).

The amylopectin fraction constitutes the remaining 70 % of the starch and is highly branched at points linked by α-1,6 bonds (Figure 1). Amylopectin has an average branch chain length of 20 to 25 glucose units and a molecular weight greater than $10^8$, making it the largest molecule in nature (Nigam and Singh, 1995). Each amylopectin molecule has one reducing end and many non-reducing ends.

**Table 3.** The amylose and amylopectin content of various starch sources.

| Starch source | % Amylose | % Amylopectin |
|---|---|---|
| Acorn | 24.0 | 76.0 |
| Banana | 16.8 | 83.2 |
| Maize | 24.0 | 76.0 |
| Amylomaize | 52.0 | 48.0 |
| Waxy maize | 0 | 100 |
| Potato | 20.0 | 80.0 |
| Rice | 18.5 | 81.5 |
| Sago | 25.9 | 74.1 |
| Wheat | 25.0 | 75.0 |

Adapted from Greenwood (1956) and Fogarty and Kelly (1979).

### 3.3 Starch hydrolysis

Starch can be degraded using acid, enzymes or a combination of both. Acid hydrolysis is often incomplete and produces condensation and degradation by-products (Moreton, 1978; Kim and Handy, 1985). The use of acid also requires corrosion resistant equipment (Jarl, 1969). Enzymatic hydrolysis has a number of advantages, including the fact that enzymes are highly specific, allowing the process of hydrolysis and the end-products to be controlled. Enzyme reactions are milder, resulting in less by-product formation and "browning" (Nigam and Singh, 1995).

#### 3.3.1 Enzymatic hydrolysis

For rapid and effective starch hydrolysis, α-amylase, glucoamylase and a debranching activity are required (De Mot and Verachtert, 1987). Enzymatic hydrolysis of starch can be divided into three main stages: (a) gelatinisation, which is the disruption of the starch granules by heat treatment at higher than 60 °C (Bentley and Williams, 1996), (b) liquefaction, which is the disruption of insoluble starch granules in an aqueous solution by heat and subsequent enzymatic hydrolysis of the starch particles (Stewart, 1987), and

(c) saccharification, which is the further hydrolysis of the oligosaccharides to fermentable sugars (Keim, 1983).

During gelatinisation, the starch granules hydrate, swell and become soluble (Guzmán-Maldonado and Paredes-López, 1995). The gelatinised starch solution has a high viscosity, which is caused by the unfolding of the branched amylopectin molecules (Bentley and Williams, 1996). This retrograde starch forms a gel, which is difficult to re-dissolve.

Liquefaction occurs when an α-amylase catalyses the hydrolysis of the α-1,4 glucosidic linkages in amylose and amylopectin in the gelatinised starch (Sheppard, 1986). This initial liquefaction is normally carried out by endo-amylases, which hydrolyse the bonds located within the starch structure, leading to a rapid decrease in the viscosity of the starch slurry (Sheppard, 1986; Norman, 1979).

Saccharification is due to the hydrolytic activity of enzymes that yield glucose units as the main reaction products. This process involves the stepwise removal of glucose molecules from the non-reducing ends of the starch molecule (Keim, 1983).

### 3.3.2 Amylolytic enzymes

Originally the term amylase was used to describe enzymes capable of hydrolysing α-1,4 glucosidic bonds of amylose, amylopectin, glycogen and their hydrolysis products (Fogarty and Kelly, 1979). Amylase enzymes that effect starch hydrolysis are now classified in the following major groups:

*α-Amylases*

Endo-amylases (also known as endoglucanases) are α-amylases, which cleave α-1,4 glucosidic bonds in amylose, amylopectin and related polysaccharides that are located in the inner region of the polysaccharide molecule, yielding oligosaccharides of varying chain length (Brown, 1979; Norman, 1979). The main products of hydrolysis are glucose, maltose, maltotriose, maltopentose and maltohexose (Norman, 1979;

Yamashita *et al.*, 1985). The α-amylase enzymes have a higher affinity for oligosaccharides with a linear segment of at least five glucose residues (Steyn and Pretorius, 1995).

The hydrolysis of starch molecules by α-amylases can be discussed in terms of two theories, that of multiple attack and that of preferred attack (Aunstrup, 1978). The multiple attack theory is based on the assumption that all the bonds in the molecule are subject to equal hydrolysis. Upon random encounter of an enzyme and a substrate, one part of the substrate is cleaved and the remainder of the substrate remains bound to the enzyme for further hydrolysis. This repeated hydrolysis takes place a number of times until that portion of the substrate is completely hydrolysed (Aunstrup, 1978). In the theory of preferred attack or multichain attack, a single hydrolytic event occurs during each encounter between the substrate and the enzyme. Differences in the hydrolytic actions are explained by the assumption that all the glycosidic bonds are not equally susceptible to enzyme hydrolysis.

Amylases used industrially may be divided into two groups, namely thermostable α-amylases, used mainly for high temperature liquefaction, and thermolabile α-amylases, used for saccharification (Norman, 1979). Different α-amylase enzymes have a different dependency on calcium ions. The more stable enzymes are stabilised by 50 $mg.l^{-1}$, compared to 100 to 150 $mg.l^{-1}$ required by the less-stable, heat-liable enzymes (Sheppard, 1986). Some bacterial α-amylase enzymes require as little as 5 mg $Ca^{2+}$. $l^{-1}$ (Guzmán-Maldonado and Paredes-López, 1995). The α-amylase enzyme catalyses hydrolysis from the non-terminal α-1,4 glycosidic bonds and is able to bypass the α-1,6 bonds of amylopectin, yielding maltose as the major end product. α-Amylase may have a regulatory role in starch degradation, as it can be considered the rate-limiting enzyme in starch hydrolysis (Dunn, 1974).

*β-Amylases*

β-Amylase (also known as exo-amylase or exoglucanase) cleaves α-1,4 glucosidic bonds in amylose, amylopectin and related polysaccharides (Aschengreen, 1969). The glucogenic exo-amylases also cleave α-1,6 glucosidic bonds in isoamylase, panose or

branched oligosaccharides, though at a slower rate. Maltogenic exo-amylases are not able to bypass α-1,6 glucosidic branching points (Fogarty and Kelly, 1980). The main end products of hydrolysis are glucose and maltose, which are removed stepwise from the non-reducing chain end (Norman, 1979). The main products of β-amylase action on starches are limit dextrin and maltose (Fogarty and Kelly, 1979). Many microorganisms are known to produce this enzyme; one of the most well known is the bacterium *Bacillus polymyxa* (Aunstrup, 1983).

*Glucoamylases*

Glucoamylase (exo-1,4-α-D-glucan glucohydrolase), which is also known as amyloglucosidase (Brown, 1979), is a glycoprotein (Aunstrup, 1983) that catalyses the removal of single glucose units from the non-reducing ends of starch (Bui *et al.*, 1996; Fogarty and Kelly, 1979). This exoamylase occurs almost exclusively in fungi (Fogarty and Kelly, 1979) and is capable of hydrolysing the α-1,6 linkages (Brown, 1979). Glucoamylase (EC 3.2.1.3) is one of the most important industrial enzymes and it is relatively cheap when calculated on an enzyme protein basis (Aunstrup, 1983). Highly saccharified starch hydrolysates and dextrose are made almost exclusively using this enzyme (Aunstrup, 1983).

Glucoamylase can also hydrolyse the α-1,6 and α-1,3 bonds, although this occurs at a slow rate and is also more active on long chain polymers than on short chains (Rielly, 1985). The rate of hydrolysis is influenced by the molecular size and structure of the substrate, as well as by the order of the bonds in the polymer (Fogarty and Kelly, 1980).

*Debranching amylases*

Debranching enzymes are able to hydrolyse α-1,6-glucosidic bonds, but at a rather slow rate. Pullulanase and isoamylase are the major debranching enzymes. Both these enzymes are directly acting enzymes capable of degrading unmodified amylopectin (Fogarty and Kelly, 1980). Isoamylase has a high affinity for amylopectin and glycogen, but a low affinity for pullulan (Fogarty and Kelly, 1980). Pullulanase, as the name indicates, has a high affinity for pullulan, producing hexa- and nona-

oligosaccharides initially and maltose as the end product. Amylose is only partially degraded to a β-limit dextrin by pullalanase (Fogarty and Kelly, 1980).

*Amylopullulanases*

The amylopullulanases comprise a unique group of amylases, often referred to as an all-in-one type of starch degrading enzyme. Amylopullulanases have the ability to hydrolyse both the α-1,4 and the α-1,6 bonds, thus allowing the debranching of amylopectin and the almost complete hydrolysis of starch (Steyn and Pretorius, 1995). The best-known example is the amylopullulanase encoded by the amylopullulanase gene *LKA1* from *Lipomyces spencermartinsiae* (Spencer-Martins and Van Uden, 1979).

Table 4 summarises the starch-degrading enzymes and their activities.

*Enzymes degrading raw starch*

Twenty-five years ago, it was thought that glucoamylases could not digest raw starch (Fogarty and Kelly, 1979). This misconception was due to the limited ability of amylase enzymes to degrade raw starch because of the lack of a raw starch-binding domain. Thus, pre-cooking at high temperatures was necessary to gelatinise the starch (Keim, 1983). However, starch fermentation without pre-cooking has received much attention as a way of saving as much as 30 to 40 % of the total energy expenditure (Mikuni *et al.*, 1987).

Enzymes with some ability to degrade raw starch, including α-amylase from *Bacillus licheniformis* and glucoamylase from *Aspergillus oryzae, Rhizopus niveus* and *Saccharomycopsis fibuligera,* have been described and isolated (Tubb, 1986). The glucoamylase, amyloglucosidase 1, from *Rhizopus* species exhibits strong debranching properties and actively degrades raw starch, with a preference for waxy starches (Fogarty and Kelly, 1979). Mikuni *et al.* (1987) observed alcohol formation by glucoamylases from *Chalara paradoxa* under fermentative conditions using raw maize starch as substrate. They reported that, apart from several technical problems, a 90 % theoretical yield of fermentation ethanol was obtained.

**Table 4.** Classification of starch-degrading enzymes.

| Enzyme | Common name | Reaction catalysed |
|---|---|---|
| Endo-1,4-α-D glucanase, Glucandehydrogenase (EC 3.2.1.1) | α-Amylase | Endohydrolyses α-1,4-glucosidic bonds |
| Exo-1,4-α-glucanase, Maltohydrolase (EC 3.2.1.2) | β-Amylase | Hydrolyses alternate glycosidic linkages in an exo-fashion. |
| α-D-glycoside glucohydrolase, Maltase (EC 3.2.1.20) | α-D-Glucosidase | Hydrolyses terminal 1,4-linked glucose residues from the non-reducing ends. |
| Amyloglucosidase, Glucan 1,4-α-glucosidase (EC 3.2.1.3) | Glucoamylase | Hydrolyses terminal 1,4-linked glucose residues successively from the non-reducing ends, releasing glucose. |
| Pullulan 4-glucano-hydrolase (EC 3.2.1.57) | Isopullulanase | Hydrolyses α-1,4-glucosidic linkages in pullulan. Little effect on starch. |
| α-Dextrin 6-glucano-hydrolase (EC 3.2.1.41) | Pullulanase | Hydrolyses 1,6-linkages of pullulan and other polysaccharides. |
| Endo α-1,6-glucanase, Glycogen 6-glucano-hydrolase (EC 3.2.1.68) | Isoamylase | Hydrolyses α-1,6-glucosidic branches of amylopectin, glycogen and other polysaccharides |
| Cyclomaltodextrinase (EC 2.4.1.54) | Cyclodextrinase | Hydrolyses cyclomaltodextrins to linear maltodextrins |
| Dextrin 6-α-D-glucan hydrolyse (EC 3.2.1.10) | Isomaltase | Hydrolyses α-1,6-D-glucosidic linkages |

Adapted from Steyn and Pretorius (1990), Nigam and Singh (1995), Fogarty and Kelly (1979), De Mot (1990) and McCann and Barnett (1986).

## 3.4 Enzyme activity and stability

The physico-chemical parameters, especially the pH and temperature, must be optimal to obtain maximal enzyme activity.

### 3.4.1 pH and temperature

The pH plays an important role in regulating the rate and effectiveness of starch hydrolysis (Drozdowixz and Jones, 1995). The optimum pH for cereal α-amylase is about 5.5 to 6.0 (Matton *et al.*, 1987). Fogarty and Kelly (1979) reported that α-amylases were stable in the pH range of 5.5 to 8.0 and remained stable under extremes of pH in the presence of a full complement of calcium. Commercial glucoamylase operates best at a pH of 4.0 to 4.5. De Mot (1990) reported that the optimum pH values were found in the range of pH 4.0 to pH 6.5. The temperature optimum for glucoamylase is between 40 and 60 °C (Fogarty and Kelly, 1979). Table 5 gives an indication of the pH ranges over which some of these amylases are active.

If the temperature of a hydrolysed starch solution drops too low, retrogradation of the starch will occur, producing an insoluble gel. The amylases required for starch hydrolysis all have specific ranges in which they operate effectively. De Mot (1990) reported that the temperature optimum for α-amylases is 40 to 50 °C and 50 to 60 °C for the glucoamylases. Table 5 gives a more detailed indication of the temperature optima for some of the starch-degrading enzymes. The estimates for the relative molecular masses of the α-amylases enzymes range between 40 000 and 80 000 (De Mot, 1990).

**Table 5.** A summary of some of the starch-degrading enzymes, their pH ranges for stability (with the pH optimum indicated in brackets), temperature optima and molecular weight.

| Source organism | Enzyme | pH range | Temperature, °C | Estimated Mr (kDa) |
|---|---|---|---|---|
| *Aspergillus niger* | α-Amylase | 4.7 - 6.0 | 60 | 60 |
| | Glucoamylase | 3.8 | 50 | 45 |
| *Aspergillus oryzae* | α-Amylase | 5.5 - 5.9 | 50 - 57 | 51 - 52.6 |
| | Glucoamylase | 4.5 - 5.0 (4.8) | 50 | 38 |
| *Aspergillus awamori* | α-Amylase | 5.0 - 9.0 (4.5) | 60 | |
| | Glucoamylase | 4.5 - 6.0 (5.0) | 55 | 125 - 140 |
| *Bacillus* | α-Amylase | 5.7 - 6.0 (5.9) | 55 - 65 | 49 |
| *amyloliquefaciens* | Glucoamylase | 6.8 | 40 | 27 |
| *Bacillus polymyxa* | β-Amylase | 7.5 | 45 | 44 |
| *Lipomyces* | α-Amylase | 4.0 - 6.5 (5.5) | 40 | 38, 65, 76 |
| *spencermartinsiae* | Glucoamylase | 4.0 - 6.5 (4.5) | 50 | 81 |
| *Lipomyces starkeyi* | α-Amylase | | | 56, 76 |
| *Schwanniomyces* | α-Amylase | 4.0 - 6.0 (5.5) | 40 - 60 | 52 - 62 |
| *occidentalis* | Glucoamylase | 5.0 - 6.0 (5.0) | 60 | 117 - 155 |
| *Saccharomycopsis* | α-Amylase | 4.5 | 40 - 50 | |
| *capsularis* | Glucoamylase | 4.5 | 40 - 50 | |
| *Saccharomycopsis* | α-Amylase | 4.5 - 5.0 | 40 | 40 - 50 |
| *fibuligera* | Glucoamylase | 4.5 - 5.8 | 40 | 55 |
| *Saccharomyces* | Glucoamylase | 4.6 | 25 | |
| *cerevisiae* var. | Debranching | 6.4 | 32 | |
| *diastaticus* | Glucoamylase | 4.6 | 32 | |

Adapted from Aunstrup (1978 and 1983), De Mot (1990), Fogarty and Kelly (1979, 1980 and 1983), Littlejohn (1973), Simões-Mendes (1984), Spencer-Martins and Van Uden (1979), Steyn and Pretorius, (1995), Wilson and Ingledew (1982).

## 3.5 Genetically engineered yeasts

During recent years, the increasing demand for ethanol has led to the development of genetically engineered strains of *S. cerevisiae* capable of a one-step bioconversion of starch. *S. cerevisiae* is the organism of choice for the production of ethanol due to the advantages discussed in Section 3.1.1. In this respect, the tendency seems to be towards the insertion of α-amylase or glucoamylase genes from bacterial, fungal or non-microbial sources into the *S. cerevisiae* genome (Table 6), because *S. cerevisiae* can be grown rapidly and to a high cell density in simple culture media. *S. cerevisiae* can be manipulated almost as easily as *Escherichia coli* and is a suitable host organism for the production of secreted as well as soluble proteins (Romanos *et al.*, 1992). However, this is not always possible: yeasts, being eukaryotic, contain introns and unique characteristics of the five-prime upstream untranslated region in the chromosome, which may affect the translation of the foreign protein (Randez-Gil and Sanz, 1993).

Protoplast fusion is a useful technique allowing the creation of novel strains with enhanced characteristics for alcohol production. Protoplast fusion is used to overcome problems, such as differences in mating types, multiplying copies of specific genes (Sakai *et al.*, 1986) or combining characteristics present in the parent strain in the new phenotype (Kavanagh and Whittaker, 1996). Sakai *et al.* (1986) used protoplast fusion to introduce additional STA genes (glucoamylase genes) into *Saccharomyces diastaticus* and then isolated hybrids showing improved ethanol production. Although starch fermentation was improved, no fusants were obtained that had adequate glucoamylase productivity for fermenting starch as rapidly as glucose. As a result, ethanol yields from starch were low. With the development of more specific means of manipulating genes, protoplast fusion is no longer the principal method for novel strain construction (Kavanagh and Whittaker, 1996).

Species of the genus *Schwanniomyces* and *Saccharomyces* are more frequently used as host organisms for foreign genes, because of an absence of pyrogenic toxins (Park *et al.*, 1993). Lately, a number of other yeast species, such as *Pichia pastoris, Kluyveromyces lactis* and species of the genus *Candida,* to name but a few, have become important host

organisms. This is mainly due to promoter strength, secretion efficiency and ease of cultivation to high cell densities (Romanos *et al.*, 1992).

**Table 6**. Recombinant yeast strains with amylase genes of various origins.

| Recipient | Gene | Origin | Reference |
|---|---|---|---|
| *S. cerevisiae* | α-Amylase | Rice | Uchiyama *et al.* (1995) |
| *S. cerevisiae* | α-Amylase | Rice | Kumagai *et al.* (1993) |
| *S. cerevisiae* | α-Amylase | Rice | Kumagai *et al.* (1990) |
| *S. cerevisiae* | α-Amylase | Wheat | Rothstein *et al.* (1987) |
| *S. cerevisiae* | α-Amylase | Barley wheat | Juge *et al.* (1993) |
| *Schizosaccharomyces pombe* | α-Amylase | Mouse | Tokunaga *et al.* (1993) |
| *S. cerevisiae* | α-Amylase | Mouse | Filho *et al.* (1986) |
| *S. cerevisiae* | α-Amylase | Human saliva | Nakamura *et al.* (1986) |
| *S. cerevisiae* | α-Amylase | *Schwanniomyces occidentalis* | Wang *et al.* (1989) |
| *S. cerevisiae* | Glucoamylase | *Lipomyces spencermartinsiae* | Steyn and Pretorius (1995) |
| *S. cerevisiae* | α-Amylase | *Aspergillus oryzae* | Randez-Gill and Sanz (1993) |
| *S. cerevisiae* | Glucoamylase | *Saccharomyces diastaticus* | Steyn and Pretorius (1991) |
| *S. cerevisiae* | Glucoamylase | *Bacillus stearothermophilus* | Nonato and Shishido (1988) |

Most yeast expression vectors have been shuttle vectors based on the multi-copy 2μ plasmid, containing a yeast promoter and terminator (Romanos *et al.*, 1992). Due to the rapid expansion in yeast genetics, our understanding of these components has increased greatly, thus allowing a variety of choices for the construction of expression vectors.

However, the insertion of a foreign gene into a expression vector does not guarantee high levels of expression, as gene expression is a multi-step process (Romanos *et al.*, 1992). Various genetic procedures have been employed for improving starch hydrolysis by *S. cerevisiae* strains, as discussed below.

### 3.5.1 Promoters

One of the most important factors influencing the expression of a foreign gene in the yeast is the level of transcription provided by the promoter (Hadfield *et al.*, 1993). Yeast promoters are highly complex, extending to over 500 base pairs containing multiple upstream activation sequences, negative regulatory sites and multiple TATAA elements. These components regulate the efficiency and accuracy of the initiation of transcription. When a heterologous gene is cloned into yeast, the natural promoter of the heterologous gene may be replaced with a yeast promoter (Cha and Yoo, 1996).

The first promoters used were glycolytic promoters, i.e. the alcohol dehydrogenase *(ADH1)*, phosphoglycerate kinase *(PGK1)* and the glyceraldehyde-3-phosphate dehydrogenase *(GAP)* promoters. Most promoters are regulated to some extent (Table 7), but the most powerful glycolytic promoters are poorly regulated (Romanos *et al.*, 1992). Hadfield *et al.* (1993) also stated that these glycolytic promoters were constitutive. Contrary to Romanos *et al.* (1992), Hadfield *et al.* (1993) stated that the transcriptional activity of the glycolytic promoters was subject to regulation, which reflected the physiological state of the cell. The promoters that were of importance to this research project, namely the *ADH1* and *PGK1* promoters, are discussed below.

#### *PGK1*

*PGK1*, encoding phosphoglycerate kinase (Park *et al.*, 1993), is one of the best-expressed genes in *S. cerevisiae*, making up about 5 % of the total protein (Heinisch and Hollenberg, 1998). It was one of the first glycolytic genes to be sequenced and much attention has been paid to its transcriptional regulation. Contradiction exists in the literature regarding the *PGK1* promoter. Sakai *et al.* (1992) stated that the *PGK1*

promoter was activated by glucose. However, Park *et al.* (1993) and Shiba *et al.* (1994) stated that the *PGK1* promoter was repressed by high glucose concentrations and derepressed at low glucose concentrations.

**Table 7.** Some promoter systems and their regulation effects.

| Promoter | Regulation |
|---|---|
| *PGK1* | Less than 20-fold induction by glucose |
| *ADH1* | Poor induction by glucose. |
| | No glucose repression. |
| *ADH2* | 100-fold repression by glucose |
| *PHO5* | 200-fold repression by inorganic phosphate |
| *SUC2* | Repressed by low glucose concentrations |
| *GAL7* | Induced by galactose, repressed by glucose |

Adapted from Romanos *et al.* (1992) and Hadfield *et al.* (1993).

***ADH1***

The yeast gene *ADH1* encodes for alcohol dehydrogenase, which catalyses the reduction of acetaldehyde to ethanol (Thomsen, 1987). The promoter region, contained on a 1.5-KB Bam HI fragment, could express foreign genes in yeasts when inserted behind this promoter (Thomsen, 1987). This promoter was previously considered to be a constitutive promoter. However, it is now known that its activity is regulated in such a way that it is decreased during growth on non-fermentable substrates and during the stationary phase (Denis *et al.*, 1983). It is not certain how this regulation is effected, but it is thought that the activation of an upstream promoter creates a longer mRNA chain from which the *ADH1* protein cannot be read (Ammer, 1983). Denis *et al.* (1983) reported that the longer mRNA chain was functional in *ADH1* production. The removal of an upstream sequence, pAAH5, from the *ADH1* promoter eliminated the inhibitory effect on *ADH1* synthesis without affecting the *ADH1* promoter activity (Beier and Young, 1982).

There have been a number of attempts in the past to introduce a foreign starch-degrading activity into non-amylolytic yeasts by recombinant DNA technology. Although there have been some successes, a number of associated problems remain. One of the most prominent problems in industrial applications is consumer acceptance. In addition, few investigations have assessed the impact of the 'genetic burden' or 'metabolic burden' on the growth and fermentation characteristics of the transformed yeast. Furthermore, the expression and secretion of a foreign gene in the new host organism needs to be optimised, which is a time-consuming and costly process.

## 4 References

**Abouzied, M. & Reddy, C. (1986).** Direct fermentation of potato starch to ethanol by co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*. *Appl Environ Microbiol,* **52**, 1055-1059.

**Abouzied, M. & Reddy, C. (1987).** Fermentation of starch to ethanol by a complementary mixture of an amylolytic yeast and *Saccharomyces cerevisiae*. *Biotechnol Lett,* **9**, 59-62.

**Ammer, G. (1983).** Expression of genes in yeast using the *ADC1* promoter. *Methods Enzymol,* **101**, 192-201.

**Aschengreen, Jr., N.H. (1969).** Microbial enzymes for alcohol production. *Process Biochem,* **4**, 23-25.

**Aunstrup, K. (1978).** Enzymes of industrial interest: traditional products. In *Annual reports on fermentation processes, Vol 2,* pp. 125-154. Edited by D. Perlman. New York: Academic Press Inc.

**Aunstrup, K. (1983).** Enzymes of industrial interest; traditional products. In *Annual reports on fermentation processes, Vol 6,* pp. 175-201. Edited by G.T. Tsao. New York: Academic Press Inc.

**Beier, D.R. & Young, E.T. (1982).** Characterisation of a regulatory region upstream of the *ADR2* locus of *Saccharomyces cerevisiae*. *Nature,* **300,** 724-728.

**Bentley, I.S. & Williams, E.C. (1996).** Starch conversion. In *Industrial Enzymology* 2nd Ed. pp. 341-357. Edited by T. Godfrey & S. West. London: Macmillan Press Inc.

**Brown, D.E. (1979).** Technology of microbial polysaccharase production. In *Microbial polysaccharides and polysaccharases.* pp. 297-326. Edited by R.C.W. Berkeley, G.W. Gooday & D. C. Ellwood. New York: Academic Press Inc.

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

**Cha, H.J. & Yoo, Y.J. (1996).** Determination of optimal glucose concentrations for glucoamylase production from plasmid-harbouring and chromosome-integrated recombinant yeasts using a *SUC2* promoter. *Process Biochem,* **31,** 499-506.

**Chakrabarti, A.C. & Storey, K.B. (1990).** Co-immobilisation of amyloglucosidae and pullulanase for enhanced starch hydrolysis. *Appl Microbiol Biotechnol,* **33,** 48-50.

**De Mot, R. (1990).** Conversion of starch by yeasts. In *Yeast, Biotechnology and Biocatalysis,* pp. 163-222. Edited by H. Verachtert & R. De Mot. New York & Basel: Marcel Dekker Inc.

**De Mot, R. & Verachtert, H. (1987).** Some microbiological and biochemical aspects of starch bioconversion by amylolytic yeasts. In *Crit Rev Biotechnol, Vol 5,* pp. 259-272. Edited by G.G. Stewart & I. Russell. Florida: CRC Press Inc.

**De Mot, R., Van Dijck, K., Donkers, A. & Verachtert, H. (1985).** Potentialities and limitations of direct alcoholic fermentation of starchy material with amylolytic yeasts. *Appl Microbiol Biotechnol* **22,** 222-226.

**Denis, C.L., Ferguson, J. & Young, E.T. (1983).** mRNA levels for the fermentative alcohol dehydrogenase of *Saccharomyces cerevisiae* decrease upon growth on a nonfermentable carbon source. *J Biol Chem,* **258,** 1165-1171.

**Drozdowixz, Y.M. & Jones, R.L. (1995).** Hormonal regulation of organic and phosphoric acid release by barley aleurone layers and scutella. *Plant Physiol,* **108,** 769-776.

**Dunn, G. (1974).** A model for starch breakdown in higher plants. *Phytochem,* **13,** 1341-1346.

**Filho, S.A., Galembeck, E.V., Faria, J.B. & Frascino, A.C.S. (1986).** Stable yeast transformants that secrete functional α-amylase encoded by cloned mouse pancreatic cDNA. *Biotechnology,* **4,** 311-315.

**Finn, R.K. (1987).** Conversion of starch to liquid sugar and ethanol. *Biotechnology,* **1,** 101-107.

**Fogarty, W.M. & Kelly, C.T. (1979).** Starch-degrading enzymes of microbial origin. *Prog Ind Microbiol,* **15,** 89-150.

**Fogarty, W.M. & Kelly, C.T. (1980).** Amylases, amyloglucosidases and related glucanases. In *Economic microbiology, microbial enzymes and bioconversions, Vol 5,* pp. 115-170. Edited by A.H. Rose. London: Academic Press Inc.

**Fogarty, W.M. & Kelly, C.T. (1983).** Microbial α-glucosidases. In *Process Biochem,* **18,** 6-12.

**Gogoi, B.K., Bezbaruak, R.L., Pillai, K.R. & Baruah, J.N. (1987).** Production, purification and characterisation of an α–amylase produced by *Saccharomycopsis fibuligera. J Appl Bacteriol,* **63,** 373-379.

**Goodwin, T.W. & Mercer, E.I. (1983).** Introduction to plant biochemistry. 2nd Ed. pp. 254-264. Oxford: Pergamon Press Inc.

**Greenwood, C.T. (1956).** Aspects of the physical chemistry of starch. In *Advances in carbohydrate chemistry,* pp. 335-393. Edited by M.L. Wolfrom & R.S. Tipson. New York: Academic Press Inc.

**Guzmán-Maldonado, H. & Paredes-López, O. (1995).** Amylolytic enzymes and products derived from starch: A review. *Critical reviews in food science and nutrition,* **35,** 373-403.

**Hadfield, C., Raina, K.K., Shashi-Meno, K. & Mount, R.C. (1993).** The expression and performance of cloned genes in yeasts. *Mycol Res,* **97,** 897-944.

**Heinisch, J.J. & Hollenberg, C.P. (1998).** Yeasts. In *Biotechnology: Biological fundamentals, Vol 1,* pp. 470-514. Edited by H-J. Rehm & G. Reed. New York: VCH (verlagsgesellschaft) Pub. Inc. (Weinheim, Germany).

**Ibragimova, S.I., Kozlov, D.G., Kartasheva, N.N., Suntsov, N.I., Efremov, B.D. & Benevolensky, S.V. (1995).** A strategy for construction of industrial strains of distiller's yeast. *Biotechnol Bioeng,* **46,** 285-290.

**Ingledew, M.W. (1987).** *Schwanniomyces*: A potential super yeast. In *CRC Crit Rev Biotechnol, Vol 5,* pp. 159-176. Edited by G. S. Graham & R. Inge. Florida: CRC Press Inc.

**Jarl, K. (1969).** Production of microbial food from low-cost starch materials and purification of industry's waste starch effluents through the Symba yeast process. *Food Technol,* **23,** 1009-1012.

**Juge, N., Sogaard, M., Chaix, J-C., Martin-Eauclaire, M-F., Svensson, B., Marchis-Mouren, G. & Guo, X-J. (1993).** Comparison of barley malt α-amylase isozymes 1 and 2: construction of cDNA hybrids by in vivo recombination and their expression in yeast. *Gene,* **130,** 159-166.

**Kavanagh, K. & Whittaker, P.A. (1996).** Application of protoplast fusion to the non-conventional yeast. *Enzyme Microbiol Technol,* **18,** 45-51.

**Keim, C.R. (1983).** Technology and economics of fermentation alcohol - an update. *Enzyme Microb Technol,* **5,** 103-114.

**Kelly, C.T., Moriarty, M.E. & Fogarty, W.M. (1985).** Thermostable extracellular α-amylase and α-glucosidase of *Lipomyces starkeyi. Appl Microbiol Biotechnol,* **22,** 352-358.

**Koenig, D.W. & Day, D.F. (1989).** Induction of *Lipomyces starkeyi* dextranase. *Appl Environ Microbiol,* **55,** 2079-2081.

**Kim, K. & Handy, M.K. (1985).** Acid hydrolysis of sweet potato for ethanol production. *Biotechnol Bioeng,* **2,** 316-320.

**Kim, K., Park, C.S. & Mattoon, J.R. (1988).** High-efficiency, one-step starch utilisation by transformed *Saccharomyces* cells which secrete both yeast glucoamylase and mouse α–amylase. *Appl Environ Microbiol,* **54,** 966-971.

**Kumagai, M.H., Shah, M., Terashima, M., Vrkljan, Z., Whitaker, J.R. & Rodriguez, R.L. (1990).** Expression and secretion of rice α-amylase by *Saccharomyces cerevisiae*. *Gene,* **94,** 209-216.

**Kumagai, M.H., Sverlow, G.G., Della-Cioppa, G. & Grill, L.K. (1993).** Conversion of starch to ethanol in a recombinant *Saccharomyces cerevisiae* strain expressing rice α-amylase from a novel *Pichia pastoris* alcohol oxidase promoter. *Biotechnology,* **11,** 606-610.

**Lewis, S.M. (1996).** Fermentation alcohol. In *Industrial Enzymology, Vol 2.1,* pp. 13-48. Edited by T. Godfrey & S. West. London: Macmillan Press Inc.

**Littlejohn, J.H. (1973).** Analyses of microbial origin. In *CRC Handbook of Microbiology: Microbial Products, Vol 3,* pp. 651-658. Edited by A.I. Laskin & H.A. Lechevalier. Cleveland, Ohio: CRC Press Inc.

**Matton, J.R., Kim, K. & Laluce, C. (1987).** The application of genetics to the development of starch-fermenting yeasts. In *CRC Crit Rev Biotechnol, Vol 5,* pp. 195-204. Edited by G.G. Stewart & I. Russell. Florida: CRC Press Inc.

**McCann, A.K. & Barnett, J.A. (1986).** The utilisation of starch by yeasts. *Yeast,* **2,** 109-115.

**Mikuni, K., Monma, M. & Kainuma, K. (1987).** Alcohol fermentation of corn starch digested by *Chalara paradoxa* amylase without cooking. *Biotechnol Bioeng,* **29,** 729-732.

**Moreton, R.S. (1978).** Growth of *Candida utilis* on enzymatically hydrolysed potato waste. *J Appl Bacteriol,* **44,** 373-382.

**Nakamura, Y., Sato, T., Emi, M., Miyanohara, A., Nishide, T. & Matsubara, K. (1986).** Expression of human salivary α-amylase gene in *Saccharomyces cerevisiae* and its secretion using the mammalian signal sequence. *Gene,* **50,** 239-245.

**Nigam, P. & Singh, D. (1995).** Enzyme and microbial systems involved in starch processing. *Enzyme Microbial Technol,* **17,** 770-778.

**Norman, B.E. (1979).** The application of polysaccharide degrading enzymes in the starch industry. In *Microbial Polysaccharides and Polysaccharases, Vol 15,* pp. 339-376. Edited by R.C.W. Berkeley, G.W. Gooday & D.C. Ellwood. New York: Academic Press Inc.

**Nonato, R.V. & Shishido, K. (1988).** α-Factor-directed synthesis of *Bacillus stearothermophilus* α-amylase in *Saccharomyces cerevisiae. Biochem Biophys Res Com,* **152,** 76-82.

**Oteng-Gyang, K., Moulin, G. & Galzy, P. (1980).** Effect of medium composition on excretion and biosynthesis of the amylases of *Schwanniomyces castellii. European J Appl Microbiol Biotechnol,* **9,** 129-132.

