Xylanase Production by a Recombinant Strain of *Saccharomyces cerevisiae*: The Effect of Carbon Catabolite Repression and Ethanol Concentration in Batch and Continuous Cultures

by

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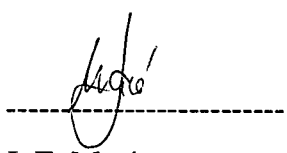
DECLARATION

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

Signature: 

J. E. Maré

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NOTE

Diverse genetic nomenclature is applied in literature. Therefore, a standardised nomenclature was used in this thesis to maintain consistency. The following examples illustrate the nomenclature used for proteins and genes in this thesis:

Genes from *Saccharomyces cerevisiae* – *URA3*, *ADH2*, *LEU2*, *SUC2* and *FUR1* (Sherman, 1991).

Genes from any microbial source, except *S. cerevisiae* – *xyn2*, *xynA*.

When genes were expressed in *S. cerevisiae* the nomenclature for genes of this microorganism was used.

Proteins from any microbial source – Ura3, Adh2, Leu2, Suc2 and Fur1 (Sherman, 1991).

Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* **194**, 3-21.

SUMMARY

Endo-β-1,4-xylanase production by a recombinant strain of *Saccharomyces cerevisiae* Y294, transformed with a *Trichoderma reesei* xylanase gene (*XYN2*), was evaluated in batch and continuous cultures. Expression of the heterologous xylanase gene by this yeast was under control of a promoter-terminator expression cassette derived from the alcohol dehydrogenase II gene (*ADH2*) of *S. cerevisiae*, which is mainly regulated through carbon catabolite repression.

The 3,5-dinitrosalicylic acid and Somogyi-Nelson assays for endo-β-1,4-xylanase activity (hereafter referred to as xylanase) in supernatants from cultures of the recombinant *S. cerevisiae* strain and in a commercial xylanase preparation were compared. These two assays gave widely differing activity values, even more so than anticipated. These huge discrepancies in activity values obtained indicated that these assays did not allow a reliable comparison of the activities of xylanolytic enzyme mixtures and preparations containing a single xylanase without accessory enzymes.

In aerobic batch culture, the highest specific rate of xylanase production (20.8 nkat.mg biomass^{-1}.h^{-1}) and activity (1 590 nkat.ml^{-1}) were recorded with glucose as carbon source. The inclusion of ethanol as carbon source, which is a standard procedure for the derepression of *ADH2*, resulted in poor xylanase production. The influence of glucose flux, glucose concentration and growth rate on xylanase production by this recombinant strain of *S. cerevisiae* Y294 was investigated in carbon-limited continuous culture and compared to results obtained with recombinant xylanase-producing *S. cerevisiae* strains H158 and CEN.PK110-6C. The latter two strains harboured the same expression cassette and xylanase gene (*XYN2*) as the recombinant *S. cerevisiae* Y294 strain. Xylanase production by strains Y294, H158 and CEN.PK110-6C was markedly reduced at glucose flux values greater than 0.98, 1.44 and 1.68 mmol glucose.g biomass^{-1}.h^{-1}, respectively. Despite the obvious strain differences, with all three yeast strains the glucose flux appeared to contribute to a greater extent towards the regulation of xylanase production than did the glucose concentration in the culture.

Xylanase production by recombinant *S. cerevisiae* strains Y294 and CEN.PK110-6C was found to be transcriptionally repressed by ethanol at and above 5.5 g ethanol.l^{-1}. This finding was surprising, since the xylanase gene was under the regulation of the *ADH2* promoter and alcohol dehydrogenase II is associated with the assimilation of ethanol by effecting the catalysis of ethanol to acetaldehyde. This finding adds a new perspective to the regulation of alchohol dehydrogenase II.

In a fed-batch culture of recombinant *S. cerevisiae* Y294, the specific rate of xylanase production was increased 2.5-fold as compared to batch cultures, through control of the growth rate at 0.1 h^{-1} by using an exponentially increasing feed rate. These observations also indicate the important role of the growth rate, and thus possibly the carbon flux, in regulating *ADH2*-mediated xylanase production.

OPSOMMING

Endo-β-1,4-xilanase produksie deur 'n rekombinante *Saccharomyces cerevisiae* Y294 stam, wat getransformeer is met 'n xilanase geen (*XYN2*) afkomstig vanaf *Trichoderma reesei*, is bestudeer in lot- en kontinue kulture. Uitdrukking van die xilanase-geen in hierdie rekombinant was onder die invloed van 'n inisieerder-termineerder uitdrukkingsvektor afgelei vanaf die alkoholdehidrogenase II (*ADH2*) geen. *ADH2* word hoofsaaklik deur koolstof kataboliet repressie gereguleer.

Die 3,5-dinitrosalisiliese suur en Somogi-Nelson metodes vir bepaling van endo-β-1,4-xilanase aktiwiteit in bostand van kulture van die rekombinante *S. cerevisiae* stam en 'n kommersiële xilanase preparaat is met mekaar vergelyk. Hierdie twee metodes het aktiwiteit-waardes wat baie van mekaar verskil het, gelewer, selfs meer as wat verwag is. Hierdie groot afwyking in aktiwiteitswaardes het aangedui dat hierdie twee metodes nie 'n vertrouenswaardige vergelyking tussen die xilanase-aktiwiteit van xilanolitiese ensiem-mengsels en preparate wat 'n enkele xilanase ensiem, sonder bykomende xilanolitiese hulp-ensieme, kon maak nie.

In aërobiese lotkulture is die hoogste spesifieke snelheid van xilanase-produksie (20.8 nkat.mg biomassa^{-1}.h^{-1}) en -aktiwiteit (1590 nkat.ml^{-1}) met glukose as koolstofbron verkry. Die insluiting van etanol as koolstofbron, wat 'n standaardprosedure vir die derepessie van *ADH2* is, het tot swak xilanase-produksie gelei. Die invloed van glukose-vloei, glukose-konsentrasie en groeisnelheid op xilanase-produksie deur hierdie rekombinante *S. cerevisiae* Y294 stam is in koolstof-beperkte kontinue kulture ondersoek en met resultate verkry vir rekombinante xilanase-produserende *S. cerevisiae* stamme H158 en CEN.PK110-6C, vergelyk. Die laasgenoemde twee stamme het dieselfde uitdrukkingsvektor en xilanase-geen as *S. cerevisiae* Y294 gehuisves. Xilanase-produksie deur stamme Y294, H158 en CEN.PK110-6C is merkwaardig verlaag wanneer die glukose vloei 0.98, 1.44 en 1.68 mmol glukose.g biomassa^{-1}.h^{-1}, respektiewelik, oorskry het. Ten spyte van die oogmerklike verskille tussen die stamme, het die glukose-vloei, eerder as die glukose-konsentrasie, in al drie die stamme oorwegend tot die regulering van xilanase produksie bygedra.

Xilanase-produksie deur die rekombinante *S. cerevisiae* stamme Y294 en CEN.PK110-6C is transkripsioneel deur etanol konsentrasies van 5.5 $g.l^{-1}$ of hoër onderdruk. Hierdie bevinding was onverwags, aangesien die xilanase-geen onder regulering van die *ADH2* promoter is. Alkoholdehidrogenase II word geassosieer met die opname van etanol deur die katalise van etanol na asethaldehied te fasiliteer. Hierdie bevinding voëg 'n nuwe dimensie toe tot die regulering van die alkohol dehidrogenase II geen.

In gevoerde lotkulture van die rekombinante *S. cerevisiae* Y294 is die spesifieke snelheid van xilanase-produksie 2.5-voudig verhoog in vergelyking met lotkulture, deur die groeisnelheid te beheer by 0.1 h^{-1}, deur gebruik te maak van 'n eksponensieel-toenemende voersnelheid. Hierdie bevindings dui daarop dat die groeisnelheid, en dus ook moontlik die glukose-vloei, 'n belangrike rol gespeel het in die regulering van *ADH2*-gefasiliteerde xilanase produksie.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Yeasts are commercially valuable to various industries, including the baking and brewing industries, the animal nutrition industry, the production of alcohol fuels and chemical feedstocks and, more recently, enzyme production (Alexander, 1986; Tøttrup and Carlsen, 1989). Hemicellulolytic enzymes have received considerable attention of researchers because of their potential application in herbivore digestion, the pulp and paper industry, feedstock and baking industries and in the recycling of photosynthetically fixed carbon through natural decay and composting (Coughlan and Hazlewood, 1993; Herrmann *et al.*, 1997). *Trichoderma reesei* is recognised as an industrially important fungus for the production of enzymes capable of hydrolysing polysaccharides abundant in woody materials, especially cellulose and xylan (Stålbrand *et al.*, 1993).

Heterologous gene expression in yeast has become a routine procedure in molecular biology. This study is on heterologous gene expression by a recombinant strain of *Saccharomyces cerevisiae*, capable of producing an endo-β-1,4-xylanase (hereafter referred to as xylanase). The expression of the xylanase gene (*XYN2* from *Trichoderma reesei*) was facilitated through a promoter-terminator expression cassette derived from the alcohol dehydrogenase II gene (*ADH2*). This recombinant strain of *S. cerevisiae* was supplied by the Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa.

The physiology of heterologous gene expression by recombinant yeast strains is often neglected by molecular biologists. This sadly leads to a paucity of information regarding mechanisms and metabolic processess, other than the genetic events, that may influence the production of heterologous proteins by a recombinant yeast strain. Rapid advancements in the field of biotechnology have created the need for quantification of metabolic processes in (recombinant) microorganisms so that they can be optimally exploited (Sonnleitner and Käppeli, 1986). The main benefits associated with accurate quantification of metabolic processes are the ability to increase product yield ($Y_{p/s}$), the specific rate of product formation (q_p), the uniformity and quality of the microbial product and the attainment of process uniformity (Sonnleitner and Käppeli, 1986).

1.2. Aim of study

The aim of this study was to determine the physiological parameters that influenced xylanase production by this recombinant *Saccharomyces cerevisiae* Y294 strain. The specific aims were sub-divided as follows:

i. A literature survey on topics related to this project, including xylanases, their biotechnological applications and factors that may influence the expression of heterologous xylanase by the recombinant *S. cerevisiae* strain.

ii. An investigation of the effect of different carbon sources and culture medium composition on xylanase production.

iii. Quantification of glucose repression and 'alcohol induction' of the *ADH2* promoter in carbon-limited chemostat cultures.

iv. Determination of whether repression was dependent on the glucose *concentration* or on the glucose *flux*.

v. Determination of plasmid stability during prolonged cultivations.

vi. Application of the data to develop a fed-batch cultivation protocol for optimum xylanase production by the recombinant *S. cerevisiae* strain.

1.3. Literature review

1.3.1. The natural occurrence and degradation of xylan

1.3.1.1 Localisation and structural composition of xylan in plants

Cellulose, hemicellulose and lignin constitute the major biopolymers found in wood. Four types of hemicelluloses are predominant in plants, namely xylan, mannan, galactan and arabinan (Whistler and Richards, 1970). Xylan is the second most abundant polysaccharide in nature and is surpassed only by cellulose in abundance (Whistler and Richards, 1970). Xylans are classified according to the nature of the linkages joining the xylose residues. β-1,3-Linked xylans are found only in marine algae, those containg a mixture of β-1,3- and β-1,4-linkages only in seaweeds and β-1,4-linked xylans occur in hardwoods, softwoods and grasses (Barry and Dillon, 1940; Dekker and Richards, 1976; Kato and Nevins, 1984; Timell, 1965). Hetero-β-1,4-D-xylans constitute the major portion of the hemicellulose-fraction in terrestial plants (Timell, 1965; Whistler and Richards, 1970). Native xylans are complex polymers containing varying amounts of arabinose, 4-O-methylglucuronic acid and acetic acid groups attached to the main xylose chain, depending on the botanical origin of the xylan (Johannson and Samuelson, 1977; Puls and Schuseil, 1993).

Xylan accounts for 10 to 35 % of the dry weight of hardwoods (angiosperms) (Puls and Schuseil, 1993). The main hemicellulose in hardwood is O-acetyl-4-O-methylglucuronoxylan (Puls and Schuseil, 1993). On average, the degree of polymerisation (DP) is 200 with 10 % of the backbone units substituted at C–2 with 4-O-methyl-α-D-glucuronic acid and 70 % of the xylopyranosyl units acetylated at C–2 and / or C–3 (Lindberg *et al.*, 1973; Puls and Schuseil, 1993). Small amounts of rhamnose and galacturonic acid may also form part of the main chain (Coughlan and Hazlewood, 1993).

Hetero-β-1,4-D-mannans (galactoglucomannan and glucomannan) comprise approximately two-thirds and arabino-4-O-methylglucuronoxylan about one-third of the total hemicellulose found in softwoods (Johannson and Samuelson, 1977). Softwoods (gymnosperms) contain 10 to 15 % arabino-4-O-methylglucuronoxylan which is located mainly in the tertiary wall (S3) of pinewood (Puls and Schuseil, 1993). Softwood xylan is not acetylated and consists of a

backbone containing β-1,4-linked xylose units, with α-1,2-linked 4-O-methylglucuronic acid and α-1,3-linked L-arabinofuranoside substituents (Joseleau *et al.*, 1992; Puls and Schuseil, 1993). The ratio of arabinose side-groups to xylose is 1 : 8 and on average, two out of ten xylose units are substituted with uronic acid (Joseleau *et al.*, 1992; Puls and Schuseil, 1993). Softwood xylans contain less α-1,2-linked 4-O-methylglucuronic acid than hardwood xylans and the L-arabinofuranosyl side-chains are linked to the main chain *via* C-2 and / or C-3 (Joseleau *et al.*, 1992; Puls and Schuseil, 1993). Some of the arabinosyl side-chains are substituted at C-5 with feruloyl or *p*-coumaroyl residues (Joseleau *et al.*, 1992; Meuller-Harvey *et al.*, 1986; Puls and Schuseil, 1993). Grass arabinoxylans differ from species to species and from tissue to tissue within the same species, regarding the proportion and composition of the xylan present (Meuller-Harvey *et al.*, 1986; Puls and Schuseil, 1993).

1.3.1.2. Enzymes necessary for the complete hydrolysis of xylan

The complete enzymatic hydrolysis of xylan into its constituent monocarbohydrates requires the synergistic action of a consortium of xylanolytic enzymes (Figure 1.1). This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure (Coughlan and Hazlewood, 1993; Jeffries, 1996). The genes encoding the full complement of enzymes involved in xylan degradation have yet to be isolated from any single microorganism (Coughlan and Hazlewood, 1993). Endo-β-1,4-xylanase is the crucial enzyme in any xylanolytic enzyme complex because this enzyme is responsible for the depolymerisation of the xylan backbone (Viikari *et al.*, 1994a, b). The majority of xylanolytic microorganisms produce more than one xylanase, which differ with respect to their physico-chemical properties, *e.g.* molecular mass (Mr), isoelectric point (pI), pH optima, temperature stability and temperature optima (Biely *et al.*, 1992). Many bacteria and fungi produce multiple isomeric forms of endoxylanase, usually as a result of multiple genes, but also through post-translational modification of a single gene product (Coughlan and Hazlewood, 1993; Sunna and Antranikian, 1997). The different isoforms may differ in their substrate specificities as seen in hydrolysis of structurally diverse native xylans (Biely, 1985).

Xylanases are similar to cellulases in several repects. Both cleave β-1,4-glycosidic bonds, thus having similar catalytic mechanisms and functionality (Coughlan and Hazlewood, 1993).

Xylanolytic organisms, with a few exceptions, are also cellulolytic and secrete complex mixtures of cellulases and xylanases simultaneously (Sunna and Antranikian, 1997). Synergisms have been observed between the backbone-degrading enzymes (xylanases and xylosidases) and the side chain cleaving enzymes (arabinofuranosidases, glucuronidases, acetyl esterases, *p*-coumaroyl esterases and feruloyl esterases), resulting in greater hydrolysis than the sum of hydrolysis caused by each individual enzyme (Viikari *et al.*, 1994a, b). This observed synergism might be due to steric hinderance caused by the substituent groups on xylanase action, or due to the fact that some of the side-group substituents are more easily liberated from short xylo-oligomers than from intact polymeric xylan (Coughlan and Hazlewood, 1993; Jeffries, 1996).

Xylanases are classified into two groups: endo-β-1,4-xylanases and exo-β-1,4-xylanases (Biely, 1985; Christakopoulos *et al.*, 1997). Exo-β-1,4-xylanases hydrolyse xylan from the non-reducing end of the polymer thus yielding only β-D-xylose as a hydrolysis product (Biely, 1985). In the remainder of this section the characteristics of endoxylanases is discussed. Endo-β-D-xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) hydrolyse xylan and xylo-oligomers with a depolymerisation (DP) of greater than 2, the affinity increasing with increasing DP (Biely *et al.*, 1985, 1997). Endoxylanases are distinguished from one another on the basis of the substrates on which they act, as well as their reaction products (Biely *et al.*, 1997). Endoxylanases are secreted extracellularly, since xylan is a large polymer incapable of crossing the cell membrane (Biely, 1985). Polymeric xylans are usually cleaved at unsubstituted regions to yield mixtures of unsubstituted xylo-oligomers, as well as short and long chain substituted xylo-oligomers (Coughlan and Hazlewood, 1993). Removal of the substituent groups by ancillary enzymes create new substrates for endoxylanase action. The majority of endo-β-xylanases can be classified into two groups (Christakopoulos *et al.*, 1997; Clarke *et al.*, 1997; Jeffries, 1996), those with low Mr / high pI values (family G / 11 xylanases) and those with high Mr / low pI values (family F / 10 xylanases). Both families contain bacterial and fungal enzymes, suggesting that the acquisition of xylanase acivity has involved, at some stage, lateral gene transfer between fungi and bacteria. (Hazlewood and Gilbert, 1992).