**Panchal, C.J., Harbison, A., Russell, I. & Stewart, G.G. (1982).** Ethanol production by genetically modified strains of *Saccharomyces. Biotechnol Lett,* **4,** 33-38.

**Park, Y.S., Shiba, S., Lijima, S. & Kobayashi, T. (1993).** Comparison of three different promoter systems for secretory α-amylase production in fed-batch cultures of recombinant *Saccharomyces cerevisiae. Biotechnol Bioeng,* **41,** 854-861.

**Randez-Gil, F. & Sanz, P. (1993).** Expression of *Aspergillus oryzae* α-amylase gene in *Saccharomyces cerevisiae. FEMS Microbiol Lett,* **112,** 119-124.

**Rielly, P.J. (1995).** Enzymatic degradation of starch. In *Starch conversion technology* pp. 101. Edited by G.M.A. Beynum & J.A. Roels. New York: Marcel Dekker Inc.

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

**Rothstein, S.J., Lahners, K.N., Lazarus, C.M., Baulcombe, D.C. & Gatenby, A.A. (1987).** Synthesis and secretion of wheat α-amylase in *Saccharomyces cerevisiae. Gene,* **55,** 353-356.

**Sá-Correia, I. & Van Uden, N. (1981).** Production of biomass and amylase by the yeast *Lipomyces spencermartinsiae* in starch-limited continuous culture. *European J Appl Microbiol Biotechnol,* **13,** 24-28.

**Sakai, T., Koo, K-I., Saitoh, K. & Katsuragi, T. (1986).** Use of protoplast fusion for the development of rapid starch-fermenting strains of *Saccharomyces diastaticus*. *Agric Biol Chem,* **50**, 297-306.

**Sakai, A., Chibazakura, T., Shimizu, Y. & Hishinuma, F. (1992).** Molecular analysis of *P0P2* gene, a gene required for glucose-derepression of gene expression in *Saccharomyces cerevisiae*. *Nucleic Acids Research,* **20,** 6227-6233.

**Sheppard, G. (1986).** The production and uses of microbial enzymes in food processing. *Prog Ind Microbiol,* **23,** 237-270.

**Shiba, S., Nishida, Y., Park, Y.S., Lijima, S. & Kobayashi, T. (1994).** Improvement of cloned α-amylase gene expression in fed-batch culture of recombinant *Saccharomyces cerevisiae* by regulating both glucose and ethanol concentrations using a fuzzy controller. *Biotechnol Bioeng,* **44,** 1055-1063.

**Sills, A.M. & Stewart, G.G. (1982).** Production of amylolytic enzymes by several yeasts species. *J Inst Brew,* **88,** 313-316.

**Simões-Mendes, B. (1984).** Purification and characterisation of the extracellular amylases of the yeast *Schwanniomyces alluvius*. *Can J Microbiol,* **30,** 1163-1170.

**Spencer-Martins, I. (1984).** Production and properties of an extracellular cyclodextrin hydrolase from the yeast *Lipomyces spencermartinsiae*. *Int J Microbiol,* **2,** 31-38.

**Spencer-Martins, I. & Van Uden, N. (1977).** Yields of yeast growth on starch. *European J Appl Microbiol Biotechnol,* **4**, 29-35.

**Spencer-Martins, I. & Van Uden, N. (1979).** Extracellular amylolytic system of the yeast *Lipomyces spencermartinsiae*. *European J Appl Microbiol Biotechnol,* **6,** 241-250.

**Steverson, E.M., Korus, R.A., Admassu, W. & Heimsch, R.C. (1984).** Kinetics of the amylase system of *Saccharomycopsis fibuliger*. *Enzyme Microbiol Technol,* **6,** 549-554.

**Steyn, A.J.C. & Pretorius, I.S. (1990).** Expression and secretion of amylolytic enzymes by *Saccharomyces cerevisiae*. *Acta Varia,* **5**, 76-126.

**Steyn, A.J.C. & Pretorius, I.S. (1991).** Co-expression of a *Saccharomyces diastaticus* glucoamylase-encoding gene and a *Bacillus amyloliquefaciens* α-amylase-encoding gene in *Saccharomyces cerevisiae*. *Gene,* **100,** 85-93.

**Steyn, A.J.C. & Pretorius, I.S. (1995).** Characterisation of a novel α-amylase from *Lipomyces spencermartinsiae* and expression of its gene (LKA1) in *Saccharomyces cerevisiae*. *Curr Genet,* **28,** 526-533.

**Stewart, G.G. (1987).** The biotechnological relevance of starch-degrading enzymes. In *Crit Rev Biotechnol, Vol 5*, pp. 89-93. Edited by G. S. Graham & R. Inge. Florida: CRC Press Inc.

**Strasser, A.W.M., Janowicz, Z.A., Roggenkamp, R.O., Dahlems, U., Weydemann, U., Merckelbach, A., Gellissen, G., Dohmen, R. J., Piontek, M., Melber, K. & Hollenberg, C.P. (1991).** Applications of genetically manipulated yeasts. In *Symp British Mycological Soc,* **18,** 161-169.

**Thomsen, K.K. (1987).** Production of α-amylase by yeast. *CRC Crit Rev Biotechnol,* **5,** 205-215.

**Tokunaga, M., Kawamura, A., Yonekyu, S., Kishida, M. & Hishinuma, F. (1993).** Secretion of mouse α-amylase from fission yeast *Schizosaccharomyces pombe*: Presence of chymostatin-sensitive protease activity in the culture medium. *Yeast,* **9,** 379-387.

**Tokunaga, M., Ishibashi, M., Tatsuda, D. & Tokunaga, H. (1997).** Secretion of mouse α-amylase from *Kluyveromyces lactis. Yeast,* **13,** 699-706.

**Tubb, R.S. (1986).** Amylolytic yeasts for commercial applications. *Trends in Biotechnol,* **4,** 98-104.

**Uchiyama, K., Ohtani, T., Morimoto, M., Shioya, S., Suga, K., Harashima, S. & Oshima, Y. (1995).** Optimisation of rice α-amylase production using temperature-sensitive mutants of *Saccharomyces cerevisiae* for the PHO regulatory system. *Biotechnol Prog,* **11,** 510-517.

**Van Dijken, J.P. & Scheffers, W.A. (1984).** Studies on alcoholic fermentation in yeasts. *Prog Ind Microbiol,* **20,** 497-506.

**Wang, T.T., Lin, L.L. & Hsu, W.H. (1989).** Cloning and expression of a *Schwanniomyces occidentalis* α-amylase gene in *Saccharomyces cerevisiae. Appl Environ Microbiol,* **55,** 3167-3172.

**Wilson, J.J., Khacharorians, G.G. & Ingledew, W.M. (1982).** *Schwanniomyces*: SCP and ethanol from starch. *Biotechnol Lett,* **5,** 333-338.

**Wilson, J.J. & Ingledew, W.M. (1982).** Isolation and characterisation of *Schwanniomyces alluvius* amylolytic enzymes. *Appl Environ Microbiol,* **44,** 301-307.

**Yamashita, I., Itoh, T. & Fukui, S. (1985).** Cloning and expression of the *Saccharomycopsis fibuligera* α-amylase gene in *Saccharomyces cerevisiae. Agric Biol Chem,* **49,** 3089-3091.

# Chapter 2

# PRELIMINARY EVALUATION OF RECOMBINANT *SACCHAROMYCES CEREVISIAE* STRAINS ON STARCH AGAR PLATES AND IN SHAKE FLASK CULTURES

**Abstract**

A number of recombinant *Saccharomyces cerevisiae* strains containing combinations of various amylase genes and promoters were evaluated with regard to their ability to ferment starch to ethanol. Notable differences in the hydrolysis zones on starch agar plates indicated that the type of starch medium used and the amylase produced significantly affected the size of the hydrolysis zones. The results from the starch agar plates were not a good indicator of the performance of the strains in liquid starch media, as was found when these strains were further evaluated in shake flasks containing a 2 % starch medium. Less than 10 g ethanol.$l^{-1}$ were produced over a 200 h incubation period. Aerobic growth yielded higher biomass concentrations and, subsequently, higher amylase activity values. An average amylase activity of 305 U.$l^{-1}$ was produced by one strain under aerobic conditions, whereas the amylase activity of most of the other strains remained low or negligible, resulting in a slow rate of starch hydrolysis and low overall biomass and ethanol production.

## 1 Introduction

Starch is the major carbohydrate in all higher plants, constituting a high percentage of the biomass of crops such as potatoes, maize and cassava (Russell *et al.*, 1986; De Menezes, 1982; De Mot and Verachtert, 1986). Starch comprises two major components, namely amylose (α-1,4-linked D-glucose residues) and amylopectin (α-1,4 and α-1,6-linked D-glucose residues) (Janse and Pretorius, 1995). For effective starch hydrolysis, the cleavage of the endo-α-1,4 glycosidic bonds by α-amylases and the hydrolysis of the α-1,6 bonds and α-1,4 linked glucose residues from the non-reducing ends by glucoamylases, as well as the hydrolysis of the α-1,6 branching points by the debranching enzymes, are required (De Mot and Verachtert, 1987; Fogarty and Kelly, 1979; Goodwin and Mercer, 1983; Norman, 1979). As an abundant renewable biopolymer, starch is used as a carbon feedstock for the production of bio-ethanol (Finn, 1987). The multi-step conversion of starch to ethanol is costly and can account for more than half of the production costs (De Mot, 1990). It would, therefore, be advantageous to use a

process in which a microorganism was capable of complete starch hydrolysis, thereby obviating the need for commercial amylases.

Of the approximately four hundred recognised yeast species twenty five percent are capable of using starch as a carbon and energy source (Sà-Correia and Van Uden, 1981; Spencer-Martins and Van Uden, 1977, 1979). This does not imply that all can hydrolyse starch efficiently, as many yeast species lack the necessary enzymes for complete starch degradation (Spencer-Martins and Van Uden, 1977, 1979). Thus, partial digestion and liquefaction by thermostable enzymes to dextrins and maltose are necessary prior steps for starch hydrolysis (Mattoon *et al.*, 1987). In respect of starch fermentation, *Saccharomyces cerevisiae*, with the exception of *Saccharomyces cerevisiae* var. *diastaticus*, which has a weak glucoamylase activity, lacks amylolytic activity and is, therefore, unable to utilise starch during the vegetative growth phase (Pretorius, 1994). The manipulation of *S. cerevisiae* to synthesise and secrete both α-amylase and glucoamylase enzymes would allow this yeast, with its strong fermentative capacity, to convert starch directly to ethanol. The optimum synthesis and secretion of these enzymes is central to the efficient single-step conversion of starch to ethanol. α-Amylase and glucoamylase genes from a wide range of origins, including rice α-amylase (Kumagai *et al.*, 1993), wheat α-amylase (Rothstein *et al.*, 1987), *Schwanniomyces occidentalis* α-amylase (Wang *et al.*, 1989), *S. diastaticus* glucoamylase and *Aspergillus* glucoamylase have been successfully expressed in *S. cerevisiae*. However, the expression of these genes was usually in a haploid yeast strain. In this study, diploid *S. cerevisiae* strains transformed with various combinations of amylase genes and promoters facilitated the direct comparison of the transformants by generating data on the fermentation parameters of these strains and identifying potential problems. Other aspects that were investigated included the rates and yields of enzyme and ethanol production, the susceptibility of amylase production to glucose repression and, finally, the determination of which transformants were most suited for the direct production of ethanol from starch.

# 2 Materials and Methods

## 2.1 Yeast strains

### 2.1.1 Strains with integrated amylase genes

The Institute for Wine Biotechnology at the University of Stellenbosch, Stellenbosch, South Africa, kindly supplied all these recombinant strains. The Belgian brewer's yeast *S. cerevisiae* Hoegaarden strain, a genetic modification of the Hoegaarden mother strain, and the recombinant *Saccharomyces cerevisiae* ATCC 4126 (LKA1) contained the LKA1 α-amylase obtained from *Lipomyces spencermartinsiae (kononenkoae)* IGC4052B. Figure 1 details the genetics of the plasmid used for these transformants. All the transformants contained the amylase DNA integrated into a chromosomal location. Since these strains were diploid, the dominant selection marker *SMR1-410* was used to indicate if the transformations had been successful (Figure 1). *SMR1-410* confers resistance to the herbicide sulfometuron methyl (N-[(4,6 dimethylpyrimidin-2-yl) aminocarbonyl]-2-methoxycarbonyl-benzene-sulfonamide), which has a very low mammalian toxicity (Casey *et al.*, 1988). The target site of this herbicide in *S. cerevisiae* is acetolactate synthase, which forms part of the biosynthetic pathway for isoleucine and valine. *SMR1-410* differs from the acetolactate synthase gene (*ILV2*) by one amino acid base pair (Casey *et al.*, 1988).

Recombinant strains of the diploid yeast strain Sigma L5366 (Liu *et al.*, 1993), which contained only the *ura3-52* marker, were used to transform the strains stell1 through 8 using the YIP5 integration plasmid. Figure 2 details the construction of these plasmids. All transformations were done using the lithium acetate method of Gietz and Schiestl (1991). The recombinant yeasts strains were numbered in the order in which they were received. Table 1 indicates the donor strains of the respective amylases and the promoters used. All LKAI α-amylase and LKAII glucoamylase genes from *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 were linked to the *PGK1*

promoter and terminator. The ALP1 α-amylase and the GLU1 glucoamylase genes from *Saccharomycopsis fibuligera* CSIR Y-0269 had their natural promoters and terminators.

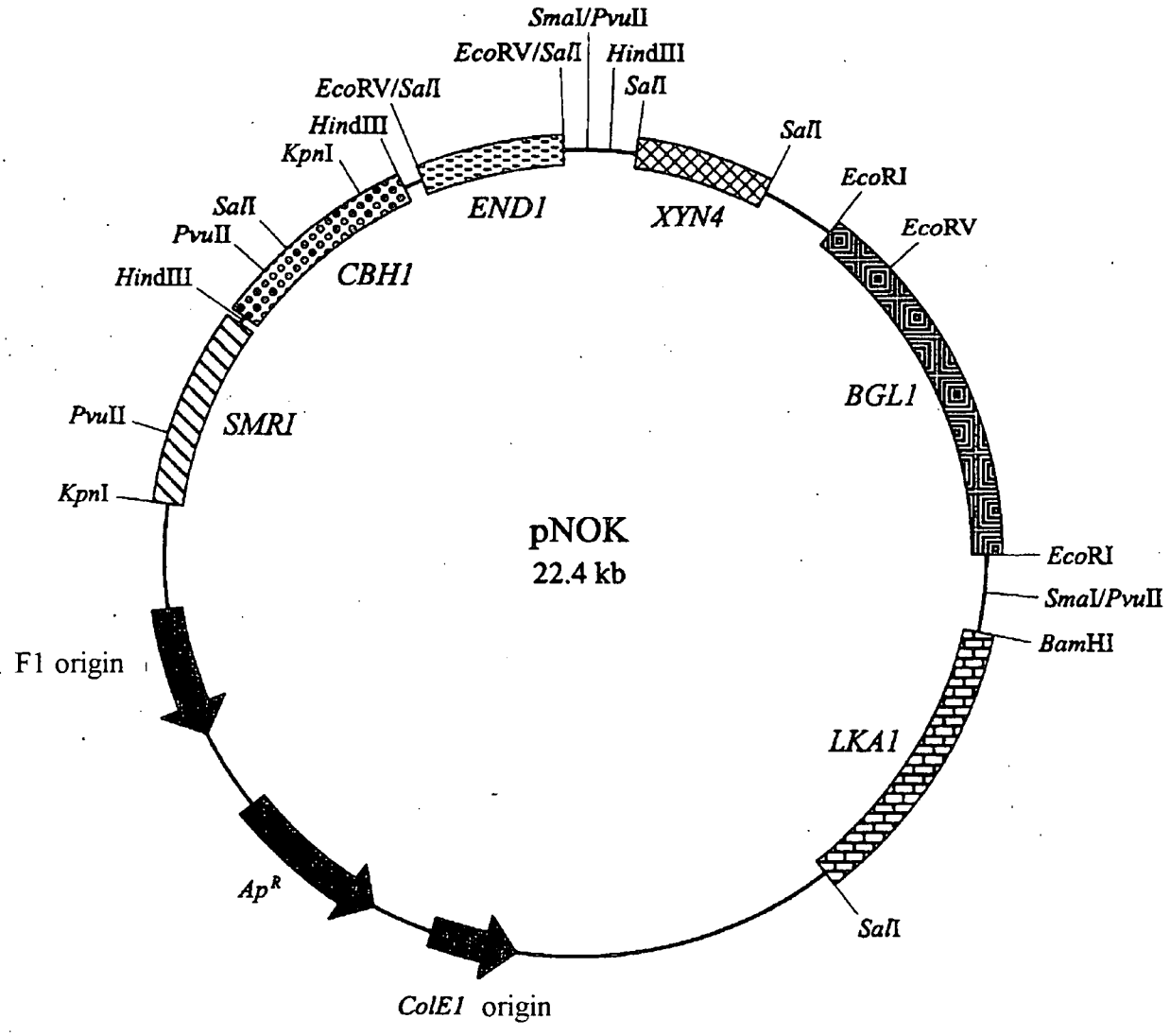



**Figure 1.** A schematic representation of the plasmid pNOK used for the construction of strains *S. cerevisiae* ATCC 4126 (LKA1) and *S. cerevisiae* Hoeg. After constructing the *E. coli-S. cerevisiae* shuttle plasmid, the LKA1 gene construct was cloned into a new shuttle plasmid generating plasmid pNOK. Adapted from Van Rensburg and Pretorius, University of Stellenbosch (personal communication).

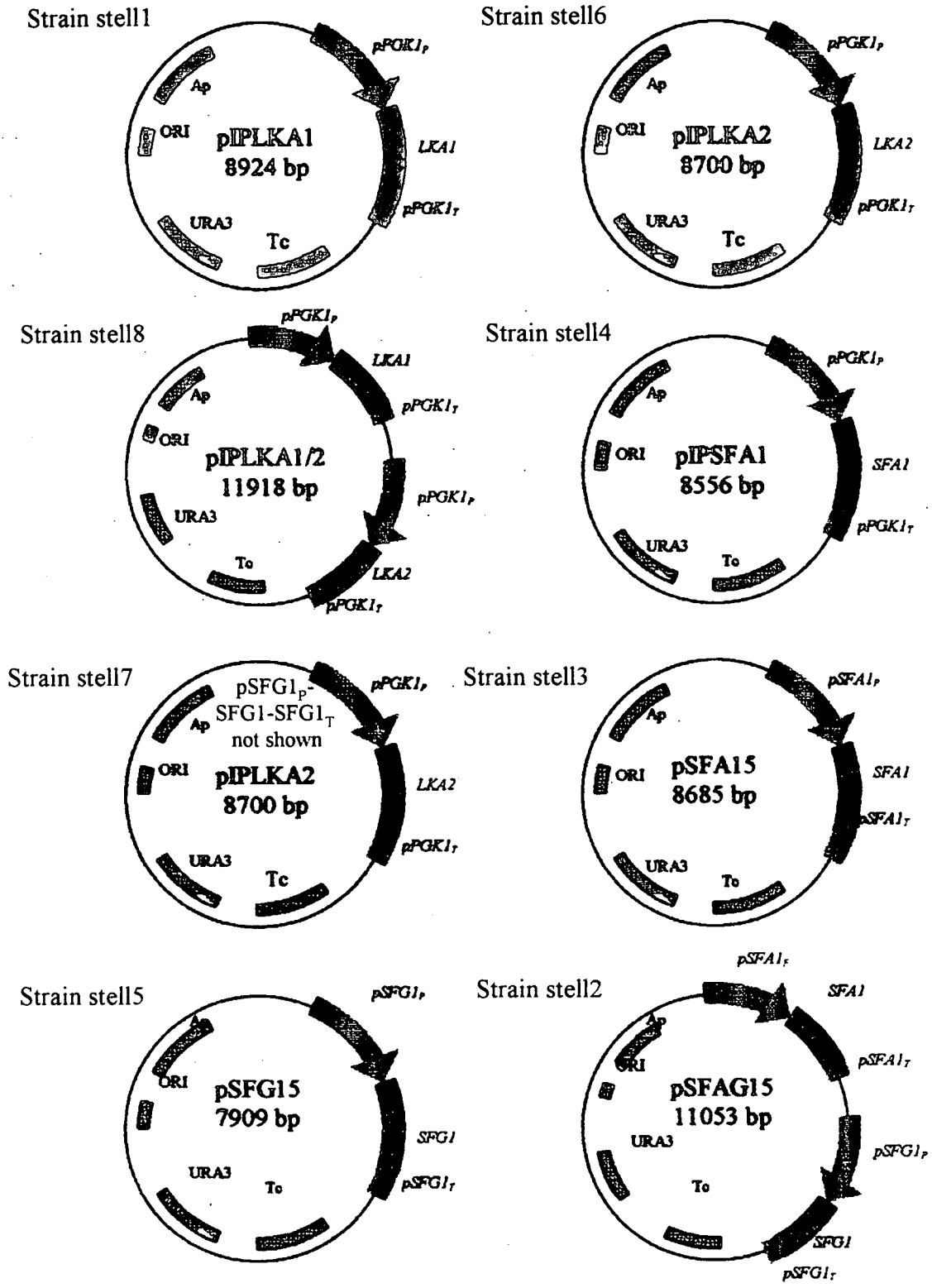



**Figure 2**. Schematic representation of the plasmids used for the construction of strains stell1 through 8. Plasmid construction remained the same for all strains; only the promoters and genes differed, as indicated in Table 1. Adapted from Eksteen *et al.* (2002). Abbreviations: (LKA1 & LKA2) *Lipomyces spencermartinsiae* glucoamylase, (SFA1) *Saccharomycopsis fibuligera* α-amylase and (SFG1) glucoamylase.

### 2.1.2 Strains transformed with non-integrating plasmids

These strains were provided by Dr J. Albertyn, University of the Free State, using the diploid Sigma L5366 strain (Liu *et al*, 1993) with the *ura3-52* marker as mother strain. The strains contained the *Saccharomycopsis fibuligera* α-amylase and glucoamylase genes in a single (pRS416) and multi-copy (pRS426) plasmid form, using their natural promoters.

### 2.1.3 Naturally amylolytic and other yeast strains

The naturally amylolytic *Schwanniomyces occidentalis* CSIR Y-993 and Y-828 strains, obtained from the yeast culture collection at the University of the Free State, were included as reference strains for the initial evaluations on the starch agar plates.

## 2.2 Culture maintenance

All strains were maintained at 4 °C on GPY agar slants comprising (per litre): 40 g glucose, 5 g peptone (Biolab Diagnostics, Midrand, South Africa), 5 g yeast extract (Biolab Diagnostics) and 20 g agar. The cultures were transferred to a fresh agar slant every four months to maintain viability and subcultures were made as required.

## 2.3 Starch agar for screening of amylase activity

A variety of starch agar media were used in the preliminary evaluation of the amylase activity of the above-mentioned yeast transformants. These included (a) **YNBS medium,** containing (per litre): 6.7 g yeast nitrogen base (Difco, Detroit, MI, USA), 10 g soluble starch, 10 g $NaH_2PO_4$, 4 g $Na_2HPO_4.H_2O$, 20 g agar. (b) **YNBS Phadebas medium,** containing (per litre): 6.7 g yeast nitrogen base (Difco), 10 g $NaH_2PO_4$, 4 g $Na_2HPO_4.H_2O$, 20 g agar and 20 Phadebas tablets (Pharmacia Diagnostics, Uppsala, Sweden). (c) **YEP Phadebas medium,** containing (per litre): 10 g yeast extract (Biolab Diagnostics), 20 g peptone (Biolab Diagnostics), 20 g agar and 20 Phadebas tablets (see

section 2.5.2 for a description of the Phadebas substrate). (d) **Starch Azure medium,** containing (per litre): 10 g yeast extract (Biolab Diagnostics); 20 g peptone (Biolab Diagnostics), 20 g agar, 2 g starch azure (Sigma, St. Louis, MO, USA).

Four sets of YNBS starch plates were prepared using soluble starch from four different suppliers, namely BDH (Poole, UK), Sigma (Lintner, soluble potato starch), Merck (Darmstadt, Germany) and Saarchem (Krugersdorp, South Africa). To determine if amylase production was repressed by glucose, 10 g of glucose.$l^{-1}$ were added to the above media. To prevent the precipitation of mineral salts, the $Na_2HPO_4.H_2O$ and $NaH_2PO_4$ were autoclaved together, but separate from the other constituents. The starch was also autoclaved separately from the other constituents, which were subsequently aseptically combined in a laminar flow cabinet. Agar plates were prepared by adding 25 ml of the sterile medium (pH 5.0) to 90 mm diameter plastic petri dishes, yielding an average agar depth of 5 mm. Point inoculations of the above-mentioned yeast strains were made and these were incubated for 7 days at 30 °C. Hydrolysis zones on the YEP Phadebas, YNBS Phadebas and starch azure plates were clearly visible due to the blue colour of the starch, thus no staining was required. The YNBS plates were stained by flooding the plates with a solution of 0.1 % $I_2$ dissolved in 1.0 % KI. The final hydrolysis zone was reported as the total diameter minus the colony diameter.

## 2.4 Batch cultivations

Strains were streaked out on GPY agar plates and incubated at 30 °C for 24 hours. Cells from these plates were used to inoculate a 250 ml Erlenmeyer flask (pre-inoculum), containing 100 ml of medium comprising (per litre): 20 g glucose, 10 g yeast extract (Biolab Diagnostics, Midrand, South Africa), 20 g peptone (Biolab Diagnostics) and 2 g $KH_2PO_4$, at pH 4.5, which was then incubated for 24 h at 30 °C on a rotary shaker. The inoculum was prepared by transferring 5 ml of the latter culture to a 250 ml Erlenmeyer flask containing 95 ml of the above medium and incubating it for 12 h at 30 °C. Aerobic flask cultivations were carried out in 1-litre Erlenmeyer flasks fitted with cotton wool plugs and containing 300 ml of the starch liquid medium, pH 4.5, comprising (per litre):

20 g Merck starch, 10 g yeast extract (Biolab Diagnostics), 20 g peptone (Biolab Diagnostics) and 2 g $KH_2PO_4$. After inoculation with a 10 % inoculum prepared as described above, these were incubated at 30 °C on a rotary shaker at 200 $r.min^{-1}$. Anoxic flask cultivations were carried out in 1-litre Erlenmeyer flasks fitted with cotton wool plugs, containing 1000 ml of the starch medium and a magnetic stirrer bar. After inoculation as above, these flasks were incubated at 30 °C on an MR 2000 magnetic stirrer (Heidolph, Germany) at 100 $r.min^{-1}$ using a Heidolph Digital 2002 revolution counter. Some of these starch cultivations were repeated with an additional 10 g of glucose.$l^{-1}$, where indicated. Samples were taken at regular intervals for assays.

## 2.5 Analytical procedures

### 2.5.1 Starch assay

The starch assay described by Horn *et al.* (1988) was slightly modified to determine the degree of starch hydrolysis. To a 500 µl aliquot of the culture supernatant, 1.25 ml of 0.5 M phosphate citrate buffer (pH 6.6) plus 10 µl of a thermostable alpha-amylase (Termamyl 120 L; Novo Nordisk A/S, Bagsværd, Denmark) was added and the test tube was boiled for 10 min before cooling to 60 °C in a water bath. The pH was subsequently adjusted to pH 4.5 by the addition of 1 ml of 0.5 M citrate buffer, pH 4.5, before adding 10 µl of glucoamylase (AMG 300L; Novo Nordisk A/S). After a 15 min incubation period, the mixture was cooled on ice and the glucose concentration determined using the peroxidase-oxidase assay (Sigma), as described in section 2.5.3 below, which was converted to a starch concentration by dividing by 1.111; the latter factor was calculated as $180n/162n$ to account for the one molecule of water required to hydrolyse each glycosidic bond in the starch molecule (Thomas *et al.*, 1996).

**Table 1**. Recombinant strains of *Saccharomyces cerevisiae* used in this investigation

| Strain | α-Amylase origin & promoter | | Glucoamylase origin & promoter | |
|---|---|---|---|---|
| stell1 [a] | *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 | *PGK1* | | |
| stell3 [a] | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural | | |
| stell4 [a] | *Saccharomycopsis fibuligera* CSIR Y-0269 | *PGK1* | | |
| stell5 [a] | | | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural |
| stell6 [a] | | | *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 | *PGK1* |
| stell2 [a] | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural |
| stell7 [a] | | | *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 | *PGK1* |
| | | | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural |
| stell8 [a] | *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 | *PGK1* | *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 | Natural |
| *S. cerevisiae* ATCC 4126 (LKA1) [a] | *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 | *PGK1* | | |
| *S. cerevisiae* Hoeg (LKA1) [a] | *Lipomyces spencermartinsiae (kononenkoae)* IGC4052 | *PGK1* | | |
| *S. cerevisiae* MC [b] | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural |
| *S. cerevisiae* SC [b] | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural | *Saccharomycopsis fibuligera* CSIR Y-0269 | Natural |

a Supplied by Institute for Wine Biotechnology, Stellenbosch

b Supplied by the University of the Free State

MC Multi-copy non-integrating plasmid

SC Single copy non-integrating plasmid

### 2.5.2 Alpha-amylase assay

Alpha-amylase activity was assayed using a slightly modified method of Horn *et al.* (1992) and the Phadebas amylase test (Pharmacia Diagnostics). The Phadebas substrate is a water-soluble cross-linked starch polymer with a blue dye intercalated between the bonds and is specific for α-amylase activity. To a 200 µl of the culture supernant, 4 ml of 0.1 M sodium acetate buffer (pH 5.0) was added to the test tube and pre-incubated at 40 °C for 5 minutes. One Phadebas tablet (Pharmacia) was added to each test tube and subsequently vortexed at one-minute intervals for 15 minutes. A blank consisting of 4.2 ml of the buffer was subjected to the same procedures. The reaction mixture supernant was read against the blank at 620 nm after terminating the reaction with 1 ml of a 0.5 M sodium hydroxide solution and centrifuging at 8000 K for 5 min. The amylase activity, in $U.l^{-1}$, was read from the standard curve supplied in the Phadebas kit. Provided the linear range of the assay is observed, amylase values in the range of 30 to 4000 $U.l^{-1}$ can be determined.

### 2.5.3 Glucoamylase activity assay

Glucoamylase activity was assayed by determining the amount of glucose liberated from a 0.5 % solution of soluble starch (Merck) dissolved in boiling 0.05 M $KH_2PO_4$-NaOH buffer (pH 5.0) and cooled to 40 °C (Wilson and Ingledew, 1982). A 5 ml aliquot of the substrate was added to 1.0 ml of the appropriately diluted prewarmed supernatant. The reaction was stopped after exactly 10 min by heating in a boiling water bath for 5 min, followed by cooling in iced water. The amount of glucose liberated was determined using a peroxidase-glucose oxidase enzyme analysis kit (Sigma). The absorbance was measured at 450 nm against a blank, which consisted of buffer and the peroxidase-glucose oxidase reagent. The enzyme activities were calculated as follows:

$$\text{Enzyme activity } (U.l^{-1}) = A \times (1000/180.16)\ \mu_{mol}/10$$

$$= \mu_{mol} \text{ glucose. } (l.min)^{-1}$$

Where A is the total glucose, calculated as follows:

$$A = (Glucose_{sample} - Glucose_{sample\ blank}\ mg.l^{-1}) \times 6$$

### 2.5.4 Glucose, ethanol and biomass

The residual glucose content of the sample or supernatants was quantified using a Sugar Analyser I high-performance liquid chromatograph (HPLC) equipped with a refractive index detector and a Waters Sugarpack I™ column (Waters Associates, Milford, MA, USA) operating at 85 °C and with an eluent (degassed water) flow rate of 0.5 ml.min$^{-1}$. Alternatively, the quantitative determination of the glucose present in the sample was determined using the peroxidase-oxidase glucose enzyme analysis kit (Sigma). The analytical procedure was carried out in accordance with the procedures set out in the pamphlet. Direct exposure to sunlight or bright daylight was avoided, as the colour reagent is light sensitive. The absorbance was determined at 450 nm against the blank, which contained water instead of the sample. This analysis was accurate for glucose values in the range of 0.25 to 3 g.l$^{-1}$.

Ethanol concentrations were determined with a Hewlett-Packard 5710A gas chromatograph with a Hewlett-Packard 3390A integrator (Hewlett-Packard, Atlanta, GA, USA), fitted with a glass column (15 m x 1.5 mm internal diameter) packed with 80-100 mesh Porapak N (Waters Associates, Milford, MA, USA) and using 50 ml of nitrogen carrier gas.min$^{-1}$ at an oven temperature of 165 °C. Growth was monitored colorimetrically using a Wissenschaftlich-Technische Werkstätten (WTW) GMBH Photolab S6 photometer (Weilheim, Germany). The biomass was gravimetrically determined in triplicate by centrifuging 10 ml culture samples at 3000 r.min$^{-1}$ for 5 min, washing twice with distilled water and drying to a constant mass at 105 °C.

## 3 Results and discussion

### 3.1 Starch agar plates

Table 2 indicates the zones of starch hydrolysis obtained with some of the amylolytic yeast strains on different starch agar media. The *Schwanniomyces occidentalis* strains, which are naturally amylolytic, yielded the largest zones, with the nett clearing zone

diameters ranging from 24.5 to 44.7 mm on the starch medium excluding glucose. All the *Schwanniomyces* strains showed significantly smaller nett hydrolysis zones, as small as 6 mm (for example, see *Schw. occidentalis* CSIR Y-993, YEP Phadebas), when glucose was included in the medium, indicating that glucose repressed amylase production. In contrast to the *Schwanniomyces* strains, the *S. cerevisiae* strains Hoeg (LKA1) and ATCC 4126 (LKA1) did not appear subject to glucose repression, as larger zones of hydrolysis were obtained with glucose inclusion. *S. cerevisiae* ATCC 4126 (LKA1) performed poorly in comparison to *S. cerevisiae* strain Hoeg (LKA1), suggesting that the *L. spencermartinsiae (kononenkoae)* α-amylase was not as effective in the ATCC strain as in the Hoeg strain. The data obtained from these preliminary starch analyses indicated that the *Saccharomyces cerevisiae* ATCC 4126 (LKA1) and *S. cerevisiae* Hoeg (LKA1) strains did not produce and/or excrete amylases as effectively as the naturally amylolytic *Schwanniomyces* strains.

Figure 3 not only illustrates the differences in hydrolytic activity between the naturally amylolytic *Schwanniomyces* strains and these recombinant *S. cerevisiae* strains, but also illustrates the differences obtained when different starch sources were used. These variations in hydrolysis activity could be attributed to the differences in the starch composition, such as a higher degree of branching, which would render the starch less susceptible to α-amylase degradation. Due to the influence of the type of medium and the type of starch used on the degree of starch hydrolysis observed, the activity index values (the ratio between the biomass and the starch hydrolysis zone), which gave an indication of the efficiency of the starch hydrolysis relative to growth, were calculated. By way of example, the *Schw. occidentalis* Y-828 strain produced similarly sized hydrolytic zones on the YNBS Phadebas, 36 and 29 mm without and with glucose respectively, due to yeast growth on glucose in the latter case. However, the activity index values showed less starch hydrolysis per biomass unit.

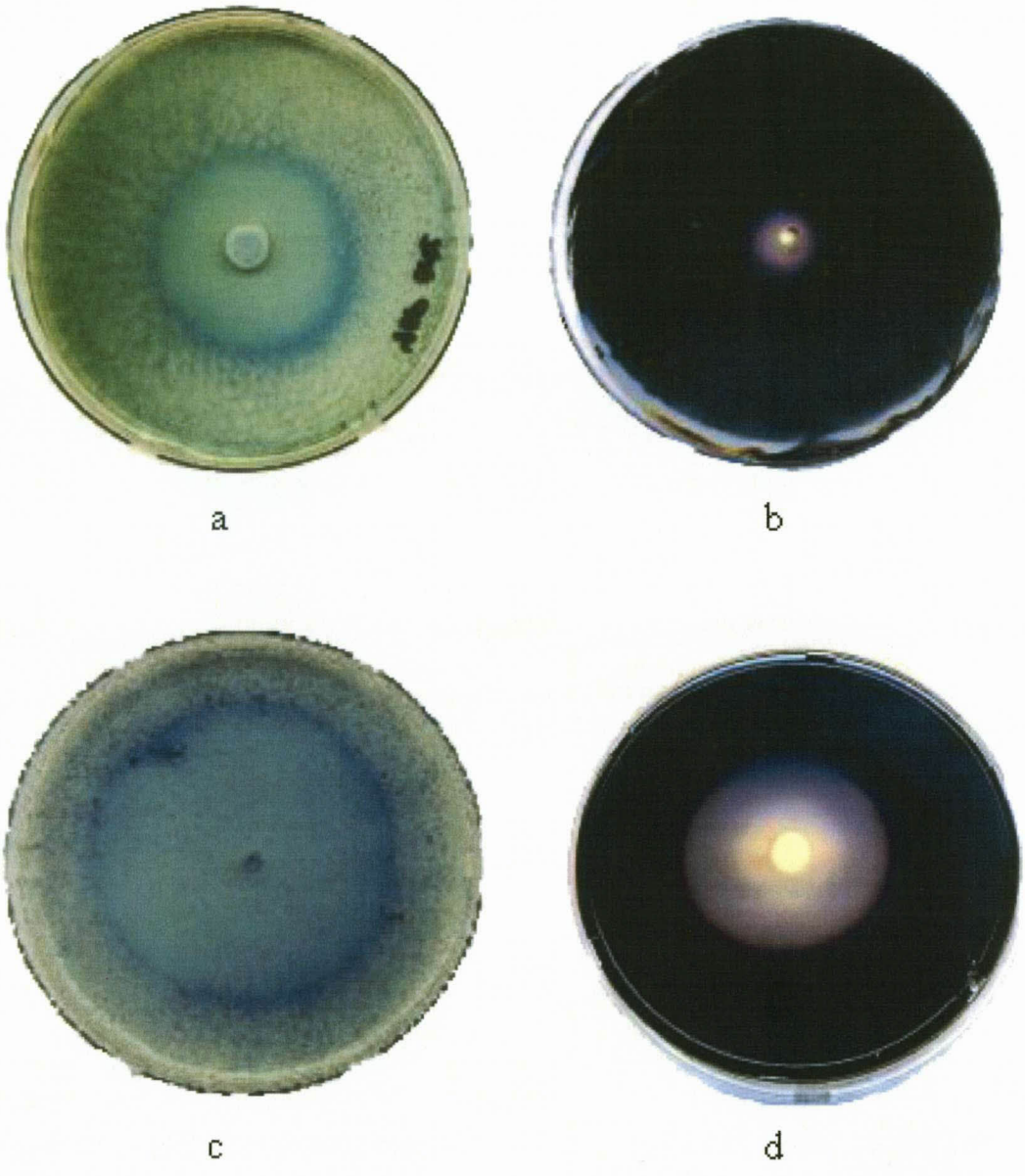



**Figure 3.** Starch agar plates showing *S. cerevisiae* ATCC 4126 (LKA1) grown on (a) YNBS Phadebas starch and (b) YNBS BDH starch and *Schw. occidentalis* CSIR Y-993 grown on (c) YNBS Phadebas starch and (d) YNBS BDH starch, all excluding glucose.

**Table 2.** Zones of starch hydrolysis obtained on various starch agar media, with (+) or without (-) added glucose, with recombinant amylolytic yeasts strains compared to *Schw. occidentalis*, incubated at 30 °C for 7 days. The hydrolysis zone values (in mm), defined as the total diameter minus the colony diameter, are the mean values of duplicate experiments. The activity index, indicated in brackets, was calculated as the [(average diameter of clearing zone)-(diameter of colony)]/diameter of colony.

| Basal medium | Starch type | Glucose addition | Yeast strains | | | |
|---|---|---|---|---|---|---|
| | | | *S. cerevisiae* Hoeg Y-0454 (LKA1) | *S. cerevisiae* ATCC 4126 (LKA1) | *Schw. occidentalis* CSIR Y-993 | *Schw. occidentalis* Y-828 |
| YNBS | Merck | - | 13 (4.3) | 7.5 (1.9) | 28.5 (3.4) | 31 (3.4) |
| YNBS | Merck | + | 24 (6) | 19 (2.7) | 22 (2.6) | 6.5 (0.8) |
| YNBS | BDH | - | 18 (3.6) | 4 (1) | 31 (3.1) | 36.5 (3.7) |
| YNBS | BDH | + | 22 (4.4) | 14 (2.3) | 22.5 (2.3) | 26.2 (2.6) |
| YNBS | Saarchem | - | 18 (6) | 11 (5.5) | 33 (4.1) | 36 (5.1) |
| YNBS | Saarchem | + | 27 (6.8) | 20 (4) | 26.3 (2.3) | 28 (3.1) |
| YNBS | Lintner | - | 16 (4) | 9 (3) | 32.5 (4.6) | 35 (3.9) |
| YNBS | Lintner | + | 19 (4.8) | 22 (4.4) | 26.6 (2.4) | 27.5 (2.8) |
| YNBS | Phadebas | - | 27 (6.8) | 22 (7.4) | 44.7 (11.8) | 36 (12) |
| YNBS | Phadebas | + | 30 (6.4) | 27 (5.6) | 23.5 (2.3) | 29 (2.9) |
| YEP | Phadebas | - | 22.5 (5) | 15 (3) | 24.5 (3.8) | 39 (4.8) |
| YEP | Phadebas | + | 27 (3.4) | 20 (2.9) | 6 (0.8) | 17.7 (1.7) |
| YEP | Azure | - | 23 (4.6) | 16 (3.2) | 31 (3.9) | 33.5 (4.5) |
| YEP | Azure | + | 27 (4.5) | 20 (2.9) | 9.2 (1.0) | 18 (1.6) |

The mean values of the starch hydrolysis zones obtained with transformants derived from the Sigma strain are given in Table 3. As stated above, Phadebas starch is specific for α-amylase, hence strains with an α-amylase gene produced visible hydrolysis zones on the Phadebas starch plates (Fig. 4). The starch hydrolysis zones of strains stell5, 6 and 7 on the Phadebas starch plates were either small or absent, indicating limited or no α-amylase activity (Fig. 4). Strains stell6 and stell7 were initially thought to contain the α-amylase gene LKAII from *Lipomyces spencermartinsiae (kononenkoae)*, but the preliminary results on the starch agar plates indicated a lack of α-amylase activity. It was subsequently confirmed that the LKAII gene was indeed a glucoamylase gene (I.S. Pretorius, University of Stellenbosch, personal communication). The small zones obtained on the α-amylase sensitive starch Azure plates further confirmed these findings.

These results showed that strains stell2 (Fig. 4), stell3, stell5 and *S. cerevisiae* SC were susceptible to glucose repression of amylase production. This indicates that the natural promoters of *Saccharomycopsis fibuligera* were repressible by glucose, whereas the *PGK1* promoter, which is known to be a constitutive promoter, was not. The activity index values further illustrate the repression of amylase production by glucose. Strains stell4, 7 and 8 produced slightly larger hydrolysis zones when glucose was added, as would be expected with an additional carbon source, resulting in more yeast biomass. Whereas strain stell4 showed no indication of glucose repression, the activity index values of strains stell7, 8 and *S. cerevisiae* MC indicated some repression, probably due to the different starch types and composition of the medium used. Strains with only a glucoamylase performed poorly, indicating that an α-amylase activity was required for complete starch hydrolysis. As with the yeast strains in Table 2, the hydrolysis zone diameter was dependent on the starch source.

These differences in the starch types could be attributed to the differences in the manufacturing methods used. The more pre-cooking and chemical pre-treatment involved, the easier the starch can be degraded. For example, the Merck starch is more complex than the other starches, having a lower solubility, with concentrated solutions gelatinising upon cooling (De Mot *et al.*, 1984).

**Table 3.** Zones of starch hydrolysis obtained on various starch agar media, with (+) or without (-) added glucose, with transformants derived from the Sigma strain, incubated at 30 °C for 7 days. The hydrolysis zone values (in mm), defined as the total diameter minus the colony diameter, are the mean values of duplicate experiments. The activity index, indicated in brackets, was calculated as the [(average diameter of clearing zone)-(diameter of colony)]/diameter of colony.

| Basal medium | Starch type | Glucose addition | Recombinant *Saccharomyces cerevisiae* strain | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | stell1 | stell2 | stell3 | stell4 | stell5 | stell6 | stell7 | stell8 | MC | SC |
| YNBS | Merck | - | 18.5 (4.5) | 28 (3.7) | 24.5 (5.4) | 28 (9.3) | 0 (0) | 4.75 (0.86) | 13.5 (2.7) | 20 (6.6) | 37 (6.2) | 29 (3.2) |
| YNBS | Merck | + | 22.5 (3.4) | 3.5 (0.5) | 11.5 (2.3) | 27.5 (9.2) | 0 (0) | 6 (0.92) | 19.5 (3) | 24.3 (3.7) | 34 (5.6) | 0 (0) |
| YNBS | BDH | - | 19 (4.5) | 29 (5.8) | 28.5 (6.3) | 29 (11.6) | 1 (0.2) | 10 (1.9) | 18.5 (4.1) | 20 (2.6) | 41.5 (7.5) | 35 (5.8) |
| YNBS | BDH | + | 22 (3.6) | 8.5 (1.4) | 10 (1.8) | 27 (12) | 0 (0) | 9.5 (1.2) | 13.5 (3.4) | 31 (5.2) | 34.5 (6.3) | 4.5 (1.1) |
| YNBS | Saarchem | - | 22 (4.6) | 11.5 (2.6) | 19.5 (3.4) | 35.5 (11.8) | 4 (0.3) | 18.5 (5.7) | 19 (5.4) | 23.8 (7.3) | 38 (6.3) | 13 (3.2) |
| YNBS | Saarchem | + | 27.5 (5.0) | 8.25 (1.4) | 14.5 (2.9) | 37.5 (15) | 0 (0) | 10.5 (1.6) | 19 (3.8) | 33 (4.4) | 17 (5.7) | 9 (1.5) |
| YNBS | Lintner | - | 22 (4.8) | 30 (7.5) | 22.5 (5) | 27 (7.7) | 3.5 (0.8) | 4 (0.9) | 11.5 (2.3) | 24.3 (2.7) | 32 (6.4) | 30 (5) |
| YNBS | Lintner | + | 28.5 (4.1) | 9.25 (1.8) | 13.5 (3.9) | 31.5 (12.6) | 0 (0) | 5.25 (0.6) | 16.5 (2.2) | 32 (2.6) | 10 (1.3) | 8 (1.1) |
| YNBS | Phadebas | - | 24.5 (3.5) | 17 (5.6) | 14 (4.7) | 26.5 (8.8) | 0 (0) | 0 (0) | 0 (0) | 27.5 (4.9) | 21 (4.6) | 17 (5.4) |
| YNBS | Phadebas | + | 31 (4.2) | 2 (0.4) | 3 (0.4) | 28 (11.2) | 0 (0) | 1 (0.2) | 0 (0) | 34.2 (5.8) | 9 (1.0) | 8 (0.7) |
| YEP | Phadebas | - | 27 (4.5) | 14.75 (2.5) | 15 (2.3) | 19.5 (3.9) | 0 (0) | 0 (0) | 0 (0) | 27.5 (3.0) | 25.5 (3.2) | 21 (2.6) |
| YEP | Phadebas | + | 27.5 (2.2) | 3.25 (0.3) | 11.25 (1.3) | 24.75 (2.6) | 0 (0) | 0 (0) | 0 (0) | 31.5 (2.7) | 18 (1.7) | 17 (1.8) |
| YEP | Azure | - | 26.3 (6.5) | 18.5 (3.1) | 14.5 (4.1) | 25 (7.1) | 0 (0) | 0 (0) | 0 (0) | 29 (4.6) | 20 (6.7) | 16 (8) |
| YEP | Azure | + | 25 (1.9) | 31.5 (3) | 6.75 (1.1) | 31.5 (4.2) | 0 (0) | 0 (0) | 0 (0) | 34 (2.6) | 17.5 (3.2) | 16 (2.5) |

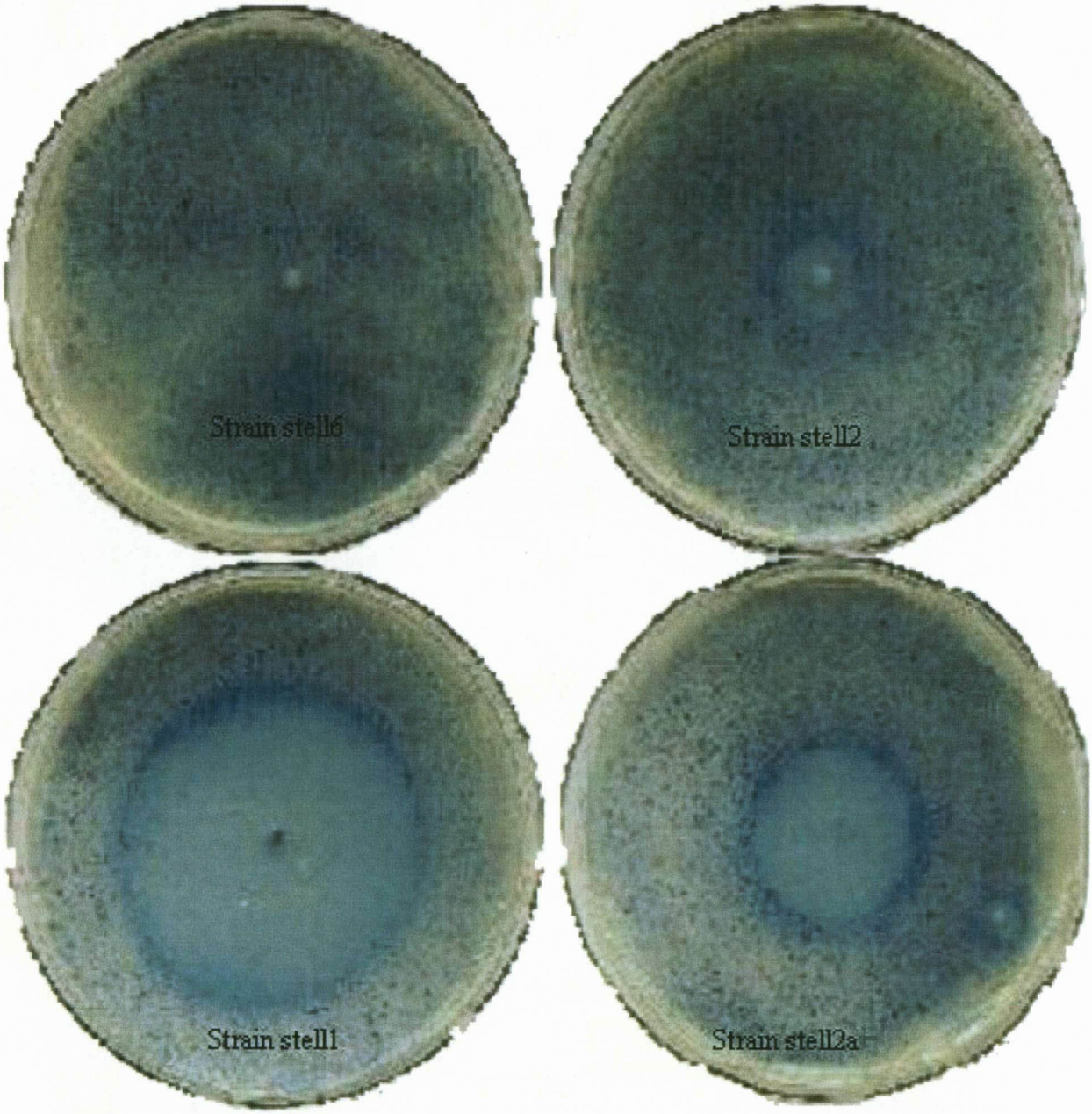



**Figure 4**. YNBS Phadebas starch agar plates showing the growth of (a) strain stell6 without glucose, (b) strain stell2 with glucose, (c) strain stell8 with glucose and (d) strain stell2 without glucose.

This probably would make the starch less susceptible to certain amylases, especially the cell-bound amylases (De Mot *et al.*, 1984). De Mot *et al.* (1984) reported that *L. spencermartinsiae, S. fibuligera* and *Schw. occidentalis* were capable of effectively hydrolysing Merck starch. The hydrolysis zones obtained with the *Schw. occidentalis* strains agreed with these findings. All recombinant strains, with the exception of the *S. cerevisiae* ATCC 4126 (LKA1), which had the LKA1 gene from *L. spencermartinsiae* with the natural promoter, performed well on the Merck starch agar plates. Strain stell1, also with the LKA1 gene, produced notably larger hydrolysis than *S. cerevisiae* ATCC 4126 (LKA1). By way of example, *S. cerevisiae* ATCC 4126 (LKA1) yielded no zone on YNBS Merck (without glucose), whereas the stell1 strain (also with the LKA1 gene) gave a net hydrolysis zone of 18.5 mm on the same medium. The molecular mass of the amylase molecule differs between species (Table 5, Chapter 1). This may impact on the size of the hydrolysis zones obtained, as the size of the enzyme molecule could directly influence the rate of diffusion through the starch agar. Thus, the diameter of the hydrolysis zone may not accurately reflect the actual amylolytic activity of the yeast in liquid media. These hydrolysis values on starch agar plates therefore are not reliable indicators of the ability of these yeasts to convert starch to ethanol or to utilise starch as a sole carbon source for growth (De Moraes *et al.*, 1995). In liquid media and under fermentation conditions, other factors, such as fermentation capacity, ethanol tolerance and the enzyme concentration, excretion and accessibility, also play an important role. However, this screening served the purpose of providing a general indication of the performance of each strain in terms of its ability to hydrolyse the starch.

## 3.2 Flask cultivations

A more rigorous evaluation of the growth and amylolytic characteristics of some of the recombinant strains were conducted in flask cultivations.

### 3.2.1 Anoxic cultivations

Figure 5 shows a typical fermentation profile of *Saccharomyces cerevisiae* Hoeg (LKA1) on glucose. These culture parameters are summarised in Table 4, which could serve as a reference for evaluating fermentation performance on starch.

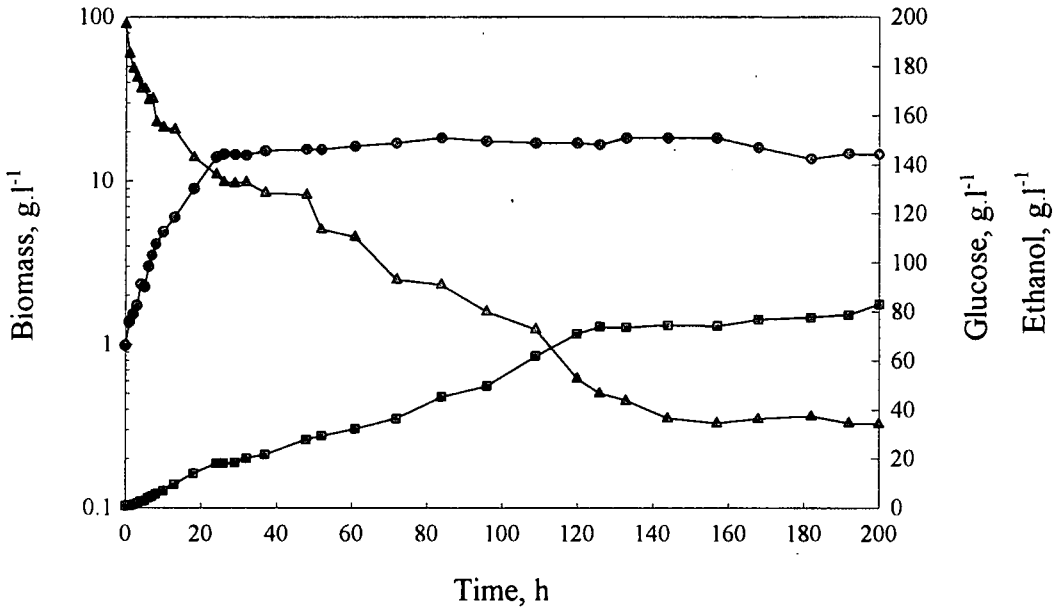



**Figure 5.** A typical fermentation profile of the Belgian brewer's yeast Hoeg (LKA1) strain, cultivated on 200 g glucose.$l^{-1}$ at pH 5.5 and 30 °C in a 1 litre Erlenmeyer flask. Symbols: biomass (●), glucose (▲), ethanol (■).

The $\mu_{max}$ value of 0.151 $h^{-1}$ was lower than the value of 0.35 $h^{-1}$ reported by Fiechter *et al.* (1981) for other strains grown on glucose under anoxic conditions. However, the high glucose concentration used here would have resulted in a decreased growth rate. The ethanol yield coefficient was close to the theoretical maximum value of 0.51, as ethanol production would be expected to be maximal under anoxic conditions because no respiration of ethanol would occur.

**Table 4.** Anoxic cultivation parameters of the recombinant Hoeg (LKA1) and *S. cerevisiae* ATCC 4126 (LKA1) strains in flask cultures using a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$ or 200 g glucose.$l^{-1}$. The mean values of duplicate experiments are shown.

| Parameter | Hoeg (LKA1) | | ATCC 4126 (LKA1) |
|---|---|---|---|
| | Glucose, 200 g.$l^{-1}$ | Merck starch, 20 g.$l^{-1}$ | Merck starch, 20 g.$l^{-1}$ |
| Cultivation time, h | 200 | 223 | 223 |
| Biomass, g.$l^{-1}$ | 14.55 | 1.4 | 1.13 |
| Ethanol, g.$l^{-1}$ | 82.93 | 4.8 | 1.3 |
| Residual glucose, g.$l^{-1}$ | 34.15 | 0 | 0 |
| Residual starch, g.$l^{-1}$ | n/a | 8.95 | 14.5 |
| E, % | 82.7 [a] | 53 [b] | 34 [b] |
| $Q_p^{max}$, g.$l^{-1}$.$h^{-1}$ | 0.801 | 0.019 | 0.00013 |
| $Y_{x/s}$ | 0.083 | 0.102 | 0.044 |
| $Y_{p/s}$ | 0.488 | 0.409 | 0.021 |
| $\mu_{max}$, $h^{-1}$ | 0.151 | 0.0048 | 0.0047 |

E [a] Efficiency of glucose utilisation; initial glucose - residual glucose /initial glucose x 100

E [b] Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100

n/a Not applicable

$Q_p^{max}$ Maximum volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time

$\mu_{max}$ Maximum specific growth rate

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g glucose equivalents utilised, calculated from the final ethanol concentration

$Y_{x/s}$ Cell yield coefficient, g dry biomass/g glucose equivalents utilised, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the yeast extract and peptone components of the medium

The recombinant Hoeg (LKA1) and *S. cerevisiae* ATCC 4126 (LKA1) strains performed poorly on starch, as was evident from the maximum specific growth rates, the final biomass produced, the low ethanol yield coefficient as well as the poor efficiency of starch hydrolysis (Table 4). Although the Hoeg strain produced up to 4.8 g ethanol.$l^{-1}$, the time required was extremely long. The recombinant ATCC 4126 strain did not yield promising results on the starch agar plates (Table 2) and gave poor overall results when cultivated in liquid media (Table 4). These results were not surprising, because both strains were transformed with only an α-amylase. Based on this poor performance, it was decided to discontinue further evaluation of these strains.

Table 5 summarises the fermentation parameters for the recombinant *S. cerevisiae* strains derived from the Sigma strain under anoxic conditions. The naturally amylolytic yeast *Schwanniomyces occidentalis* CSIR Y993 was included as a reference strain. Using transformants of the same parental strain facilitated a better comparative evaluation of the different amylase genes and promoters used. All these transformants grew in the starch medium.

Little or no ethanol assimilation was expected under these conditions. Although up to 8.3 g of ethanol.$l^{-1}$ was produced, the fermentation times were extremely long, exceeding 200 h, at which point the fermentations were stopped. Strain stell3 performed considerably better than strain stell4 with respect to the degree of starch hydrolysis, maximum ethanol and biomass concentration (Table 5). Both strains stell3 and 4 had been transformed with the α-amylase gene from *S. fibuligera,* but with different promoters. This direct comparison indicated that a higher α-amylase activity was obtained using the natural promoter than when the *PGK1* promoter was used, even though the latter promoter is regarded as a powerful constitutive promoter. This difference was not evident from the screening on the starch agar plates. Furthermore, a direct comparison of strains stell1 and 4, which both used the *PGK1* promoter, clearly demonstrated that the α-amylase gene from *L. spencermartinsiae* resulted in much better starch hydrolysis and ethanol production that the corresponding gene from *S. fibuligera.*

A similar comparison between strain stell6 and strain stell5 indicated that the *L. spencermartinsiae* glucoamylase in combination with the *PGK1* promoter was more

efficient than the *S. fibuligera* glucoamylase with the natural promoter. The relatively good results obtained with strains stell5 and 6 were unexpected and could have been due to the fact that an α-1,6 debranching activity is usually associated with a glucoamylase, giving a relatively high degree of starch hydrolysis, albeit at a slow rate (Guzmán-Maldonado and Paredes-López, 1995). Strain stell2 (α-amylase and a glucoamylase from *S. fibuligera*, natural promoter) and strain stell8 (α-amylase and a glucoamylase from *L. spencermartinsiae*, *PGK1* promoter) both degraded the starch effectively. Although the production of biomass and ethanol were comparable, strain stell8 performed significantly better in respect of enzyme production and efficiency of starch hydrolysis than strain stell2.

Similarly, the data obtained with *S. cerevisiae* strains MC and SC was comparable to strains stell2 and 8 in terms of biomass and ethanol production. However, in contrast to strain stell2, these strains (*S. fibuligera* α-amylase on a non-integrating plasmid) produced detectable α-amylase activity values. Despite this, these values were lower than those obtained with strain stell8, further indicating that, in these constructs, the transformation of *S. cerevisiae* with the amylolytic genes from *L. spencermartinsiae* yielded better results than with the amylolytic genes from *S. fibuligera*.

The above data, most notably the $\mu_{max}$ and biomass values, indicated strain stell7 as the best strain for starch saccharification and fermentation. Strain stell7, despite having two glucoamylase genes and no α-amylase, gave a high percentage and rate of starch hydrolysis, which could be explained by the debranching activity of the LKAII glucoamylase (Spencer-Martins and Van Uden, 1979). The growth rate of strain stell7 was considerably higher than that of the other strains; this probably reflected the rate of glucose liberation from starch. Furthermore, the rate of volumetric ethanol production (an important parameter for an industrial process) by strain stell7 was much higher than that of the other strains, again indicative of more rapid glucose liberation. *Schw. occidentalis* performed poorly, as an overall optimum dissolved oxygen tension of at least 40 % has been shown to be required for simultaneous biomass and amylase production (Horn *et al.*, 1992).

**Table 5**. Anoxic cultivation parameters of different recombinant yeast strain in flasks cultures using a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$. The mean values of duplicate experiments are shown.

| Parameter | Recombinant *S. cerevisiae* strain | | | | | | | | | | *Schwanniomyces occidentalis* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | stell1 | stell2 | stell3 | stell4 | stell5 | stell6 | stell7 | stell8 | MC | SC | CSIR Y-993 |
| Cultivation time, h | 202.3 | 202.3 | 202.3 | 202.3 | 202.3 | 200 | 200 | 200 | 200 | 200 | 200 |
| Biomass, g.$l^{-1}$ | 1.82 | 2.48 | 1.76 | 0.66 | 1.76 | 2.28 | 2.91 | 2.24 | 2.33 | 2.42 | 0.51 |
| Ethanol, g.$l^{-1}$ | 6.0 | 7.4 | 6.6 | 1.2 | 7.5 | 7.2 | 8.3 | 7.27 | 7.0 | 6.4 | 0.75 |
| Glucose, g.$l^{-1}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E, % | 68 | 84 | 59 | 25 | 69 | 70 | 92 | 97 | 98.7 | 93.8 | 26 |
| Glucoamylase, U.$l^{-1}$ | 0 | 20 | 0 | 0 | 11 | 90 | 100 | 55 | 104 | 76 | 0 |
| Glucoamylase, U.g biomass$^{-1}$ [a] | 0 | 8.1 | 0 | 0 | 6.3 | 39.5 | 34.3 | 24.6 | 44.6 | 31.4 | 0 |
| α-Amylase activity, U.$l^{-1}$ | 78 | 0 | 0 | 0 | 0 | 0 | 0 | 74 | 38 | 30 | 0 |
| α-Amylase, U.g biomass$^{-1}$ [a] | 42.9 | 0 | 0 | 0 | 0 | 0 | 0 | 33 | 16.3 | 12.4 | 0 |
| $Y_{x/s}$ | 0.054 | 0.084 | 0.068 | 0.032 | 0.062 | 0.073 | 0.109 | 0.138 | 0.142 | 0.119 | 0.068 |
| $Y_{p/s}$ | 0.302 | 0.459 | 0.431 | 0.036 | 0.388 | 0.361 | 0.394 | 0.416 | 0.359 | 0.336 | 0.013 |
| $Q_p^{max}$, g.$l^{-1}$.$h^{-1}$ | 0.048 | 0.084 | 0.073 | 0.034 | 0.045 | 0.079 | 0.108 | 0.097 | 0.129 | 0.094 | 0.028 |
| $\mu_{max}$, $h^{-1}$ | 0.004 | 0.018 | 0.003 | 0.003 | 0.007 | 0.048 | 0.092 | 0.014 | 0.087 | 0.017 | 0.018 |

[a] Specific enzyme activity per gram biomass produced

E Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100

$Q_p^{max}$ Maximum volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time

$Y_{x/s}$ Cell yield coefficient, g dry biomass/g starch, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the yeast extract and peptone components of the medium

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g starch utilised, calculated from final ethanol concentration produced

$\mu_{max}$ Maximum specific growth rate

### 3.2.2 Aerobic cultivations

Although not conducive to ethanol production, a series of cultivations were executed under aerobic conditions with 2 % starch as the main carbon source to maximise biomass and amylase production, as well as to evaluate the effect of aerobic vs. anoxic conditions on enzyme production. Even though all strains yielded higher biomass values with minimal or no ethanol production, the amylase activities remained negligible (Table 6). Strain stell3 (α-amylase from *S. fibuligera*, natural promoter) hydrolysed 81 % of the starch after 200 h, whereas strain stell4 (α-amylase from *S. fibuligera, PGK1* promoter) hydrolysed only 24 % after 200 h. This again confirmed the data obtained under anoxic conditions, which indicated that the natural promoter resulted in a higher activity. A comparison of the α-amylase activity values of strains stell1 and 8 (α-amylase from *L. spencermartinsiae*) with the values obtained with strains stell3 and 4 (α-amylase from *S. fibuligera*) again indicated that the α-amylase gene from *L. spencermartinsiae* produced higher values of α-amylase activity (Table 6). However, these values were lower than those obtained with the naturally amylolytic *Schwanniomyces occidentalis* strain. These very low enzymes activity values could have been due to a number of reasons, such as low levels of excretion, binding of the enzyme to the substrate or inadequate control of the cultivation conditions in the shake flasks.

In terms of biomass production and percentage of starch hydrolysed, strains stell2, 7 and 8 performed the best. Strain stell7, despite having two glucoamylase genes and no α-amylase gene, gave results similar to strain stell8 (having a glucoamylase and an α-amylase gene). Strain stell7 gave the highest biomass yield coefficient, 0.544, and the highest glucoamylase activity, 400 $U.l^{-1}$. As with the anoxic cultivations, strains stell5 and 6 (both with only a glucoamylase) performed surprisingly well and yielded results comparable to those of the transformants with both amylases in terms of efficiency of starch hydrolysis, the maximum specific growth rate and the final biomass concentration.

Figures 7, 8 and 9 show that, under aerobic conditions, the rates of growth were significantly higher than under anoxic conditions. Due to the increased oxygen supply,

the increased biomass concentrations resulted in improved enzyme activity values and subsequent increased rates of starch hydrolysis and growth. However, the specific enzyme activity values did not improve (Table 6).

The limited α-amylase activity and the low glucoamylase activities caused the slow rate of starch hydrolysis, which in turn resulted in the low biomass and ethanol yields. The α-amylase activity values obtained with most strains were below the detection limit (30 $U.l^{-1}$), except with strains stell1 (Figure 6) and 8 (Tables 5 and 6). De Mot and Verachtert (1986) reported α-amylase values of 860 $U.l^{-1}$ for the *L. spencermartinsiae (kononenkoae)* IGC 4052-B strain, obtained under aerobic conditions using soluble Merck starch as the main carbon source. The highest α-amylase activity value recorded was 305 $U.l^{-1}$ obtained with strain stell8 under aerobic conditions, less than half that reported by De Mot and Verachtert (1986). Similarly, Birol *et al.* (1998) reported higher glucoamylase activity values of between 345 and 1053 $U.ml^{-1}$ for similar recombinant strains.

Dhawale *et al.* (1982) and Horn *et al.* (1991) reported an exponential correlation between the nett clearing zones on the starch agar plates and the enzyme activities in liquid culture. No linear or exponential correlation was observed between either the nett clearing zones, the total hydrolysis area or the activity index values and the total amylase activity (Figs. 10a, 10b and 10c). The data obtained under anoxic conditions yielded profiles similar to those shown for the aerobic conditions. The correlation between the starch clearing zones and the amylolytic activity may be species specific and thus not a good selection parameter for use between different species. It was evident that the starch agar plates did not give a reliable indication of the ability of the different transformants to hydrolyse starch, although the limited number of data points may have contributed to the lack of correlation. Figure 10b suggest a possible correlation between the nett starch clearing zone areas ($mm^2$) and the glucoamylase activity obtained in liquid cultures, as indicated by the broken line; however, the few data points are insufficient to verify this. A similar correlation was observed between the nett clearing zones diameters (mm) as depicted in Fig 10a.