β-D-Xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) hydrolyse xylo-oligosaccharides to xylose, thus forming a crucial component of microbial xylanolytic systems (Kristufek *et al.*,

1995). Xylosidases are mono or dimeric proteins located extracellularly or cell-bound, depending on the organism and cultivation time (Coughlan and Hazlewood, 1993). The activity of xylosidases towards xylo-oligomers decrease with increasing DP, resulting in almost complete inactivity towards polymeric xylan (Biely, 1985; Kristufek *et al.*, 1995).

α-L-Arabinofuranosyl residues are widely distributed in plant heteropolysaccharides, including arabinoxylan. α-L-Arabinofuranosyl residues occur as side-groups in arabinoxylan (Johannson and Samuelson, 1977). These molecules are linked to the β-1,4-xylopyranosyl backbone *via* α-1,3 and α-1,2 linkages (Johannson and Samuelson, 1977; Puls and Schuseil, 1993). Arabinose side-groups can hamper the enzymatic hydrolysis of hemicelluloses used as substrates in various industrial applications, such as improvement of animal feed digestibility (Margolles-Clark *et al.*, 1996). Depending on substrate specificity, enzymes hydrolysing L-arabinose linkages have been classified as α-L-arabinofuranosidases (EC 3.2.1.55) and endo-1,5-α-L-arabinases (EC 3.2.1.99). α-L-Arabinofuranosidases are either mono-, di-, tetra or octameric structures, capable of hydrolysing terminal nonreducing α-1,2-, α-1,3- and α-1,5-L-arabinofuranosyl residues (Coughlan and Hazlewood, 1993; Margolles-Clark *et al.*, 1996). Endo-1,5-α-L-arabinases hydrolyses endo-1,5-α-L-arabinofuranosidic linkages of arabinans, but have no activity against terminal nonreducing L-arabinofuranosyl residues (Margolles-Clark *et al.*, 1996). The α-L-arabinofuranosidases are divided in two additional groups, *i.e.* the *Streptomyces purpuracens* and *Aspergillus niger* type arabinofuranosidases. The *A. niger* type arabinofuranosidases are active on small synthetic substrates and are capable of hydrolysing arabinosyl side groups from arabinans, arabinogalactans and arabinoglucuronoxylans (Margolles-Clark *et al.*, 1996). The *S. purpuracens*-type arabinofuranosidases act only on low molecular weight α-L-arabinosides and arabinose-containing oligosaccharides (Margolles-Clark *et al.*, 1996).

The relatively large glucuronic acid side groups attached to C-2 of the xylopyranose residues in the xylan backbone hinders the action of other xylanolytic enzymes (Siika-aho *et al.*, 1994). Ester linkages between acid constituents in xylan and lignin are the most common type of linkage found between these two polymers in native wood (Siika-aho *et al.*, 1994). Therefore, α-glucuronidases capable of cleaving 4-O-methyl glucuronic acid side groups are essential for the total hydrolysis of wood xylans (Siika-aho *et al.*, 1994; Sunna and Antranikian, 1997). The hydrolysis of 4-O-methylglucuronoxylan by xylanases prior to incubation with α-

glucuronidases yield better substrates or is sometimes even a prerequisite for to the action of glucuronidases (Coughlan and Hazlewood, 1993; Sunna and Antranikian, 1997).

Two types of esterases are important for the complete hydrolysis of acetylglucuronoxylan, *i.e.* acetylxylan esterases and non-specific acetyl esterases (Biely, 1985; Jeffries, 1996). Acetyl esterases release acetate from acetylated xylo-oligomers, with acetylxylobiose being the best substrate, but they are inactive against acetylglucuronoxylan (Jeffries, 1996; Siika-aho *et al.*, 1994). Acetate is released from the C-3 position of the xylopyranose residues, indicating regioselectivity. The co-operative action of xylanase and xylosidase, together with acetyl esterases are thus required for the hydrolysis of acetylglucuronoxylan (Biely, 1985). Acetylxylan esterases are capable of liberating acetic acid substituents directly from polymeric xylans such as acetylglucuronoxylan, as well as from acetylated xylo-oligomers (Siika-aho *et al.*, 1994; Sunna and Antranikian, 1997).

The term phenolic acid esterase refers to two additional types of enzymes necessary for the complete hydrolysis of xylans, *i.e.* ferulic acid esterases and *p*-coumaroyl esterases (Jeffries, 1996). Ferulic acid esterases and *p*-coumaroyl esterases liberate ferulate and *p*-coumarate, respectively, from soluble acid-sugar esters in xylo-oligomeric substrates (Coughlan and Hazlewood, 1993; Sunna and Antranikian, 1997). *Aspergillus niger* produces a non-specific esterase capable of releasing ferulic, *p*-coumaric and acetic acid from acetylated xylo-oligomers (Coughlan and Hazlewood, 1993; Sunna and Antranikian, 1997).

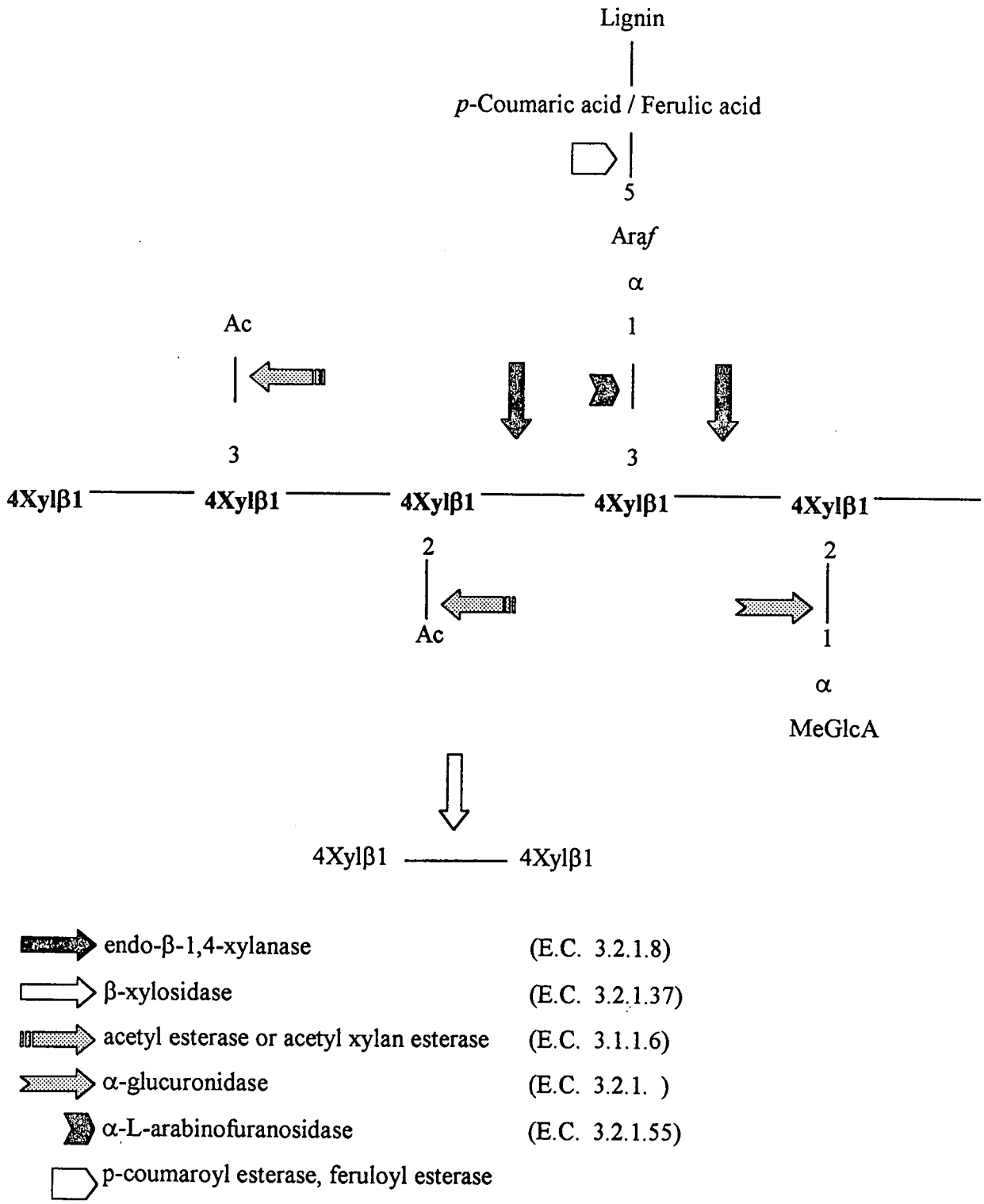


Figure 1.1. A hypothetical plant heteroxylan fragment and the sites of attack by xylanolytic enzymes. MeGlcA, 4-O-methyl glucuronic acid; Ara*f*, α-L-arabinofuranosyl; Ac, acetic acid and 4Xylβ1, β-1,4-linked D-xylose residue (Adapted from Biely *et al.*, 1985, 1992).

1.3.1.3. Xylanase production by *Trichoderma reesei*

Trichoderma species, especially *Trichoderma reesei*, are industrially important fungi capable of producing both cellulolytic and xylanolytic enzymes (Bailey *et al.*, 1993). Apart from being a potent xylanase-producing fungus, *T. reesei* also produces a battery of ancillary hydrolases such as a β-1,4-xylosidase (Herrmann *et al.*, 1997; Margolles-Clark *et al.*, 1996), five β-mannanases (Buchert *et al.*, 1993; Stålbrand *et al.*, 1993), an α-L-arabinofuranosidase (Margolles-Clark *et al.*, 1996), an α-glucuronidase (Siika-aho *et al.*, 1994), two acetylxylan esterases (Siika-aho *et al.*, 1994) and two acetyl esterases (Coughlan and Hazlewood, 1993).

T. reesei produces two major xylanases (Xyn1 and Xyn2) with different pH optima, pH stability, isoelectric points and substrate specificities (Buchert *et al.*, 1992, 1994a; Clarke *et al.*, 1997). Both these xylanases belong to the low molecular weight β-glycanase family G (11), of which the amino acid sequences are highly conserved (Törrönen *et al.*, 1994; Törrönen and Rouvinen, 1997). Both Xyn1 and Xyn2 are small proteins with molecular masses of 19 and 21 kDa consisting of 178 and 190 amino acids, respectively (Buchert *et al.*, 1992; Törrönen *et al.*, 1994). The isoelectric point for Xyn1 is pH 5.2 and for Xyn2 it is pH 9.0 (Törrönen *et al.*, 1994). Xyn2 is a single-domain polypeptide containing two β-sheets, one α-helix and 15 β-strands (Törrönen *et al.*, 1994). The catalytic site of Xyn2 is in the form of a cleft and the putative catalytic residues, Glu 86 and Glu 177, are highly conserved among family G (11) xylanases (Törrönen *et al.*, 1994; Törrönen and Rouvinen, 1997). It has been estimated that four xylose units can fit into the cleft (Törrönen *et al.*, 1994). *T. reesei* hydrolyses unsubstituted xylan slowly with the formation of small amounts of xylotriose and larger amounts of longer xylo-oligomers. However, substituted xylan is hydrolysed more efficiently, resulting in xylotriose as the main reaction product (Buchert *et al.*, 1994a; Törrönen *et al.*, 1994). Xyn2 accounts for more than 50% of the xylanolytic activity of *T. reesei* when grown in xylan-containing media (Buchert *et al.*, 1994a; Törrönen *et al.*, 1994; Törrönen and Rouvinen, 1997).

The xylanolytic complex of filamentous fungi has been studied in some detail. Xylan, xylobiose, xylose, β-methyl-D-xyloside (a synthetic analogue of xylobiose) and higher homologous oligosaccharides (Simão *et al.*, 1997b) have been found to induce the xylanolytic enzyme system of *Aspergillus terreus* (Hrmová *et al.*, 1991), *Aspergillus sydowi* and

Aspergillus tamarii (Simão *et al.*, 1997a, b). By contrast, the xylanolytic system of *Trichoderma reesei* is induced by xylan and xylobiose, but not by xylose (Biely, 1992; Simão *et al.*, 1997a, b). Alkyl-, aryl- and methyl-xylosides are ineffective in inducing xylanase activity in *T. reesei* (Coughlan and Hazlewood, 1993). However, in all cases studied thus far, the synthesis of xylanases was repressed by glucose (Bahkali, 1996; Biely, 1985; Simão *et al.*, 1997a). The induction of xylanase biosynthesis by xylan occurs through soluble low molecular mass catabolites which are generated from xylan by the action of constitutively produced extracellular or cell-bound xylanolytic enzymes (Biely, 1985; Coughlan and Hazlewood, 1993; Prade, 1995; Simão *et al.*, 1997a). Upon liberation of these low molecular weight catabolites, the full xylanolytic capacity of the microorganism is induced.

Xylanase production by *T. reesei* is regulated on the transcriptional level and it differs in respect of *xyn1* and *xyn2*. The *xyn1* promoter is active when xylan and / or xylose is present in the fermentation broth and is silenced in the presence of glucose (Biely, 1985; Zeilinger *et al.*, 1996). The *xyn2* promoter enables low basal transcription regardless of the carbon source, but transcription is greatly enhanced in the presence of xylan, xylobiose, sophorose and cellobiose (Zeilinger *et al.*, 1996). A constitutive protein complex binds to a CCAAT motif located –216 bp upstream of the *xyn2* promoter and is responsible for the basal transcriptional level. In the presence of an inducer compound, an induced protein complex forms that binds to the CCAAT motif in a different manner than the constitutive protein complex, resulting in induction of *xyn2* transcription (Zeilinger *et al.*, 1996).

Production of cellulases and xylanases by *T. reesei* are under separate control mechanisms, therefore virtually cellulase-free xylanolytic enzyme preparations can be obtained from this microorganism through manipulation of the medium composition and cultivation conditions (Bailey *et al.*, 1993; Gamerith *et al.*, 1992; Suh *et al.*, 1988). When *T. reesei* was cultivated on a xylan-based medium at a high pH (minimum pH 6 to 7), a high xylanase production with a low production of cellulase was obtained (Bailey *et al.*, 1993). Gamerith *et al.* (1992) obtained xylanase activities of up to 6668 $nkat.ml^{-1}$ with the ratio of carboxymethylcellulase (CMCase) : xylanase less than 0.015 : 1 when *T. reesei* was grown on hemicellulose substrates obtained from viscose fibre production. In literature xylanase activities of up to 55934 $nkat.ml^{-1}$ have been reported with immobilised cells (Haapala *et al.*, 1994). However, a significant amount of cellulase (12167 $nkat.ml^{-1}$) was also produced, yielding a ratio of xylanase to CMCase of 4.589 : 1.

1.3.2. Industrial applications of xylanases

In general, microbial biotechnology is directed towards the improvement of resource utilisation, optimisation of current processess through the addition of microbially-derived enzymes, the production of flavour compounds, polysaccharides, pigments and antioxidants, as well as reducing the environmental impact of large-scale, well-established industries (Knorr and Sinskey, 1985). During the past two decades, numerous applications have been found for endoxylanases. In this section only the three main areas where xylanases have made an impact in industry will be discussed, although the reader should take note that many other interesting areas for xylanase application exist (Table 1.1). Apart from their important role in the hydrolysis of xylan-containing raw materials used in various industries, xylanases have also been found to play important physiological roles in plant tissues. Xylanases are involved in fruit softening, seed germination and plant defence systems (Deising and Mendgen, 1991; Prade, 1995). Microbial xylanases have the ability to induce ethylene synthesis in plant cells, thus acting as elicitors of the plant defence systems (Apel *et al.*, 1993; Deising and Mendgen, 1991). This recently new role of xylanases in plants might give rise to new applications for xylanases (Prade, 1995).

Table 1.1. Industrial applications of xylanolytic enzymes (Coughlan and Hazlewood, 1993).

Application
Elucidation of the structure of complex xylans
Extraction of juices, flavours, spices, oils and pigments
Clarification of juices and wines
Production of modified xylans as bulking agents used in food processing
Production of sweeteners (xylitol) or flavours from xylan
Modification of cereal flours to enhance the volume, texture and staling properties of bread
Improvement of the nutritional value of silage, wheat- and rye-based animal feedstuffs
Retting of flax, hemp, jute, sisal and bast
Saccharification of agricultural and forestry residues for fermentation to fuels and chemical feedstocks
Prebleaching of pulp during paper manufacturing
Refining of dissolving pulp for the production of viscose rayon, cellulose esters and ethers

1.3.2.1. Pulp and paper industry

Today's consumer increasingly demands products of high quality produced through processess that have little or no impact on the environment. This is due to the global trend in realising that the resources of our planet are limited and should be utilised in a responsible manner. Apart from public perception, governments worldwide are also increasing the restrictions placed on effluents, toxic gases and by-products resulting from industrial activities that are deleterious to the environment. Like any other large-scale industry, the pulp and paper industry is thus also faced with having to reduce their environmental impact (Viikari *et al.*, 1994b).