A comparison of the fermentations on glucose (Fig. 5 & Table 4) with the data obtained on starch (Table 5) revealed the shortcomings of these recombinant strains, as an extremely long fermentation time of 200 h was required to hydrolyse 20 g starch.$l^{-1}$ due to the low levels of enzyme activity; this fermentation time was comparable to the time required to produce 8 % ethanol from 200 g glucose.$l^{-1}$ (Table 4). Although the ethanol yield coefficients were acceptable in some cases, these final ethanol concentrations of up to 8.3 g.$l^{-1}$ need to be about 10-fold higher for an economically viable ethanol process. Commercial ethanol production would require the production of 6 to 12 g.$l^{-1}$ (w/v) in a relatively short fermentation time (Keim, 1983).

**Table 6.** Aerobic cultivation parameters after 200 h of different recombinant yeast strains in flasks cultures using a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$. The mean values of duplicate experiments are shown.

| Parameter | Recombinant *S. cerevisiae* strain | | | | | | | | | | *Schwanniomyces occidentalis* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | stell1 | stell2 | stell3 | stell4 | stell5 | stell6 | stell7 | stell8 | MC | SC | CSIR Y-993 |
| Biomass, $g.l^{-1}$ | 9.58 | 12.56 | 10.84 | 2.15 | 11.31 | 10.70 | 11.70 | 10.32 | 10.30 | 9.20 | 8.54 |
| Ethanol, $g.l^{-1}$ | 0.2 | 0.3 | 0.2 | 0.1 | 1.5 | 0.1 | 0.69 | 0.12 | 0 | 0 | 0.13 |
| Glucose, $g.l^{-1}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E, % | 76 | 94 | 81 | 25 | 79 | 76 | 93 | 97 | 98 | 98.0 | 94 |
| Glucoamylase, $U.l^{-1}$ | 0 | 80 | 0 | 0 | 53 | 340 | 400 | 360 | 398 | 305 | 540 |
| Glucoamylase, U.g biomass$^{-1}$ [a] | 0 | 6.4 | 0 | 0 | 4.7 | 31.8 | 34.2 | 34.88 | 38.6 | 33.2 | 63.2 |
| α-Amylase activity, $U.l^{-1}$ | 81 | 0 | 35 | 17 | 0 | 0 | 0 | 305 | 204 | 181 | 364.3 |
| α-Amylase, U.g biomass$^{-1}$ [a] | 8.46 | 0 | 3.23 | 7.9 | 0 | 0 | 0 | 29.55 | 21.9 | 19.7 | 42.66 |
| $Y_{x/s}$ | 0.440 | 0.522 | 0.495 | 0.252 | 0.557 | 0.491 | 0.544 | 0.499 | 0.538 | 0.536 | 0.387 |
| $Y_{p/s}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.011 |
| $Q_p^{max}$, $g.l^{-1}.h^{-1}$ | 0.033 | 0 | 0.003 | 0 | 0.007 | 0.008 | 0.012 | 0.009 | 0.008 | 0.003 | 0.000 |
| $\mu_{max}$, $h^{-1}$ | 0.030 | 0.180 | 0.254 | 0.123 | 0.243 | 0.249 | 0.300 | 0.361 | 0.272 | 0.192 | 0.190 |

[a] Specific enzyme activity per gram biomass produced

E Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100

$Q_p^{max}$ Maximum volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time

$Y_{x/s}$ Cell yield coefficient, g dry biomass/g starch, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the yeast extract and peptone components of the medium

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g starch utilised, calculated from final ethanol concentration produced

$\mu_{max}$ Maximum specific growth rate

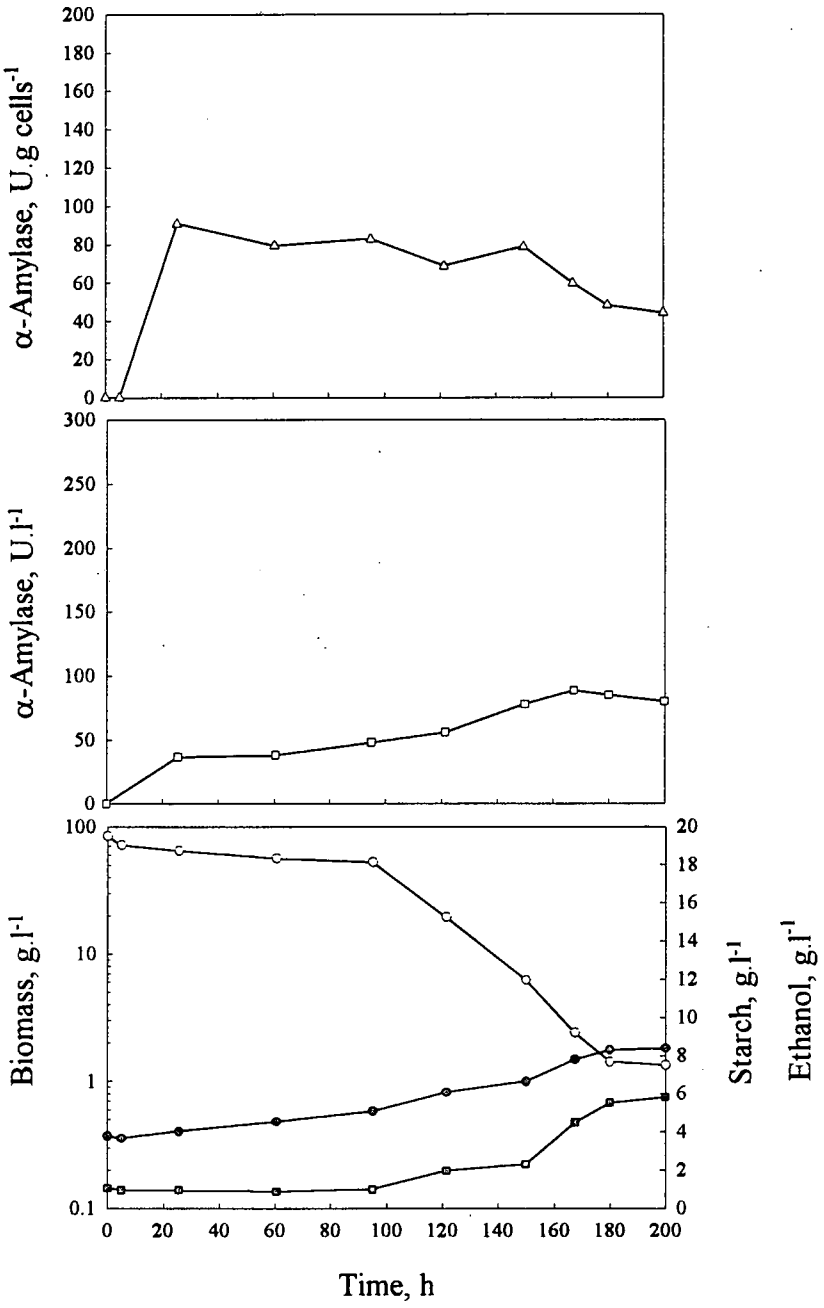



**Figure 6.** Aerobic cultivation profile of the recombinant *S. cerevisiae* strain stell1 in a shake flask with a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$. Symbols: biomass (●), starch (○), ethanol (■), α-amylase (□), specific α-amylase activity (△).

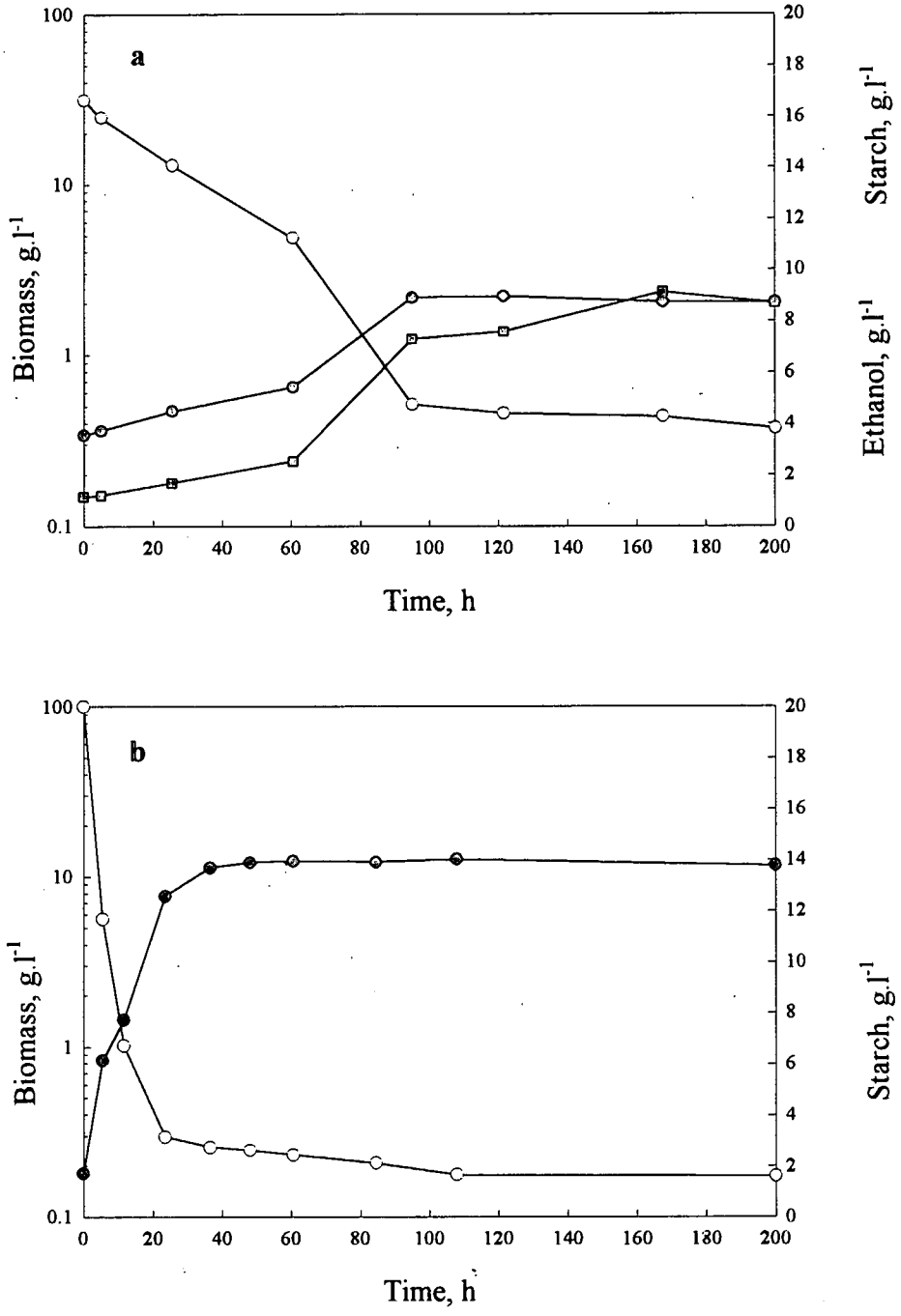



**Figure 7.** Anoxic (a) and aerobic (b) cultivation profiles of the recombinant *S. cerevisiae* strain_stell2 in a shake flask with a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$. Symbols: biomass (●), starch (○), ethanol (■).

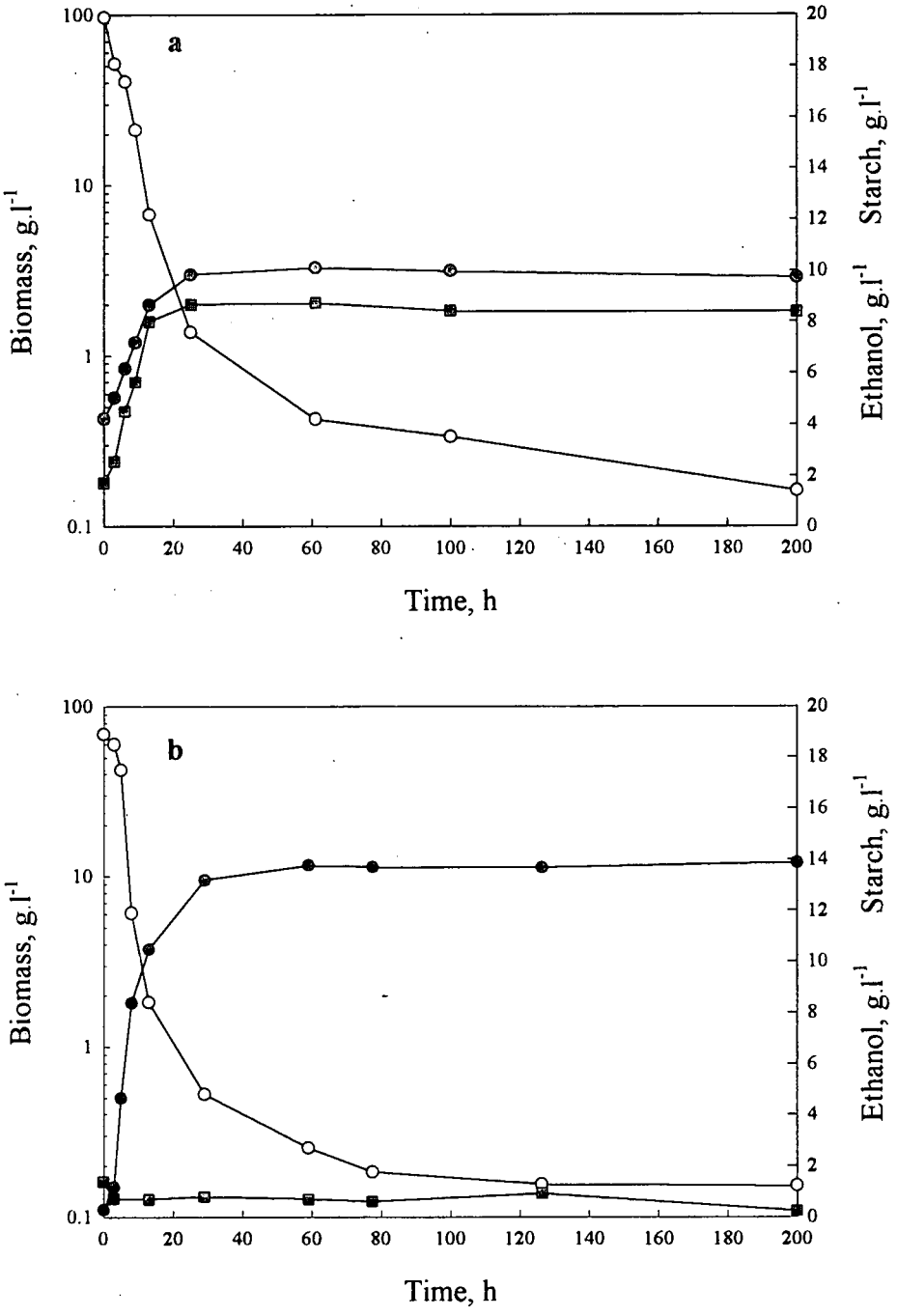



**Figure 8.** Anoxic (a) and aerobic (b) cultivation profiles of the recombinant *S. cerevisiae* strain_stell7 in a shake flask using a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$. Symbols: biomass (●), starch (○), ethanol (■).

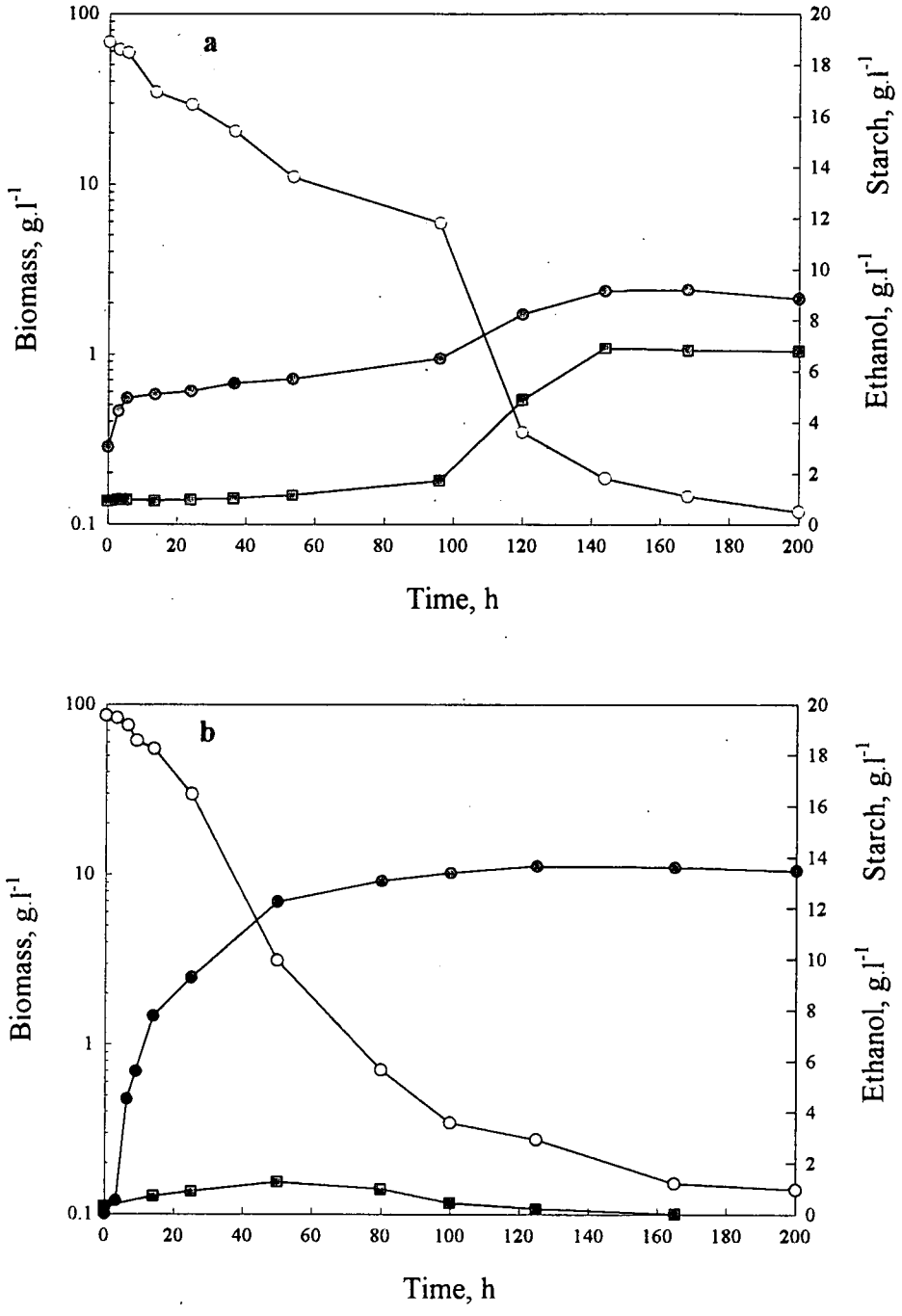



**Figure 9.** Anoxic (a) and aerobic (b) cultivation profiles of the recombinant *S. cerevisiae* strain_stell8 in a shake flask using a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$. Symbols: biomass (●), starch (○), ethanol (■).

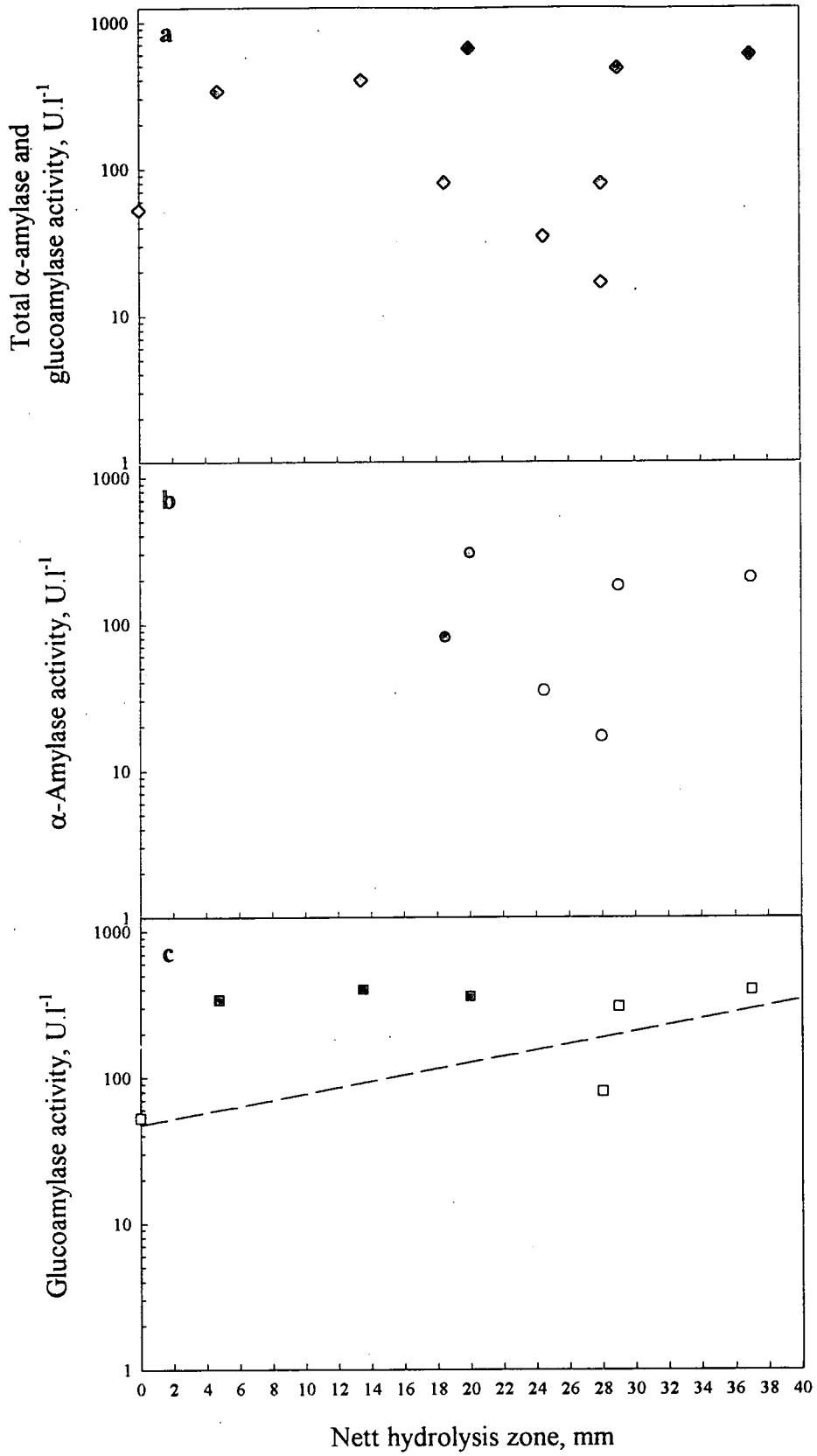



**Figure 10a**. Correlation between (A) total amylase activities, (B) the α-amylase activities and (C) the glucoamylase activities obtained in liquid cultures and the nett starch clearing zone diameters (mm) obtained on Merck starch agar plates. Symbols: Total amylolytic activity (◆), *L. spencermartinsiae* α-amylase activity (●), *S. fibuligera* α-amylase activity (○), *L. spencermartinsiae* glucoamylase (■), *S. fibuligera* glucoamylase activity (□).

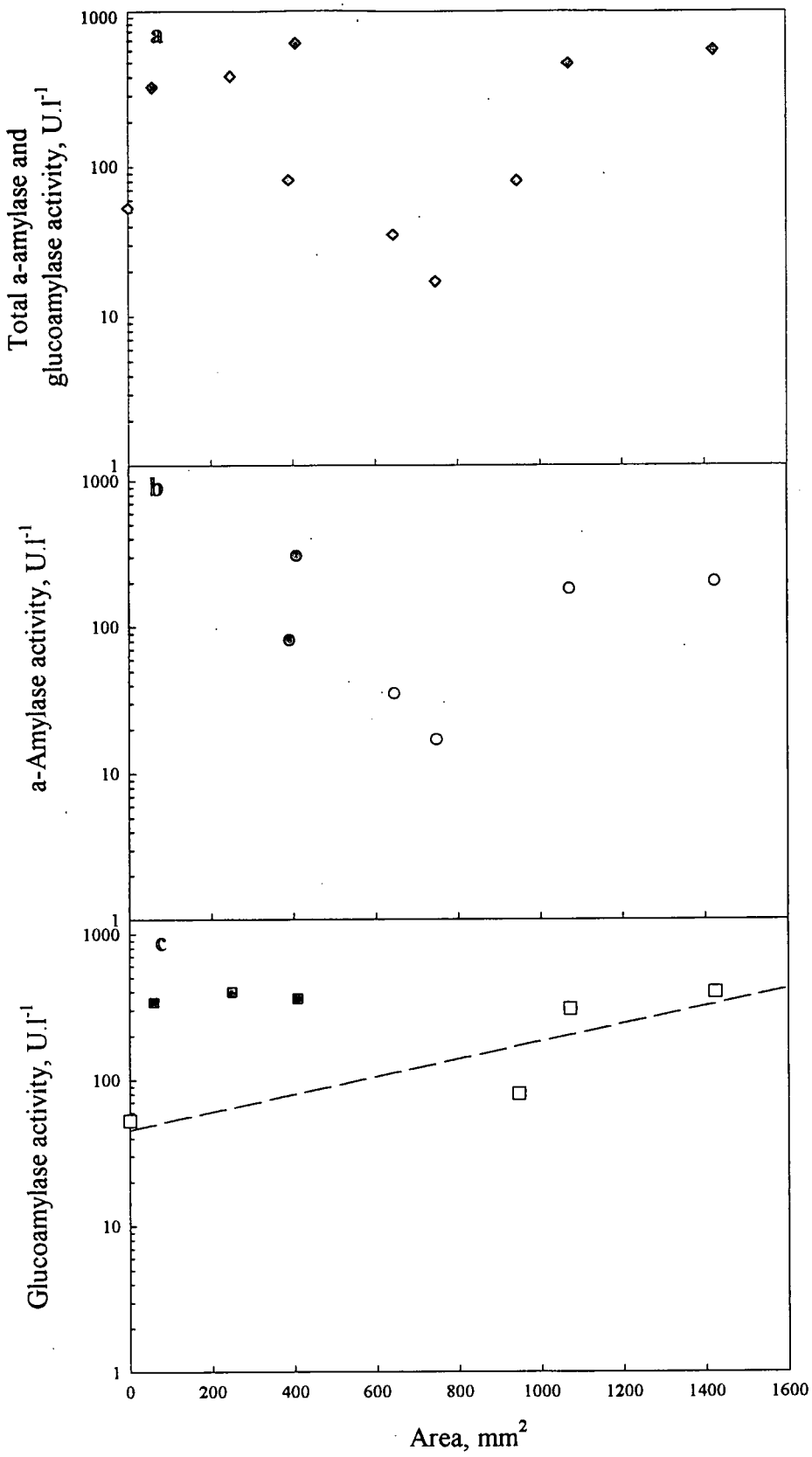



**Figure 10b**. Correlation between (A) total amylase activities, (B) the α-amylase activities and (C) the glucoamylase activities obtained in liquid cultures and the nett starch clearing zone areas ($mm^2$) obtained on Merck starch agar plates. Symbols: Total amylolytic activity (◆), *L. spencermartinsiae* α-amylase activity (●), *S. fibuligera* α-amylase activity (○), *L. spencermartinsiae* glucoamylase (■), *S. fibuligera* glucoamylase activity (□).

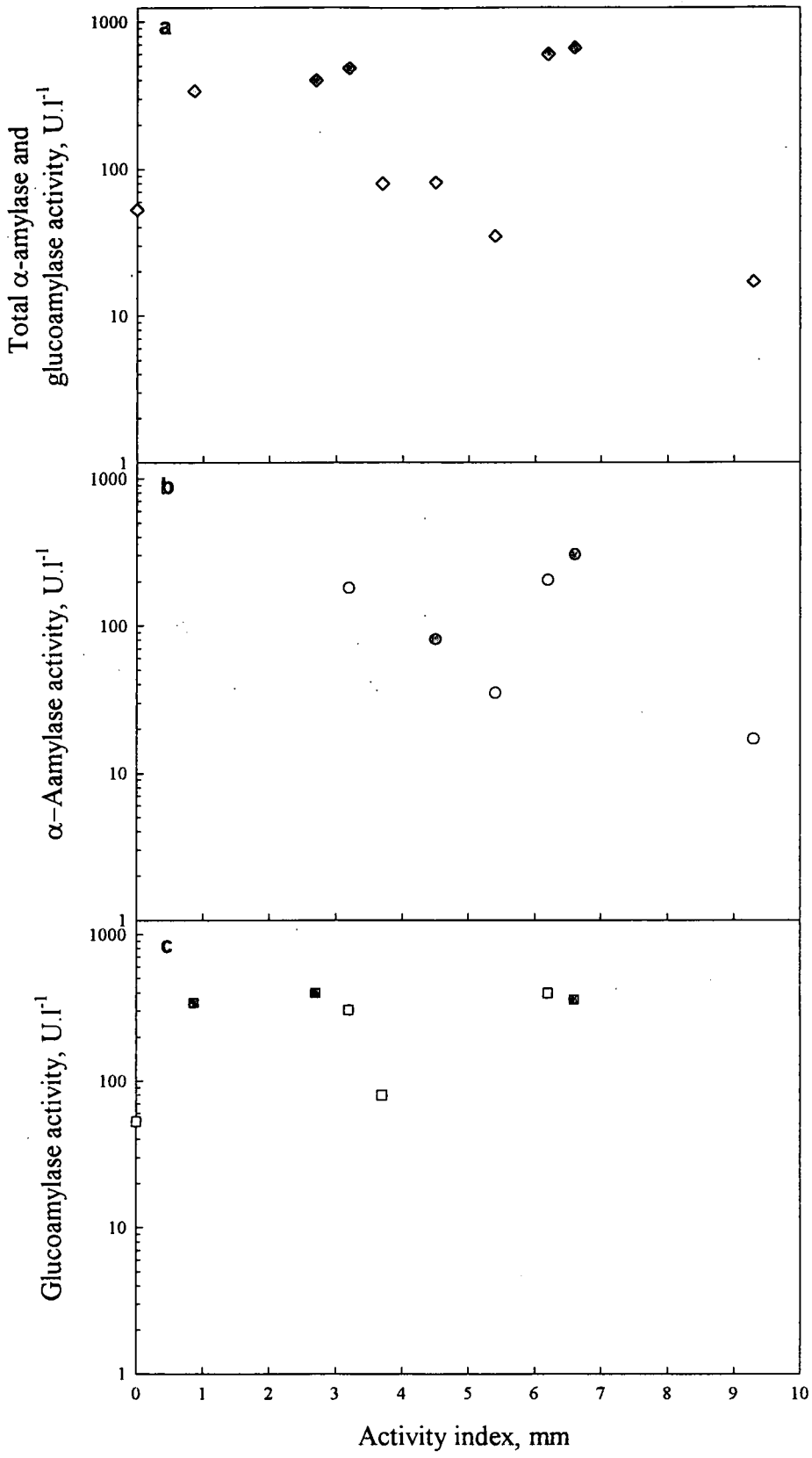



**Figure 10c**. Correlation between (A) total amylase activities, (B) the α-amylase activities and (C) the glucoamylase activities obtained in liquid cultures and the activity index values obtained on Merck starch agar plates. Symbols: Total amylolytic activity (◆), *L. spencermartinsiae* α-amylase activity (●), *S. fibuligera* α-amylase activity (○), *L. spencermartinsiae* glucoamylase (■), *S. fibuligera* glucoamylase activity (□).

# 4 References

**Birol, G., Önsan, Z.İ., Kirdar, B. & Oliver, S.G. (1998).** Ethanol production and fermentation characteristics of recombinant *Saccharomyces cerevisiae* strains grown on starch. *Enzyme Microbiol Technol,* **22,** 672-677.

**Casey, G.P., Xiao, W. & Rank, G.H. (1988).** A convenient dominant selection marker for gene transfer in industrial strains of *Saccharomyces* yeast: SMR1 encoded resistance to the herbicide sulfometuron methyl. *J Inst Brew,* **94,** 93-97.

**De Menezes, T.J.B. (1982).** Starchy materials for alcohol fuel production. *Process Biochem,* **17,** 32-35.

**De Moraes, L.M.P., Astolfi-filho, S. & Oliver, S.G. (1995).** Development of yeast strains for the efficient utilisation of starch: evaluation of constructs that express α-amylase and glucoamylase separately or as bifunctional proteins. *Appl Microbiol Biotechnol,* **43,** 1067-1076.

**De Mot, R. (1990).** Conversion of starch by yeasts. In *Yeast, biotechnology and biocatalysis,* pp. 163-222. Edited by H. Verachtert & R. De Mot. New York and Basel: Marcel Dekker, Inc.

**De Mot, R., Andries, K. & Verachtert, H. (1984).** Comparative study of starch degradation and amylase production by ascomycetous yeast species. *System Appl Microbiol,* **5,** 106-118.

**De Mot, R. & Verachtert, H. (1986).** Enhanced production of extracellular α-amylase and glucoamylase by amylolytic yeasts using α-cyclodextrin as carbon source. *Appl Microbiol Biotechnol,* **24,** 459-462.

**De Mot, R. & Verachtert, H. (1987).** Some microbiological and biochemical aspects of starch bioconversion by amylolytic yeasts. In *Crit Rev Biotechnol, Vol 5,* pp. 259-272. Edited by G. S. Graham & R. Inge. Florida: CRC Press Inc.

**Dhawale, M.R., Wilson, J.J., Khachatourians, G.G. & Ingledew, W.M. (1982).** Improved method for the detection of starch hydrolysis. *Appl Environ Microbiol,* **44,** 747-750.

**Eksteen, J.M., Van Rensburg, P., Cordero Otero, R. & Pretorius, I.S. (2002).** The evaluation and comparison of recombinant *S. cerevisiae* strains expressing α-amylase and/or glucoamylase from different yeast organisms for the efficient utilisation of starch. *Appl Environ Microbiol,* submitted.

**Fiechter, A., Fuhrmann, G.F. & Käppeli, O. (1981).** Regulation of glucose metabolism in growing yeast cells. *Adv Microbiol Physiol,* **22,** 123-183.

**Finn, R. (1987).** Conversion of starch to liquid sugar and ethanol. In *Microbial genetic engineering and enzyme technology,* pp. 101-107. Edited by C.P. Hollenberg & H. Sahm. Stuttgart: Gustav Fischer.

**Fogarty, W.M. & Kelly, C.T. (1979).** Starch-degrading enzymes of microbial origin. *Prog Ind Microbiol,* **15,** 89-150.

**Gietz, R.D. & Schiestl, R.H. (1991).** Applications of high efficiency lithium acetate transformations of intact yeast cells using single-stranded nucleic acid as carrier. *Yeast,* **7,** 253-263

**Goodwin, T. W. & Mercer, E.I. (1983).** Introduction to plant biochemistry. 2nd Ed. pp. 254-264. Oxford: Pergamon Press Inc.

**Guzmán-Maldonado, H. & Paredes-López, O. (1995).** Amylolytic enzymes and products derived from starch: A review. *Critical reviews in food science and nutrition,* **35,** 373-403.

**Horn, C.H., Du Preez, J.C. & Lategan, P.M. (1988).** Protein enrichment of banana plant wastes by yeast cultivation. *Biol Wastes,* **24,** 127-136.

**Horn, C.H., Du Preez, J.C. & Kilian, S.G. (1991).** Selective isolation of amylase derepressed mutants of *Schwanniomyces occidentalis*. *System Appl Microbiol,* **14,** 299-304.

**Horn, C.H., Du Preez, J.C. & Kilian, S.G. (1992).** Amylase production by a *Schwanniomyces occidentalis* mutant in chemostat culture. *Appl Microbiol Biotechnol,* **37,** 147-151.

**Janse, B.J.H. & Pretorius, I.S. (1995).** One-step enzymatic hydrolysis of starch using a recombinant strain of Saccharomyces cerevisiae producing α-amylase, glucoamylase and pullulanase. *Appl Microbiol Biotechnol,* **42,** 878-883.

**Keim, C.R. (1983).** Technology and economics of fermentation alcohol - an update. *Enzyme Microbiol Technol,* **5,** 103-104.

**Kumagai, M.H., Sverlow, G.G., Della-Cioppa, G. & Grill, L.K. (1993).** Conversion of starch to ethanol in a recombinant *Saccharomyces cerevisiae* strain expressing rice α-amylase from a novel *Pichia pastoris* alcohol oxidase promoter. *Biotechnol,* **11,** 606-610.

**Liu, H., Styles, C.A. & Fink, G.R. (1993).** Elements of the yeast pheromone response pathway required for filamentous growth of diploids. *Science,* **262,** 1741-1744.

**Mattoon, J.R., Kim, K. & Laluce, C. (1987).** The application of genetics to the development of starch-fermenting yeasts. In *CRC Crit Rev Biotechnol, Vol 5,* 195-204. Edited by G.G. Stewart & I. Russell. Florida: CRC Press Inc.

**Norman, B.E. (1979).** The application of polysaccharide degrading enzymes in the starch industry. In *Microbial polysaccharides and polysaccharases, Vol 15,* pp. 339-376. Edited by R.C.W. Berkeley, G.W. Gooday & D.C. Ellwood. New York: Academic Press Inc.

**Pretorius, I.S. (1994).** Die genetiese manipulasie van die gis *Saccharomyces cerevisiae* vir die moontlike omskakeling van polisakkariededryke landbougewasse en nywerheidsafval na enkelselproteïen en brandstofetanol. *SA Tydskrif vir Natuurwetenskap & Tegnologie,* **13,** 66-79.

**Rothstein, S.J., Lahners, K.N., Lazarus, C.M., Baulcombe, D.C. & Gatenby, A.A. (1987).** Synthesis and secretion of wheat α-amylase in *Saccharomyces cerevisiae*. *Gene,* **55,** 353-356.

**Russell, I., Crumplen, C.M., Jones, R.M. & Stewart, G.G. (1986).** Efficiency of genetically engineered yeast in the production of ethanol from dextrinized cassava starch. *Biotechnol Lett,* **8,** 169-174.

**Sá-Correia, I. & Van Uden, N. (1981).** Production of biomass and amylase by the yeast *Lipomyces kononenkoae* in starch-limited continuous culture. *European J Appl Microbiol Biotechnol,* **13,** 24-28.

**Spencer-Martins, I. & Van Uden, N. (1977).** Yields of yeast growth on starch. *European J Appl Microbiol Biotechnol,* **4,** 29-35.

**Spencer-Martins, I. & Van Uden, N. (1979).** Extracellular amylolytic system of the yeast *Lipomyces kononenkoae*. *European J Appl Microbiol Biotechnol,* **6,** 241-250.

**Thomas, K.C., Hynes, S.H. & Ingledew, W.M. (1996).** Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Process Biochem,* **13,** 321-331.

**Wang, T.T., Lin, L.L., Hsu, W.H. (1989).** Cloning and expression of a *Schwanniomyces occidentalis* α-amylase gene in *Saccharomyces cerevisiae*. *Appl Environ Microbiol,* **55,** 3167-3172.

**Wilson, J.J. & Ingledew, W.M. (1982).** Isolation and characterisation of *Schwanniomyces alluvius* amylolytic enzymes. *Appl Environ Microbiol,* **44,** 301-307.

Chapter 3

# AMYLASE PRODUCTION AND STARCH HYDROLYSIS BY SELECTED RECOMBINANT *SACCHAROMYCES CEREVISIAE* STRAINS IN BENCH-TOP AND 15-L BIOREACTORS

**Abstract**

Six recombinant strains of *Saccharomyces cerevisiae*, transformed with heterologous yeast amylase genes, were selected for evaluation in 2-l bench-top bioreactors using media containing 20 g of starch.$l^{-1}$. Under anoxic conditions, a maximum of 8.1 g of ethanol.$l^{-1}$ was produced after a lengthy fermentation. Aerobic cultivation resulted in higher biomass concentrations and, therefore, higher volumetric amylase activity values and higher rates of starch hydrolysis, but no ethanol production. Similar results were obtained for cultivations conducted at pH 4.5 and pH 5.5. A comparison between fermentations on starch and glucose further suggested that the rate of starch hydrolysis and not the fermentative capacity of these recombinant strains was the rate-limiting factor. Four of these strains were further evaluated in 15-l bioreactors using media containing 55 g of starch.$l^{-1}$. In all cases, the volumetric amylase activity was higher than in the bench-top reactors, due to a higher biomass concentration. A maximum of 21 g of ethanol.$l^{-1}$ was produced by strain stell7 within the relatively short time of 120 h. Strain stell8 was characterised by a curious multi-phasic growth profile and a low α-amylase activity that appeared only late in the cultivation.

## 1 Introduction

A number of recombinant *Saccharomyces cerevisiae* strains transformed with different combinations of amylase genes and promoters were subjected to preliminary evaluation on starch agar plates and in liquid starch media in shake flasks (Chapter 2). Based on these results and considering their genetic make up, transformants were selected for further evaluation in 2-l bench-top and 15-l bioreactors under anoxic and aerobic conditions, facilitating stricter control of the cultivation conditions. Strains stell2 and 8 contained both the α-amylase and the glucoamylase genes from *Saccharomycopsis fibuligera* (with its natural promoters) and *Lipomyces spencermartinsiae (kononenkoae)* (under the control of *PGK1* promoter) respectively. Strain stell7, which had a unique combination of two glucoamylase genes, performed surprisingly well in the preliminary evaluations. Strain stell1 exhibited the strongest amylase activity of the strains

containing only an α-amylase gene. Additionally, the *S. cerevisiae* strains MC and SC, both with the α-amylase and the glucoamylase genes from *Saccharomycopsis fibuligera* (with their natural promoters), were included in these evaluations.

The use of optimal cultivation conditions is essential to ensure amylase activity values that allow a rapid and effective rate of starch hydrolysis. A factor with a great influence on the production and effectiveness of enzymes, and thus the on rate of starch hydrolysis, is the pH (Brown, 1979; Drozdowixz and Jones, 1995). Most yeasts exhibit optimum growth at pH values between 4.5 and 6.5, and nearly all can grow in more acid or alkaline media (Walker, 1998). Calleja *et al.* (1987) postulated that the excretion of amylases was pH dependent, as the amylases first accumulated in the periplasm, where the pH may be presumed to be similar to that of the external medium. Ruohonen *et al.* (1991) reported only transient detection of *Bacillus* α-amylase during the early exponential growth phase of the recombinant *Saccharomyces cerevisiae* host due to a decrease in the pH of the medium. Similarly, Calleja *et al.* (1987) reported a rapid and irreversible inactivation of *Schwanniomyces alluvius* α-amylase at pH values below pH 3. The optimal pH values of various yeast α-amylases and glucoamylases have been reported to be between 4 and 6 (Chapter 1, Section 3.5.1). Steyn and Pretorius (1995) reported an optimal pH range of 3.8 to 4.5 for *Lipomyces spencermartinsiae* IGC4052 α-amylase, with a stability range from pH 3 to pH 8 (Figure 1a). *Saccharomycopsis fibuligera* has an optimum pH range of 4.5 to 5.8 (De Mot, 1990). Considering the optimum pH values for both the *L. spencermartinsiae* and the *S. fibuligera* amylases, pH 4.5 and pH 5.5 were selected for liquid media cultivations. The use of bioreactors provided a number of advantages over the use of conventional shake flasks, especially regarding the monitoring and control of the pH, temperature and dissolved oxygen tension during the cultivation. Another factor that can significantly affect the rate of amylase production is the vector or plasmid copy number and the stability thereof (Hadfield *et al.*, 1993). Thus, cultivations using two different medium compositions, as well as other aspects, including plasmid stability and evaluation at two pH values, were investigated.

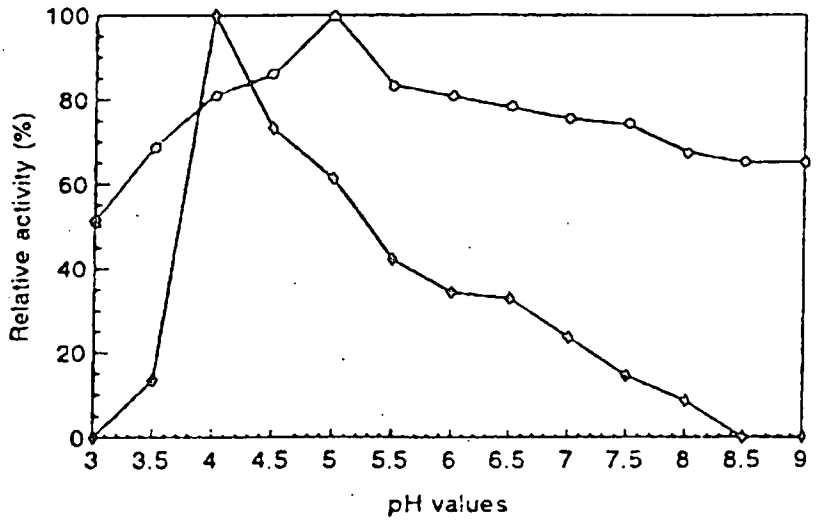



**Figure 1a.** The activity and stability profile of the *Lipomyces spencermartinsiae* α-amylase. Symbols: activity (◆), stability (●). Adapted from Steyn and Pretorius (1995).

# 2 Materials and methods

## 2.1 Yeast strains

The strains stell2 (α-amylase and glucoamylase from *S. fibuligera*, natural promoters), stell8 (α-amylase and glucoamylase *L. spencermartinsiae (kononenkoae)*, *PGK1* promoters), stell7 (two glucoamylase genes from *S. fibuligera* and *L. spencermartinsiae (kononenkoae)*) and stell1 (α-amylase from *L. spencermartinsiae (kononenkoae)*, *PGK1* promoter) used in this investigation were kindly supplied by the Institute for Wine Biotechnology at the University of Stellenbosch. Dr J. Albertyn, from the University of the Free State provided the *Saccharomyces cerevisiae* strains with multi and single copy plasmids. All strains were maintained as described under materials and methods in Chapter 2, Section 2.2.

## 2.2 Cultivation

*2-l Multigen Bioreactor*

Anoxic and aerobic batch cultivations were carried out in 2-litre stirred tank reactors (Multigen F-2000; New Brunswick Scientific, Edison, NJ, USA), each fitted with an exhaust gas condenser (a reflux cooler), a polarographic oxygen electrode (Ingold AG, Urdorf, Switzerland) and a pH electrode (Mettler Toledo, Halstead, U.K.). Medium A comprised (per litre): 20 g starch (Merck, Darmstadt, Germany), 20 g peptone (Biolab Diagnostics, Midrand, South Africa), 10 g yeast extract (Biolab Diagnostics) and 2 g $KH_2PO_4$. Dow Corning 1520 (EU) silicone antifoam (Dow Corning, Seneffe, Belgium) was added at 0.75 ml per litre of medium to suppress foaming. Further addition was done as required during the cultivation. All cultivations were carried out at 30 °C with the pH controlled at pH 4.5 or pH 5.5 (as indicated) by automatic titration with 3 M KOH or manual titration with 3 M $H_2SO_4$. The dissolved oxygen tension was maintained above 20 % of saturation, using an aeration rate of 700 to 1000 $ml.min^{-1}$ and manual adjustment of the stirrer speed between 300 and 800 $r.min^{-1}$. The anoxic cultivations were not completely anaerobic as the headspace was not flushed with

nitrogen gas, but the air supply was shut off and a low stirrer speed of 300 $r.min^{-1}$ was maintained to minimise surface aeration while still facilitating mixing.

The inoculum was prepared by streaking out the strain on GPY agar plates and incubating at 30 °C for 24 h. Cells from these plates were used to inoculate a 250 ml Erlenmeyer flask (pre-inoculum) containing 100 ml of medium A, which comprised (per litre): 10 g glucose, 10 g yeast extract (Biolab Diagnostics, Midrand, South Africa), 20 g peptone (Biolab Diagnostics) and 2 g $KH_2PO_4$, adjusted to pH 4.5 and incubated for

24 h at 30 °C on a rotary shaker. The inoculum was prepared by transferring 5 ml of the latter culture to a 250 ml Erlenmeyer flask containing 95 ml of the above medium and incubating it for 12 h at 30 °C. This entire inoculum was transferred aseptically to a sterile 250 ml flask fitted with a side connector, silicon rubber tubing and an inoculation glass bell for the inoculation process.

*14-l and 15-l Bioreactors*

Anoxic and aerobic evaluations were performed in a 14-l Chemap 3000 stirred tank reactor (STR) (Chemap AG, Volketswil, Switzerland) fitted with three disk turbine impellers, using a working volume of 10.5 l. Alternatively, a 15-l Biostat C STR (B. Braun Biotech International, Melsungen, Germany) fitted with two disk turbine impellers and a single marine propeller, with a working volume of 10.5 l, was used. After *in situ* sterilisation of the bioreactor containing 10 l of medium, the pH was adjusted to pH 5.5 and a 500 ml inoculum was added aseptically. The dissolved oxygen in the medium was monitored with a polarographic $pO_2$ electrode (Mettler, Toledo, Halstead, UK) and maintained at or above 20 % of saturation by manually increasing the air flow rate, which was varied using an aeration rate from 1 to 10 $l.min^{-1}$, and automatic adjustment of the stirrer speed between 300 and 1400 $r.min^{-1}$. The pH was monitored with an Ingold pH electrode and maintained at pH 5.5 by automatic titration with 3 N KOH or 3 N $H_2SO_4$. Medium B comprised (per litre): 55 g starch (Merck), 0.25 g citric acid, 5 g peptone (Biolab Diagnostics), 5 g yeast extract (Biolab Diagnostics), 6 g $(NH_4)_2SO_4$, 2.5 g $KH_2PO_4$, 0.05 g $MgSO_4.7H_2O$, 0.03 g $CaCl_2.2H_2O$,

0.5 ml trace element solution (according to Du Preez and Van der Walt, 1983) and 0.75 ml Dow corning 1520 Silicone antifoam (Dow corning, Seneffe, Belgium). All cultivations were carried out at 30 °C. As with the bench-top bioreactors, the headspace was not flushed with nitrogen gas, but the air supply was terminated and the stirrer speed maintained at 300 $r.min^{-1}$ to minimise surface aeration while facilitating mixing.

The preparation of the pre-inoculum followed the same procedure as for the 2-l bioreactors, but a pH of 5.5 was used. The inoculum was prepared by transferring 25 ml of the pre-inoculum to two 1-l Erlenmeyer flask containing 225 ml of medium A and then incubating for 24 h at 30 °C. The contents of these two flasks were combined aseptically in a sterile 1-l inoculation flask, fitted with a side connector, silicone rubber tubing and an inoculation needle.

## 2.3 Analyses

The biomass and ethanol concentrations, as well as the extracellular amylase activities of each sample, were analysed as described in Chapter 2, Section 2.5. For economical and convenience reasons, the starch concentrations of the samples were determined using the iodine staining method of Randez-Gill and Sanz (1993) and not by the enzymatic procedure described in Chapter 2, Section 2.5.1. A 100 µl aliquot of the sample was added to a test tube containing 900 µl of 0.15 % iodine and a 1.5 % KI solution. A ten-fold dilution was made and the absorbance was read at 550 nm. The resulting absorbance was converted to starch concentration using a standard curve prepared with standards consisting of (per litre) 20, 15, 10, 5 and 2 g of Merck starch and subjected to the same analytical procedure as the samples. Samples taken for glucose analysis during the 15-l bioreactor cultivations were inactivated by the prior addition of 500 µl of a 5 N HCl solution to the sampling bottle. Glucose concentrations were determined as in Chapter 2, using the enzymatic peroxidase-oxidase assay (Sigma, St. Louis, MO, USA). All statistical analyses were done using the GraphPad™ Instat® version 3.05 programme (GraphPad Software Inc., San Diego, USA).

## 2.4 Plasmid stability and loss

The replica plating technique was used to determine the degree of plasmid loss. An appropriate dilution series was prepared using sterile PBS (phosphate buffered saline) with the pH adjusted to 7.4 with HCl. PBS contained (per litre): 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$ and 0.24 g $KH_2PO_4$ (Sambrook *et al.*, 1989). A 100 µl aliquot of the appropriate dilution was distributed evenly over a GPY agar plate using the hockey stick method. After a 48 h incubation period at 30 °C, the colonies were transferred to the *-ura3* agar plates by replica plating, using the sterile velvet squares stretched over a circular block, and incubated for 24 h at 30 °C. The *-ura3* agar plates contained (per litre): 40 g glucose, 5 g peptone (Biolab Diagnostics), 5 g yeast extract (Biolab Diagnostics), 20 g agar, 0.03 g L-leucin, 0.03 g L-tyrosine, 0.02 g L-histidine and 0.6 g CSM (-His-Leu-Trp-Ura). All the plasmids contained the *-ura3* marker, thus the colonies retaining the plasmids were present on the *-ura3* agar plates. The genetic constructs of the plasmids are detailed in Chapter 2, Fig 1 and 2.

# 3 Results and discussion

## 3.1 2-l bench top bioreactor cultivations

The theoretical maximum biomass yield coefficient on glucose is 0.511 (Thomas *et al.*, 1996). On starch, using a factor of 1.111 to convert starch to glucose units (Thomas *et al.*, 1996), this corresponds to a maximum yield coefficient of 0.567 g.g starch$^{-1}$. However, the observed biomass yield coefficients obtained with these recombinant strains were significantly higher, probably because of the high concentration of yeast extract and peptone in the medium, which also served as carbon source for biomass formation. Therefore, cultivations were carried out in the absence of starch and glucose to determine the biomass yield on the non-starch components of the medium (Table 1). Although growth was extremely slow, strains stell2 and stell7 produced *ca.* 0.55 g biomass.l$^{-1}$ from the peptone and yeast extract components of medium A. Strain stell8, grown on medium B, produced *ca.* 0.46 g biomass.l$^{-1}$, but with a higher growth rate due

adjust the biomass yield coefficients so as to more accurately reflect the biomass production on starch as a substrate.

**Table 1**. Cultivation parameters of selected recombinant *S. cerevisiae* strains derived from the Sigma mother strain, with only the peptone and yeast extract components of the medium as carbon source, after 24 h. The mean values of duplicate experiments are shown.

| Parameter | Anoxic Medium A | | Aerobic Medium B |
|---|---|---|---|
| | stell2 | stell7 | stell8 |
| Biomass, $g.l^{-1}$ | 0.55 | 0.57 | 0.46 |
| Ethanol, $g.l^{-1}$ | 0.82 | 0.80 | 0 |
| $\mu_{max}$, $h^{-1}$ | 0.009 | 0.008 | 0.277 |

$\mu_{max}$ Maximum specific growth rate

## 3.2 Effect of transformation and pH on growth characteristics

### 3.2.1 Growth on glucose

The extent to which the pH value and transformation affected these recombinant strains was investigated by growing the transformants and the Sigma parental strain with glucose as the main carbon source at pH 4.5 and pH 5.5 (Table 2). The $\mu_{max}$ values in the cultivations at pH 4.5 ranged from 0.124 to 0.263 $h^{-1}$, which were generally lower than the $\mu_{max}$ values ranging from 0.341 to 0.381 $h^{-1}$ recorded at pH 5.5. Although the specific growth rate on glucose was almost double that obtained on starch (Table 3), it was still lower than the values for *S. cerevisiae* reported elsewhere (Rieger *at al.*, 1983). Similarly, Fiechter *et al.* (1981) and De Kock and Du Preez (2000) reported a $\mu_{max}$ value of 0.45 $h^{-1}$ for aerobic growth on glucose by different strains of *S. cerevisiae*. The $\mu_{max}$ value of the Sigma parental strain was 0.365 $h^{-1}$, suggesting that this strain may have been a weaker strain. Despite the aerobic conditions, small amounts of ethanol were produced, as reflected by the low ethanol yield coefficients. This ethanol fermentation was probably due to the Crabtree effect (Fiechter *et al.*, 1981; Walker, 1998). Although

differences in the maximum specific growth rates were observed between pH 4.5 and pH 5.5, differences between the Sigma parental strain and the *S. cerevisiae* transformants at each pH value were statistically not significant, as P values greater than 0.5 were obtained using ANOVA with a 95 % confidence interval, indicating that transformation with the foreign amylase genes did not impair the growth rate.

**Table 2.** Aerobic cultivation parameters of selected yeast strains, in 2-l bioreactor cultures using a yeast extract/peptone medium containing 20 g glucose.$l^{-1}$ after 24 hours. The mean values of duplicate experiments are shown.

| Parameter | *S. cerevisiae* strains | | | | | |
|---|---|---|---|---|---|---|
| | Sigma strain | stell2 | stell7 | stell8 | MC | SC |
| **pH 5.5** | | | | | | |
| Biomass, g.$l^{-1}$ | 7.20 | 9.06 | 8.36 | 8.79 | 8.81 | 8.29 |
| Ethanol, g.$l^{-1}$ | 2.7 | 1.08 | 1.11 | 1.29 | 0.90 | 0.96 |
| Glucose, g.$l^{-1}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| $Y_{x/s}$ | 0.297 | 0.435 | 0.388 | 0.412 | 0.434 | 0.391 |
| $Y_{p/s}$ | 0.109 | 0.039 | 0.041 | 0.055 | 0.034 | 0.029 |
| $\mu_{max}$, $h^{-1}$ | 0.365 | 0.381 | 0.341 | 0.372 | 0.321 | 0.319 |
| **pH 4.5** | | | | | | |
| Biomass, g.$l^{-1}$ | 6.99 | 7.53 | 7.36 | 7.23 | 7.45 | 7.39 |
| Ethanol, g.$l^{-1}$ | 0.92 | 1.07 | 1.86 | 1.56 | 1.05 | 1.04 |
| Glucose, g.$l^{-1}$ | 0.5 | 0 | 0.3 | 0.5 | 0.1 | 0.9 |
| $Y_{x/s}$ | 0.322 | 0.371 | 0.373 | 0.342 | 0.348 | 0.360 |
| $Y_{p/s}$ | 0.047 | 0.055 | 0.028 | 0.080 | 0.053 | 0.047 |
| $\mu_{max}$, $h^{-1}$ | 0.220 | 0.263 | 0.253 | 0.255 | 0.245 | 0.251 |

$Y_{x/s}$ Cell yield coefficient, g dry biomass/g glucose utilised, calculated from final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the yeast extract and peptone components of the medium

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g glucose utilised, calculated from the final ethanol concentration

$\mu_{max}$ Maximum specific growth rate

### 3.2.2 Growth on starch

In view of the increased biomass concentrations and growth rates obtained at pH 5.5 using glucose as carbon source (Table 2), additional cultivations using strain stell2 were carried out at pH 5.5 with starch as the main carbon source. Despite its ability to grow on starch, strain stell2 did not show any detectable levels of amylase activity. As discussed above, the pH can affect enzyme production and thus, by culturing this strain at other pH values, it was hoped to improve the amylase activities.

*Anoxic cultivations*

*pH 4.5*

In terms of starch hydrolysis, ethanol production and final biomass yields, strains stell1, 2, 7, 8 and *S. cerevisiae* strains MC and SC were comparable (Table 3). Initial evaluations on the starch agar plates indicated that strain stell7 lacked α-amylase activity. Cultivations in a liquid starch medium further confirmed the lack of α-amylase activity, but despite this strain stell7 performed surprisingly well when compared to the other transformants. Strain stell8, despite having the ideal gene combination, did not excel above the other strains. Figures 1, 2 and 3 showed notable differences in the rates of starch hydrolysis, ethanol and biomass production between strains stell2, 7 and 8. These graphical representations, as well as the data in Table 1, show that strain stell7 was superior to the other strains in terms of the growth rate and rate of starch hydrolysis. The performance of strain stell1 was comparable to the other strains, despite having only an α-amylase. The high percentage of starch hydrolysed (94 %) corresponded with substantial ethanol production (6.19 $g.l^{-1}$), indicating that this α-amylase was effective in terms of starch degradation. The performance of *S. cerevisiae* strains MC and SC were comparable to that of the other strains, but with lower biomass production. Contrary to strain stell2, both these strains produced detectable α-amylase activity values, but with lower specific activities than strains stell1 and stell8. *S. cerevisiae* strains stell2, MC and SC all contained the same α-amylase and glucoamylase from *S. fibuligera*.

All the strains produced more biomass than in the shake flask cultures, undoubtedly due to an increased efficiency of starch hydrolysis. Strain stell7 showed only a slight increase in the percentage of starch hydrolysis and ethanol production under these conditions, resulting in a small increase in the product yield coefficient. The maximum specific growth rate ($\mu_{max}$) of strain stell2 was higher than in the shake flasks (Table 5, Chapter 2), but was still significantly lower than the reported $\mu_{max}$ of *S. cerevisiae* (Fiechter *et al.*, 1981). This increase in $\mu_{max}$ corresponded with an increase in the percentage of starch hydrolysed as well as an increase in biomass and ethanol production. Despite this, the α-amylase activity of strain stell2 remained below the detection limit of the assay. Strain stell1 showed the largest increase in the efficiency of starch hydrolysis, from 68 % in the shake flasks to 94 % in these bioreactors cultures, also with an increase in $\mu_{max}$ value, but with a decreased specific α-amylase activity.

Strain stell2 (*S. fibuligera* amylases, natural promoter) gave the highest ethanol yield coefficient of 0.470, but produced no detectable α-amylase, whereas strains stell1 and stell8 (*L. spencermartinsiae* α-amylase, *PGK1* promoter) produced low α-amylase values of 77.6 and 131 $U.l^{-1}$ respectively under anoxic conditions. Direct comparison of strains stell1 and stell8 with strain stell2 indicated that the LKA1 gene from *L. spercermartinsiae* gave a higher extracellular α-amylase activity than the *S. fibuligera* α-amylase gene.

*pH 5.5*

A comparison of the growth parameters of strain stell2 obtained at pH 4.5 with those obtained at pH 5.5 (Table 3) revealed a small decrease in biomass production and efficiency of starch hydrolysis. The maximum specific growth rate at pH 5.5 was lower. The α-amylase activity remained at or below the detection limit of the Phadebas assay (30 $U.l^{-1}$), while the glucoamylase activity showed no notable improvement from the cultivations carried out at pH 4.5.

**Table 3.** Anoxic cultivation parameters of selected recombinant *S. cerevisiae* yeast strains in 2-l bioreactor cultures using a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$, after 200 h. The mean values of duplicate experiments are shown.

| Parameter | pH 4.5 | | | | | | pH 5.5 |
|---|---|---|---|---|---|---|---|
| | stell1 | stell2 | l7 | stell8 | MC | SC | stell2 |
| Biomass, g.$l^{-1}$ | 3.21 | 3.55 | 3.39 | 3.77 | 2.65 | 2.52 | 3.01 |
| Ethanol, g.$l^{-1}$ | 6.19 | 7.8 | 8.1 | 7.1 | 7.3 | 6.2 | 6.34 |
| Glucose, g.$l^{-1}$ | 0.18 | 0.24 | 0.22 | 0 | 0 | 0 | 0 |
| E, % | 94 | 92 | 93 | 100 | 98 | 95 | 85 |
| Glucoamylase, U.$l^{-1}$ | 0 | 35 | 115 | 53 | 109 | 74 | 32 |
| Glucoamylase, U.g biomass$^{-1}$ [a] | 0 | 9.9 | 35.1 | 14.1 | 44.5 | 32.9 | 10.6 |
| α-Amylase, U.$l^{-1}$ | 77.6 | 0 | 0 | 131 | 42 | 34 | 0 |
| α-Amylase, U.g biomass$^{-1}$ [a] | 24.2 | 0 | 0 | 34.7 | 15.9 | 13.6 | 0 |
| $Y_{x/s}$ | 0.112 | 0.121 | 0.176 | 0.152 | 0.101 | 0.090 | 0.110 |
| $Y_{p/s}$ | 0.321 | 0.470 | 0.432 | 0.320 | 0.365 | 0.336 | 0.296 |
| $Q_p^{max}$, g.$l^{-1}$.$h^{-1}$ | 0.089 | 0.067 | 0.299 | 0.157 | 0.129 | 0.116 | 0.025 |
| $\mu_{max}$, $h^{-1}$ | 0.043 | 0.109 | 0.107 | 0.068 | 0.092 | 0.082 | 0.091 |

[a] Specific enzyme activity, expressed as units per gram biomass

E Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100

$Q_p^{max}$ Maximum volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time

$Y_{x/s}$ Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the yeast extract and peptone components of the medium

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g starch utilised, calculated from the final ethanol concentration

$\mu_{max}$ Maximum specific growth rate

*Aerobic cultivations*

*pH 4.5*

Although there was an increase in the volumetric amylase activities under aerobic conditions (Table 4) compared to the anoxic cultures (Table 3), the specific activities (expressed on a biomass basis) were similar to those obtained under anoxic conditions, indicating that an increased oxygen supply did not improve amylase production by these transformants *per se*. Strain stell7 again proved superior in terms of biomass and glucoamylase activity, resulting in a rapid initial rate of starch hydrolysis, hydrolysing *ca.* 68 % of the starch within 30 h (Fig. 2). In contrast to strain stell7, strain stell8 gave complete starch hydrolysis due to the presence of an α-amylase, albeit at a much slower rate (Fig. 3). The more rapid rate of starch hydrolysis by the double glucoamylase combination of strain stell7, rather than by the glucoamylase and α-amylase combination in strain stell8, was contrary to what was expected. As before, *S. cerevisiae* strains MC and SC, transformed with the same amylases from *S. fibuligera* as strain stell2, produced detectable α-amylase activities, but with lower specific activities than strains stell1 and 8, which contained the *L. spencermartinsiae* α-amylase. Although strain stell2 produced hydrolysis zones on the Phadebas plates, the lack of detectable α-amylase activity remained evident throughout these cultivations.

The cultivation results (Table 4) were similar to the data obtained from the shake flask cultures, but with improved maximum specific growth rates for all the strains except strains stell7 and 8. As before, the rate of starch hydrolysis was much higher under aerobic conditions (but with minimal ethanol production) due to a higher biomass production, and thus higher volumetric amylase activities facilitated by the increased oxygen supply.

In terms of amylase production and rates of starch hydrolysis, the LKA1 α-amylase and the LKAII glucoamylase genes from *L. spencermartinsiae* were superior to the *S. fibuligera* amylase genes. However, strain stell8, which had the α-amylase and glucoamylase combination from *L. spencermartinsiae*, yielded disappointing results.

*pH 5.5*

As with the anoxic cultivations, strain stell2 exhibited a small decrease in biomass production and efficiency of starch hydrolysis when grown at pH 5.5 (Table 4). The maximum specific growth rate was lower at pH 5.5. While the glucoamylase activity improved slightly from 67 to 74 $U.l^{-1}$, the α-amylase activity was below the detection limit of the Phadebas assay (30 $U.l^{-1}$). Strain stell2 was excluded from the subsequent 15-l bioreactor cultivations, as the data from the shake flasks and the 2-l bioreactor cultivations indicated poor amylase production, which resulted in a slow rate of starch hydrolysis and an extended fermentation time.