The essence of pulp bleaching is to remove as much lignin as possible from the pulp, since residual lignin results in the pulp having a brownish colour (Buchert *et al.*, 1994b). Organic chlorine compounds are formed during the chemical bleaching of pulp (Buchert *et al.*, 1994b). These compounds arise from the reactions between the residual lignin present in the pulp fibres and the chlorine used for bleaching (Buchert *et al.*, 1994b; Viikari *et al.*, 1994b). Chlorine gas (Cl_2), and to a lesser extent chlorine dioxide (ClO_2), are responsible for the presence of toxic, mutagenic, adsorbable organic halogens (AOX) or total organically bound chlorine (TOCl) and dioxins in the effluents of pulp bleaching plants (Buchert *et al.*, 1994b; Eriksson, 1991; Senior and Hamilton, 1992; Smith *et al.*, 1994; Tolan and Foody, 1995; Viikari *et al.*, 1994b). Therefore, researchers are seeking methods for producing pulps using non-polluting chemicals, as well as trying to develop more efficient pulping methods to reduce the amount of residual lignin passing to the bleaching stage and to find alternative bleaching methods (Tolan and Foody, 1995; Viikari *et al.*, 1994b).

Enzymatic bleaching, in particular xylanase-aided bleaching, has been found to be the most promising of the new bleaching technologies (Table 1.2). The idea of enzyme-aided bleaching originated from Viikari *et al.* (1986) at the VTT Biotechnical Laboratory in Finland. The effectiveness of xylanase-aided bleaching has been evaluated in three aspects. Firstly, an increase in the amount of solubilised sugars is observed after incubation of xylanase with pulp (Prade, 1995). Secondly, pulps exhibit increased bleachability using conventional bleaching chemicals after xylanase treatment (Viikari *et al.*, 1994a) and thirdly, xylanases facilitated the removal of lignin-carbohydrate complexes (LCC's) from pulp (Prade, 1995; Yang and Erikkson, 1992). Today, xylanase-aided bleaching is used in several kraft pulp mills all over

the world (Vicuña *et al.*, 1995) and several commercial xylanase preparations are available (Table 1.3).

Table 1.2. Technologies available to reduce the amount of dioxins produced as a result of using a bleaching sequence which include active chlorine (Cl_2). (Adapted from Tolan and Foody, 1995).

Technology	**Effect on pulp mill operation**
Increased ClO_2 substitution of Cl_2	High capital expenditure for generator upgrade Possible increase in bleaching cost
Extended delignification	High capital expenditure, retrofits can be difficult
Oxygen delignification	High capital expenditure
Ozone	High capital expenditure, very new technology
Peroxide in alkali stages	Low capital expenditure, but usually more expensive than chlorine
Xylanase enzymes	Low capital expenditure Can be used in combination with other technologies

Table 1.3. Commercial xylanase preparations available for enzymatic bleaching of pulps (Vicuña *et al.*, 1995; Viikari *et al.*, 1994b).

Product name	Application pH	Application temperature (°C)	Supplier
Irgazyme 10	5-7	35-55	Gennencor International
Irgazyme 40	6-8	35-70	Gennencor International
Cartazyme HS	3-5	30-50	Sandoz Chemicals
Cartazyme HT	5-8	60-70	Sandoz Chemicals
Ecopulp	5-6	50-55	Alco, Ltd.
Xylanase	5-6	55	Iogen Corp.
Xylanase	7-8	55	Iogen Corp.
VAI-xylanase	6-7.5	65-75	Voest-Alpine
Pulpzyme HB	6-8	50-55	Novo-Nordisk

The most important criterion for xylanase preparations is no or very low cellulase activity, since contaminating cellulase activity has been found to be deleterious to pulp fibre strength (Bailey *et al.*, 1993; Tolan, 1995). The use of xylanases during the bleaching of kraft (sulphate) pulps were unable to facilitate the production of fully bleached pulp with chlorine-free chemicals (Viikari *et al.*, 1994b). However, xylanases enabled a reduction in the amount of chlorine (Cl_2 and / or ClO_2) necessary to reach the desired target pulp brightness after bleaching or a higher pulp brightness could be achieved using the same amount of chlorine (Buchert *et al.*, 1994b; Viikari *et al.*, 1994a, b). Savings in chlorine consumption of up to 30 % has been reported (Buchert *et al.*, 1992), as well as reductions in the AOX of up to 50 % in the resulting effluents, after pretreatment of pulps with xylanase (Bernier *et al.*, 1994). Several published results have also appeared that discuss the use of xylanases in elemental chlorine free (ECF) and total chlorine free (TCF) bleaching sequences (Gamerith *et al.*, 1992; Paiva *et al.*, 1997; Tolan and Foody, 1995).

Oxygen and ozone are also under investigation to reduce and/or eliminate chlorine compounds used in pulp bleaching (Paiva *et al.*, 1997). These compounds have, however, been found to cause some cellulose degradation through the oxidative attack of cellulose (Gamerith *et al.*, 1992). Xylanases have been shown to improve the bleachability of both unbleached and oxygen-bleached hardwood pulp, as well as oxygen-bleached softwood pulp (Yang and Eriksson, 1992). Degradation of carbohydrates (including cellulose) were even more pronounced with either hot or cold caustic extraction. Xylanases enable the specific removal of xylan, which avoids losses in pulp yield and quality. The major drawbacks of enzyme-aided bleaching are the availability, cost and quality standard of enzyme preparations (Gamerith *et al.*, 1992). These factors are of particular importance in chemical pulp production, where an additional target of pulp bleaching is the selective removal of hemicellulose (Gamerith *et al.*, 1992).

Hemicelluloses are extensively modified during the production of kraft pulp (Buchert *et al.*, 1995). Xylan is partly solubilised in the pulping liquor during the heating period of kraft cooking at high alkaline pH (Buchert *et al.*, 1995; Viikari *et al.*, 1994a, b). As the cooking is continued, the alkalinity decreases and degraded xylan of low depolymerisation precipitates onto the surface of the cellulose microfibrils (Buchert *et al.*, 1995). This precipitated xylan is almost crytalline due to the removal of the side-groups (arabinose and glucuronic acid) present on the 1,4-xylopyranosyl chains during the cooking process (Buchert *et al.*, 1995; Viikari *et*

al., 1994a,b). The precipitated xylan is thought to physically restrict the removal of high molecular weight lignin from the pulp fibres during the subsequent bleaching stages (Viikari *et al.*, 1994a,b). Two hypotheses exist for the mechanism of xylanase-aided bleaching. Firstly, xylanase could enhance bleaching by rendering the fibre structure more porous and permeable, thus aiding the extraction of lignin (Viikari *et al.*, 1994a,b). Secondly, xylanases could increase the extractability of lignin by reducing the amount of lignin-carbohydrate complexes (LCC) present in the pulp fibres (Viikari *et al.*, 1994a,b; Yang and Eriksson, 1992). In addition, the complete removal of hemicellulose (including xylan), chemically or enzymatically, is not always the main aim of (bio)bleaching (see below), since hemicelluloses improve interfibre bonding during paper manufacturing (Viikari *et al.*, 1994b).

The kraft process of chemical pulping is the predominant method world wide. However, the sulphite method of cooking is still important, especially in dissolving pulp production (Christov and Prior, 1993). Dissolving (sulphite) pulp is used for the production of viscose rayon, cellulose esters and ethers (Hinck *et al.*, 1985). Other products derived from dissolving pulp include carboxymethyl cellulose, plastics, lacquers, cellophane packaging, sponges, sausage castings, photographic films and cigarette filters (Hinck *et al.*, 1985). In contrast to pulps used for the production of paper, the presence of hemicelluloses are undesirable in dissolving pulp (Annergren and Rydholm, 1959; Hinck *et al.*, 1985). The hemicelluloses (mainly xylan) remaining in the bleached pulp cause problems in the later alkalisation and spinning stages of the viscose process (Buchert *et al.*, 1995; Hinck *et al.*, 1985). The selective removal of xylan from sulphite dissolving-grade pulps by xylanases could benefit viscose rayon manufacturing and increase the quality of the final product (Annergren and Rydholm, 1959; Hinck *et al.*, 1985).

Due to the specificity of enzymes, they can also be used as analytical tools to aid in fibre characterisation (Buchert *et al.*, 1995). Xylanases attack only pulp xylan without solubilising the other carbohydrates present under mild hydrolysis conditions, thus no additional destruction of the solubilised carbohydrates or fibres occur. Through combination of enzymatic peeling of fibres with powerful analytical techniques, such as NMR spectroscopy, novel information can be obtained regarding the structure of pulp fibres (Buchert *et al.*, 1995). This is useful to determine the effects of cooking time and type of bleaching chemicals used on the structure of surface xylan, as well as the structure and amount of side-groups (Buchert *et al.*, 1995).

Xylanases, especially Xyn2, produced by *T. reesei* have been found to be effective in biobleaching (Bailey *et al.*, 1993). Treatment of softwood pulp with crude culture filtrate and the purified Xyn2 (pI 9) xylanase (Table 1.4) resulted in solubilisation of 0.64 % and 0.58 % of the pulp, respectively (Bailey *et al.*, 1993). These authors found a significant increase in pulp brightness after enzymatic treatment, with only a slight reduction in viscosity (indicating that the culture filtrate was sufficiently free of cellulase and thus suitable for use in pulp treatments).

Table 1.4. Peroxide delignification of softwood kraft pulp treated with concentrated *Trichoderma reesei* culture filtrate (unpurified xylanase) produced on xylan and with a purified pI 9 (Xyn2) xylanase of the same organism (Bailey *et al.*, 1993).

Enzyme	**Solubilized sugars (% of dry weight)**				**Kappa number**	**Brightness (%)**	**Viscosity ($dm^3.kg^{-1}$)**
	DNS	**HPLC**					
		Glu	**Xyl**	**Ara**			
Unpurified xylanase	0.64	<0.02	0.68	<0.02	16.0	50.8	940
Xyn2 (pI 9)	0.58	<0.02	0.66	<0.02	16.1	50.7	960
Reference	0.10	<0.02	0.04	<0.02	18.4	48.5	960

The xylanase dosage was 500 $nkat.g^{-1}$ pulp.

Reference pulp was treated under the same conditions but without addition of enzyme.

DNS, reducing sugars by the DNS method; HPLC, analysis of monomers after secondary enzymatic hydrolysis of the solubilized oligomers: Glu, glucose; Xyl, xylose; Ara, arabinose.

1.3.2.2. Food and animal feed industry

Animal feed industry. Animal feed is supplemented with enzymes for two reasons. Firstly, enzyme supplementation degrades soluble fibre with anti-nutritional properties or improves the apparent metabolisable energy (AME) of cereals and, secondly, it supplements the animal's own digestive enzymes during maturation (Cowan, 1996). Cereals and vegetable protein raw materials used in animal feed can be subdivided into four main groups on the basis of their fibre composition (Table 1.5). The choice of enzymes to be used depends on the type and relative percentage of the raw materials in the feed (Chesson, 1993). It is thus clear that enzymes should be matched with the raw materials used in order to increase the efficiency and economic viability of enzyme addition.

In general, commercial pig and poultry farmers use cereal-based feeds. Thus, the cost and nutritive value of cereals are important factors determining profitability of enzyme addition (Chesson, 1993). The non-starch polysaccharide (NSP) content of cereals (*e.g.* β-glucan and arabinoxylan) have been found to vary with variety, climatic conditions and region where the cereals were cultivated (Annison, 1993; Chesson, 1993; Cowan, 1996). By-products from the milling and agricultural industries can also be upgraded by supplementation with enzymes. Treatment of wheat millings with xylanases and a combination of xylanases and proteases have been shown to improve protein availability and energy liberation (Cowan, 1996). The remainder of this section will focus on the benefits of xylanase-based enzyme preparations used in pig and poultry feed.

Table 1.5. Classification of raw materials into four groups based on their fibre composition. (Adapted from Chesson, 1993; Cowan, 1996; Graham and Inborr, 1992).

Group	Raw materials	Main non-starch polysaccharide (NSP)	Main enzyme(s) used in supplementation
1	Barley, oats	β-Glucan	β-glucanases
2	Wheat, rye, triticale	Arabinoxylan (pentosan)	β-xylanases
3	Sorghum (white), maize	-	α-amylases
4	Vegetable protein	Galactosaccharides and pectic materials	α-galactosidases, proteases, pectinases, phytases

The use of xylanase-based multi-enzyme products to supplement pig and poultry diets based on wheat, rye and triticale has come of age (Chesson, 1993; Graham and Inborr, 1992). These kinds of enzyme products also contain other enzymes such as β-glucanases, pectinases, α-amylases, phytases, carboxymethylcellulases (CMC'ases) and proteases, since feeds consist of a mixture of cereals and other feedstuffs (Chesson, 1993; Cowan, 1996; Graham and Inborr, 1992; GrootWassink *et al.*, 1989).

The AME value of some cereals, especially of wheat, is highly variable and sometimes markedly lower than would be expected from their nutrient content (Annison, 1993; Chesson, 1993; Cowan, 1996). Arabinoxylans have been implicated in reducing the AME of wheat, rye and triticale, the causing of digestive disorders and faecal litter problems (Annison, 1993; Graham and Inborr, 1992). The inclusion of xylanases in feeds containing a significant amount of arabinoxylan improved the digestion and absorption of starch, fats, protein, sodium, ammonium and calcium salts (Annison, 1993; Bedford and Classen, 1992; Graham and Inborr, 1992; GrootWassink *et al.*, 1989). The main findings supporting the hypothesis of the anti-nutritive effect of soluble NSP cell wall components of wheat (mainly highly branched arabinoxylan) were: (1) wheat AME values correlated negatively with soluble NSP levels, (2) addition of NSP to broiler diets depressed the AME of the diets, (3) enzymatic degradation of the cell wall polysaccharides *in situ* raised the AME values and (4) addition of purified wheat-arabinoxylan depressed the AME of wheat in a dose-dependent manner (Annison, 1993; Bedford and Classen, 1992). The improvement of nutrient utilisation by the addition of xylanases to feed was attributed to two factors. Firstly, the hydrolysis of arabinoxylan resulted in reduced viscosity of the intestinal tract, allowing more efficient digestion and absorption of nutrients in the small intestine (Bedford and Classen, 1992; GrootWassink *et al.*, 1989). Secondly, fibre polysaccharides (*e.g.* arabinoxylan) are associated with cell walls, which encapsulates other nutrients. By rendering the cell walls more permeable, nutrients were more readily available to the animal's own digestive enzymes (Graham and Inborr, 1992).

When piglets were fed feed supplemented with a xylanase-based multi-enzyme preparation, significant improvements in average daily live weight gain and feed conversion efficiency could be observed (Graham and Inborr, 1992). In comparison with the control group, the experimental group reached their target live weight earlier with less variation in final live weight (Graham and Inborr, 1992). The reduced variation in final live weight suggested that enzyme supplementation of feed benefited the poorer performing piglets more (Graham and

Inborr, 1992). Interestingly, enzyme-supplemented feed also caused a reduction in the frequency and severity of diarrhoea (resulting from improved feed digestibility and reduction in the negative effects of anti-nutritional factors), neonatal mortality, as well as the necessity of antibiotic treatments (Chesson, 1993; Cowan, 1996; Graham and Inborr, 1992).

Enzyme supplementation of broiler chicken feed improved feed conversion efficiency as a result of more efficient digestion and absorption of nutrients in the small intestine (Bedford and Classen, 1992). Broiler chickens fed rye as a cereal source fail to thrive due to the presence of arabinoxylan that increase digesta viscosity and consequently reduce digestibility of nutrients (Bedford and Classen, 1992; GrootWassink *et al.*, 1989). However, addition of a xylanase-based enzyme preparation to a rye-based feed was shown to effectively improve growth performance of the chickens (GrootWassink *et al.*, 1989). Xylanase-based products have also been shown to improve litter quality by reducing the viscosity and water-binding capacity of the excreta as a result of polysaccharide (arabinoxylan) degradation (Graham and Inborr, 1992). The addition of xylanases to a wheat-based feed has been shown to significantly increase the AME value of the feed, especially of feeds containing wheat with a low-AME value (Chesson, 1993).

Supplementation of wheat-based turkey poult diets with xylanases improved the live weight gain and feed conversion efficiency of the birds, especially during the early parts of the feed programme (Cowan, 1996). Thus, the positive effects of xylanase addition decreased as the turkeys aged. Laying hens fed barley, oats or wheat-based diets exhibited an increased frequency in contamination of eggs with fecal excreta or 'dirty eggs' (Chesson, 1993). Supplementation of barley and wheat with β-glucanases and β-xylanases, respectively, decreased the frequency of egg contamination (Cowan, 1996). Eggs marked with droppings have a reduced market value.

The survival of enzyme activities present in above-mentioned coctails is important. Chesson (1993) found that enzyme activity was sufficiently retained during feed preparation (*e.g.* pelletting of feed), as well as within the host (Cowan, 1996). The use of enzyme 'cocktails' has come of age in various parts of the world and numerous enzyme supplementations, especially formulated for the feed industry, are now readily available (Chesson, 1993).

Food industry. People are constantly looking for methods and additives that will either improve dough processing or the quality of the baked products. A baking process is evaluated on the basis of criteria such as dough handling (machinability) and process yield. The quality of a baked product is evaluated on appearance (volume, colour, texture) and eating properties (crump elasticity, flavour, aroma). Xylanases from *Aspergillus niger* var. *awamori* have been found to substantially improve bread volume, reduce the stickyness of dough and improve the crump stucture of bread (Maat *et al.*, 1992). In addition, partial hydrolysis of xylan to xylo-oligosaccharides are used as moisture-preserving food additives (Biely *et al.*, 1992). Other biotechnological applications of xylanases in the food industry include liquefaction of coffee mucilage, alteration of the rheological and organoleptic properties of musts and wines and the extraction of pigments and flavour compounds (Coughlan, 1992).