**Table 4.** Aerobic cultivation parameters of selected recombinant *S. cerevisiae* strains in 2-l bioreactor cultures using a yeast extract/peptone medium containing 20 g Merck starch.$l^{-1}$, after 200 h. The mean values of duplicate experiments are shown.

| Parameter | pH 4.5 | | | | | | pH 5.5 |
|---|---|---|---|---|---|---|---|
| | stell1 | stell2 | stell7 | stell8 | MC | SC | stell2 |
| Biomass, g.$l^{-1}$ | 10.96 | 12.95 | 12.94 | 13.49 | 11.23 | 10.30 | 12.30 |
| Ethanol, g.$l^{-1}$ | 0 | 0.15 | 0.40 | 0 | 0 | 0 | 0.27 |
| Glucose, g.$l^{-1}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E, % | 78 | 95 | 93 | 100 | 97 | 96 | 93 |
| Glucoamylase, U.$l^{-1}$ | 0 | 67 | 430 | 278 | 374 | 320 | 74 |
| Glucoamylase, U.g biomass$^{-1\,a}$ | 0 | 5.2 | 33.2 | 21.3 | 33.3 | 30.1 | 6.0 |
| α-Amylase, U.$l^{-1}$ | 252 | 0 | 0 | 441 | 224 | 194 | 0 |
| α-Amylase, U.g biomass$^{-1\,a}$ | 22.9 | 0 | 0 | 30.4 | 19.9 | 18.2 | 0 |
| $Y_{x/s}$ | 0.648 | 0.581 | 0.605 | 0.632 | 0.523 | 0.501 | 0.549 |
| $Y_{p/s}$ | 0 | 0 | 0.004 | 0 | 0 | 0 | 0.022 |
| $Q_p^{max}$, g.$l^{-1}$.$h^{-1}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $\mu_{max}$, $h^{-1}$ | 0.102 | 0.211 | 0.240 | 0.233 | 0.227 | 0.198 | 0.119 |

[a] Specific enzyme activity, expressed as units per gram biomass

E Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100

$Q_p^{max}$ Maximum volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time

$Y_{x/s}$ Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the yeast extract and peptone components of the medium

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g starch utilised, calculated from the final ethanol concentration

$\mu_{max}$ Maximum specific growth rate

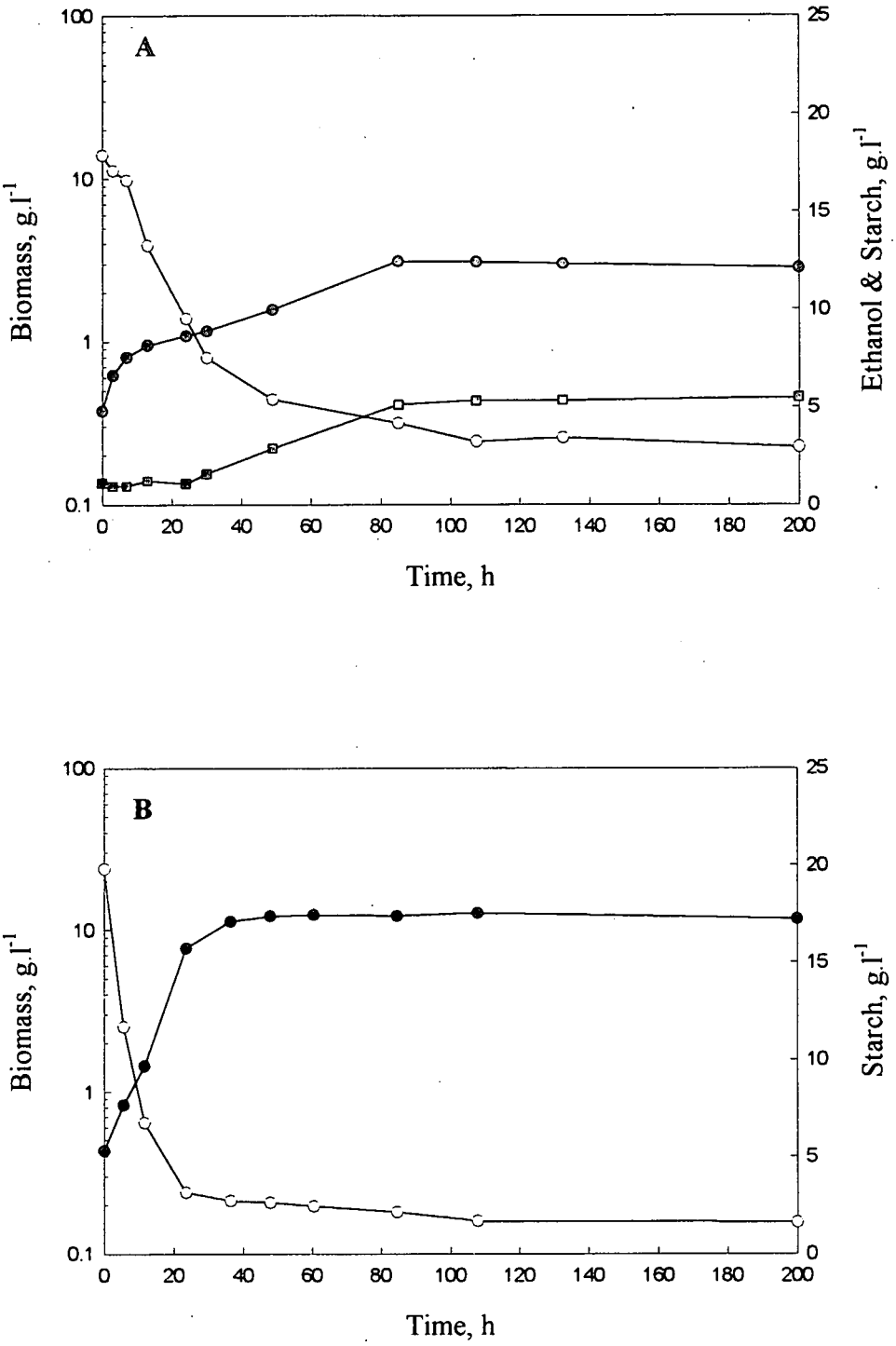



**Figure 1.** Anoxic (A) and aerobic (B) cultivation profiles of the recombinant *S. cerevisiae* strain stell2 in a 2-l bioreactor, using a yeast extract/peptone medium containing 20 g starch.$l^{-1}$. Symbols: biomass (●), ethanol (■) and starch (○).

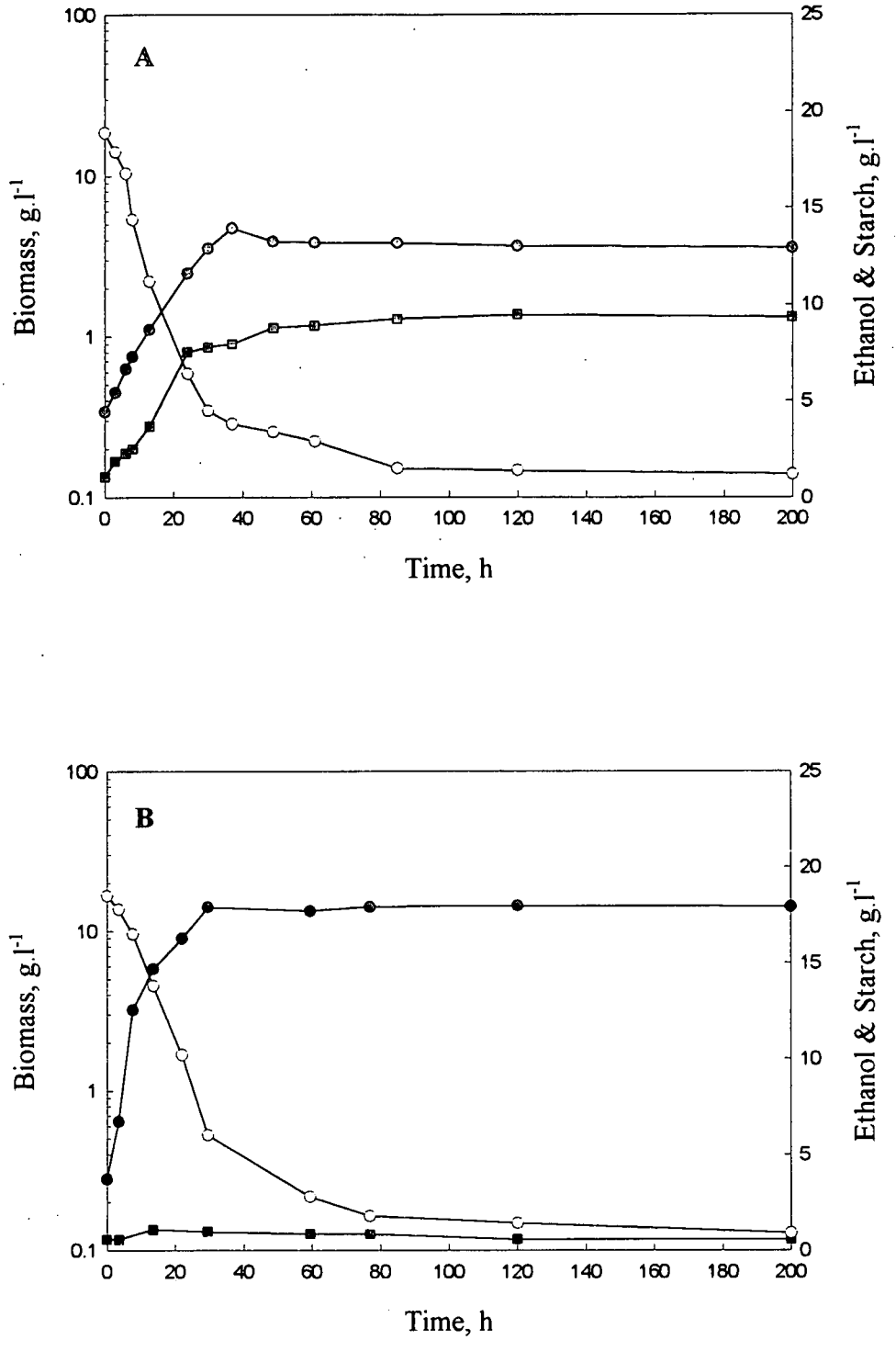



**Figure 2.** Anoxic (A) and aerobic (B) cultivation profiles of the recombinant *S. cerevisiae* strain stell7 in a 2-l bioreactor, using a yeast extract/peptone medium containing 20 g starch.$l^{-1}$. Symbols: biomass (●), ethanol (■) and starch (○).

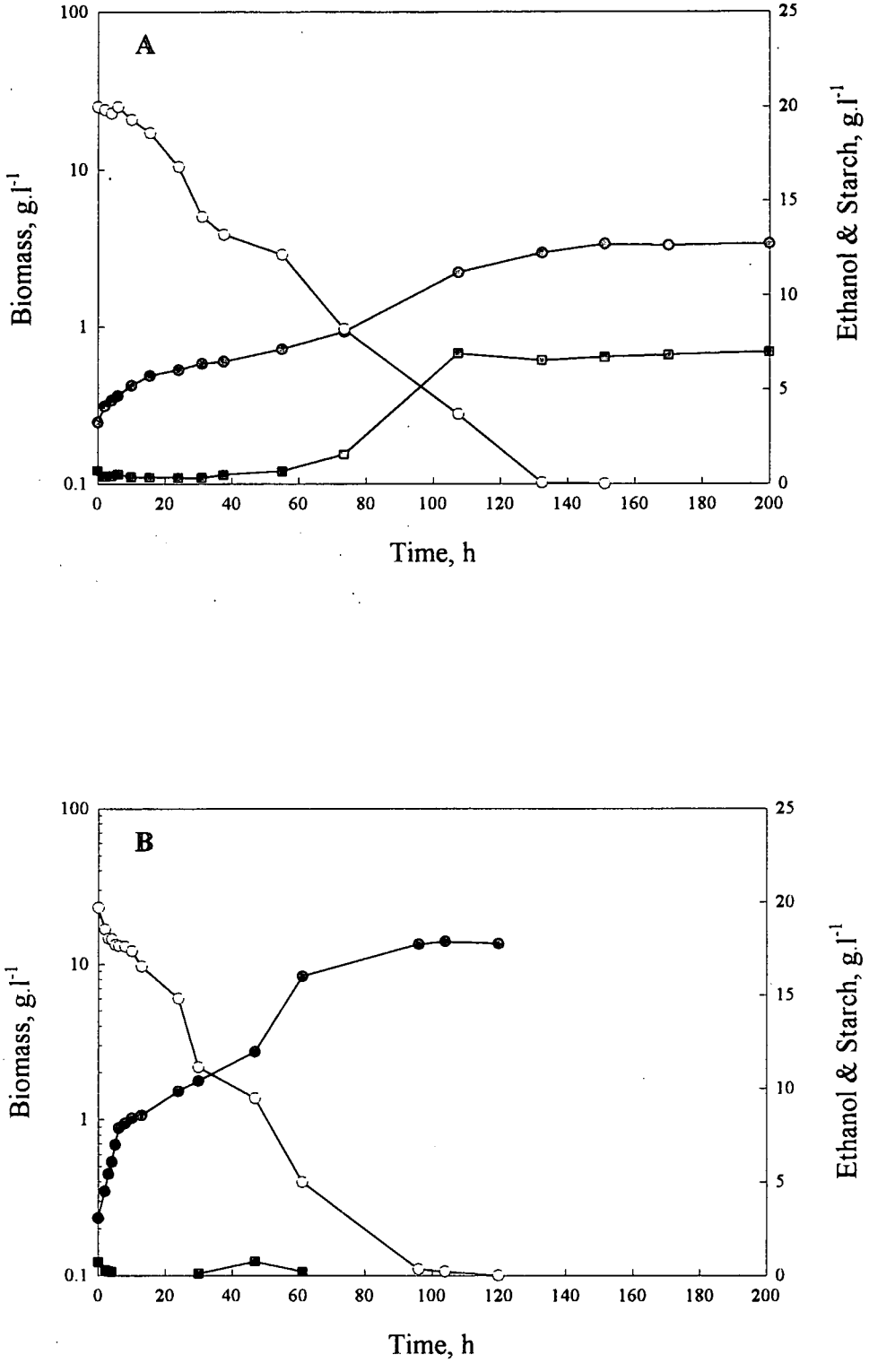



**Figure 3.** Anoxic (A) and aerobic (B) cultivation profiles of the recombinant *S. cerevisiae* strain stell8 in a 2-l bioreactor, using a yeast extract/peptone medium containing 20 g starch.$l^{-1}$. Symbols: biomass (●), ethanol (■) and starch (○).

### 3.3 15-l Bioreactor cultivation

To confirm the results obtained in the shake flask and 2-l bioreactor cultures, 15-l bioreactor cultivations were carried out. Additionally, a higher starch concentration, a more defined medium (medium B) and a pH of 5.5 were used. Problems encountered during the 2-l bioreactor cultivations, such as a limited sampling volume, which prevented frequent sampling and enzyme analysis, could be overcome by using the 15-l bioreactors, which allowed a more detailed cultivation profile. For the cultivations performed in the 14-l and 15-l stirred tank reactors, only certain strains, namely stell6, 7, 8 and *S. cerevisiae,* were evaluated, as these strains were the most promising.

*Aerobic cultivations*

Table 5 summarises the culture parameters for the various strains under aerobic conditions. The performance of strains stell6 and stell7, which both lack an α-amylase, was comparable in terms of their rates of starch hydrolysis and final glucoamylase activity (Figs. 4 and 5). The higher volumetric rates of glucoamylase production by strains stell6 and 7 allowed these strains to hydrolyse the starch in almost half the time required by stell8 and in a similar time interval as those reported by Marín *et al.* (2001). These authors reported on an industrial baker's yeast strain transformed with the *SWA2* amylase gene from *Schwanniomyces occidentalis* fused to the *ADH1* promoter. Using the latter transformant, a 95 % hydrolysis of a 2 % starch solution was obtained within 35 h under aerobic conditions. However, strains stell6 and 7 gave *ca.* 95 % hydrolysis within 30 h, suggesting that these strains were more efficient in terms of starch hydrolysis than those reported by Marín *et al.* (2001).

Although the glucoamylase activity of strain stell7 increased more rapidly than that of strain stell6 (Figs. 4 and 5), this had little effect on the overall rate and efficiency of starch hydrolysis. Strain stell7 grew notably faster than strain stell6, however. The higher glucoamylase activity values caused little significant enhancement of starch hydrolysis, although it appears that the presence of the additional *S. fibuligera* glucoamylase in strain stell7 improved the growth rate and biomass yield on starch. No

ethanol was produced under aerobic cultivations, as there was little or no glucose accumulation in the medium to cause the Crabtree effect.

A multi-phasic growth profile was obtained with strain stell8 (Fig. 6). A duplicate experiment, starting with a different colony of the same yeast transformant, yielded a similar multi-phasic growth profile. Cultivation on all media components excluding any carbon source, i.e. starch or glucose (Table 1), suggested that growth phase one indicated in Fig. 6 was due to growth on the amino acids and other medium components and not on the starch, as the maximum specific growth rates were similar. Furthermore, the starch hydrolysis curve (Fig. 6) indicated little if any starch hydrolysis during this time, substantiating this observation. The second growth phase, and most notably the slowest growth phase, extended over a period of approximately 55 h. Growth phase three was characterised by a rapid increase in biomass and amylase production, with a correspondingly rapid decrease in starch concentration. The slow starch hydrolysis during the first 60 h was curious, considering that this transformant possessed the same glucoamylase gene as strain stell6 and an additional α-amylase.

The biomass yield coefficients were lower than those obtained in the 2-l bioreactor (Tables 3 and 4) and shake flask cultures (Tables 6, Chapter 2). Strains stell6 and 7 showed an increase in specific amylase production (Table 7) when compared to the shake flask cultures (Table 6, Chapter 2), whereas strains stell8 and *S. cerevisiae* MC yielded comparable results.

**Table 5.** Aerobic cultivation parameters of selected recombinant *S. cerevisiae* strains in 15-l bioreactor cultures using medium B containing 55 g Merck starch.$l^{-1}$.

| Parameter | Recombinant *S. cerevisiae* strain | | | |
|---|---|---|---|---|
| | stell6 | stell7 | stell8 | MC |
| Time, h | 70 | 80 | 150 | 80 |
| Biomass, g.$l^{-1}$ | 19.7 | 27.97 | 16.45 | 19.24 |
| Ethanol, g.$l^{-1}$ | 0 | 0 | 0 | 0 |
| Glucose, g.$l^{-1}$ | 0 | 0 | 0 | 0 |
| E, % | 100 | 99.7 | 100 | 98 |
| Glucoamylase, U.$l^{-1}$ | 1386 | 1543 | 581 | 742 |
| Glucoamylase, U.g biomass$^{-1}$ [a] | 70.9 | 55.2 | 35.3 | 38.6 |
| α-Amylase, U.$l^{-1}$ | N.A. | N.A. | 500 | 458.4 |
| α-Amylase, U.g biomass$^{-1}$ [a] | N.A. | N.A. | 30.4 | 23.8 |
| $Y_{x/s}{}^{1}$ | 0.373 | 0.524 | 0.303 | 0.366 |
| $Y_{x/s}{}^{2}$ | 0.358 | 0.514 | 0.293 | 0.355 |
| $Y_{p/s}$ | 0 | 0 | 0 | 0 |
| $Q_p{}^{max}$, g.$l^{-1}$.$h^{-1}$ | 0 | 0 | 0 | 0 |
| $\mu_{max}$, $h^{-1}{}_1$ | N.A. | N.A. | 0.237 | 0.102 |
| $\mu_{max}$, $h^{-1}{}_2$ | 0.154 | 0.21 | 0.096 | 0.114 |
| $\mu_{max}$, $h^{-1}{}_3$ | N.A. | N.A. | 0.181 | N.A. |
| Plasmid stability, % | 98 | 99 | N.D. | 94 |

[a] Specific enzyme activity, expressed as units per gram biomass

E Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100

N.A. Not applicable

N.D. Not determined

$Q_p{}^{max}$ Maximal volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time

$Y_{x/s}{}^{1}$ Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration produced, with no correction for biomass produced from other media components

$Y_{x/s}{}^{2}$ Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the amino acid constituents of the medium

$\mu_{max\,1}$ Maximum specific growth rate on a medium excluding the starch (growth phase one)

$\mu_{max\,2}$ Maximum specific growth rate for the initial growth on starch (growth phase two)

$\mu_{max\,3}$ Maximum specific growth rate for the second growth phase on starch (growth phase three)

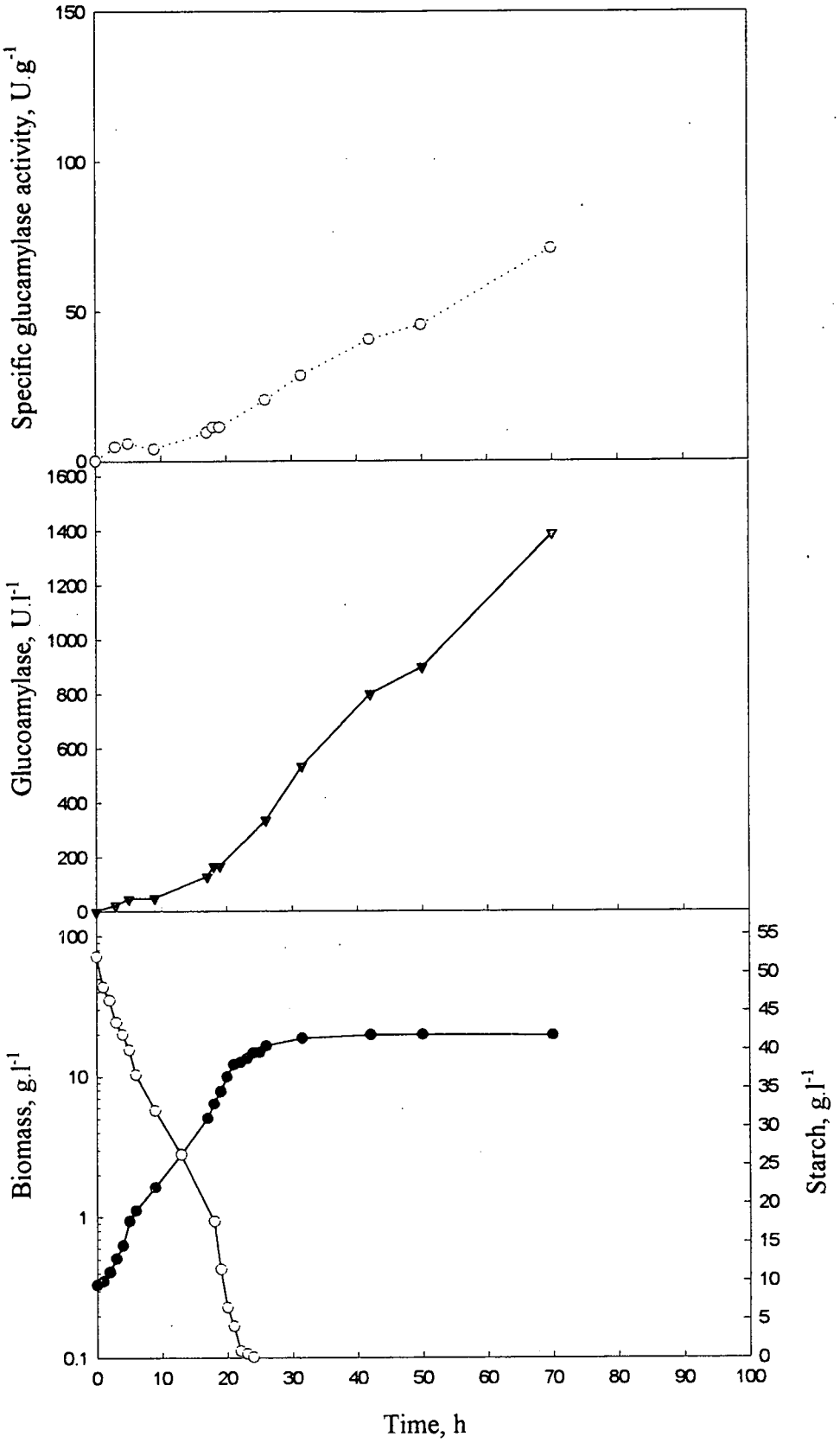



**Figure 4**. Aerobic cultivation profile of the recombinant *S. cerevisiae* strain stell6 in a 15-l bioreactor with medium B containing 55 g Merck starch.$l^{-1}$. Symbols: biomass (●), starch (○), glucoamylase (▼), specific glucoamylase activity (···○···).

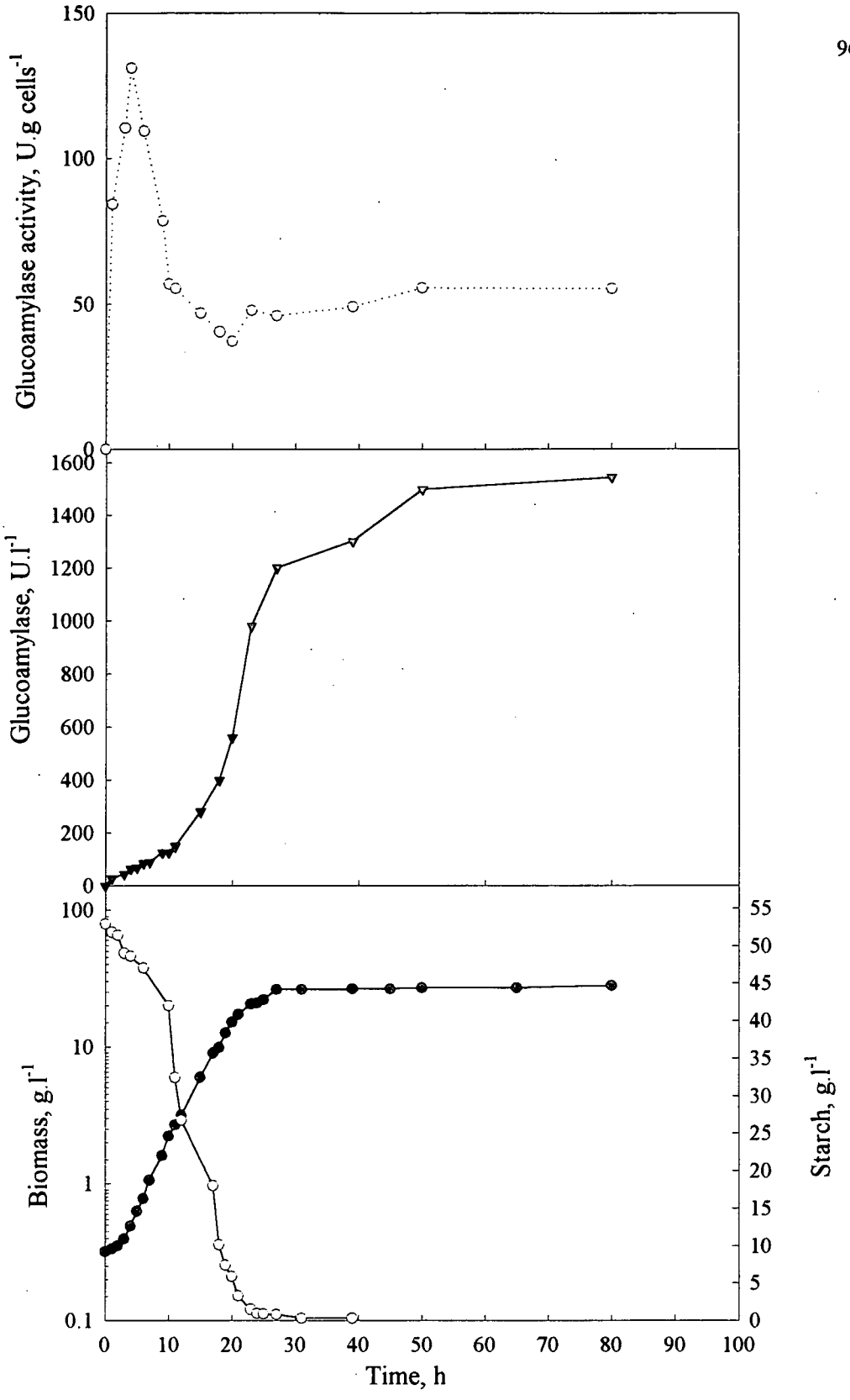



**Figure 5**. Aerobic cultivation profile of the recombinant *S. cerevisiae* strain stell7 in a 15-l bioreactor with medium B containing 55 g Merck starch.$l^{-1}$. Symbols: biomass (●), starch (○), glucoamylase (▼), specific glucoamylase activity (···○···).

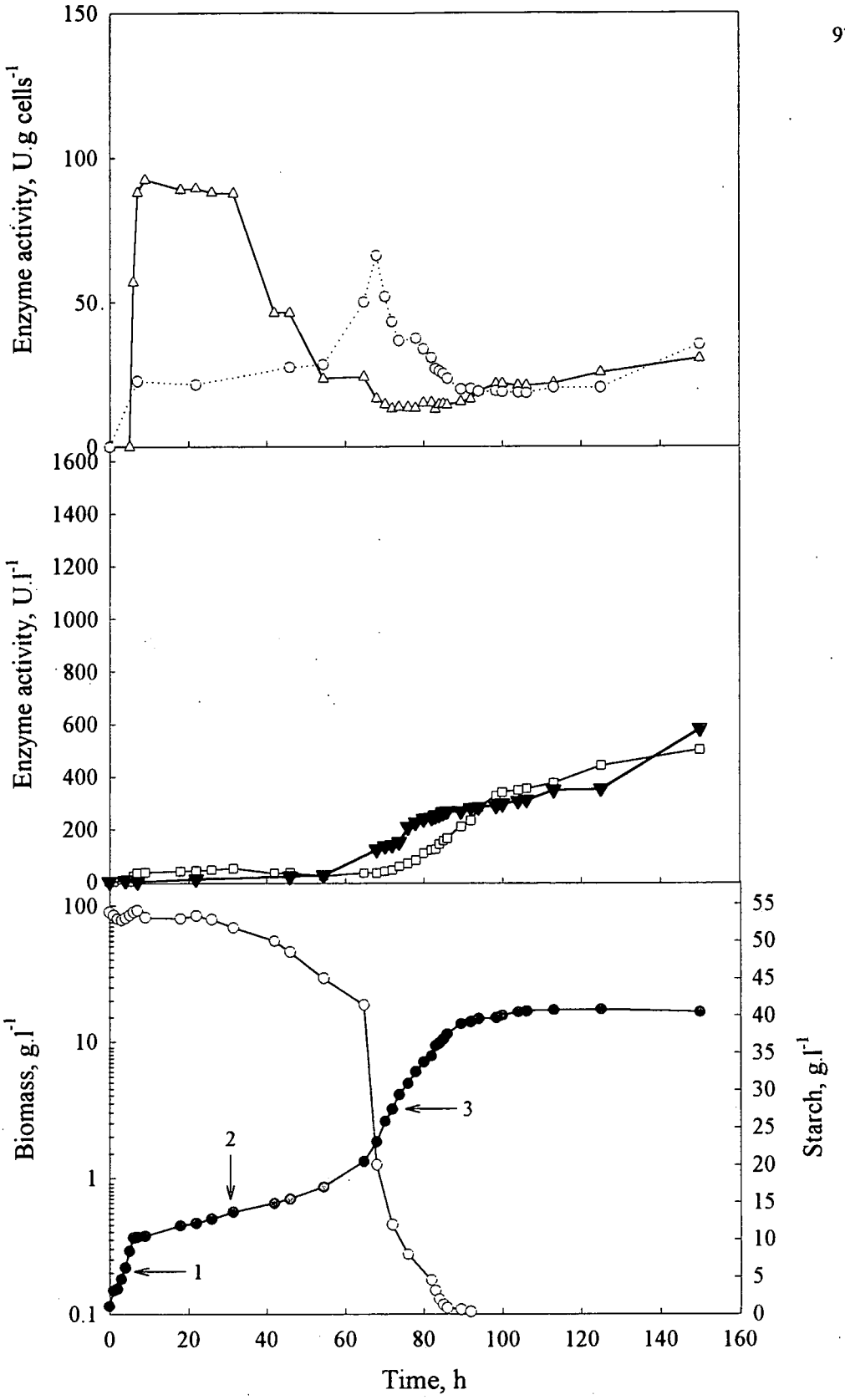



**Figure 6**. Aerobic cultivation profile of the recombinant *S. cerevisiae* strain stell8 in a 15-l bioreactor with medium B containing 55 g Merck starch.$l^{-1}$. Symbols: biomass (●), starch (○), α-amylase (□), specific α-amylase activity (△), glucoamylase (▼), specific glucoamylase activity (···○···). Numbers 1 to 3 denote the different growth phases as discussed in the text and indicated in Table 5.

*Anoxic cultivations*

Table 6 summarises the culture parameters obtained under anoxic conditions. Although strains stell6 and 7 yielded comparable biomass concentrations, the ethanol and glucoamylase levels produced by strain stell7 were markedly higher (Figs. 7 and 8). The glucoamylase activity values of strain stell8 were notably lower than those reported for strains stell6 and 7, resulting in an extended fermentation time with lower biomass and ethanol production (Fig. 9). *S. cerevisiae* strain MC yielded lower values in terms of biomass, ethanol, α-amylase and glucoamylase production in comparison to strains stell6, 7 and 8 (Table 6). With all the strains, the residual glucose in the medium was non-detectable.

In terms of the time required, starch hydrolysis by strains stell6 and 7 was *ca.* three-fold slower compared to the aerobic cultivations. This was mainly due to the lower biomass concentrations and concomitant lower volumetric enzyme activity. Although the multi-phasic growth profile of strain stell8 that was obtained was not as evident as under aerobic conditions, the first phase of growth was still characterised by a conspicuous absence of detectable amylase activity (Fig. 9). The $\mu_{max}$ values and the biomass yield coefficients of strain stell6 and 7 were similar to those reported by Birol *et al.* (1998). They reported $\mu_{max}$ values of between 0.024 and 0.035 $h^{-1}$ and biomass yield coefficients of between 0.060 and 0.121 g biomass.g substrate$^{-1}$ for a recombinant *S. cerevisiae* strain bearing a double cassette plasmid with the *Aspergillus awamori* glucoamylase and the *Bacillus subtilis* α-amylase, grown under conditions similar to those reported here.

Thus far, strains stell6 and 7, possessing the *L. spencermartinsiae* glucoamylase gene, and strain stell1, possessing the *L. spencermartinsiae* α-amylase gene, were far superior in terms of amylase production and total starch hydrolysis to the other recombinant strains evaluated here. However, despite having a combination of both the *L. spencermartinsiae* amylases, strain stell8 performed poorly. As with the aerobic cultivations, the contribution of the *S. fibuligera* glucoamylase towards total starch hydrolysis appeared minor. Horn *et al.* (1991) reported α-amylase activity values of

1680 $U.ml^{-1}$ for a *Schw. occidentalis* mutant, obtained under aerobic cultivation using a similar α-amylase assay procedure. Even under aerobic conditions, the highest α-amylase activity recorded during this investigation was 500 $U.l^{-1}$ produced by strain stell8, which is more than 3000-fold lower. These low amylase activity values resulted in an extended fermentation time, with $\mu_{max}$ values and biomass yield coefficients lower than those reported by Birol *et al.* (1998).

In marked contrast to the aerobic cultivations, the specific glucoamylase activity (on a biomass basis) produced by strain stell7 was almost double that of strain stell6 (Table 6); in the case of both strains the specific activities were much higher than under aerobic cultivations (Table 5). An investigation into plasmid stability indicated that the number of recombinant colony forming units (CFU) that grew on the *–ura3* agar plates was almost 100 %, indicating no loss of the integrating plasmids containing the amylase genes in strains stell6, 7 and 8 (Tables 5 & 6). The *S. cerevisiae* strain MC had a non-integrating plasmid in a multicopy form, which might explain the small decrease in the percentage of colony forming units (CFUs) containing the plasmid (Tables 5 & 6). This suggested that the poor amylase production by these strains was not due to a loss of the foreign α-amylase and glucoamylase genes from the host cells.