1.3.2.3. Production of fuel ethanol from lignocellulosic waste

Probably the most compelling stimilus for research on hemicellulolytic enzymes was the oil crisis of the seventies, which sparked the search for alternative sources of liquid fuel and chemical feedstocks (Coughlan, 1992). Available plant biomass from fuel crops, agricultural and forestry residues and wastes are generated in vast tonnages annually and contain one or more of three major complex carbohydrates, *i.e.* cellulose, starch and xylan. Xylan represents a large fraction of biomass that could be used for the production of fuel alcohol and chemical feedstock molecules (Hayn *et al.*, 1993; Parisi and Parisi, 1989). Economic viability of biomass-based fuel alcohol production depends on the effective bioconversion of cellulose, but effective conversion of the xylan component will further enhance the economic gains of such a process (Hayn *et al.*, 1993). The development of microorganisms that have cellulolytic, amylolytic and xylanolytic properties, as well as a high tolerance to ethanol and osmotic stress, have interested various researchers worldwide (Alexander, 1986). In view of the above, the production and sale of cellulolytic and hemicellulolytic enzymes would be of considerable economic importance (Coughlan and Hazlewood, 1993). However, due to economic considerations the bioconversion of biomass has yet to be realised (Coughlan, 1992). A biological process for fuel-ethanol production from lignocellulose requires: (1) delignification to liberate cellulose and hemicellulose from LCC's, (2) depolymerisation of cellulose and hemicellulose into their coresponding monomeric sugars and (3) fermentation of the mixed sugars, comprising hexose and pentose sugars, to ethanol (Lee, 1997).

Hydrolysis of xylan by xylanases and xylosidases yield high levels of xylose, without the by-product formation associated with acid hydrolysis (Suh *et al.*, 1988). The first step in the xylose-degradation pathway is the convertion of xylose to xylulose. Bacteria convert xylose to xylulose in a one-step reaction catalysed by xylose isomerase. Yeasts, however, use a two-step process. Firstly, xylose is reduced to xylitol by xylose reductase and then converted to xylulose by xylitol dehydrogenase (Alexander, 1986). This two-step process is very demanding on the NAD(P)H / NAD(P) pools in the cells and is, therefore, a possible limitation in xylose degradation (Alexander, 1986).

No known yeast possesses all the characteristics necessary for industrial-scale fermentation of lignocellulolytic materials. The ideal organism should be capable of a high ethanol productivity and yield, pentose and hexose fermentation, as well as being resistant to inhibitors produced during the pretreatment and hydrolysis of lignocellulose (Hayn *et al.*, 1993). *Pichia stipitis*, *Candida shehatae*, *Pachysolen tannophilus* and *Fusarium oxysporon* are capable of fermenting D-xylose to ethanol. However, none of them possess all the desired characteristics for industrial-scale application (Hahn-Hägerdal *et al.*, 1994; Spencer-Martins, 1994).

A recombinant *Saccharomyces cerevisiae* strain capable of efficient conversion of xylan or xylose to ethanol (Figure 1.2) might be realised in the forseeable future. *S. cerevisiae* is incapable of fermenting xylose, but can ferment xylulose, an isomer of xylose (Hahn-Hägerdal *et al.*, 1994). In theory, the genes encoding xylan-degradation and the isomerisation of xylose to xylulose can be cloned into *S. cerevisiae*, yielding a strain with the desired characteristics for lignocellulose fermentation. Inhibition of yeast growth on lignocellulotic hydrolysates could partially be overcome by de-toxifying the substrate, allowing the yeast to adapt, using a continuous process or a combination of these procedures (Lee, 1997). Although present *S. cerevisiae* strains ferment xylose poorly, it still remains a novel possibility for commercial xylose fermentation (Hahn-Hägerdal *et al.*, 1994).

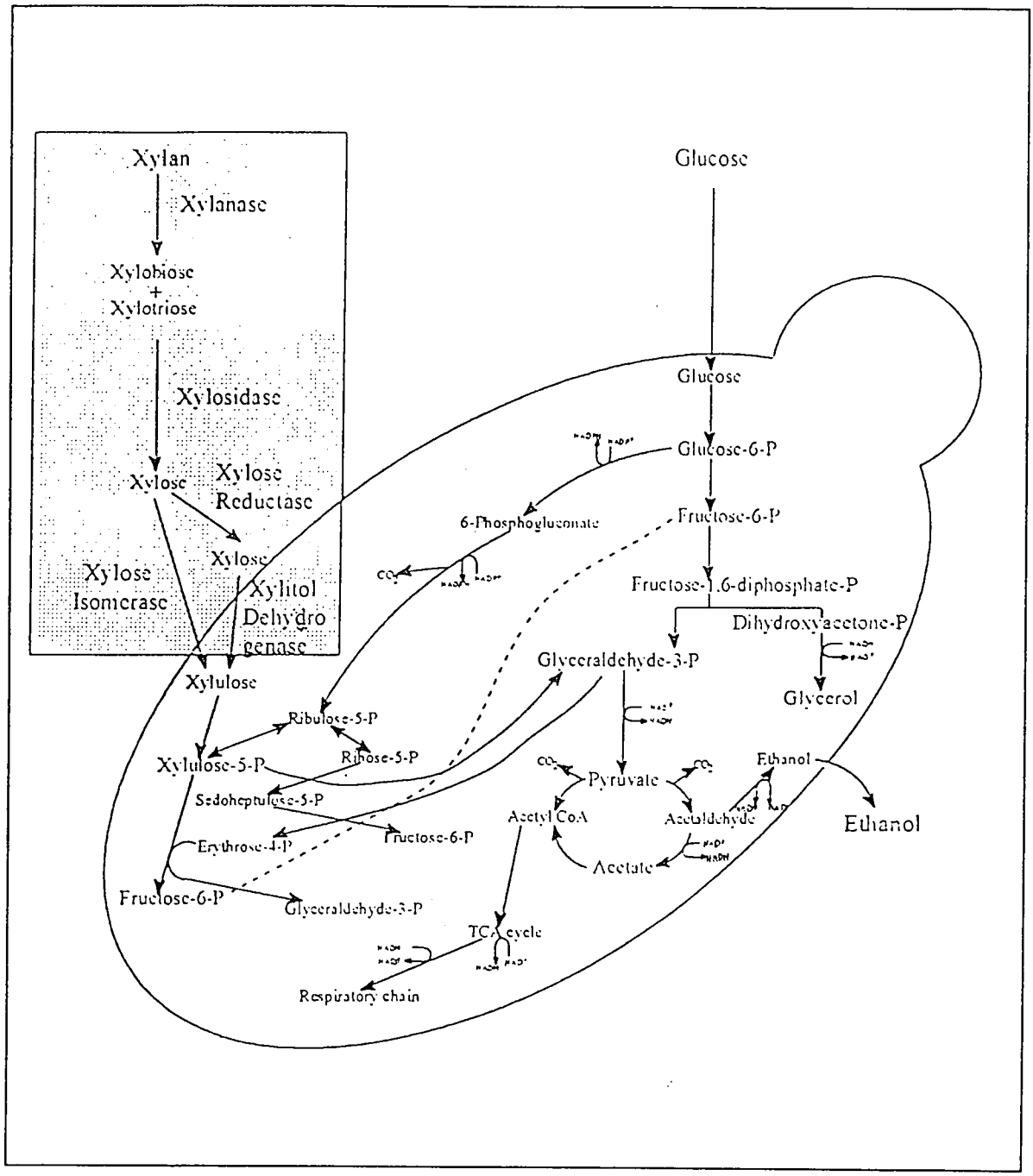


Figure 1.2. The proposed pathway for xylose and glucose metabolism in *Saccharomyces cerevisiae* when genes encoding heterologous xylanase, xylosidase and either xylose reductase / xylitol dehydrogenase or xylose isomerase are integrated into the yeast genome. The grey box indicates the action of the heterologous enzymes in such a yeast strain when grown on xylan (adapted from Hahn-Hägerdal *et al.*, 1994; Luttig, 1997).

1.3.3. Heterologous protein production by *Saccharomyces cerevisiae*

Heterologous gene expression in yeasts has become a well documented routine procedure (see Gancedo, 1992; 1998; Klein *et al.*, 1998; Loison *et al.*, 1986; Romanos *et al.*, 1992 and references cited therein). Yeasts are unicellular, ontogenic, eukaryotic microorganisms capable of performing eukaryotic processing steps on the heterologous polypeptide(s) expressed by them (Gellissen and Hollenberg, 1997). Yeasts are suitable hosts for the expression of heterologous (eukaryotic) proteins for a number of reasons. Firstly, yeasts are capable of rapid growth on low-cost media (Romanos *et al.*, 1992). Secondly, high expression levels of heterologous protein can be obtained and, thirdly, post-translational modifications and efficient secretion of the heterologous proteins are possible (Faber *et al.*, 1995; Sudbery, 1996). *S. cerevisiae* was the first yeast species to be exploited for heterologous gene expression due to its strong historical background in the baking and brewing industries (Faber *et al.*, 1995). Yeasts have been the choice organism of researchers to produce pharmaceutical proteins, because yeasts do not contain toxic cell wall pyrogens (as in *Escherichia coli*) nor oncogenic or viral DNA as found in mammalian cells (Loison *et al.*, 1986; Romanos *et al.*, 1992; Tøttrup and Carlsen, 1989).

1.3.3.1. Factors influencing secretion of heterologous proteins by *Saccharomyces cerevisiae*

Efficient production of heterologous proteins in *S. cerevisiae* can be obtained by cloning the foreign gene of interest into a plasmid vector, which replicates at a high copy number or which is integrated into the genome (Buckholz and Gleeson, 1991; Loison *et al.*, 1986). To ensure that the plasmid is stably inherited, a selection system has to be used to ensure counterselection of cells which have lost plasmid-borne information (Buckholz and Gleeson, 1991). The most commonly used selection systems employ an auxotrophic host strain with a single-gene defect, with the plasmid providing the complementing function (Buckholz and Gleeson, 1991; Loison *et al.*, 1986; Romanos *et al.*, 1992). Selection of plasmid-bearing cells is achieved through cultivation of the recombinant cells in a minimal medium deficient of any molecules that can cure the cells of their auxotrophy (Buckholz and Gleeson, 1991; Loison *et al.*, 1986).

Protein secretion in yeast is directed by an amino-terminal signal (prepro) sequence which mediates co-translational translocation into the endoplasmic reticulum (ER). An appropriate leader sequence (prepro elements) is necessary for post-translational modifications and secretion of eukaryotic proteins (Romanos *et al.*, 1992; Sudbery, 1996). This is achieved by fusing the protein sequence to an N-terminal leader sequence that directs the nascent polypeptide to the secretory apparatus. Heterologous protein may be secreted using either a foreign signal, often derived from the protein being secreted, or a yeast signal. The MFα1 leader sequence from *S. cerevisiae* functions in all the yeasts (Gellissen and Hollenberg, 1997; Tøttrup and Carlsen, 1989).

Apart from MFα1, the genuine leader sequence of the heterologous protein to be expressed could also sometimes (not always) be used. However, due to the difficulty in predicting whether a particular foreign signal sequence will function in yeast, the use of homologous signal sequences from acid phophatase (*PHO5*), invertase (*SUC2*) or mating factor α-1 (*MFα1*) have been frequently used for heterologous protein secretion in *S. cerevisiae* (Tøttrup and Carlsen, 1989). The signal peptide is removed by a signal peptidase followed by the addition of N- (asparagine-linked) and O- (serine or threonine) linked glycosyl structures. Proteins are then transported to the Golgi-apparatus where modifications to the glycosyl structures take place (Sudbery, 1996). The last stage encompasses the packaging of proteins into secretory vesicles which are delivered to the cell surface. Problems in the secretory process may occur at any stage of heterologous protein secretion. The most common problems encountered include improper folding and disulphide bond formation, retention of heterologous proteins in the ER, Golgi-apparatus or cell wall, degradation of retained heterologous proteins in the ER, aberrant processing and hyperglycosylation (Sudbery, 1996). Hyperglycosylation is increasingly being considered as a major drawback to the secretion of therapeutic glycoproteins by *S. cerevisiae*, since it could change the functionality or immunogenecity of the protein (Kukuruzinska, 1987; Romanos *et al.*, 1992; Sudbery, 1996).

1.3.3.2. Strategies to improve secretion of heterologous proteins by *Saccharomyces cerevisiae*

Screening has frequently resulted in the isolation of 'super-secreting' recombinant yeast strains (Romanos *et al.*, 1992). Smith *et al.* (1985) employed a mutagenesis approach in

conjunction with a rapid screening procedure to isolate 'super' calf prochymosin-secreting colonies. Two 'super-secreting' strains were identified and designated as *ssc1* and *ssc2* (Smith *et al.*, 1985). The secretion of the heterologous calf prochymosin was increased from less than 1 % to more than 80 % of the amount of protein produced (Smith *et al.*, 1985). Selection directed towards identifying mutants that are resistant to the toxic effects of a foreign protein has also proved to be successful. Shuster *et al.* (1989) exploited the slow growth rate of human insulin-like growth factor 1 (IGF-1)-expressing cells through the isolation of faster growing IGF-1-expressing cells which accumulated in a population of IGF-1-expressing cells under selection. Mutations at a single locus (*HPX1*) resulted in increased resistance to IGF-1 toxicity as well as increased IGF-1 production (Shuster *et al.*, 1989). Thirdly, heterologous protein production can also be improved by decreasing the cultivation temperature. Tøttrup and Carlsen (1989) found an increase in the yield of heterologous superoxide dismutase-human proinsulin fusion protein (SOD-PI) upon changing the cultivation temperature from 30 to 26 °C and concluded that the increased yield achieved was due to decreased proteolytic activity at the lower temperature.

1.3.3.3. Effect of medium composition and cultivation protocol on growth and heterologous protein production

Synthesis of a cloned gene product depends on both genetic (plasmid stability, copy number, promoter strength) and environmental factors (growth rate, medium composition) (Chiruvolu *at al.*, 1996; Marquet *et al.*, 1987). A major disadvantage of using a recombinant yeast strain for heterologous protein production may be plasmid stability. Therefore; integrated expression vectors are gaining in popularity (Chiruvolu *at al.*, 1996). Although plasmid loss can be reduced by using a selective medium for growth, it often results in a lower growth rate, cell yield and overall productivity (Chiruvolu *at al.*, 1996; Lee *et al.*, 1986). The specific growth rate can significantly influence plasmid stability (Chiruvolu *at al.*, 1996; Lee *et al.*, 1986). The medium composition, therefore, also plays a critical role in the design of a process for heterologous protein production by a recombinant yeast strain.

Chiruvolu *at al.* (1996) descibed the production of heterologous ovine interferon-τ in *S. cerevisiae*, using an *ADH2 / GAPDH* hybrid promoter in fed-batch cultures. The recombinant was grown under glucose limitation to reduce ethanol accumulation and, during the production

period, ethanol was continuously fed to the bioreactor at a rate of 1 $g.l^{-1}.h^{-1}$. They concluded that the use of a complex medium (YEP) improved plasmid stabibility, growth rate and ovine interferon-τ production as compared to minimal media with selective pressure (Chiruvolu *at al.*, 1996). By contrast, Lee *et al.* (1986) found plasmid stability to be lower in complex media as compared to selective media. However, complex media facilitated a higher rate of growth and hepatitis B surface antigen production and were, therefore, more suitable for heterologous protein production as compared to the selective medium (Lee *et al.*, 1986).

1.3.3.4. Other yeasts employed in the production of heterologous proteins

In addition to *S. cerevisiae*, yeast host-vector systems have also been developed for the methylotrophic yeasts *Hansenula polymorpha*, *Pichia pastoris* and *Candida boidinii* as well as for the budding yeast *Kluyveromyces lactis* (Buckholz and Gleeson, 1991; Gellissen and Hollenberg, 1997; Sudbery, 1996). This is due to the fact that heterologous proteins expressed in *S. cerevisiae* often exhibited organism-specific limitations such as a lack of strong, tightly-regulated promoters, poor secretion efficiency of proteins larger than 30 kDa, instability of the vector in the production strain, hyperglycosylation of foreign proteins and inability to reach high cell densities in fed-batch cultures (Faber *et al.*, 1995; Romanos *et al.*, 1992). The methylotrophs and *K. lactis* have been shown to secrete heterologous proteins at least as efficiently as *S. cerevisiae* and, in many instances, a higher yield of heterologous protein was obtained which was also of a better quality (Gellissen and Hollenberg, 1997). In all the above-mentioned non-*Saccharomyces* yeasts, strong inducible promoters have been isolated from their special metabolic pathways. The commercial production of a huge number of heterologous proteins using these yeast species has been described in literature (see Faber *et al.*, 1995; Gellissen and Hollenberg, 1997; Romanos *et al.*, 1992 and the references therein).

Methylotrophic yeasts are capable of utilising methanol as their sole source of carbon and energy (Faber *et al.*, 1995). To date, all the methylotrophic yeasts identified belong to the genera *Hansenula*, *Pichia*, *Candida* and *Torulopsis* (Faber *et al.*, 1995). The isolated genes encoding the key enzymes in the methanol-oxidising pathway of the methylotrophic yeasts have been found to provide strong inducible promoters capable of facilitating efficient expression of heterologous proteins (Gellissen and Hollenberg, 1997). The key enzymes of the methanol-oxidising pathway have been found to constitute up to 60 to 80 % of the total

intracellular protein content of methanol-grown cells (Faber *et al.*, 1995; Gellissen and Hollenberg, 1997). The first step in methanol utilisation is the conversion of methanol to formaldehyde, catalysed by alcohol oxidase. *P. pastoris* contains two highly homologous alcohol oxidase genes, namely *aox1* and *aox2* (Gellissen and Hollenberg, 1997). In *H. polymorpha*, a single methanol oxidase gene (*mox*) exists (Gellissen and Hollenberg, 1997). The alcohol oxidase genes are induced to considerable levels in methanol-containing media and in the case of *H. polymorpha*, considerable expression of *mox* can also be obtained in media supplemented with low levels of glycerol to facilitate derepression of *mox* (Gellissen and Hollenberg, 1997). Formaldehyde can either enter the assimilatory pathway directly to be used for biomass production or it can be oxidised through two dehydrogenase reactions to CO_2, the latter step being catalysed by formate dehydrogenase (Gellissen and Hollenberg, 1997). The use of *aox1*, *mox* and *fmd* (encoding formate dehydrogenase) promoter sequences has resulted in efficient hererologous gene expression in *P. pastoris* and *H. polymorpha* (Faber *et al.*, 1995; Gellissen and Hollenberg, 1997; Sudbery, 1996). Commercial production of heterologous proteins by these two methylotrophs is now common practise (see review by Faber *et al.*, 1995). Apart from cytosolic accumulation and secretion of heterologous proteins, methylotrophic yeasts can store heterologous proteins in peroxisomes. This compartimentalisation of foreign proteins is especially advantageous if the protein of interest is highly susceptible to proteolytic degradation or when it is toxic to the host (Faber *et al.*, 1995; Sudbery, 1996).

K. lactis is able to utilise lactose as sole carbon and energy source. Enzymes necessary for the metabolism of lactose are strongly induced in lactose-containing media, *e.g* containing whey (Gellissen and Hollenberg, 1997). Lactose uptake is facilitated by lactose permease (encoded by the *lac12* gene) and converted to glucose and galactose by β-galactosidase, encoded by *lac4* (Gellissen and Hollenberg, 1997). The *lac4* gene is strongly expressed in the presence of lactose and galactose, thus serving as a promising element for heterologous gene expression (Gellissen and Hollenberg, 1997; Romanos *et al.*, 1992).

Yarrowia lipolytica is a dimorphic yeast which has been commercially exploited for the production of citric acid, 2-keto glutarate, erythritol, mannitol and iso-propyl malate (Romanos *et al.*, 1992). In addition, *Y. lipolytica* is capable of converting fatty acids and alkanes into alcohol and the production of single cell protein from *n*-paraffins (Buckholz and Gleeson, 1991; Romanos *et al.*, 1992). The unique genotype of this organism has also been

exploited for obtaining powerful, tight regulatory promoters which could be successfully exploited for heterologous protein production (Buckholz and Gleeson, 1991).

The use of the fission yeast *Schizosaccharomyces pombe* has been relatively limited with regard to heterologous protein production (Buckholz and Gleeson, 1991). This is due to the fact that little fermentation technology has been developed for this organism and relatively few inducible promoters are present in this organism (Buckholz and Gleeson, 1991; Romanos *et al.*, 1992).

1.3.4. Genetic manipulation of *Saccharomyces cerevisiae* to degrade xylan

1.3.4.1. Heterologous protein expression in *Saccharomyces cerevisiae* using the *ADH2* promoter

ADH2, SUC2 and *CYC1* encoding alcohol dehydrogenase II, invertase and iso-1-cytochrome c, respectively, are examples of promoters primarily regulated by glucose repression. *ADH2* is a powerful, tightly regulated promoter that has been used for the expression of heterologous proteins such as toxic insulin-like growth factor 1 (IGF-I) (Price *et al.*, 1990; Shuster, 1989). *ADH2* is repressed over 100-fold by glucose (Shuster, 1989).

Cousens *et al.* (1987) described the production of human pro-insulin of up to 100 $mg.l^{-1}$ by *S. cerevisiae*, using a regulated hybrid promoter (*ADH2 / GAPDH*) consisting of the regulatory region of *ADH2* fused to the 3' region of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Chiruvolu *et al.* (1996) descibed the production of heterologous ovine interferon-τ in *S. cerevisiae*, using an *ADH2 / GAPDH* hybrid promoter in fed-batch cultures. The recombinant was grown under glucose limitation to reduce ethanol accumulation, which results in a lower biomass yield. During the production period, ethanol was continuously fed to the bioreactor at a rate of 1 $g.l^{-1}.h^{-1}$ (Chiruvolu *et al.*, 1996)

Tøttrup and Carlsen (1989) developed a fed-batch cultivation process for the production of SOD-pro-insulin using an *ADH2 / GAPDH* hybrid promoter. In these experiments, a constant, empirically-determined glucose feed was used, which resulted in a glucose excess during most of the growth phase with a low residual glucose concentration (20 $mg.l^{-1}$) during the later

stages as the cell density increased (Tøttrup and Carlsen, 1989). Upon changing from growth on glucose to an ethanol feed, the concentration of SOD-pro-insulin increased from 1.2 $g.l^{-1}$ to 1.6 $g.l^{-1}$ (Tøttrup and Carlsen, 1989). These authors concluded that the relatively small increase (25%) in product concentration was due to partial repression of the promoter by the residual glucose (20 $mg.l^{-1}$) present in the medium.

Several studies described the cloning and expression of cellulolytic and hemicellulolytic enzymes in *S. cerevisiae*, directed towards producing recombinant yeast strains capable of a one-step polysaccharide fermentation to yield commercially important commodities (Table 1.6). However, in none of these studies (Luttig *et al.*, 1997; van Rensburg *et al.*, 1997, 1998) were fermentation trials carried out to study the physiology of foreign gene expression. In conclusion, fed-batch cultivations using an ethanol feed during the production period was the most successful to facilitate *ADH2* or *ADH2 / GAPDH*-regulated heterologous protein production by *S. cerevisiae* (Chiruvolu *et al.*,1996; Cousens *et al.*, 1987; Tøttrup and Carlsen, 1989).

Table 1.6. Expression of heterologous enzymes in *Saccharomyces cerevisae* regulated by an *ADH2* promoter or a hybrid promoter using the regulatory sequence of *ADH2*.

Heterologous protein(s)	**Conc.**	**Reference**
Bacillus subtilis endo-β-1,3-1,4-glucanase and *Butyvibrio fibrisolvens* endo-β-1,4-glucanase	-	Van Rensburg *et al.*, 1997
Butyvibrio fibrisolvens endo-β-1,4-glucanase	-	Van Rensburg *et al.*, 1998
Aspergillus kawachii endo-β-xylanase	-	Luttig *et al.*, 1997
Aspergillus niger var. *awamori* endo-β-xylanase	-	Luttig *et al.*, 1997
Human insulin-like growth factor 1 (IGF-I)	-	Shuster, 1989
Human proinsulin as SOD-PI[a]	1500 $mg.l^{-1}$	Tøttrup and Carlsen, 1989[b]
Ovine interferon-τ	49 $mg.l^{-1}$	Chiruvolu *et al.*, 1996[b]
Human proinsulin	100 $mg.l^{-1}$	Cousens *et al.*, 1987[b]

[a] SOD-PI, human superoxide dismutase-human proinsulin fusion product

[b] *ADH2/GAPDH* hybrid promoters were used

1.3.4.2. Function and regulation of *ADH2* in *Saccharomyces cerevisiae*

Alcohol dehydrogenases catalyse the reversible transfer of hydrogen to the carbonyl group of aldehydes and related compounds, making them ubiquitous in the majority of eukaryotes and in some prokaryotes (Ciriacy, 1997). Alcohol dehydrogenases play an important physiological role in *S. cerevisiae* and related species. *S. cerevisiae* utilises almost all available sugar fermentatively, regardless of oxygen availability. Adh1 catalyses the reduction of acetaldehyde to ethanol, regenerating the glycolytic NAD^+ (Russell *et al.*, 1983; Saliola *et al.*, 1990). Upon depletion of the fermentable hexose sugar, oxidative assimilation of the accumulated ethanol follows, which is catalysed by Adh2 (Denis *et al.*, 1981; Russell and Hall, 1983). Thus, alcohol dehydrogenases serve to link oxidative and fermentative metabolism in *S. cerevisiae*, allowing optimal utilisation of the available hexose sugar (Figure 1.3) (Ciriacy, 1997). The remainder of this section will focus on the molecular mechanisms controlling *ADH2* expression. The regulation of *ADH1* is also briefly discussed.

Both Adh1 and Adh2 are located in the cytoplasm. Adh1 and Adh2 are homotetrameric structures, requiring bound zinc (Zn^{2+}) near its catalytic centers, as well as a second zinc per subunit for stabilisation of the tertiary structure (Ciriacy, 1997). Adh3 (mADH) is associated with the mitochondrion; however, Adh3 cannot functionally substitute the cytoplasmic Adh isozymes since cytoplasmic NADH cannot enter the mitochondrial matrix (Ciriacy, 1997; Russell and Hall, 1983; Saliola *et al.*, 1990). Apart from the three major alcohol dehydrogenases mentioned above, three additional alcohol dehydrogenases have been identified by genetic means, namely Adh4, Adh5 and Adh6 (Ciriacy, 1997; Lutstorf and Megnet, 1968; Saliola *et al.*, 1990). Interestingly, Adh4 and Adh5 resemble bacterial alcohol dehydrogenases more than their yeast counterparts and are, therefore, considered candidates for horizontal gene transfer between yeast and bacteria (Ciriacy, 1997). The function and significance of the last three alcohol dehydrogenases have not yet been elucidated, since they are not expressed during normal growth conditions (Ciriacy, 1997).

The levels of Adh1 and Adh2 isozymes are primarily regulated by the presence of fermentable hexose sugars *via* regulation of *ADH1* (*ADC1*) and *ADH2* (*ADR2*) transcription (Denis *et al.*, 1981; Karnitz *et al.*, 1992). Adh1 production is induced five to ten-fold by fermentable hexose sugars, *e.g.* glucose (Ciriacy, 1997; Saliola *et al.*, 1990). The molecular basis and regulation of *ADH1* expression have not been fully unravelled yet. A conserved upstream

activating sequence, UAS_{RPG} located at –664 bp, seem to be essential for high-level *ADH1*-expression (Ciriacy, 1997). Deletion of UAS_{RPG} does not change the regulatory pattern of *ADH1* expression, but reduces expression several-fold (Ciriacy, 1997). UAS_{RPG} binds a general transcriptional activator Rap1, as well as the positively *trans*-activating Gcr1 protein and together this tenary complex is essential for activation of *ADH1* transcription (Ciriacy, 1997).

The regulation of the *ADH2* gene has been extensively studied in order to elucidate the molecular mechanisms responsible for carbon catabolite repression. In contrast to the regulation of most genes subject to carbon catabolite repression, there is no evidence for a Mig1 binding site in the *ADH2* promoter region (Gancedo, 1998). In addition, it appears that, except for Snf1 and Reg1, most of the elements that control the expression of carbon catabolite repressible genes are not operating on *ADH2* (Gancedo, 1998). *ADH2* is expressed only in the absence or depletion of a fermentable hexose sugar and is not induced by ethanol (Ciriacy, 1975; Denis and Malvar, 1990; La Grange *et al.*, 1996; Romanos *et al.*, 1992; Tøttrup and Carlsen, 1989) as is stated occasionally (Ciriacy, 1997). Two major, separate regulatory pathways regulate *ADH2* expression. The first pathway involves the activator Ccr4 and its negative effectors Cre1 and Cre2 (Denis, 1984; Denis and Malvar, 1990). The *CRE* genes are suggested to comprise a general transcriptional control system in yeast (Denis and Malvar, 1990). The second pathway, specific for regulation of *ADH2*, involve the transcriptional activator Adr1 (Ciriacy,1975; Denis *et al.*, 1981; Denis and Malvar, 1990; Karnitz *et al.*, 1992). The two pathways function independently from each other, but both are required for full *ADH2* expression (Denis, 1984; Denis and Malvar, 1990).

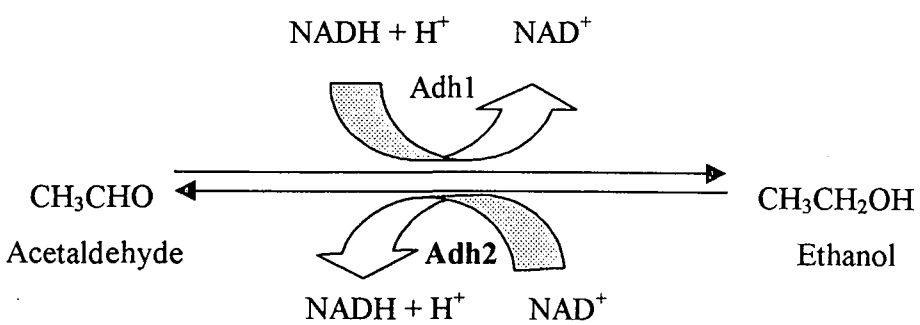


Figure 1.3. A schematic illustration of the function of Adh1 and Adh2 during sugar metabolism in *S. cerevisiae*.

Adr1 is the major transcriptional activator of *ADH2* and positively regulates *ADH2* derepression (Ciriacy, 1975; Ciriacy, 1979; Denis *et al.*, 1981; Irani *et al.*, 1987). The binding of Adr1 to upstream activation sequence 1 (UAS1) of *ADH2* is essential for derepression of *ADH2* transcription (Ciriacy, 1997; Denis *et al.*, 1981; Irani *et al.*, 1987). Adr1 activation of *ADH2* is mediated through UAS1, a 22-bp perfect inverted repeat and the lack of *ADH2* expression during carbon catabolite repression is due to the absence of bound Adr1 to UAS1 (Irani *et al.*, 1987; Shuster *et al.*, 1986). The transcriptional activation function of ADR1 is controlled by two means. Firstly, cAMP-dependent phosphorylation (cAPK) of Adr1 at serine residue 230 is responsible for the down-regulation of its transcriptional activation function (Ciriacy, 1997; Denis and Audino, 1991; Karnitz *et al.*, 1992). In *S. cerevisiae*, *TPK1*, *TPK2* and *TPK3* encode the cAPK catalytic subunits, whereas the cAPK regulatory subunit is encoded by *BCY1* (Denis and Audino, 1991). A glucose-dependent signal, transmitted through the adenyl cyclase effectors Cdc25, Ras1 and Ras2 mediates the activation of the cAPK phosphorylation system during growth on glucose (Denis and Audino, 1991). Secondly, *ADR1* mRNA translation is regulated in a glucose-dependent manner, even though *ADR1* transcription is only slightly affected by carbon catabolite repression (Ciriacy, 1979; Ciriacy, 1997; Shuster *et al.*, 1986). Glucose seems to affect the translation rate of *ADR1* mRNA in *S. cerevisiae* (Vallari *et al.*, 1992). The concentration of Adr1 was at least 10-fold higher in ethanol-grown cultures than in glucose-grown cultures (Vallari *et al.*, 1992). However, under these conditions, the level of *ADR1* mRNA was only two-fold higher in ethanol-grown cultures than in glucose-grown cultures and the stability (half-life) of Adr1 itself was found to be more or less the same. Therefore, Vallari *et al.* (1992) concluded that the decreased levels of Adr1 was a result of decreased *ADR1* mRNA translation (Adr1 synthesis) in glucose-grown cultures of *S. cerevisiae* as compared to ethanol-grown cultures.

Sch9, a cAMP-independent protein kinase, can functionally substitute cAPK activity in *S. cerevisiae* when over-expressed (Ciriacy, 1997; Denis and Audino, 1991). Initially, it was thought that Sch9 down-regulated Adr1 function in the same manner as cAPK. Denis and Audino (1991) showed that Sch9 did not down-regulate Adr1 function as does cAPK, but rather functioned independently of cAPK and Adr1 as an activator of *ADH2* expression. *CCR1* (*SNF1*) and *CAT1* encoded another yeast protein kinase which is required for the transcription of various glucose-repressed genes, including *ADH2* (Denis and Audino, 1991; Denis *et al.*, 1981). Ccr1 and Sch9 protein kinases control factors required for *ADH2* regulation that act independently of Adr1 and constitute a separate, non-parallel pathway to

that involving cAPK in the regulation of *ADH2* (Denis and Audino, 1991). In addition to UAS1, a second UAS element (UAS2) exists, which is located immediately downstream of UAS1 (Ciriacy, 1997). However, it remains unclear whether any of the known regulatory factors for *ADH2* expression acts through UAS2 (Ciriacy, 1997). Many of these factors (Swi1, 2, 3; Ccr1, 4; Cre1, 2; Sch9; Adr6,7,8,9; Ssn20) are possibly part of the basic transcriptional machinery and may not be *ADH2*-specific or control factors that act through other activation sequences (Ciriacy, 1997; Denis and Audino, 1991; Denis and Malvar, 1990; Denis *et al.*, 1981; Karnitz *et al.*, 1992). Mutations in *CRE1* result in an altered cell morphology (Denis, 1984), suggesting that the *CRE* genes are part of the regulatory mechanism of a variety of genes (Denis and Malvar, 1990). However, the molecular signals that initiate repression / derepression of *ADH2* remain elusive (Ciriacy, 1997).