**Table 6**. Anoxic cultivation parameters of selected recombinant *S. cerevisiae* strains in 15-l bioreactor cultures using medium B containing 55 g Merck starch.$l^{-1}$.

| Parameter | Recombinant *S. cerevisiae* strain | | | |
|---|---|---|---|---|
| | Stell6 | Stell7 | Stell8 | MC |
| Time, h | 140 | 120 | 200 | 165 |
| Biomass, g.$l^{-1}$ | 4.47 | 4.15 | 3.34 | 2.43 |
| Ethanol, g.$l^{-1}$ | 16.7 | 21.2 | 17.2 | 12.9 |
| E, % | 100 | 100 | 99.9 | 97 |
| Glucoamylase, U.$l^{-1}$ | 780 | 1340 | 145 | 136 |
| Glucoamylase, U.g biomass$^{-1\,a}$ | 178.5 | 322.5 | 43.4 | 55.9 |
| α-Amylase, U.$l^{-1}$ | N.A. | N.A. | 111.4 | 41.1 |
| α-Amylase, U.g biomass$^{-1\,a}$ | N.A. | N.A. | 33.4 | 16.9 |
| $Y_{x/s}{}^{1}$ | 0.082 | 0.076 | 0.056 | 0.042 |
| $Y_{x/s}{}^{2}$ | 0.071 | 0.066 | 0.046 | 0.031 |
| $Y_{p/s}$ | 0.327 | 0.401 | 0.312 | 0.258 |
| $Q_p^{max}$, g.$l^{-1}$.$h^{-1}$ | 0.311 | 0.562 | 0.214 | 0.253 |
| $\mu_{max}$, $h^{-1}{}_1$ | N.A. | N.A. | 0.018 | 0.029 |
| $\mu_{max}$, $h^{-1}{}_2$ | 0.056 | 0.082 | 0.0184 | 0.045 |
| $\mu_{max}$, $h^{-1}{}_3$ | N.A. | N.A. | N.A. | N.A. |
| Plasmid stability, % | 98 | 98 | 99 | 93 |

| | |
|---|---|
| a | Specific enzyme activity, expressed as units per gram biomass |
| E | Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100 |
| N.A. | Not applicable |
| $Q_p^{max}$ | Maximal volumetric rate of ethanol production, calculated from the maximum slope of ethanol produced |
| $Y_{x/s}{}^{1}$ | Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration produced, with no correction for biomass produced from other media components. |
| $Y_{x/s}{}^{2}$ | Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the amino acid constituents of the medium |
| $\mu_{max}$, $h^{-1}{}_1$ | Maximum specific growth rate on a medium excluding the starch (growth phase one) |
| $\mu_{max}$, $h^{-1}{}_2$ | Maximum specific growth rate for the initial growth on starch (growth phase two) |
| $\mu_{max}$, $h^{-1}{}_3$ | Maximum specific growth rate for the second growth phase on starch (growth phase three) |

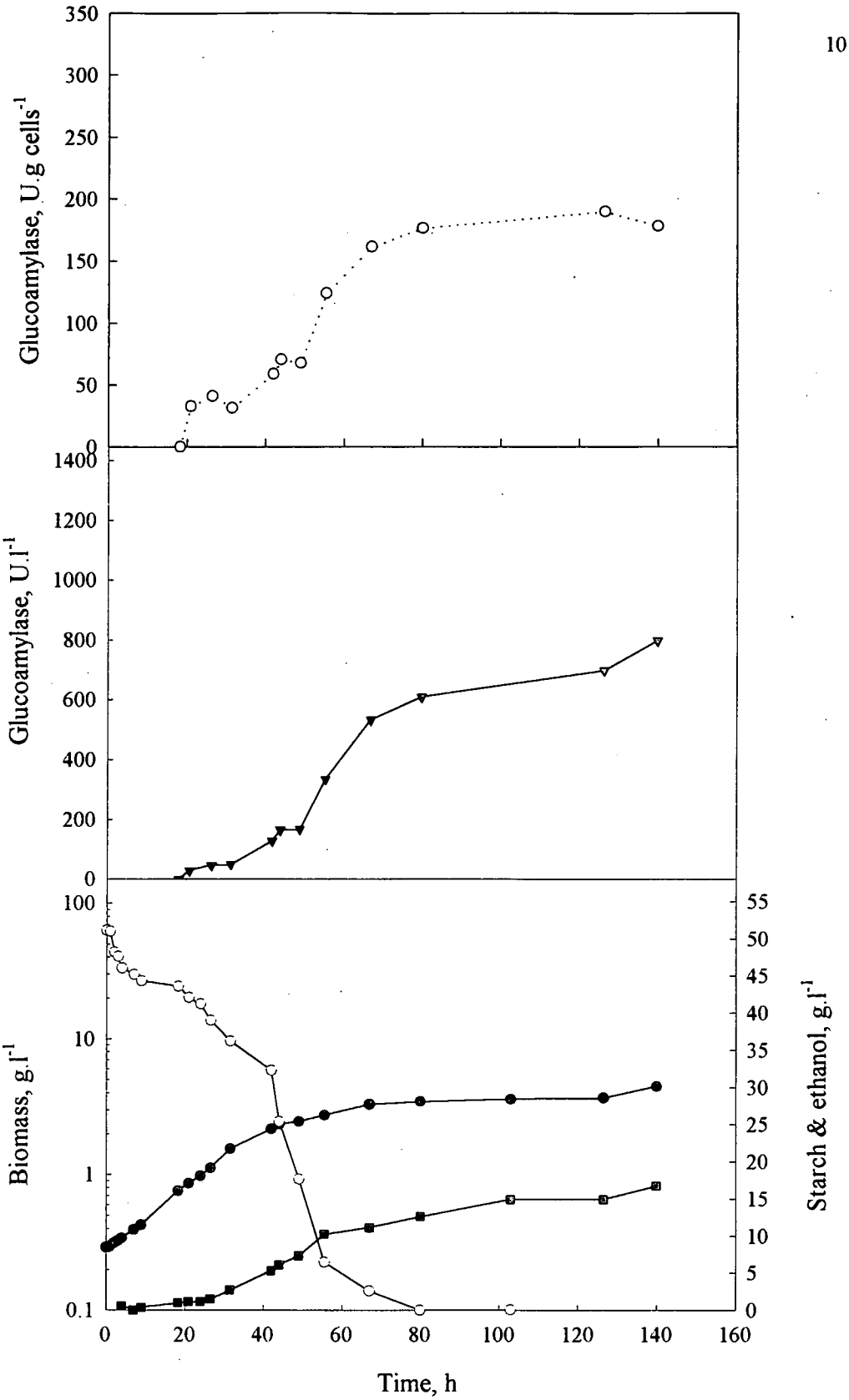



**Figure 7.** Anoxic cultivation profile of the recombinant *S. cerevisiae* strain stell6 in a 15-l bioreactor with medium B containing 55 g Merck starch.$l^{-1}$. Symbols: biomass (●), ethanol (■), starch (○), glucoamylase (▼), specific glucoamylase activity (···○···).

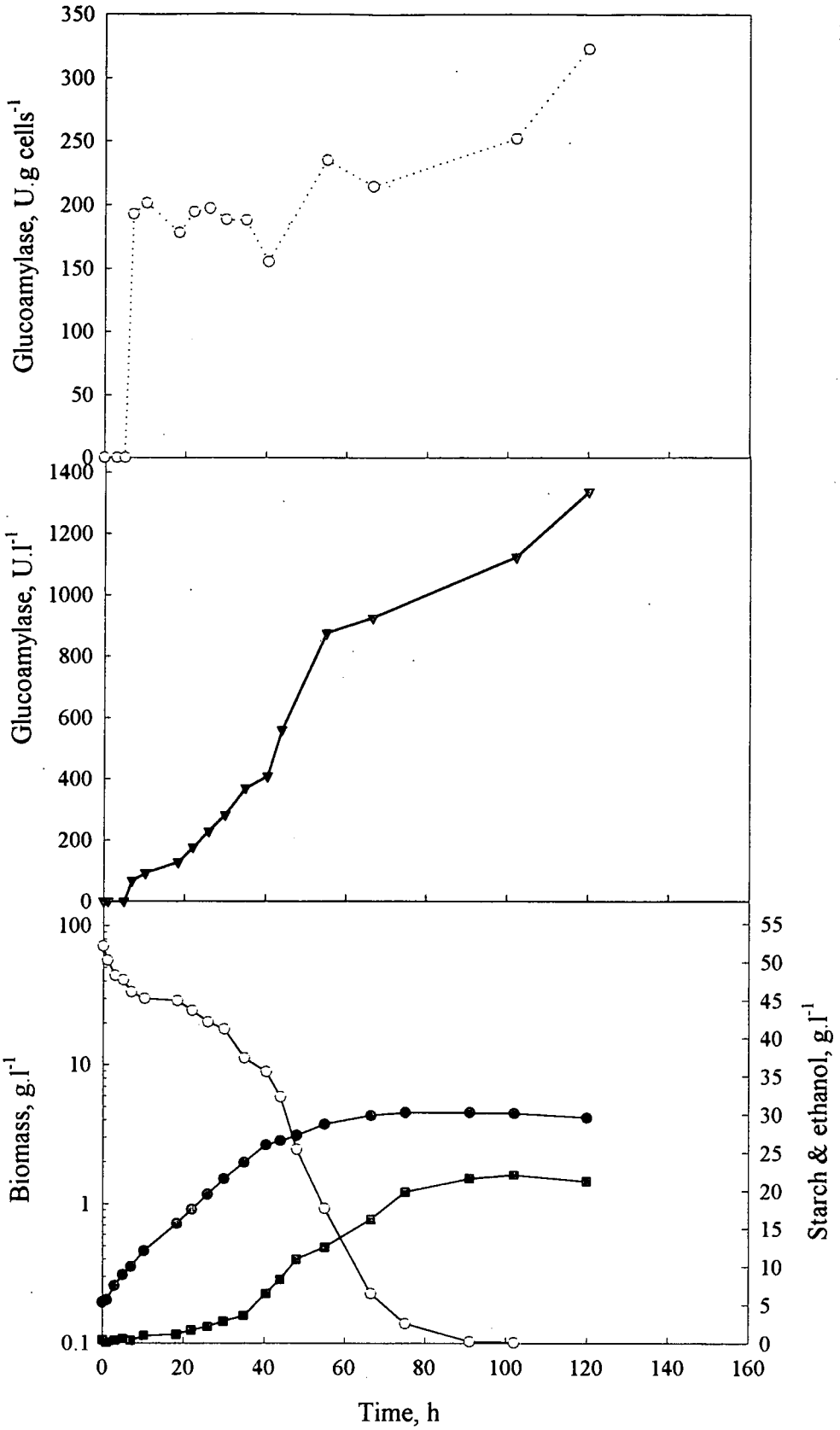



**Figure 8**. Anoxic cultivation profile of the recombinant *S. cerevisiae* strain stell7 in a 15-l bioreactor with medium B containing 55 g Merck starch.$l^{-1}$. Symbols: biomass (●), ethanol (■), starch (○), glucoamylase (▼), specific glucoamylase activity (···○···).

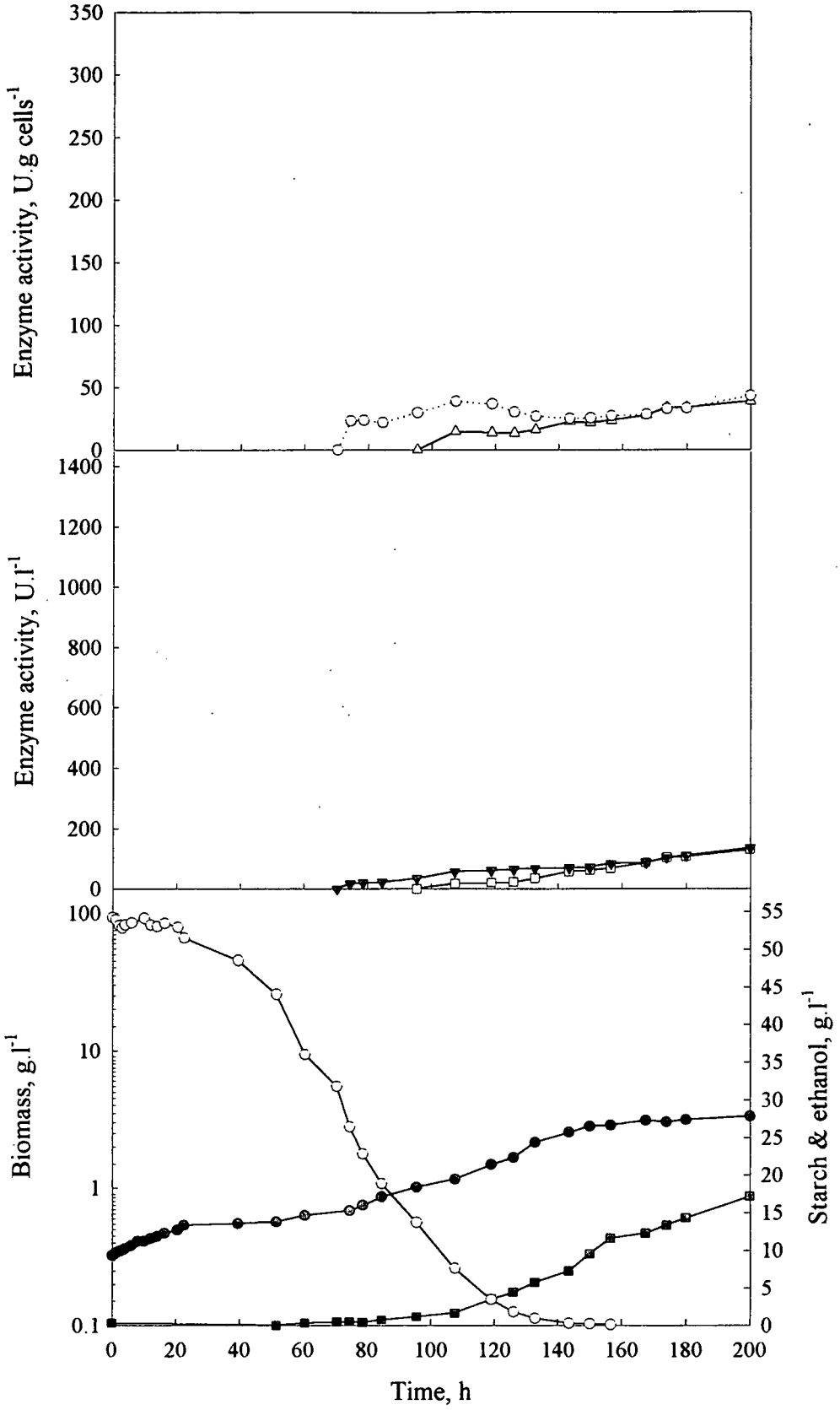



**Figure 9**. Anoxic cultivation profile of the recombinant *S. cerevisiae* strain stell8 in a 15-l bioreactor with medium B containing 55 g Merck starch.$l^{-1}$. Symbols: biomass (●), ethanol (■), starch (○), α-amylase (□), specific α-amylase activity (△), glucoamylase (▼), specific glucoamylase activity (···○···).

### 3.4 Efficiency of fermentation

Despite almost complete starch hydrolysis by most strains, the time required for the completion of hydrolysis was long. The suitability of these recombinant strains for the industrial fermentation of starch to ethanol would be facilitated by a direct comparison with fermentations on glucose. The parameters obtained in the 15-l bioreactor cultures using medium A and B, with glucose as carbon source, are summarised in Table 7.

As expected, the maximum specific growth rates and biomass yields on glucose were much higher than those obtained on starch (Tables 3 and 7). However, the ethanol yield coefficients and the maximal volumetric rate of ethanol production obtained on the starch and glucose were comparable, suggesting that these recombinant strains were not limited in terms of ethanol fermentation. The rate-limiting step in terms of starch fermentation was the conversion of the starch to fermentable sugars, which was determined by the rate and quantity of amylase production.

**Table 7.** Cultivation parameters of the recombinant *Saccharomyces cerevisiae* strain stell8 in anoxic 15-l bioreactor cultures using two different media.

| Parameter | Medium | | |
|---|---|---|---|
| | Medium B<br>55 g glucose.$l^{-1}$ | Medium A<br>55 g glucose.$l^{-1}$ | Medium A<br>20 g glucose.$l^{-1}$ |
| Time, h | 29 | 30 | 25 |
| Biomass, g.$l^{-1}$ | 5.15 | 6.75 | 2.25 |
| Ethanol, g.$l^{-1}$ | 17.80 | 17.52 | 7.2 |
| pH | 5.52 | 5.51 | 5.50 |
| Glucose, g.$l^{-1}$ | 0 | 0 | 0 |
| $Y_{x/s}$ | 0.094 | 0.116* | 0.085* |
| $Y_{p/s}$ | 0.326 | 0.350 | 0.340 |
| $Q_p^{max}$ g.$l^{-1}$.$h^{-1}$ | 0.543 | 0.495 | 0.375 |
| $\mu_{max}$, $h^{-1}$ | 0.440 | 0.391 | 0.235 |

| | |
|---|---|
| $Y_{x/s}$ | Cell yield coefficient, g dry biomass/g glucose utilised, calculated from the final biomass concentration produced |
| $Y_{p/s}$ | Ethanol yield coefficient, g ethanol/g glucose utilised, calculated from the final ethanol concentration produced |
| $Q_p^{max}$ | Maximal volumetric rate of ethanol production, calculated from the maximum slope of ethanol produced. |
| * | Corrected for an average biomass production of 0.55 g dry biomass/g glucose utilised |
| $\mu_{max}$ | Maximum specific growth rate |

## 4 References

**Birol, G., Önsan, Z.İ., Kirdar, B. & Oliver, S.G. (1998).** Ethanol production and fermentation characteristics of recombinant *Saccharomyces cerevisiae* strains grown on starch. *Enzyme Microbiol Technol,* **22,** 672-677.

**Brown, D.E. (1979).** Technology of microbial polysaccharase production. In *Microbial polysaccharides and polysaccharases.* pp. 297-326. Edited by R.C.W. Berkeley, G.W. Gooday & D. C. Ellwood. New York: Academic Press Inc.

**Calleja, G.B., Levy-Rick, S.R., Nasim, A. & Lusena, C.V. (1987).** Extracellular amylases of starch-fermenting yeast: pH effect on export and residence time in the periplasm. *Crit Rev Biotechnol,* **5,** 177-184.

**De Kock, S.H., Du Preez, J.C. & Kilian, S.G. (2000).** Anomalies in the growth kinetics of *Saccharomyces cerevisiae* strains in aerobic chemostat cultures. *J Ind Microbiol,* **24,** 231-236.

**De Mot, R. (1990).** Conversion of starch by yeasts. In *Yeast, Biotechnology and Biocatalysis,* pp. 163-222. Edited by H. Verachtert & R. De Mot. New York & Basel: Marcel Dekker Inc.

**Drozdowixz, Y.M. & Jones, R.L. (1995).** Hormonal regulation of organic and phosphoric acid release by barley aleurone layers and scutella. *Plant Physiol,* **108,** 769-776.

**Du Preez, J.C. & Van der Walt, J.P. (1983).** Fermentation of D-xylose to ethanol by a strain of *Candida shehatae*. *Biotechnol Lett,* **5,** 357-362.

**Fiechter, A., Fuhrmann, G.F. & Käppeli, O. (1981).** Regulation of glucose metabolism in growing yeast cells. *Adv Microbiol Physiol,* **22,** 123-183.

**Hadfield, C., Raina, K.K., Shashi-Meno, K. & Mount, R.C. (1993).** The expression and performance of cloned genes in yeasts. *Mycol Res,* **97,** 897-944.

**Horn, C.H., Du Preez, J.C. & Kilian, S.G. (1991).** Selective isolation of amylase derepressed mutants of *Schwanniomyces occidentalis*. *System Appl Microbiol,* **14,** 299-304.

**Marín, D., Jiménez, A. & Lobato, M.F. (2001).** Construction of an efficient amylolytic industrial yeast strain containing DNA exclusively derived from yeast. *FEMS Microbiol Lett,* **201,** 249-253.

**Randez-Gill, F. & Sanz, P. (1993).** Expression of *Aspergillus oryzae* α-amylase gene in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett,* **112,** 119-124.

**Rieger, M., Käppeli, O. & Fiechter, A. (1983).** The role of limited respiration in the incomplete oxidation of glucose by *Saccharomyces cerevisiae*. *J Gen Microbiol,* **129,** 653-661.

**Ruohonen, L., Penttilä, M. & Keränen, S. (1991).** Optimisation of *Bacillus* α-amylase production by *Saccharomyces cerevisiae*. *Yeast,* **7,** 337-346.

**Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989).** Preparation of reagents and buffers used in molecular cloning. In *Molecular cloning: A laboratory manual* 2nd Ed. pp. B.12. New York: Cold Spring Harbor Laboratory Press.

**Steyn, A.J.C. & Pretorius, I.S. (1995).** Characterisation of a novel α-amylase from *Lipomyces kononenkoae* and expression of its gene (LKA1) in *Saccharomyces cerevisiae*. *Curr Genet,* **28,** 526-533.

**Thomas, K.C., Hynes, S.H. & Ingledew, W.M. (1996).** Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Process Biochem,* **13,** 321-331.

**Walker, G.M. (1998).** Yeast growth and yeast metabolism. In *Yeast physiology and biotechnology*, pp. 101-255. New York: John Wiley & Sons, Inc.

Chapter 4

# INVESTIGATION INTO FACTORS LIMITING AMYLASE PRODUCTION BY SELECTED RECOMBINANT *SACCHAROMYCES CEREVISIAE* STRAINS

**Abstract**

The slow rate of starch hydrolysis, due to limited amylase production, was the rate-limiting step in the saccharification and fermentation of starch by the recombinant strains stell2 and stell8. Data suggested that the low activity of the extracellular amylases was not due to intracellular enzyme accumulation, nor due to extracellular protease activity. Neither cultivation at two different pH values nor the addition of a surfactant improved amylase production. Delayed amylase production resulted in a multi-phasic growth profile of one of the strains, strain stell8. Northern blot analysis of the LKAII glucoamylase mRNA did not clearly indicate that the addition of glucose induced the *PGK1* promoter, but suggested that a low level of transcription resulted in the low extracellular amylase activity values.

## 1 Introduction

Poor amylase production by these recombinant strains of *Saccharomyces cerevisiae* resulted in a slow rate of starch hydrolysis concomitant with a slow growth rate and an extended fermentation time (Chapters 2 and 3). Since no detectable levels of α-amylase activity were obtained with strain stell2, the possibility of proteolytic activity was considered.

Proteases, also known as peptidases, are enzymes associated with protein digestion whose catalytic activity on the peptide bonds results in the formation of peptides and amino acids (North, 1982). They occupy an important role with respect to their application in the industrial and physiological areas (Neurath, 1989). Previously, proteases were classified according to molecular size, charge or substrate specificity (Neurath, 1989). Classification is now based on a comparison of the chemical nature of the active sites, the mechanism of action or the type of reaction catalysed and their three-dimensional structure (Neurath, 1989; Rao *et al.*, 1998). Proteases can be broadly classified into exopeptidases and endopeptidases (Neurath, 1989). In terms of their actions, proteases can be subdivided into limited and unlimited proteases. As the name suggests, the limited proteases cleave specific peptide bonds and play a pivotal role in

the formation of a target protein (Neurath, 1989). Unlimited proteases, on the other hand, degrade proteins into their amino acid constituents. To date, six families of proteases are recognised (Table 1). These families can further be classified according to their physical *in vitro* properties, such as pH activity range (North, 1982).

Gimenez *et al.* (1999) reported that *Saccharomyces cerevisiae* produced a number of vacuolar proteases, including serine, aspartyl and metallo-proteases, resulting in the degradation of the enzymes when the cells were disrupted. Additionally, Aunstrup (1978) reported that the synthesis of yeast protease in *Candida lipolytica* was stimulated by a poor carbon source inducing weak catabolite repression. The obvious solution would be to introduce protease inhibitors. Protease inhibitors can be subdivided into two distinct groups: site-specific inhibitors, which usually cause an irreversible modification of the amino acid sequence of the active site, and non-specific inhibitors, many of which occur naturally (Neurath, 1989). However, the various protease cocktails that are available commercially may be toxic to the yeast cells (Gimenez *et al.*, 1999). Additionally, the short half-life of most protease inhibitors may result in residual proteolytic activity in the latent state, which reappears later during purification (Gimenez *et al.*, 1999).

**Table 1.** Classification of the families of proteolytic enzymes

| Family | Protease |
|---|---|
| *Endopeptidase* | |
| Serine protease I | Chymotrypsin |
| Cysteine proteases | Papain |
| Metallo-proteases II | Thermolysin |
| Aspartic proteases | Penicillopepsin |
| *Exopeptidase* | |
| Serine protease II | Subtilisin |
| Metallo-proteases I | Bovine carboxypeptidase A |

Adapted from Neurath (1989).

Alternatively, the non-detectable α-amylase activity in the recombinant strains investigated here could be the result of phase separation, which occurs when water-soluble polymers and a salt solution are mixed (Albertsson, 1986). In such a system, the macromolecules are partitioned to one side, with smaller molecules being distributed between the phases. This can cause the cells to be completely separated into one phase, usually the bottom phase, with the amylases being evenly distributed or separated into the top phase, thus reducing their effectiveness (Ramgren *et al.*, 1988). Surfactants such as Trixon X-100, Tween 80, CTAB (cetylammoniumbromide) and SDS (sodiumdodecylsulphate) have been used to reduce the surface tension in media and improved enzyme production (Ramgren *et al.*, 1988). The stimulatory effect of surfactants on the enzyme production by mesophilic microorganisms is well documented, although the mode of action has not been determined (Thies *et al.*, 1994). Similarly, Arnesen *et al.* (1998) reported increased α-amylase production by *Thermomcyes lanuginosus* when supplemented with Tween 80, while Kim *et al.* (1998) observed a two-fold increase in *Thermomonospora fusca* endoglucanase production by the recombinant *Streptomyces lividans* strain with the addition of Tween 80.

Another important factor that influences the gene expression is the additional stress placed on the cells due to the synthesis of the heterologous protein. Gene expression in yeasts is most frequently regulated at the level of transcription via promoter control (Romanos *et al.*, 1992; Hadfield *et al.*, 1993). One of the most extensively used promoters in foreign gene expression is the *PGK1* promoter (Hadfield *et al.*, 1993). Marquet *et al.* (1987) found that the action of the *PGK1* promoter could be regulated by the addition of glucose. Activation of the *PGK1* promoter by glucose, causing a sudden increase in enzyme activity, i.e. the induction of this promoter, is a concept supported by a number of authors (Sakai *et al.*, 1992; Hadfield *et al.*, 1993) and opposed by others (Park *et al.*, 1993; Shiba *et al.*, 1994). The aerobic growth of strain stell8 exhibited a curious multi-phasic profile (Chapter 3). One hypothesis to account for this observation is that the *PGK1* promoter is induced by glucose, which will explain the time delay observed for amylase production by this strain. Intracellular accumulation due to poor secretion as a result of glycosylation is another plausible explanation for the low extracellular enzyme activity.

To investigate the cause of the low α-amylase activity of these recombinant strains, strain stell2, containing the α-amylase and glucoamylase genes from *Saccharomycopsis fibuligera* (with their natural promoters), was selected, as it exhibited no detectable α-amylase activity. Other possibilities, namely degradation of the enzyme by proteolytic activity, intracellular enzyme accumulation, phase separation of the substrate and enzyme due to surface tension and a lack of enzyme production resulting from constraints on a molecular level, were also investigated.

## 2 Materials and methods

### 2.1 Yeast strains

Strain stell2, transformed with both the α-amylase and the glucoamylase genes from *Saccharomycopsis fibuligera* (with natural promoters), and strain stell8, with *Lipomyces spencermartinsiae* α-amylase and glucoamylase (under control of the *PGK1* promoter), were kindly provided by the Institute for Wine Biotechnology at the University of Stellenbosch, Stellenbosch, South Africa. Strains stell2 and 8 were maintained as described under materials and methods in Chapter 2, Section 2.2.

### 2.2 Cultivation

*2-l Multigen bioreactor*

Batch cultivations were carried out in 2-litre stirred tank reactors (Multigen F-2000; New Brunswick Scientific, Edison, NJ, USA), using the same medium composition (medium A) and cultivation conditions as described in Chapter 3, Section 2.2.

*15-l Bioreactor cultivation*

Aerobic cultivations were performed in a 15-l Biostat C bioreactor (B. Braun Biotech International, Melsungen, Germany), as described in Chapter 3, Section 2.2. Cultivation conditions and medium composition remained the same (medium B), except for the pulse addition of 3 g glucose.$l^{-1}$, as indicated. Samples taken between 20 h and 30 h were dialised (see section 2.3 below) to prevent glucose interference with the glucoamylase assay.

### 2.3 Analyses

The biomass and ethanol concentrations, as well as the extracellular amylase activities of each sample, were analysed as described in Chapter 2, Section 2.5. The starch concentrations of the samples were determined using the iodine staining method of

Randez-Gill and Sanz (1993), as noted in Chapter 3, Section 2.3. Statistical analyses were done as described in Chapter 3, Section 2.3. Dialysis was done using cellulose dialysis tubing (Sigma) and a 0.5 M phosphate buffer utilising three volume changes.

### 2.4 Northern hybridisation

The total RNA was isolated according to the method of Köhrer and Domdey (1990), electrophoresed in formaldehyde agarose gels and blotted onto Magnacharge nylon membranes (MSI, Micron, Separations Inc., Westborough, MA, USA). The bound RNA was hybridised to the $^{32}$P-labelled probes used by La Grange *et al.* (1996) (kindly supplied by the University of Stellenbosch) and to $^{32}$P-labelled *ACT1* (used as internal standard), using the High Prime labelling kit of Boehringer (Boehringer Mannheim GmbH, Mannhein, Germany) according to the instructions of the manufacturer.

### 2.5 Intracellular enzyme activity

Cells from 10 ml samples were harvested by centrifugation and the supernatant was used for the determination of extracellular enzymatic activity. The pellet was washed twice with sterile physiological saline solution. After the addition of 1 ml 20 mM imidazol buffer (pH 7) and an equal amount of glass beads (425 to 600 µm diameter; Sigma, St. Louis, MO, USA), the cells were vortexed for one min, followed by cooling on ice for a further minute. The glass beads were pre-washed in 5 N HCl, followed by washing with distilled water until the pH was neutralised. This vortexing process was repeated five to eight times or until the cells were disrupted, as verified by microscopy. This was followed by centrifugation for 5 min to remove the cell debris and the glass beads. This supernatant was evaluated for intracellular enzyme activity.

### 2.6 Extracellular protease activity

Extracellular proteolytic activity was evaluated according to Ferreira *et al.* (1999). Using 1 % casein in 0.1 M sodium phosphate buffer, pH 7.0, as substrate, 1 ml of the substrate was incubated at 40 °C for 1 hour with 1 ml of appropriately diluted culture supernatant. The reaction was terminated by the addition of 2 ml of 0.3 M

trichloroacetic acid. After mixing on a vortex mixer, the tubes were centrifuged at 3000 $r.min^{-1}$ for 10 min. The released tyrosine was estimated using the micro BCA™ protein assay reagent kit (Pierce, Separations Scientific), with tyrosine as a standard.

## 3 Results and discussion

### 3.1 Factors that may affect the extracellular amylase activity of strain stell2

Figure 1 profiles the cultivation of strain stell2 in a 2-l bioreactor with and without Tween 80 supplementation. The culture profiles were similar, with no ethanol produced due to the aerobic conditions (Table 1). Despite almost complete starch hydrolysis (*ca.* 90 %) within 40 hours, α-amylase values remained below or close to 30 $U.l^{-1}$, the lower limit of the Phadebas assay method. Although the specific amylase activity values in both cultures increased slightly during the first 20 h, the volumetric α-amylase and glucoamylase activities increased very slowly. Statistical analysis of the final glucoamylase activity values, using the unpaired t test, showed that the results obtained with and without Tween 80 supplementation were not significant ($P > 0.05$).

Assays at regular intervals during aerobic and anoxic cultivations of strain stell2 on 20 g $starch.l^{-1}$ revealed no proteolytic activity. Although *S. cerevisiae* has been reported to produce proteolytic enzymes (Gimenez *et al.*, 1999), this broad range assay did not indicate any detectable proteolytic activity. The analysis for intracellular amylase accumulation also resulted in non-detectable amylase values. These results showed that the amylase was not being degraded by extracellular proteolytic activity and was not accumulating intracellularly, nor did a reduction in surface tension increase amylase activity.

### 3.2 Promoter influence on amylase production by strain stell8

Aerobic cultivation of strain stell8 showed a curious multi-phasic growth profile, accompanied by a delayed production of detectable α-amylase activity (Chapter 3). This sudden increase in amylase production, resulting in an increased rate of starch

hydrolysis with concomitant increased growth rate, suggested that gene regulation might be implicated. As stated earlier, strain stell8 had the α-amylase and the glucoamylase from *L. spencermartinsiae* under the control of the *PGK1* promoter and terminator. As discussed above, a contradiction exists in the literature regarding the induction of the *PGK1* promoter.

**Table 1.** Aerobic cultivation parameters of the recombinant *Saccharomyces cerevisiae* strain stell2 in 2-l bioreactor cultures using medium A containing 20 $g.l^{-1}$ Merck starch. Data indicates the mean values of duplicate experiments obtained after 200 h.

| Parameter | No Tween 80 | Tween 80 |
|---|---|---|
| Biomass, $g.l^{-1}$ | 12.95 | 12.44 |
| Ethanol, $g.l^{-1}$ | 0 | 0 |
| Glucose, $g.l^{-1}$ | 0 | 0 |
| E, % | 95 | 95.4 |
| Glucoamylase, $U.l^{-1}$ | 67 | 79 |
| Glucoamylase, U.g biomass$^{-1\,a}$ | 5.2 | 6.4 |
| α-Amylase, $U.l^{-1}$ | 0 | 0 |
| α-Amylase, U.g biomass$^{-1\,a}$ | 0 | 0 |
| $Y_{x/s}$ | 0.581 | 0.596 |
| $Y_{p/s}$ | 0 | 0 |
| $Q_p^{max}$, $g.l^{-1}.h^{-1}$ | 0 | 0 |
| $\mu_{max}$, $h^{-1}$ | 0.211 | 0.203 |

[a] Specific enzyme activity, expressed as units per gram biomass

E Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100

$Q_p^{max}$ Maximal volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g glucose equivalents utilised, calculated from the final ethanol concentration produced

$Y_{x/s}$[2] Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the amino acid components of the medium

$\mu_{max}$ Maximum specific growth rate

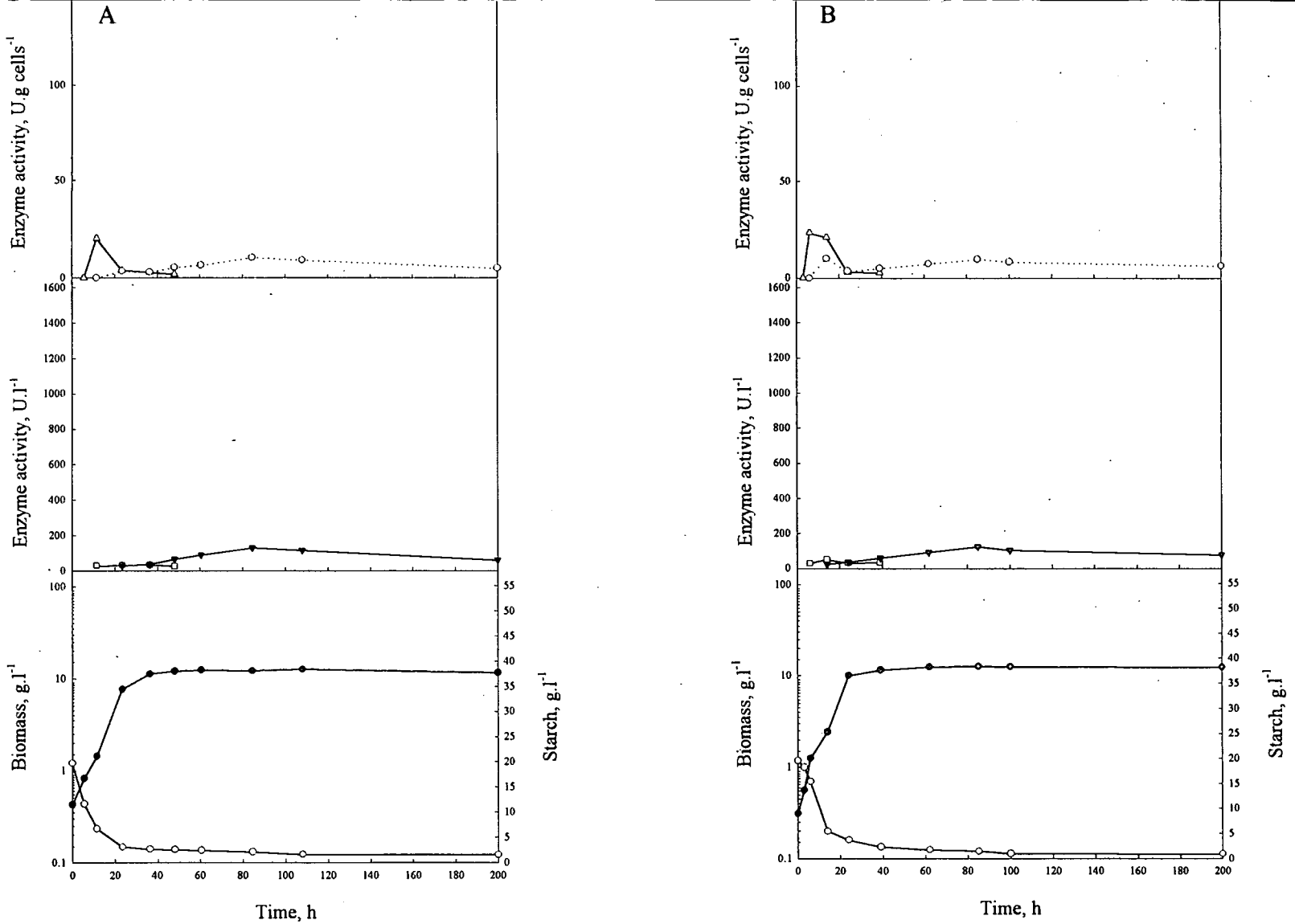



**Figure 1.** Aerobic cultivation profile of the recombinant *S. cerevisiae* strain stell2 in 2-l bioreactors (A), and with Tween 80 supplementation (B). Symbols: biomass (●), starch (○), α-amylase (□), specific α-amylase activity (△), glucoamylase (▽), specific glucoamylase activity (···○···).