1.3.4.3. Xylanase expression in *Saccharomyces cerevisiae*

Loison *et al.* (1986) described an autoselection system, suitable for heterologous protein production in complex media without the need for selection of the plasmid-bearing cells. The selection system was based on the production of uridine-5'-monophosphate (UMP) in *S. cerevisiae*. UMP can be produced *via* two biosynthetic routes. Firstly, UMP can be synthesized through the decarboxylation of ortidine-5'-monophosphate (the *de novo* pathway) or secondly, through the direct conversion of uracil to UMP in the salvage pathway (Loison *et al.*, 1986). The decarboxylation activity is encoded by *URA3* and the uracil phosphoribosyltransferase activity by *FUR1*. Yeast strains lacking both activites, *ura3fur1* double mutants, are thus non-viable since no other pathway exists for UMP synthesis. Yeast strains containing the *ura3fur1* genotype can be cured upon transformation with a plasmid containing either *URA3* or *FUR1* and the selection of plasmid-cured cells is independent of the growth medium used (Loison *et al.*, 1986). Futhermore, no detectable loss of the plasmid-linked phenotype could be observed during growth of the recombinant yeast strain in complex media for more than 30 generations (Loison *et al.*, 1986).

La Grange *et al.* (1996) cloned and expressed *XYN2* (encoding an endo-β-xylanase, Xyn2, of *Trichoderma reesei*) in *S. cerevisiae*. Expression of *XYN2* was obtained with multi-copy plasmids harbouring a promoter-terminator expression cassette derived from alcohol dehydrogenase II (*ADH2*) (La Grange *et al.*, 1996). The selection system used was based on

that described by Loison *et al.* (1986), thus facilitating β-xylanase production in complex growth media without risking loss of the episomal plasmid (La Grange *et al.*, 1996).

The recombinant *S. cerevisiae* Y294 (*fur1*::*LEU2* pDLG5) was capable of producing significant amounts of β-xylanase (1200 nkat.ml^{-1}) in complex medium (YPD) with 8 g galactose.l^{-1} as carbon source (La Gange *et al.*, 1996). Xylanase activity reached a maximum at *ca.* 80 h of cultivation (La Gange *et al.*, 1996). The recombinant Xyn2 significantly differed from the native *T. reesei* Xyn2 only in respect of molecular mass (Table 1.7). Xyn2 produced by the recombinant *S. cerevisiae* Y294 strain had a higher molecular mass (27.5 kDa) than *T. reesei* Xyn2 (21 kDa), due to N-glycosylation of the β-xylanase secreted by *S. cerevisiae* (La Gange *et al.*, 1996). Hyperglycosylation of foreign proteins expressed in *S. cerevisiae* is not uncommon (La Gange *et al.*, 1996). The same yeast strain and expression system, but with the constitutive phosphoglycerate kinase (*PGK1*) promoter governing the expression of xylanase, produced only 160 nkat.ml^{-1} of xylanase activity in complex medium with 8 g glucose.l^{-1} as carbon source. The reason for this low activity obtained with what is generally regarded as a powerful promoter is unknown.

Marquet *et al.* (1987) used a *ura3 fur1* double-mutant of *S. cerevisiae* for heterologous human α_1-antitrypsin (α_1-AT) production. The only difference with regard to heterologous xylanase expression in *S. cerevisiae* (La Grange *et al.*, 1996) was that Marquet *et al.* (1987) used a constitutive phosphoglycerate kinase (PGK) promotor in the *URA3*-bearing plasmids for regulating the expression of α_1-AT. In continuous culture at a constant dilution rate, the recombinant *S. cerevisiae* strain stably expressed α_1-AT for at least 150 generations without the appearance of plasmid-cured cells (Marquet *et al.*, 1987). They also showed that between dilution rates of 0.1 to 0.32 h^{-1}, the plasmid copy-number remained constant, regardless of the dilution rate used (Marquet *et al.*, 1987).

Table 1.7. Molecular characteristics of Xyn2 produced by *Trichoderma reesei* and the recombinant Xyn2 produced by *Saccharomyces cerevisiae* Y294. (Adapted from La Grange *et al.*, 1996).

Property	*Trichoderma reesei*	*Saccharomyces cerevisiae*
Molecular mass (kDa)	20 to 21	27.5
Optimum pH	5	6
Optimum temperature (°C)	56 to 60	60
Temperature stability (60 min) (°C)	NA	50[a]
Number of glycosylation sites	3	3
Glycosylation	No	Yes

[a] More than 90 % activity remained.

NA, data not available

1.3.4.4. Glucose metabolism and carbon catabolite repression in *Saccharomyces cerevisiae*

Glucose metabolism. The glucose-sensitive (Crabtree-positive) yeast *S. cerevisiae* exhibits remarkable metabolic flexibility (Cortassa and Aon, 1998). In aerobic batch culture ethanol is produced in the presence of excess glucose (Alexander and Jeffries, 1990; Fiechter and Seghezzi, 1992). The transient accumulation of ethanol under aerobic conditions has been referred to as the 'short-term Crabtree-effect' (Cortassa and Aon, 1998; Petrik *et al.*, 1983). Upon depletion of glucose, the produced ethanol is utilised, a phenomenon known as diauxic growth (Beck and von Meyenburg, 1968; Fiechter *et al.*, 1981; Rieger *et al.*, 1983). In continuous culture at low dilution rates, S. *cerevisiae* exhibits oxidative glucose assimilation without any ethanol being produced (Alexander and Jeffries, 1990; Beck and von Meyenburg, 1968). However, when the dilution rate is increased to above a certain critical value, glucose is metabolised respiro-fermentatively (oxido-reductively) and this is sometimes referred to as the 'long-term Crabtree-effect' (Cortassa and Aon, 1998; Fiechter and Seghezzi, 1992; Petrik *et al.*, 1983; Rieger *et al.*, 1983). The highest dilution rate that allows purely respiratory glucose metabolism and above which respiro-fermentative metabolism is observed, is called the critical dilution rate (D_c).

In carbon-limited continuous culture of *S. cerevisiae*, the specific rate of O_2 uptake and CO_2 evolution increases linearly up to D_c, after which the specific rate of CO_2 production increases dramatically and the specific rate of O_2 consumption remains relatively constant (Beck and von Meyenburg, 1968; Petrik *et al.*, 1983; Postma *et al.*, 1989). The D_c value at which ethanol is produced is strain dependent (Sierkstra *et al.*, 1992b). In addition, above D_c the biomass yield decreases steadily and the respiratory quotient increases with increasing dilution rate (D) (Alexander and Jeffries, 1990; Beck and von Meyenburg, 1968; Rieger *et al.*, 1983). Rieger *et al.* (1983) observed that *S. cerevisiae* could grow fully derepressed in continuous culture, thus indicating that repression of respiration is not caused by the substrate *per se*, but rather by the metabolic flux generated by the rate of substrate uptake.

Several hypothesis have been proposed to explain the 'Crabtree-effect': (1) Barford (1990) explained the 'Crabtree-effect' in terms of a kinetic limitation in the provision of respiratory intermediates to and from the mitochondria. It was also postulated that *S. cerevisiae* could undergo 'respiratory adaptation' or a gradual derepression of respiration as a result of an increased capacity of the respiratory intermediate transport proteins of the mitochondrial membranes (Barford, 1981; 1984; 1990; Barford and Hall, 1979). (2) Rieger *et al.* (1983) observed a limited respiratory capacity (a maximum oxygen uptake rate of 8 $mmol.g^{-1}.h^{-1}$) by *S. cerevisiae* in carbon–limited continuous culture and suggested that it was an inherent feature of the organism. Sonnleitner and Käppeli (1986) supported this hypothesis, but added the concept of an 'overflow' or 'supracritical' carbon flow at the level of pyruvate. (3) Postma *et al.* (1989) concluded that alcohol formation above D_c was not primarily due to a limited respiratory capacity in *S. cerevisiae*, but rather due to the production of weak organic acids that had an uncoupling effect on respiration. (4) Another hypothesis concerns the flux distribution of pyruvate at the branch point between oxidative and fermentative metabolism, involving the mitochondrial pyruvate dehydrogenase and the cytosolic pyruvate decarboxylase (van Hoek *et al.*, 1998; van Urk *et al.*, 1989). (5) Cortassa and Aon (1998) provided the latest hypothesis for the occurrence of the 'Crabtree' or glucose effect. They regard the dilution rate at which fermentative metabolism of glucose in continuous culture occurs as dependent on the catabolite repression features of each *S. cerevisiae* strain.

Carbon catabolite repression. Carbon catabolite repression can be exerted not only by glucose, but also by mannose, sucrose, fructose, galactose and maltose (Gancedo, 1992). Some genes are repressed by all the above-mentioned sugars, while others are only repressed

by glucose. Therefore, care should be taken to define carbon catabolite repression caused by a specific sugar (*e.g.* glucose repression). The remainder of this section discusses carbon catabolite repression in the yeast *S. cerevisiae*, as effected by glucose. Glucose-repressible genes include genes encoding enzymes involved in gluconeogenesis, respiration, the Krebs cycle, mitochondrial development and the utilisation of alternate carbon sources other than glucose, fructose or mannose (Klein *et al.*, 1998; Meijer *et al.*, 1998; Petrik *et al.*, 1983). The severity of glucose repression is highly dependent on the yeast strain used and the enzyme under investigation (Gancedo, 1992). Glucose repression can affect enzyme levels in several ways (Figure 1.4). The main effect of glucose repression is to halt or cause a decrease in the rate of transcription of a particular gene (Klein *et al.*, 1998; Meijer *et al.*, 1998). Secondly, glucose could reduce the stability (half-life) of the corresponding mRNA (Gancedo, 1998; Klein *et al.*, 1998). It is uncommon for glucose to influence the translation rate of mRNA (Gancedo, 1998) in *S. cerevisiae*, although this has been observed for Adr1, the transcriptional activator of *ADH2* (see section 1.3.4.2., Function and regulation of *ADH2* in *Saccharomyces cerevisiae*). Lastly, glucose could also increase the degradation rate of the corresponding protein (enzyme), referred to catabolite inactivation (Gancedo, 1992, 1998; Klein *et al.*, 1998). Thus, for different glucose repressible genes, the mechanism of regulation may differ (Gancedo, 1992).

A 'signal' is produced when glucose is present in the medium and this 'signal' must be received and transduced to slow down or stop the transcription of glucose-repressible genes (Gancedo, 1992). It is not exactly known how glucose exerts its repressive effects on glucose-repressible genes. Currently it is believed that the produced 'signal' does not directly interact with DNA, but rather causes alterations of regulatory proteins (Gancedo, 1992). Glucose can either activate a repressor protein or inhibit an activator protein (Gancedo, 1992). Glucose repression in *S. cerevisiae* is not associated with low intracellular cAMP levels as was found in *Escherichia coli* (Gancedo, 1992). This conclusion was derived from the observation that cAMP does not have a uniform effect on different genes subject to glucose repression, *e.g.* *GAL1*, *SUC2* and *ADH2* (Gancedo, 1992). Glucose repression was not dependent on any specific transporter (Hxt1 to Hxt7), but the extent of repression correlated well with the glucose uptake ability of a particular yeast strain (Gancedo, 1998).

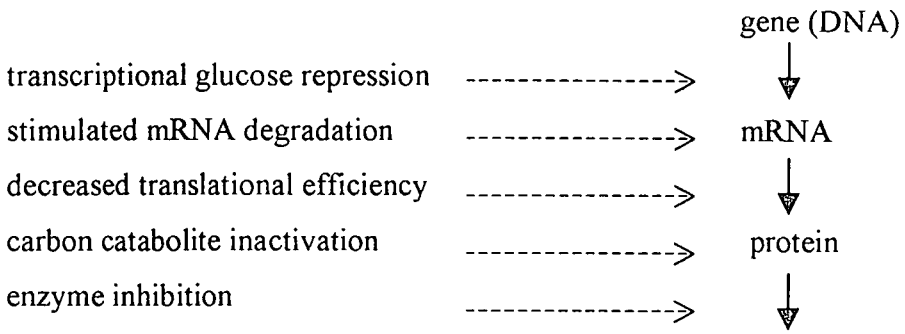


Figure 1.4. Negative glucose control mechanisms (Adapted from Klein *et al.*, 1998).

Currently, it is envisaged that the 'signal' for glucose repression is produced at or above the level of glucose-6-phosphate (Gancedo, 1992). The observations that a mutant strain defective in hexokinase isozyme 2 (Hxk2) failed to exhibit glucose repression, that galactose and maltose were unable to cause glucose repression and that a glucose analogue, 2-deoxyglucose, which can be phosphorylated after uptake but not metabolised, repressed the invertase gene (*SUC2*), support this view (Meijer *et al.*, 1998). In view of this, it has been suggested that the generation of the initial signal is caused either by the extracellular or intracellular glucose concentration or by an increased glucose flux through yeast's metabolic pathways (Meijer *et al.*, 1998).

It is important to note that the majority of studies conducted to elucidate the mechanisms involved in carbon catabolite repression was done with mutant strains grown in shake flasks under ill-defined physiological conditions (Table 1.8) (Sierkstra *et al.*, 1992a, b). During batch growth of *S. cerevisiae*, continuous changes in the growth rate, glucose concentration, as well as glucose flux occur (Meijer *et al.*, 1998). Although many factors involved with the regulation of carbon catabolite repression have been successfully identified by this means, it is desirable to investigate carbon catabolite repression in continuous cultures so that the exact physiological state of the yeast is known (Sierkstra *et al.*, 1992b). Therefore, relatively few data are available on the kinetics and sequence of events observed that quantitively describe a classical repression / derepression event.

The mere presence of glucose is incapable of causing glucose repression, suggesting that a minimum threshold concentration of extracellular or intracellular glucose or a metabolite thereof is involved in the generation of the initial signal (Meijer *et al.*, 1998; Sierkstra *et al.*,

1992b; 1994). However, the metabolic carbon flux determines the *in vivo* metabolite concentrations, which could constitute the signal mediating glucose repression. Sierkstra *et al.* (1992b) also concluded that the Crabtree effect in *S. cerevisiae* in glucose-limited continuous culture was not related to glucose repression. An inverse relationship was observed between the growth rate and transcription of *ADH1 / ADH2* and phosphoglucomutase (*GAL5*) in glucose and nitrogen-limited continuous cultures of *S. cerevisiae* (Postma *et al.*, 1989; Sierkstra *et al.*, 1994). Sierkstra *et al.* (1994) concluded that the contribution of growth rate to the regulation of *ADH2* was at least as important as regulation by glucose repression. By contrast, the mRNA levels of the other glycolytic enzymes, namely hexokinase 2 (*HXK2*), pyruvate decarboxylase (*PDC1*), pyruvate dehydrogenase (*PDA1*) and phosphoglucoisomerase (*PGI1*) remained constant, whereas glucose repression at every dilution rate was confirmed by the absence of invertase (*SUC2*) and hexokinase 1 (*HXK1*) mRNA (Sierkstra *et al.*, 1994). Recently, Meijer *et al.* (1998) concluded that glucose-repressible invertase (*SUC2*) of *S. cerevisiae* in nitrogen-limited continuous culture was regulated by the external glucose concentration and no relationship was found between glucose repression and the glucose flux. In these experiments, glucose repression of *SUC2* was initiated at external glucose concentrations between 2.5 and 3.2 $g.l^{-1}$ glucose at every dilution rate (Meijer *et al.*, 1998). From the above it is evident that glucose repression differs with respect to the gene involved and that no conclusive evidence exists regarding whether glucose repression is caused by the glucose concentration or glucose flux.

Table 1.8. Media and cultivation conditions used in studies of the regulation of *ADH2* on molecular level in *S. cerevisiae*.

Medium	Carbon sources used for repression / derepression of *ADH2*		Type and duration of cultivation	Reference
	Repression	Derepression		
YEP[a]	20 / 80 g.l^{-1} glucose	30 g.l^{-1} ethanol	Batch, 12 – 16 h	Ciriacy, 1979
YEP[a]	80 g.l^{-1} glucose	30 g.l^{-1} ethanol	Batch, overnight	Denis, 1984
YEP[a]	10 g.l^{-1} glucose	20 g.l^{-1} ethanol	Batch[b], not indicated	Denis *et al.*, 1981
YEP[a]	-	30 g.l^{-1} ethanol	Batch[b], not indicated	Denis and Audino, 1991
YEP[a] /CAA[c]	80 g.l^{-1} glucose	30 g.l^{-1} ethanol	Batch[b], not indicated	Denis and Malvar, 1990
YEP[a]	50 g.l^{-1} glucose	30 g.l^{-1} ethanol	Batch, 24 h	Irani *et al.*, 1987
YEP[a]	70 to 80 g.l^{-1} glucose	20 to 30 g.l^{-1} ethanol	Batch[b], not indicated	Saliola *et al.*, 1990
YEP[a]	30 g.l^{-1} glucose	30 g.l^{-1} ethanol	Batch, 24 h	Shuster *et al.*, 1986
YEP[a]	80 g.l^{-1} glucose	30 g.l^{-1} ethanol	Batch, overnight	Vallari *et al.*, 1992

YEP[a], medium consisting of yeast extract and peptone

Batch[b], shake-flask cultivations with no defined cutivation time indicated

CAA[c], casamino acids used for medium supplementation

SD[d], yeast synthetic medium containing yeast nitrogen base (without amino acids)

Batch[e], cells were grown to mid-exponential phase

1.4. References

Alexander, M.A. & Jeffries, T.W. (1990). Respiratory efficiency and metabolic partitioning as regulatory phenomena in yeasts. *Enzyme Microb Technol* **12**, 2-19.

Alexander, N.J. (1986). Genetic manipulation of yeasts for ethanol production from xylose. *Food Technol* 7, 99-103.

Annergren, G.A. & Rydholm, S.A. (1959). On the behaviour of hemicelluloses during sulphite pulping. *Svensk Papperstidn* **62**, 737-746.