To investigate this hypothesis, strain stell8 was again subjected to aerobic cultivation in a 15-l bioreactor as described above, with a pulse addition of 3 g glucose.$l^{-1}$ at 20 hours. The cultivation of strain stell8 with the glucose spike is profiled in Fig 3 and the cultivation parameters are summarised in Table 2. The aerobic cultivation data of strain stell8 (Chapter 3) was included in Table 2 for the purposes of comparison. The increase in biomass due to growth on glucose resulted in an increase in amylase activity and thus shifted the growth phase on starch forward by *ca.* 20 h, resulting in a reduction in the total fermentation time. The glucose pulse resulted in a sharp increase in the glucoamylase profile, followed by a curious decrease in activity, resulting in low final values. Figure 2 shows that, as with the other aerobic cultivations, there was an initial growth phase (growth phase one) on the non-starch medium components (as discussed in Chapter 3), followed by a short period of very slow growth (growth phase two). A high growth rate was noted as a result of the growth on glucose and subsequent slower growth on the starch.

One of the main elements influencing the degree of gene expression is the level of transcription initiated by the promoter (Hadfield *et al.*, 1993). The increased glucoamylase activity values obtained after glucose addition indicated induction of the *PGK1* promoter. Selected samples were dialysed to confirm that no residual glucose interfered with the glucose produced by the action of glucoamylase on the assay substrate. Investigations by Northern blot analysis of the LKAII glucoamylase mRNA of strain stell8 showed constant, but very low, levels of mRNA, suggesting no induction of the *PGK1* promoter (Figure 3). However, one point to be noted here is the very low levels of glucoamylase activity. The values reported here were expressed in $U.l^{-1}$, as they were too low to be expressed in $U.ml^{-1}$. Despite no clear indication via Northern blot analysis, the glucoamylase activity values increased, indicating that the additional glucose did improve the activity values. These low mRNA levels of glucoamylase may indicate a problem with enzyme production on a molecular level or other associated problems, which will require further investigation.

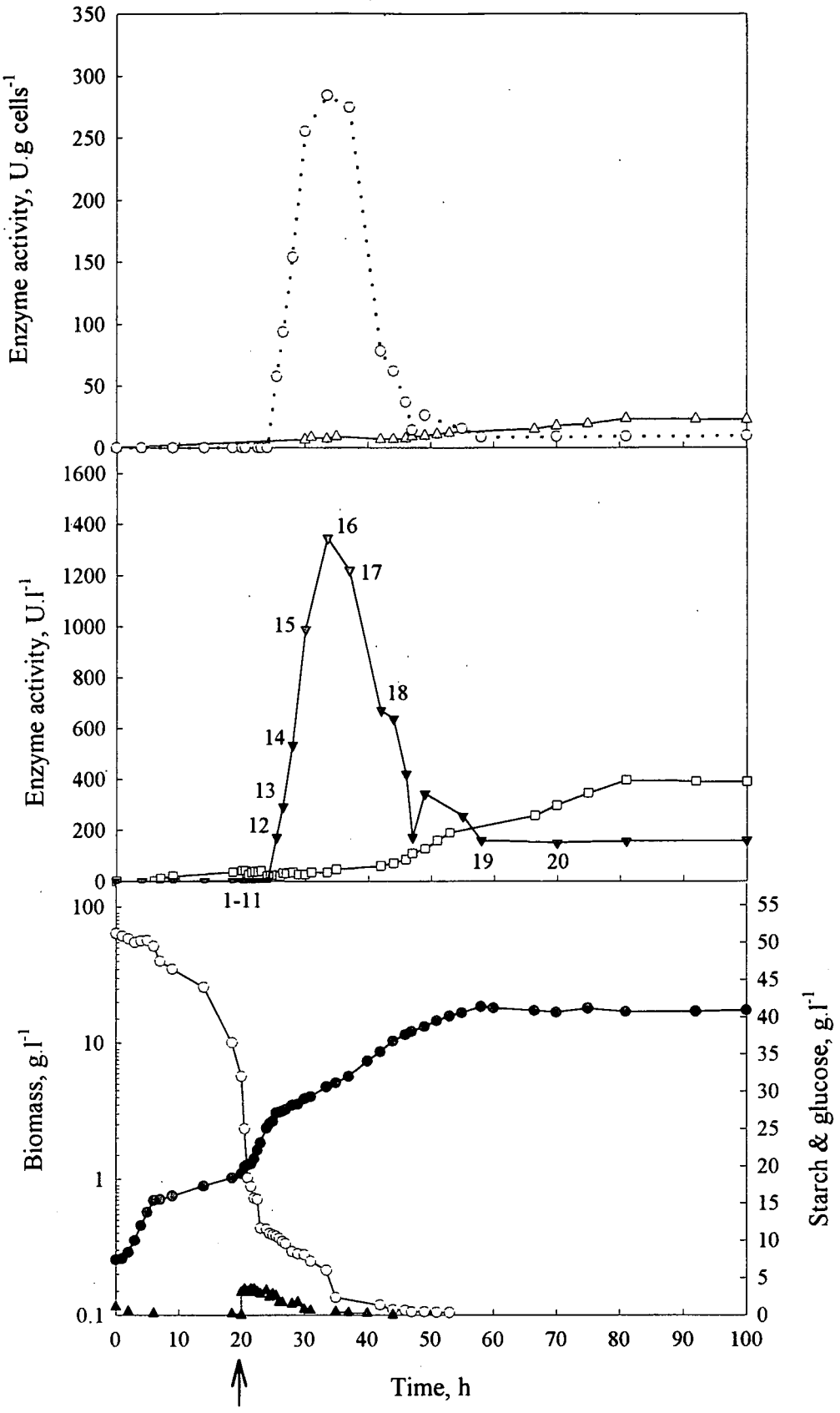
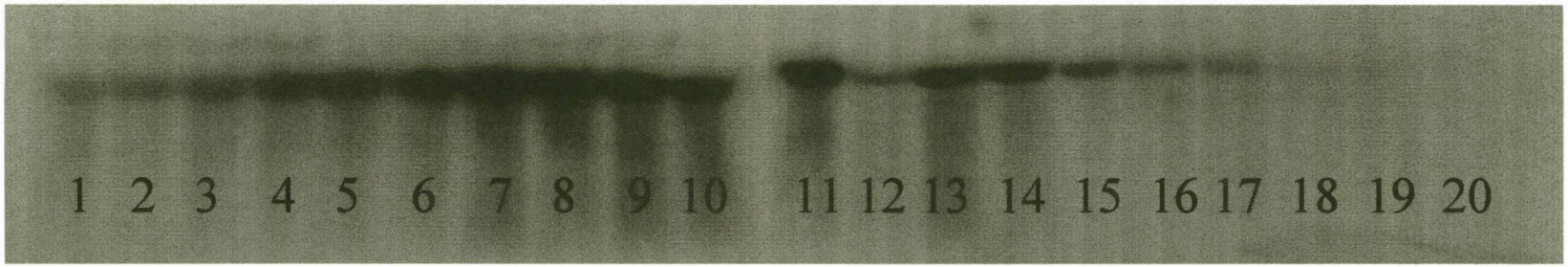



**Figure 2.** Aerobic cultivation profile of the recombinant *S. cerevisiae* strain stell8 in a 15-l bioreactor with medium B containing 55 g Merck starch.$l^{-1}$. A glucose spike (3 g.$l^{-1}$) was introduced at 20 hours, as indicated by the arrow. Symbols: biomass (●), starch (○), α-amylase (□), specific α-amylase activity (△), glucoamylase (▼), specific glucoamylase activity (···○···).

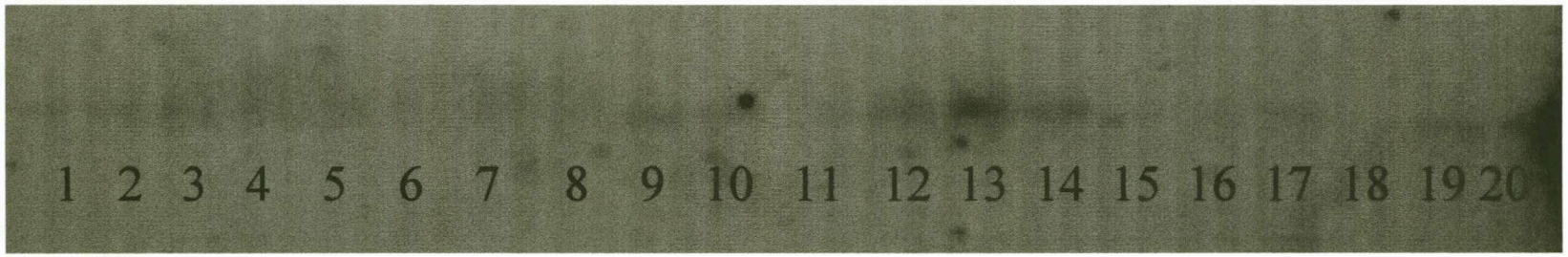
**Table 2.** Aerobic cultivation parameters of the recombinant *Saccharomyces cerevisiae* strain stell8 in aerobic 15-l bioreactor cultures using medium B containing 55 g Merck starch.$l^{-1}$

| Parameter | No glucose | | Glucose spike |
|---|---|---|---|
| | Peak value, 150 h | Value, 100 h | Value, 100 h |
| Biomass, $g.l^{-1}$ | 16.45 | 14.95 | 17.33 |
| Ethanol, $g.l^{-1}$ | 0 | 0 | 0 |
| Glucose, $g.l^{-1}$ | 0 | 0 | 0 |
| E, % | 100 | 100 | 100 |
| Glucoamylase, $U.l^{-1}$ | 581 | 294.6 | 160 |
| Glucoamylase, U.g biomass$^{-1\,a}$ | 35.3 | 18.9 | 9.5 |
| α-Amylase, $U.l^{-1}$ | 500 | 339.3 | 398.4 |
| α-Amylase, U.g biomass$^{-1\,a}$ | 30.4 | 22.7 | 23 |
| $Y_{x/s}{}^{1}$ | 0.303 | 0.273 | 0.333 |
| $Y_{x/s}{}^{2}$ | 0.293 | 0.265 | 0.322 |
| $\mu_{max}$, $h^{-1}{}_{1}$ | 0.237 | 0.237 | 0.230 |
| $\mu_{max}$, $h^{-1}{}_{2}$ | 0.096 | 0.096 | 0.044 |
| $\mu_{max}$, $h^{-1}{}_{3}$ | 0.181 | 0.181 | 0.205 |
| Plasmid stability, % | N.D. | N.D. | 98 |

| | |
|---|---|
| [a] | Specific enzyme activity, expressed as units per gram biomass |
| E | Efficiency of starch utilisation; initial starch - residual starch/initial starch x 100 |
| N.D. | Not determined |
| $Q_p^{max}$ | Maximal volumetric rate of ethanol production, calculated from the maximum slope of ethanol vs. time |
| $Y_{x/s}{}^{1}$ | Cell yield coefficient, g dry biomass/g starch utilised, calculated from the final biomass concentration produced, with no biomass correction for biomass produced from other media components |
| $Y_{x/s}{}^{2}$ | Cell yield coefficient, g dry biomass/g starch plus glucose utilised, calculated from the final biomass concentration and corrected for an average of 0.55 g biomass.$l^{-1}$ produced from the amino acid components of the medium |
| $\mu_{max\,1}$ | Maximum specific growth rate on all media components excluding the starch (growth phase one) |
| $\mu_{max\,2}$ | Maximum specific growth rate for the initial growth on starch (growth phase two) |
| $\mu_{max\,3}$ | Maximum specific growth rate for the second growth phase on starch (growth phase three) |




*ACT1* mRNA




*GLU1* mRNA

**Figure 3.** Northern blot analysis of the *Lipomyces spencermartinsiae* LKAII glucoamylase mRNA obtained from the recombinant strain stell8. Samples taken at regular intervals are indicated by the numbers 1 to 20 and correspond with the numbering indicated on the cultivation profile in Figure 2. The international standard *ATC1* was included as the control.

## 4 References

**Albertsson, P-A. (1986).** Liquid polymer phase systems. In *Partition of cells and macromolecules; distribution and fractionation of cells, mitochondria, chloroplasts, viruses, proteins, nucleic acids and antigen-antibody complexes in aqueous polymer two phase systems 3rd Ed,* pp. 18-56. New York: John Wiley & Sons, Inc.

**Arnesen, S., Eriksen, S.H., Olsen, J. & Jensen, B. (1998).** Increased production of α-amylase from *Thermomyces lanuginosus* by the addition of Tween 80. *Enzyme Microbiol Technol,* **23,** 249-252.

**Aunstrup, K. (1978).** Enzymes of industrial interest; traditional products. In *Annual reports on fermentation processes, Vol 2,* pp. 125-154. Edited by D. Perlman. New York: Academic Press Inc.

**Ferreira, G., Boer, C.G. & Peralta, R.M. (1999).** Production of xylanolytic enzymes by *Aspergillus tamarii* in solid-state fermentation. *FEMS Microbiol Lett*, **173**, 335-339.

**Gimenez, J.A., Monkovic, D.D. & Dekleva, M.L. (1999).** Identification and monitoring of protease activity in recombinant *Saccharomyces cerevisiae. Biotechnol Bioeng,* **67,** 245-251.

**Hadfield, C., Raina, K.K., Shashi-Meno, K. & Mount, R.C. (1993).** The expression and performance of cloned genes in yeasts. *Mycol Res,* **97,** 897-944.

**Kim, E., Shin, D., Irwin, D.C. & Wilson, D.B. (1998).** Fed-batch production of *Thermomonospora fusca* endoglucanase by recombinant *Streptomyces lividans. Biotechnol Bioeng,* **60,** 70-76.

**Köhrer, K. & Domdey, H. (1990).** Preparation of high molecular weight RNA. *Methods in Enzymology*, **185**, 398-405.

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

**Marquet, M., Alouani, S., Haas, M.L., Loison, G. & Brown, S.W. (1987).** Double mutants of *Saccharomyces cerevisiae* harbour stable plasmids: stable expression of a eukaryotic gene and the influence of host physiology during continuous culture. *J Biotechnol*, **6,** 135-145.

**North, M.J. (1982).** Comparative biochemistry of the proteinases of Eucaryotic microorganisms. *Microbiol Rev*, **46,** 308-340.

**Neurath, H. (1989).** The diversity of proteolytic enzymes. In *Proteolytic enzymes: a practical approach,* pp. 1-10. Edited by R.J. Beynon & J.S. Bond. Oxford: University Press Inc.

**Park, Y.S., Shiba, S., Lijima, S. & Kobayashi, T. (1993).** Comparison of three different promoter systems for secretory α-amylase production in fed-batch cultures of recombinant *Saccharomyces cerevisiae*. *Biotechnol Bioeng,* **41,** 854-861.

**Ramgren, M., Anderson, E. & Hahn-Hägerdal, B. (1988).** α-Amylase production with *Bacillus subtilis* in the presence of PEG and surfactants. *Appl Microbiol Biotechnol,* **29,** 337-340.

**Randez-Gil, F. & Sanz, P. (1993).** Expression of *Aspergillus oryzae* α-amylase gene in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett,* **112,** 119-124.

**Rao, M.B., Tanksale, A.M., Ghatge, M.S. & Deshpande, V.V. (1998).** Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev,* **62,** 597-635.

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

**Sakai, A., Chibazakura, T., Shimizu, Y. & Hishinuma, F. (1992).** Molecular analysis of *POP2* gene, a gene required for glucose-derepression of gene expression in *Saccharomyces cerevisiae*. *Nucleic Acids Research,* **20,** 6227-6233.

**Shiba, S., Nishida, Y., Park, Y.S., Lijima, S. & Kobayashi, T. (1994).** Improvement of cloned α-amylase gene expression in fed-batch culture of recombinant *Saccharomyces cerevisiae* by regulating both glucose and ethanol concentrations using a fuzzy controller. *Biotechnol Bioeng,* **44,** 1055-1063.

**Thies, E., Jenkins, T. & Stutzenberger, F. (1994).** Effects of the detergent Tween 80 on *Thermomonospora curvata*. *World J Microbiol Biotechnol,* **10,** 657-663.

# Chapter 5

# GENERAL DISCUSSION AND CONCLUSIONS

The main objective of this project was to characterise several *Saccharomyces cerevisiae* strains that had been transformed with amylase genes with regard to the relevant kinetic and stoichiometric parameters to assess their potential for commercial ethanol production. The data generated would provide the basis for the application of a transformant with potential for ethanol production on an industrial scale, utilising renewable carbon feedstocks.

The *S. cerevisiae* transformants, supplied by the Institute for Wine Biotechnology, University of Stellenbosch, originated from the diploid "Sigma" *S. cerevisiae* mother strain or the Hoegaarden mother strain, allowing direct comparison of a number of combinations of amylase genes and promoters. Amylase genes obtained from *Lipomyces spencermartinsiae* and *Saccharomycopsis fibuligera* were transformed into the Sigma strains using an integrating plasmid in a single copy form. Two additional recombinant strains, produced at the University of the Free State, also originated from the diploid "Sigma" *S. cerevisiae* mother strain and contained the *S. fibuligera* α-amylase and glucoamylase in a multi-plasmid copy (MC) and a single-plasmid copy (SC) form.

*Preliminary evaluations on starch agar plates*

Starch hydrolysis by strains stell2, stell3, stell5 and *S. cerevisiae* strains MC and SC were repressed by the inclusion of glucose in the medium, suggesting that the natural promoter of the *S. fibuligera* α-amylase gene was susceptible to glucose repression. The medium composition, together with the type of starch used, had an effect on the final diameter of the hydrolysis zones on the agar plates. This was illustrated by the larger zones of hydrolysis produced by most transformants on Lintner starch agar plates in comparison to those Merck starch agar plates. The Phadebas starch agar plates proved useful for the verification of transformation with an α-amylase. This was clearly illustrated by the inability of strains stell5, 6 and 7, all lacking an α-amylase, to produce hydrolysis zones on the Phadebas starch agar plates. Furthermore, the Phadebas starch agar plates revealed that the LKAII gene from *Lipomyces spencermartinsiae*, initially thought to be an α-amylase, was in fact a glucoamylase. Strains stell1 and 8, containing the LKA1-encoded

α-amylase from *L. spencermartinsiae,* yielded the best results on the Phadebas starch agar plates.

The above data suggested that the *L. spencermartinsiae* amylase gene products were superior to the *S. fibuligera* amylases. However, starch plates are not a reliable means of evaluating the ability of a transformant to convert starch to ethanol or to utilise starch as a sole carbon source for growth. This is because contributing factors, such as fermentation capacity, ethanol tolerance and the enzyme concentration, excretion and accessibility, play an important role in submerged cultivations (De Moraes *et al.*, 1995). In contrast to the data reported by Dhawale *et al.* (1982) and Horn *et al.* (1991), no linear relationship was observed between the hydrolysis zones obtained on the starch agar plates and the amylase activity obtained in liquid media cultivations. This could have been due to, at least in part, the differing molecular weights and diffusion characteristics of the amylases from different yeast species.

*Submerged cultivations on starch*

Aerobic and anoxic shake flask cultivations yielded poor results because of very low extracellular amylase activities. The extended fermentation times experienced with strains stell3, 4 and 5 gave reason to eliminate these strains from further evaluations. With all the strains, aerobic cultivations on starch were associated with an increased biomass production and minimal or no ethanol production. Although the volumetric amylase activity values of these strains were higher in shake flask cultures, the specific enzyme activity values, expressed as activity per unit biomass, were little affected by the aerobic cultivation. Curiously, strain stell2, which was transformed with both an α-amylase and a glucoamylase from *Saccharomycopsis fibuligera* (natural promoters), showed no detectable α-amylase values under anoxic or aerobic cultivations, despite being capable of starch hydrolysis, albeit at a slow rate.

Attempts to improve α-amylase production by means of Tween 80 supplementation, as well as cultivations at pH 4.5 and 5.5, failed to improve the extracellular levels of the α-amylase, and the differences between the fermentation parameters at pH 4.5 and 5.5

were not statistically significant. Apparently the low amylase activities were not due to intracellular accumulation of the α-amylase nor due to extracellular proteolytic activity. Only strains stell1 and 8, which had been transformed with a *L. spencermartinsiae* α-amylase gene, exhibited any detectable α-amylase activity under anoxic conditions. This again suggested that the amylase genes from *S. fibuligera* were not as effective as those from *L. spencermartinsiae* in these genetic constructs.

Strain stell7, which had been transformed with a double glucoamylase gene combination, performed unexpectedly well. This strain produced the highest biomass concentration (27.97 $g.l^{-1}$) and glucoamylase activity (1543 $U.l^{-1}$) of all the recombinant strains evaluated. Further evaluation in 14 and 15-l bioreactors again resulted in a high biomass production, effective starch hydrolysis (100 % conversion within 30 h of aerobic cultivation) and glucoamylase production by stell7. Strain stell8, despite having the most promising combination of α-amylase and glucoamylase genes (from *L. spencermartinsiae* with the *PGK1* promoter), yielded a weak amylolytic activity, accompanied by a delayed production thereof, and produced a curious multiphasic growth profile under aerobic conditions.

*Promoter influence on amylase production*

Hadfield *et al.* (1993), Marquet *et al.* (1987) and Sakai *et al.* (1992) supported the idea that increased glucose concentrations optimised the activity of the *PGK1* promoter, whereas Park *et al.* (1993) and Shiba *et al.* (1994) opposed the idea. The pulse addition of glucose to a bioreactor culture of strain stell8 resulted in a sharp peak in glucoamylase activity, but no similar increase in α-amylase activity was observed. Northern analysis of the glucoamylase (LKAII) mRNA did not indicate detectable induction of the *PGK1* promoter by the glucose pulse, however, but did reveal very low mRNA levels. Thus, induction may well have taken place, but the mRNA levels remained too low to indicate this clearly on the Northern blots. Although no direct comparison could be made, the low levels of mRNA obtained with the LKAII glucoamylase might suggest that the limiting factor in terms of amylase production could be on a transcriptional level or may have been associated with some other aspect of protein turnover.

*General conclusions*

All the recombinant stains performed better in the bioreactor cultures than in the shake flask cultures, probably due to the better regulated cultivation conditions. The natural promoters of *S. fibuligera* appeared to be repressed by glucose, whereas the *PGK1* promoters did not appear to be affected by elevated glucose levels. The amylase genes of *L. spencermartinsiae* were more effective in terms of starch degradation than those from *S. fibuligera.* Even though strain stell7 had only two glucoamylase genes and no α-amylase gene, it showed promising results.

Despite a novel approach involving the cloning of amylase genes into a diploid *S. cerevisiae* strain, the volumetric rates of amylase production remained low, resulting in extended fermentation times and thus precluding the use of these strains on an industrial scale. Although *S. cerevisiae* is favoured for genetic engineering due to its GRAS (generally regarded as safe) status, well characterised genetics, ease of manipulation and lack of interfering introns, it is not entirely an optimal host for large scale protein production. Factors such as the lack of strong, tightly regulated promoters, hyperglycosylation and the need for fed-batch fermentation to attain high cell densities and thus high product production are limitations to the exploitation of *S. cerevisiae* for the production of heterologous proteins (Romanos *et al.*, 1992). Furthermore, high-level expression of foreign proteins can place a significant metabolic burden on the host cell, reducing its growth rate and the efficiency of the gene expression (Romanos *et al.*, 1992).

**References**

**De Moraes, L.M.P., Astolfi-filho, S. & Oliver, S.G. (1995).** Development of yeast strains for the efficient utilisation of starch: evaluation of constructs that express α-amylase and glucoamylase separately or as bifunctional proteins. *Appl Microbiol Biotechnol,* **43,** 1067-1076.

**Dhawale, M.R., Wilson, J.J., Khachatourians, G.G. & Ingledew, W.M. (1982).** Improved method for the detection of starch hydrolysis. *Appl Environ Microbiol,* 44, 747-750.

**Hadfield, C., Raina, K.K., Shashi-Meno, K. & Mount, R.C. (1993).** The expression and performance of cloned genes in yeasts. *Mycol Res,* **97,** 897-944.

**Horn, C.H., Du Preez, J.C. & Kilian, S.G. (1991).** Selective isolation of amylase derepressed mutants of *Schwanniomyces occidentalis*. *Syst Appl Microbiol,* 14, 299-304.

**Marquet, M., Alouani, S., Haas, M.L., Loison, G. & Brown, S.W. (1987).** Double mutants of *Saccharomyces cerevisiae* harbour stable plasmids: stable expression of a eukaryotic gene and the influence of host physiology during continuous culture. *J Biotechnol,* **6,** 135-145.

**Park, Y.S., Shiba, S., Lijima, S. & Kobayashi, T. (1993).** Comparison of three different promoter systems for secretory α-amylase production in fed-batch cultures of recombinant *Saccharomyces cerevisiae*. *Biotechnol Bioeng,* **41,** 854-861.

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

**Sakai, A., Chibazakura, T., Shimizu, Y. & Hishinuma, F. (1992).** Molecular analysis of *P0P2* gene, a gene required for glucose-derepression of gene expression in *Saccharomyces cerevisiae*. *Nucleic Acids Research,* **20,** 6227-6233.

**Shiba, S., Nishida, Y., Park, Y.S., Lijima, S. & Kobayashi, T. (1994).** Improvement of cloned α-amylase gene expression in fed-batch culture of recombinant *Saccharomyces cerevisiae* by regulating both glucose and ethanol concentrations using a fuzzy controller. *Biotechnol Bioeng,* **44,** 1055-1063.

# Chapter 6

## SUMMARY

The direct fermentation of starch to ethanol using an amylase-producing yeast is of interest as an alternative to the conventional fermentation processes, which utilise commercial amylases. Starch is an abundant renewable biopolymer, comprising two major components, namely amylose (α-1,4-linked D-glucose residues) and amylopectin (α-1,4 and α-1,6-linked D-glucose residues), typically constituting 80 % of cereal starches. Efficient starch degradation, therefore, necessitates the use of α- and glucoamylases, together with an α-1,6 debranching activity. Naturally amylolytic yeasts are not suited to fermentations, whereas *Saccharomyces cerevisiae*, known for its strong fermentation capacity, lacks amylolytic activity (with the exception of *S. cerevisiae* var. *diastaticus*, which has weak glucoamylase activity). Consequently, the diploid "Sigma" strain of *S. cerevisiae* was transformed with different combinations of amylase genes from *Lipomyces spencermartinsiae* (with the *PGK1* promoter), and *Saccharomycopsis fibuligera* (with its natural promoters) using an integrating plasmid in a single copy form. These recombinant strains, provided by the University of Stellenbosch, were evaluated in respect of their ability to ferment starch to ethanol. Recombinant strains of *S. cerevisiae* containing the *S. fibuligera* amylase genes with non-integrating plasmids in a multi-copy and a single copy form, provided by the University of the Free State, were also evaluated.

Notable differences in the hydrolysis zones on starch agar plates indicated that the type of starch medium used and the amylase produced exerted a significant effect. The dimensions of these hydrolysis zones were a poor indicator of the performance of the strains in liquid starch media. Anoxic cultivations in shake flasks and in 2-l bioreactors containing a 2 % starch medium yielded less than 10 g ethanol.$l^{-1}$ over a 200 h incubation period. Aerobic growth yielded more biomass and, therefore, higher amylase values and higher rates of starch hydrolysis, but with no detectable amounts of ethanol. Initial evaluations indicated poor amylase activity, particularly with strains containing the *S. fibuligera* amylase genes. The limited amylase production by a strain containing the *S. fibuligera* amylase genes was not due to proteolytic activity or intracellular enzyme accumulation. Strains containing the *L. spencermartinsiae* amylase genes gave the best overall results. However, a strain containing a combination of both α-amylase and glucoamylase yielded disappointing results. The curious multi-phasic growth profile obtained with this strain, accompanied by a delay in amylase production, suggested

regulation of the *PGK1* promoter by glucose. However, Northern blot analyses indicated very low levels of glucoamylase mRNA, with no clear indication of induction of the *PGK1* promoter. A recombinant strain (strain stell7) with both glucoamylase genes (LKAII from *L. spencermartinsiae* and GLU1 from *S. fibuligera*) proved to be the most promising. Further evaluation in 15-l bioreactors resulted in the production of *ca.* 21 g ethanol.$l^{-1}$ from 55 g starch.$l^{-1}$ by strain stell7. Despite a relatively high α-amylase activity of 500 U.$l^{-1}$ after 150 h, the slow rate of enzyme production remained the rate-limiting step. Although some of these recombinant strains were capable of complete starch hydrolysis, the slow rate of starch saccharification and the concomitant low ethanol productivity rendered these strains unattractive for commercial application.

**Key words:** Recombinant, *Saccharomyces cerevisiae*, amylase, starch

# Chapter 7

# SAMEVATTING

Die gebruik van 'n amilase-produserende gis in die direkte fermentasie van stysel na etanol is van belang as 'n alternatief vir die konvensionele fermentasieproses wat van kommersiële amilase-ensieme gebruik maak. Stysel is 'n volop-beskikbare, hernieubare biopolimeer bestaande uit twee hoofkomponente, naamlik amilose (α-1,4-gekoppelde D-glukose residue) en amilopektien (α-1,4 en α-1,6-gekoppelde D-glukose residue), wat tipies 80 % van graanstysels verteenwoordig. Effektiewe stysel-afbraak noodsaak dus die gebruik van α- en glukoamilases, tesame met 'n α-1,6 onttakkingsaktiwiteit. Amilolitiese giste is van nature nie geskik vir fermentasie nie, terwyl *Saccharomyces cerevisiae*, bekend vir 'n sterk fermentatiewe kapasiteit, nie amilolitiese aktiwiteit besit nie (met uitsondering van *S. cerevisiae* var. *diastaticus*, wat 'n swak amilolitiese aktiwiteit besit). Gevolglik is die diploïde "Sigma" stam van *S. cerevisiae* met verskillende kombinasies van die amilase-gene afkomstig van *Lipomyces spencermartinsiae* (met die *PGK1* promotor) en *Saccharomycopsis fibuligera* (met die natuurlike promotors) getransformeer, deur gebruik te maak van 'n integrerende plasmied in 'n enkelkopie formaat. Die rekombinante stamme, wat deur die Universiteit van Stellenbosch voorsien is, is met betrekking tot hul vermoë om stysel tot etanol te fermenteer, geëvalueer. Rekombinante stamme van *S. cerevisiae*, wat die *S. fibuligera* amilase-gene met die nie-integrerende plasmied in 'n veelvuldige kopie-formaat bevat het, is deur die Universiteit van die Vrystaat verskaf en is ook geëvalueer.

Merkwaardige verskille in die hidrolitiese sones op die styselagarplate was 'n aanduiding dat die tipe styselmedium, sowel as die amilase wat geproduseer word, 'n betekenisvolle effek uitoefen. Die afmetings van hierdie hidrolitiese sones het 'n onbetroubare aanduiding van die werksverrigting van die stamme in vloeibare styselmedium gegee. Minder as 10 g etanol.$l^{-1}$ is gedurende anoksiese kwekings in skudflesse en in 2-l bioreaktors met 'n 2 % styselmedium na 'n inkubasietydperk van 200 h gelewer. Aërobiese kweking het meer biomassa, en dus hoër amilasewaardes en hoër styselhidrolise tempo's, gelewer, maar met geen waarneembare hoeveelhede etanol nie. Aanvanklike evaluasies het swak amilase-aktiwiteit aangedui, in besonder α-amilase aktiwiteit, vir die stamme met die amilase-gene vanaf *S. fibuligera*. Die beperkte amilase-produksie deur 'n stam met die *S. fibuligera* amilase-gene was nie as gevolg van proteolitiese aktiwiteit of intrasellulêre ensiem-akkumulasie nie. Stamme

met die *L. spencermartinsiae* amilase-gene het oor die algemeen die beste resultate gelewer. 'n Stam wat 'n kombinasie van beide α-amilase- en glukoamilase-gene bevat, het egter teleurstellende resultate gelewer. Die merkwaardige multi-fase groeiprofiel wat met hierdie stam verkry is, tesame met 'n vertraging in amilase-produksie, het die regulering van die *PGK1*-promoter deur glukose geïmpliseer. 'Northern blot' analises het egter baie lae vlakke van glukoamilase-mRNA aangedui, met geen duidelike aanduiding van induksie van die *PGK1* promoter nie. 'n Rekombinante stam (stam stell7) met beide glukoamilase gene (LKAII vanaf *L. spencermartinsiae* en GLU1 vanaf *S. fibuligera*) was die belowendste stam. Verdere evaluasie in 15-l bioreaktors het gelei tot die produksie van *ca.* 21 g etanol.$l^{-1}$ deur stam stell7 vanaf 55 g stysel.$l^{-1}$. Ondanks 'n relatief hoë α-amilase aktiwiteit van 500 U.$l^{-1}$ na 150 h, het die stadige tempo van ensiemproduksie die snelheidsbeperkende stap gebly. Alhoewel sommige van hierdie rekombinante stamme tot volledige styselhidrolise in staat was, maak die stadige styselhidrolise en die gepaardgaande lae etanolproduksie hierdie stamme onaantreklik vir kommersiële toepassing.

**Sleutelwoorde:** Rekombinant, *Saccharomyces cerevisiae*, amilase, stysel.