Annison, G. (1993). The role of wheat non-starch polysaccharides in broiler nutrition. *Aust J Agric Res* **44**, 405-422.

Apel, P.C., Panaccoine, D.G., Holden, F.R. & Walton, J.D. (1993). Cloning and targeted gene disruption of *XYL1* a beta-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. *Mol Plant-Microbe Interact* **6**, 467-473.

Bahkali, A.H. (1996). Influence of various carbohydrates on xylanase production in *Verticellium tricorpus*. *Bioresource Technol* **57**, 265-268.

Bailey, M.J., Buchert, J. & Viikari, L. (1993). Effect of pH on production of xylanase by *Trichoderma reesei* on xylan- and cellulose-based media. *Appl Microbiol Biotechnol* **40**, 224-229.

Barford, J.P. (1981). A mathematical model for the aerobic growth of *Saccharomyces cerevisiae* with a saturated respiratory capacity. *Biotechnol Bioeng* **23**, 1735-1762.

Barford, J.P. (1984). The control of respiration and fermentation in yeasts – A unified approach. Paper, *Seventh International Biotechnology Symposium*, New-Delhi, India. pp 19-25.

Barford, J.P. (1990). A general model for aerobic yeast growth: Continuous culture. *Biotechnol Bioeng* **35**, 921-927.

Barford, J.P. & Hall, R.J. (1979). An examination of the Crabtree-effect in *Saccharomyces cerevisiae* : the role of respiratory adaptation. *J Gen Microbiol* **114**, 267-275.

Barry, V.C. & Dillon, T. (1940). The occurrence of xylan in marine algae. *Nature* 146, 620.

Beck, C. & von Meyenburg, H.K. (1968). Enzyme pattern and aerobic growth of *Saccharomyces cerevisiae* under various degrees of glucose limitation. *J Bacteriol* **96**, 479-486.

Bedford, M.R. & Classen, H.L. (1992). The influence of dietary xylanase on intestinal viscosity and molecular weight ditribution of carbohydrates in rye-fed broiler chicks. In Visser, J., Beldman, G., Kusters van Someren, M.A. & Voragen, A.G.J. (Eds), *Xylans and xylanases*. Elsevier Science, Amsterdam. pp 361-370.

Bernier Jr, R.L., Gray, A.L., Moser, G.P., Hamilton, J., Roberge, M. & Senior, D.J. (1994). Fate of residual xylanase after treatment and bleaching of softwood kraft pulp. *Bioresource Technol* **50**, 79-83.

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

Biely, P., Vršanská, M., Tenkanen, M. & Kleupfel, D. (1997). Endo-β-1,4-xylanase families: differences in catalytic properties. *J Biotechnol* **57**, 151-166.

Biely, P., Vršanská, M. & Kucár, Š. (1992). Identification and mode of action of endo-(1→4)-β-xylanases. In Visser, J., Beldman, G., Kusters van Someren, M.A. & Voragen, A.G.J. (Eds), *Xylans and xylanases*. Elsevier Science, Amsterdam. pp 81-93.

Buchert, J., Kantelinen, A., Suurnäkki, A., Viikari, L. & Janson, J. (1995). Evaluation of the effects of hemicellulases on the bleachability of sulphite pulps. *Holzforchung* **49**, 439-444.

Buchert, J., Ranua, M., Kantelinen, A. & Viikari, L. (1992). The role of two *Trichoderma reesei* xylanases in the bleaching of pine kraft pulp. *Appl Microbiol Biotechnol* **37**, 825-829.

Buchert, J., Ranua, M., Siika-aho, M., Pere, J. & Viikari, L. (1994a). *Trichoderma reesei* cellulases in the bleaching of kraft pulp. *Appl Microbiol Biotechnol* **40**, 941-945.

Buchert, J., Salminen, J., Siika-aho, M., Ranua, M. & Viikari, L. (1993). The role of *Trichoderma reesei* xylanase and mannanase in the treatment of softwood kraft pulp prior to bleaching. *Holzforchung* **47**, 473-478.

Buchert, J., Teleman, A., Harjunpää, V., Tenkanen, M., Viikari, L. & Vuorinen, T. (1995). Effect of cooking and bleaching on the structure of xylan in conventional pine kraft pulp. *Tappi J* **78**, 125-130.

Buchert, J., Tenkenen, M., Kantelinen, A. & Viikari, L. (1994b). Application of xylanases in the pulp and paper industry. *Bioresource Technol* **50**, 65-72.

Buckholz, R.G. & Gleeson, A.G. (1991). Yeast systems for the commercial production of heterologous proteins. *Bio/Technology* **9**, 1067-1072.

Chesson, A. (1993). Feed enzymes. *Animal Feed Sci Technol* **45**, 65-79.

Chiruvolu, V., Stratton, J.M., Ott, T.L., Bazer, F.W. & Meagher, M.M. (1996). Effect of medium composition on growth, plasmid stability and ovine interferon-τ production in *Saccharomyces cerevisiae*. *J Ferment Bioeng* **82**, 565-569.

Christakopoulos, P., Nerinckx, W., Kekos, D., Macris, B. & Claeyssens, M. (1997). The alkaline xylanase III from *Fusarium oxysporum* F3 belongs to family F / 10. *Carbohydrate Res* **302**, 191-195.

Christov, L.P. & Prior, B.A. (1993). Xylan removal from dissolving pulp using enzymes of *Aureobasidium pullulans*. *Biotechnol Lett* **15**, 1269-1274.

Ciriacy, M. (1975). Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae.* II. Two loci controlling synthesis of the glucose-repressible ADH II. *Mol Gen Genet* **138,** 157-164.

Ciriacy, M. (1979). Isolation and characterization of further *cis*- and *trans*-acting regulatory elements involved in the synthesis of glucose-repressible alcohol dehydrogenase (ADHII) in *Saccharomyces cerevisiae. Mol Gen Genet* **176,** 427-431.

Ciriacy, M. (1997). Alcohol dehydrogenases. In Zimmermann, F.K. & Entian, K.-D. (Eds), *Yeast sugar metabolism : Biochemistry, genetics, biotechnology and applications.* Technomic publishing company Inc., Lancaster, U.S.A. pp 213-223.

Clarke, J.H., Rixon, J.E., Ciruela, A., Gilbert, H.J. & Hazlewood, G.P. (1997). Family-10 and family-11 xylanases differ in their capacity to enhance the bleachability of hardwood and softwood paper pulps. *Appl Microbiol Biotechnol* **48**, 177-183.

Cortassa, S & Aon, M.A (1998). The onset of fermentative metabolism in continuous cultures depends on the catabolite repression properties of *Saccharomyces cerevisiae. Enzyme Microbial Technol* **22**, 705-712.

Coughlan, M.P. (1992). Towards an understanding of the mechanism of action of main chain-hydrolyzing xylanases. In Visser, J., Beldman, G., Kusters van Someren, M.A. & Voragen, A.G.J. (Eds), *Xylans and xylanases.* Elsevier Science, Amsterdam. pp 111-139.

Coughlan, M.P. & Hazlewood, G.P. (1993). β-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. *Biotechnol Appl Biochem* **17**, 259-289.

Cousens, L.S., Shuster, J.R., Gallegos, C., Ku, L., Stempien, M.M., Urdea, M.S., Sanchez-Pescador, R., Taylor, A. & Tekamp-Olson, P. (1987). High level expression of proinsulin in the yeast, *Saccharomyces cerevisiae. Gene* **61,** 265-275.

Cowan, W.D. (1996). Animal feed. In Godfrey, T. & West, S. (Eds), *Industrial Enzymology.* Macmillan Press Ltd, London. pp. 74-82.

Deising, H. & Mendgen, K. (1991). Developmental control of enzyme production and cell wall modification in rust fungi, and defence reactions of the host plant. In Stahl, U. & Tudzynski, P. (Eds), *Molecular biology of filamentous fungi*. VCH-Verlag, Weinham, FRG. pp 27-44.

Dekker, R.F.H. & Richards, G.N. (1976). Hemicellulases : their occurrence, purification, properties and mode of action. *Adv Carbohydr Chem Biochem* **32**, 277-352.

Denis, C.L. (1984). Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II. *Genetics* **108,** 833-844.

Denis, C.L. & Audino, D.C. (1991). The CCR1 (SNF1) and SCH9 protein kinases act independently of cAMP-dependent protein kinase and the transcriptional activator ADR1 in controlling yeast *ADH2* expression. *Mol Gen Genet* **229,** 395-399.

Denis, C.L., Ciriacy, M. & Young, E.T. (1981). A positive regulatory gene is required for accumulation of the functional messenger RNA for the glucose-repressible alcohol dehydrogenase from *Saccharomyces cerevisiae. J Mol Biol* **148,** 355-368.

Denis, C.L. & Malvar, T. (1990). The *CCR4* gene from *Saccharomyces cerevisiae* is required for both nonfermentative and *spt*-mediated gene expression. *Genetics* **124,** 283-291.

Eriksson, K.-E.L. (1991). Biotechnology: three approaches to reduce the environmental impact of the pulp and paper industry. *Sci Progress* **75**, 175-189.

Faber, K.N., Harder, W. & Veenhuis, M. (1995). Review: Methylotrophic yeasts as factories for the production of foreign proteins. *Yeast* **11**, 1331-1344.

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

Fiechter, A. & Seghezzi, W. (1992). Regulation of glucose metabolism in growing yeast cells. *J Biotechnol* **27**, 27-45.

Gamerith, G., Groicher, R., Zeilinger, S., Herzog, P. & Kubicek, C.P. (1992). Cellulase-poor xylanases produced by *Trichoderma reesei* RUT C-30 on hemicellulose substrates. *Appl Microbiol Biotechnol* **38**, 315-322.

Gancedo, J.M. (1992). Carbon catabolite repression in yeast. *Eur J Biochem* **206**, 297-313.

Gancedo, J.M. (1998). Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* **62**, 334-361.

Gellissen, G. & Hollenberg, C.P. (1997). Application of yeasts in gene expression studies: a comparison of *Saccharomyces cerevisiae, Hansenula polymorpha* and *Kluyveromyces lactis* – a review. *Gene* **190**, 87-97.

Graham, H. & Inborr, J. (1992). Application of xylanase-based enzymes in commercial pig and poultry production. In Visser, J., Beldman, G., Kusters van Someren, M.A. & Voragen, A.G.J. (Eds), *Xylans and xylanases.* Elsevier Science, Amsterdam. pp 81-93.

GrootWassink, J.W.D., Campbell, G.J. & Classen, H.L. (1989). Fractionation of crude pentosanase (arabinoxylanase) for improvement of the nutritional value of rye diets for broiler chickens. *J Sci Food Agric* **46**, 289-300.

Haapala, R., Linko, S., Parkkinen, E. & Suominen, P. (1994). Production of endo-1,4-β-glucanase and xylanase by *Trichoderma reesei* immobilized on polyurethane foam. *Biotechnol Techniques* **8**, 401-406.

Hahn-Hägerdal, B., Jeppsson, H., Skoog, K. & Prior, B.A. (1994). Biochemistry and physiology of xylose fermentation by yeast. *Enzyme Microb Technol* **16**, 933-942.

Hayn, M., Steiner, W., Klinger, R., Steinmüller, H., Sinner, M. & Esterbauer, H. (1993). Basic research and pilot studies on the enzymatic conversion of lignocellulosics. In Saddler, J.N. (Eds), *Bioconversion of forest and agricultural plant residues.* CAB International, Wallingford, UK. pp 33-72.

Hazlewood, G.P. & Gilbert, H.J. (1992). The molecular architecture of xylanases from *Pseudomonas fluorescens* subsp. *cellulosa*. In Visser, J., Beldman, G., Kusters van Someren, M.A. & Voragen, A.G.J. (Eds), *Xylans and xylanases*. Elsevier Science, Amsterdam. pp 259-273.

Herrmann, M.C., Vrsanska, M., Jurickova, M., Hirsch, J., Biely, P. & Kubicek, C.P. (1997). The β-D-xylosidase of *Trichoderma reesei* is a multifunctional β-D-xylan xylohydrolase. *Biochem J* **321**, 375-381.

Hinck, J.F., Casebier, R.L. & Hamilton, J.K. (1985). Dissolving pulp manufacture. In Ingruber, O.V., Kocurec, M.J. & Wong, A. (Eds), *Pulp and paper manufacture*, Vol 4. CPPA Technical section, Montreal. pp 213-243.

Hrmová, M., Petráková, E. & Biely, P. (1991). Induction of cellulose- and xylan-degrading enzyme systems in *Aspergillus terreus* by homo- and heterodisaccharides composed of glucose and xylose. *J Gen Microbiol* **137**, 541-547.

Irani, M., Taylor, W.E. & Young, T.E. (1987). Transcription of the *ADH2* gene in *Saccharomyces cerevisiae* is limited by positive factors that bind competitively to its intact promoter region on multi-copy plasmids. *Mol Cell Biol* **11,** 3060-3069.

Jeffries, T.W. (1996). Biochemistry and genetics of microbial xylanases. *Curr Opinion Biotechnol* 7, 337-342.

Johannson, M.H. & Samuelson, O. (1977). Reducing end groups in birch xylan and their alkaline degradation. *Wood Sci Technol* **11**, 251-263.

Joseleau, J.P., Contat, J. & Ruel, K. (1992). Chemical structure of xylans and their interaction in the plant cell walls. In Visser, J., Beldman, G., Kusters van Someren, M.A. & Voragen, A.G.J. (Eds), *Xylans and xylanases*. Elsevier Science, Amsterdam. pp 1-15.

Karnitz, L., Morrison, M. & Young, E.T. (1992). Identification and characterization of three genes that affect expression of *ADH2* in *Saccharomyces cerevisiae*. *Genetics* **132**, 351-359.

Kato, Y. & Nevins, D. (1984). Enzymatic dissociation of Zea shoot cell wall polysaccharides (III) : purification and partial characterization of an endo-(1,4)-β-xylanase from a *Bacillus subtilis* enzyme preparation. *Plant Physiol* **75**, 753-758.

Klein, C.J.L., Olsson, L., & Nielsen, J. (1998). Glucose control in *Saccharomyces cerevisiae*: the role of *MIG1* in metabolic functions. *Microbiol* **144**, 13-24.

Knorr, D. & Sinskey, A.J. (1985). Biotechnology in food production and processing. *Yeast* **229**, 1224-1229.

Kristufek, D., Zeilinger, S. & Kubicek, C.P. (1995). Regulation of β-xylosidase formation by xylose in *Trichoderma reesei*. *Appl Microbiol Biotechnol* **42**, 713-717.

Kukuruzinska, M.A., Bergh, M.L.E. & Jackson, B.L. (1987). Protein glycosylation in yeast. *Ann Rev Biochem* **56,** 915-944.

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.

Lee, G.M., Song, K.B., Rhee, S.K. & Han, M.H. (1986). Plasmid maintenance and growth of recombinant *Saccharomyces cerevisiae* producing hepatitis B virus surface antigen. *Biotechnol Lett* **8**, 385-390.

Lee, J. (1997). Biological conversion of lignocellulosic biomass to ethanol. *J Biotechnol* **56**, 1-24.

Lindberg, B., Rosell, K.-G. & Svensson, S. (1973). Position of the O-acetyl groups in birch xylan. *Svensk Papperstidn* **76**, 30-32.

Loison, G., Nguyen-Juilleret, M., Alouani, F. & Marquet, M. (1986). Plasmid-transformed *URA3FUR1* double-mutants of *S cerevisiae*: an auto-selection system applicable to the production of foreign proteins. *Bio/Technology* **4,** 433-437.

Lutstorf, U. & Megnet, R. (1968). Multiple forms of alcohol dehydrogenase in *Saccharomyces cerevisiae. Arch Biochem Biophys* **126**, 933-944.

Luttig, M., Pretorius, I.S. & van Zyl, W.H. (1997). Cloning of two β-xylanase-encoding genes from *Aspergillus niger* and their expression in *Saccharomyces cerevisiae*. *Biotechnol Lett* **19**, 411-415.

Maat, J., Roza, M., Verbakel, J., Stam, H., da Silva, M.J.S., Bosse, M., Egmond, M.R., Hagemans, M.L.D., van Gorcom, R.F.M., Hessing, J.G.M., van der Hondel, C.A.M.J.J. & van Rotterdam, C. (1992). Xylanases and their application in bakery. In Visser, J., Beldman, G., Kusters van Someren, M.A. & Voragen, A.G.J. (Eds), *Xylans and xylanases*. Elsevier Science, Amsterdam. pp 349-360.

Margolles-Clark, E., Tenkanen, M., Nakari-Setälä, T. & Penttilä, M. (1996). Cloning of genes encoding α-L-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **62**, 3840-3846.

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.

Meijer, M.M.C., Boonstra, J., Verkleij, A.J. & Verrips, C.T. (1998). Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than the glucose flux. *J Biological Chem* **273,** 24102-24107.

Mueller-Harvey, I., Hartley, R.D., Harris, P.J. & Curzon, E.H. (1986). Linkage of ρ-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr Res* **148**, 71-85.

Paiva, T.C.B., Ferraz, A. & Durán, N. (1997). Biotreatment of a pulp mill EOP bleachery effluent with the fungi *Penicillium janthinellum* and *Lentinus edodes*. Paper, *Proceedings of the 5th Brazilian Symposium on the Chemistry of Lignins and other Wood Components*, Brazil. pp 10-12.

Parisi, F. & Parisi, M.A. (1989). Lignocellulosic materials : economic evaluation of pretreatment and hydrolysis procedures. In Coughlan, M.P. (Eds), Enzyme systems for lignocellulose degradation. Elsevier Applied Science, London. pp 243-255.

Petrik, M., Käppeli, O. & Fiechter, A. (1983). An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: Involvement of short- and long-term regulation. *J Gen Microbiol* **129**, 43-49.

Postma, E., Verduyn, C., Scheffers, A. & van Dijken, J.P. (1989). Enzymatic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Sacharomyces cerevisiae*. *Appl Environ Microbiol* **55**, 468-477.

Prade, R.A. (1995). Xylanases: from biology to biotechnology. *Biotechnol Gen Eng Rev* **13**, 101-131.

Price, V.L., Taylor, W.E., Clevenger, A., Worthington, M. & Young, E.T. (1990). Expression of heterologous proteins in *Saccharomyces cerevisiae* using the *ADH2* promoter. *Methods Enzymol* **185,** 308-318.

Puls, J. & Schuseil, J. (1993). Chemistry of hemicelluloses : relationship between hemicellulose structure and enzymes required for hydrolysis. In Coughlan, M.P. & Hazlewood, G.P. (Eds), *Hemicellulose and hemicellulases*. Portland Press, London. pp 1-28.

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.

1 151 215 25

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

Russell, P.R. & Hall, B.D. (1983). The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe. J Biol Chem* **258,** 143-149.

Saliola, M., Shuster, J.R. & Falcone, C. (1990) The alcohol dehydrogenase system in the yeast, *Kluyveromyces lactis. Yeast* **6,** 193-204.

Senior, D.J. & Hamilton, J. (1992). Use of xylanases to decrease the formation of AOX in kraft pulp bleaching. *J Pulp Paper Sci* **18,** 165–168.

Shuster, J., Yu, J., Cox, D., Chan, R.V.L., Smith, M. & Young, E. (1986). *ADR1*-mediated regulation of *ADH2* requires an inverted repeat sequence. *Mol Cell Biol* **6,** 1894-1902.

Shuster, J.R. (1989). Regulated transcriptional systems for the production of proteins in yeast: regulation by carbon source. In Barr, P.J., Brake, A.J. & Valenzuela, P. (Eds), *Yeast Genetic Engineering.* Butterworths, pp. 83-108.

Shuster, J.R., Moyer, D.L., Lee, H., Dennis, A., Smith, B. & Merrywheather, J.P. (1989). Yeast mutants conferring resistance to toxic effects of cloned human insulin-like growth factor 1. *Gene* **83,** 81-87.

Sierkstra, L.N., Nouwen, N.P., Verbakel, J.M.A. & Verrips, C.T. (1992a). Analysis of glucose repression in *Saccharomyces cerevisiae* by pulsing glucose to a galactose-limited continuous culture. *Yeast* **8,** 1077-1087.

Sierkstra, L.N., ter Schure, E.G., Verbakel, J.M.A. & Verrips, C.T. (1994). A nitrogen-limited, glucose-repressed, continuous culture of *Saccharomyces cerevisiae. Microbiol* **140,** 593-599.

Sierkstra, L.N., Verbakel, J.M.A. & Verrips C.T. (1992b). Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**, 2559-2566.

Siika-aho, M., Tenkanen, M., Buchert, J., Puls, J. & Viikari, L. (1994). An α-glucuronidase from *Trichoderma reesei* RUT C-30. *Enzyme Microb Technol* **16**, 813-819.

Simão, R.C., Souza, C.G.M. & Peralta, R.M. (1997a). The use of methyl β-D-xyloside as a substrate for xylanase production by *Aspergillus tamarii*. *Can J Microbiol* **43**, 56-60.

Simão, R.C., Souza, C.G.M. & Peralta, R.M. (1997b). Induction of xylanase in *Aspergillus tamarii* by methyl β-D-xyloside. *Appl Microbiol Biotechnol* **47**, 267-271.

Smith, R.A., Duncan, M.J. & Moir, D.T. (1985). Heterologous protein secretion from yeast. *Science* **229**, 1219-1224.

Smith, T.J., Wearne, R.H. & Wallis, A.F.A. (1994). Factors influencing the amounts of chlorinated phenols formed during bleaching of eucalypt kraft pulps. *Holzforchung* **48**, 125-132.

Sonnleitner, B. & Käppeli, O. (1986). Growth of *Saccharomyces cerevisiae* is controlled by its limited respiratory capacity: Formulation and verification of a hypothesis. *Biotechnol Bioeng* **28**, 927-937.

Spencer-Martins, I. (1994). Transport of sugars in yeasts: implications in the fermentation of lignocellulosic materials. *Bioresource Technol* **50**, 51-57.

Stålbrand, H., Siika-aho, M., Tenkanen, M. & Viikari, L. (1993). Purification and characterization of two β-mannanases from *Trichoderma reesei*. *J Biotechnol* **29**, 229-242.

Sudbery, P.E. (1996). The expression of recombinant proteins in yeasts. *Curr Opinion Biotechnol* 7, 517-524.

Suh, D.H., Becker, T.C., Sands, J.A. & Montenecourt, B.S. (1988). Effects of temperature on xylanase secretion by *Trichoderma reesei*. *Biotechnol Bioeng* **32**, 821-825.

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

Timell, T.E. (1965). Wood hemicelluloses, Part I. *Carbohydr Chem* **19**, 247-302.

Tolan, J.S. (1995). Pulp quality impact of contaminating cellulase in commercial xylanase preparations. *J Pulp Paper Sci* **21**, 132-137.

Tolan, J.S. & Foody, B.E. (1995). Novel enzyme treatment for dioxin-free bleaching of kraft pulp with high kappa factor and low ClO_2 substitution. *J Pulp Paper Sci* **21**, 1991-195.

Törrönen, A., Harkki, A. & Rouvinen, J. (1994). Three-dimensional structure of endo-1,4-β-xylanase II from *Trichoderma reesei*: two conformational states in the active site. *EMBO J* **13**, 2493-2501.

Törrönen, A. & Rouvinen, J. (1997). Structural and functional properties of low molecular weight endo-1,4-β-xylanases. *J Biotechnol* **57**, 137-149.

Tøttrup, H.V. & Carlsen, S. (1989). A process for the production of human proinsulin in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **35**, 339-348.

Vallari, R.C., Cook, W.C., Audino, D.C., Morgan, M.J., Jensen, D.E., Laudano, A.P. & Denis, C.L. (1992). Glucose repression of the yeast *ADH2* gene occurs through multiple mechanisms, including control of the protein synthesis of its transcriptional activator, ADR1. *Mol Cell Biol* **12**, 1663-1673.

Van Hoek, P., Flikweert, M.T., van der Aart, J.M., Steensma, H.Y., van Dijken, J.P. & Pronk, J.T. (1998). Effects of pyruvate decarboxylase overproduction on flux distribution at the pyruvate branch point *in Saccharomyces cerevisiae*. *Appl Environ Microbiol* **64**, 2133-2140.

Van Rensburg, P., van Zyl, W.H. & Pretorius, I.S. (1997). Over-expression of the *Saccharomyces cerevisiae* exo-β-1,3-glucanase gene together with the *Bacillus subtilis* endo-β-1,3-1,4-glucanase gene and the *Butyrivibrio fibrisolvens* endo-β-1,4-glucanase glucanase gene in yeast. *J Biotechnol* **55**, 43-53.

Van Rensburg, P., van Zyl, W.H. & Pretorius, I.S. (1998). Engineering yeast for efficient cellulose degradation. *Yeast* **14**, 67-76.

Van Urk, H., Schipper, D., Breedveld, G.J., Mak, P.R., Scheffers, W.A. & van Dijken, J.P. (1989). Localization and kinetics of pyruvate-metabolizing enzymes in relation to aerobic alcoholic fermentation in *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621. *Biochim et Biophys Acta* **992**, 78-86.

Vicuña, R., Oyarzún, E. & Osses, M. (1995). Assessment of various commercial enzymes in the bleaching of radiata pine kraft pulps. *J Biotechnol* **40**, 163-168.

Viikari, L., Kantelinen, A., Buchert, J. & Puls, J. (1994a). Enzymatic accessibility of xylans in lignocellulosic materials. *Appl Microbiol Biotechnol* **41**, 124-129.

Viikari, L., Kantelinen, A., Sundquist, J. & Linko, M. (1994b). Xylanases in bleaching: from an idea to the industry. *FEMS Microbiol Rev* **13**, 335-350.

Viikari, L., Ranua, M., Kantelinen, A., Linko, M. & Sundquist, J. (1986). Bleaching with enzymes. Paper, *Proceedings of the 3th international conference on biotechnology in the pulp and paper industry*, Stockholm, Sweden. pp. 67-69.

Whistler, R.L. & Richards, E.L. (1970). Hemicelluloses. In Pigman, W. & Horton, D. (Eds), *The Carbohydrates*. Academic Press, New York. pp 447-469.

Yang, J.L. & Eriksson, K.-E.L. (1992). Studies of bleachability of xylanase-treated kraft pulps. Paper, *Proceedings of the 5th international conference on biotechnology in the pulp and paper industry*, Kyoto, Japan. pp 75-81.

Zeilinger, S., Mach, R.L., Schindler, M., Herzog, P. & Kubicek, C.P. (1996). Different inducibility of expression of the two xylanase genes xyn1 and xyn2 in *Trichoderma reesei*. *J Biol Chem* **271**, 25624-25629.

CHAPTER 2

A COMPARISON OF THE 3,5-DINITROSALICYLIC ACID (DNS) AND SOMOGYI-NELSON ASSAYS FOR DETERMINING ENDO-β-1,4-XYLANASE ACTIVITY

An abridged version of this chapter has been prepared for submission to Biotechnology Letters

2.1. Abstract

Using a commercial xylanase preparation, the DNS assay for reducing sugars gave xylanase activity values up to 9.7-fold higher than obtained with the Somogyi-Nelson assay. However, with supernatants from cultures of a recombinant *Saccharomyces cerevisiae* strain producing only an endo-β-xylanase with no accessory enzymes, the DNS assay gave xylanase activity values of up to 74-fold higher than the Somogyi-Nelson assay. In the absence of xylanolytic accessory enzymes, the Somogyi-Nelson assay yielded extremely low activity values. The xylanase activity values obtained with oats spelts xylan as substrate were significantly higher than the values obtained with birchwood xylan, suggesting that oats spelts xylan was more readily hydrolysed. Centrifugation of the xylan substrate was essential when using the Somogyi-Nelson assay due to interference by the insoluble xylan fraction. The above huge discrepancies in activity values indicate that these assays did not allow a reliable comparison of the activities of xylanolytic enzyme mixtures and preparations containing only one endo-β-1,4-xylanase without accessory enzymes.

2.2. Introduction

Xylanase activity is usually assayed by determining the number of D-xylose equivalents released during incubation of the enzyme with its substrate. The two most commonly used methods for determining reducing sugars are the Somogyi-Nelson (Nelson, 1944; Somogyi, 1952) and the 3,5-dinitrosalicylic acid (DNS) assays (Bailey *et al.*, 1992; Miller, 1959). These methods are effective in determining xylanase activity values and the extent of xylan hydrolysis (Bailey *et al.*, 1992). The Somogyi-Nelson method has been shown to be more sensitive than the DNS method, but always yields lower values for enzymatic activities (Bailey *et al.*, 1992; Breuil and Saddler, 1985; Jeffries *et al.*, 1998) because the DNS reagent itself causes partial hydrolysis of oligosaccharides, thus increasing the number of free reducing groups in the colour reaction (Bailey *et al.*, 1992). Another major disadvantage of the DNS method is the non-stoichiometry of the colour response (Bailey, 1988). Furthermore, the actual reducing hemiacetal groups are overestimated by the DNS method, due to an increase in the colour intensity of oligomeric sugars with increasing degree of polymerisation (DP) (Breuil and Saddler, 1985; Khan, 1986).

However, despite the apparent superiority of the Somogyi-Nelson method over the DNS method (Breuil and Saddler, 1985), the latter was recommended by an International Energy Agengy (IEA) network collaboration programme headed by Bailey *et al.* (1992). The standard deviation for a single sample distributed to 20 laboratories for determining xylanase activity was 108 % of the mean (Bailey *et al.*, 1992). However, after standardisation of the method and substrate (birchwood xylan), the standard deviation was reduced to 17 % of the mean after rejection of two outliers, thus allowing meaningful comparison of results obtained in different laboratories (Bailey *et al.*, 1992). It has been recommended that reducing sugar assays can only be used as broad indicators of enzymatic activity (Jeffries *et al.*, 1998). Other existing xylanase assays, *i.e.* the viscosimetric xylanase assay, Remazol Brilliant Blue-xylan (RBB-xylan) assay, the turbidometric assay and the nephelometric assay of xylanase activity are usually used when the enzyme preparations have a high reducing sugar background or when their performance on insoluble xylan is investigated (Bailey *et al.*, 1992; Ghose and Bisaria, 1987; Sengupta *et al.*, 1987; Wang and Broda, 1992).

In a recent comparative study by Jeffries *et al.* (1998) it was found that : (1) the Somogyi-Nelson and DNS assays did not give similar responses to xylo-oligosaccharides of increasing DP, (2) on a molar basis the Somogyi-Nelson assay was less reactive and that the DNS assay was more reactive towards xylo-oligosaccharides of increasing DP, (3) it was more accurate to use xylobiose or xylotriose, rather than xylose as a standard and (4) that a meaningful analysis of xylanase kinetics, even by ion chromatography assays, was not feasible.

In this chapter a comparison of the DNS and Somogyi-Nelson assays is made for xylanase activity in supernatants from cultures of a recombinant *Saccharomyces cerevisiae* strain and a commercial xylanase preparation. These two assays gave widely differing activity values, even more so than anticipated.

2.3. Materials and Methods

2.3.1 Yeast strains

A xylanase-producing recombinant *S. cerevisiae* Y294 strain, transformed with the *XYN2* gene from *Trichoderma reesei* (La Grange *et al.*, 1996), was obtained from the Department of

Microbiology, University of Stellenbosch, Stellenbosch, South Africa and maintained on Difco yeast nitrogen base (Difco Laboratories, Detroit, MI, USA) agar slants containing 10 g glucose.l^{-1} at 4 °C. The culture was transferred to a fresh agar slant every four months.

2.3.2. Inoculum, medium and cultivation conditions

Cells from from a 24 h YPD agar plate, containing (per litre): 10 g yeast extract, 20 g peptone, 10 g glucose and 12 g agar at pH 5.5 were used to inoculate an Erlenmeyer flask containing the same medium (without agar). The glucose was autoclaved separately in distilled water and added aseptically to the sterile basal medium. After incubation for 24 h at 30 °C on a rotary shaker, 5 ml of the latter culture was transferred to a 500 ml shake flask containing 95 ml of above medium and incubated for 12 h at 30 °C. A 20 ml aliquot of this culture served as inoculum for aerobic flask cultivations in 1 litre Erlenmeyer flasks containing 200 ml of the above broth and fitted with cotton wool plugs. These were incubated at 30 °C on a rotary shaker at 200 r.min^{-1}.

2.3.3. Analyses

Whole broth samples were collected on ice, centrifuged at 3000 r.min^{-1} for 5 min and stored at –20 °C until xylanase assays were performed. For comparative purposes, the Somogyi-Nelson (Nelson, 1944; Somogyi, 1952) and the DNS assays (Bailey *et al.*, 1992) for reducing sugars, using D-xylose (Sigma, St. Louis, MO, USA) as standard, were used. A 1 % solution of birchwood xylan (Sigma) and oats spelts xylan (Sigma) were, respectively, used as substrate. The enzymatic reactions were performed with uncentrifuged as well as centrifuged substrates. A commercial xylanase preparation, Cartazyme HS-10, was obtained from Clariant UK Ltd (Horsforth, Leeds, UK) and diluted 1000-fold prior to assay. All enzymatic reactions were carried out under saturating conditions for 15 minutes at 60 °C and pH 6. One unit of activity (nkat) was defined as the amount of enzyme that released 1 nmol of xylose equivalents per second per ml of culture filtrate. Enzymatic hydrolysis products were analysed by high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) in a Dionex chromatography system, using a CarboPac™ PA-10 column (Dionex Corporation, CA, USA). D-Xylose and xylobiose (Sigma) were included as standards.

2.4. Results and Discussion

Xylanase assays were performed on a commercial enzyme preparation from *Trichoderma reesei*, Cartazyme HS-10 (Figure 2.1), and on the supernatants of a glucose-grown culture of the recombinant *S. cerevisiae* strain (Figure 2.2), using the Somogyi-Nelson and DNS assays with two different xylan substrates. *Trichoderma reesei* produces two major xylanases, Xyn1 and Xyn2, both belonging to the low molecular weight β-glycanase family G (see Chapter 1.3.1.3., Xylanase production by *Trichoderma reesei*). Both oats spelts and birchwood xylan had to be centrifuged prior to performing the Somogyi-Nelson assay due to the interference of the insoluble xylan fraction with the spectrophotometric determination of reducing sugars. However, birchwood and oats spelts xylan were totally solubilised during the DNS assay and did not require centrifugation.

The highest xylanase activity recorded for Cartazyme HS-10 (86 500 ± 6 150 nkat.ml^{-1}) was with the DNS method and oats spelts xylan as substrate (Figure 2.1). Using this method as recommended by Bailey *et al.* (1992) with birchwood xylan (not centrifuged) as substrate, the Cartazyme HS-10 activity was 49 900 ± 5 450 nkat.ml^{-1}. With centrifuged birchwood or oats spelts xylan as substrates, the respective xylanase activities obtained using the DNS method were 5.5 and 9.7-fold higher than with the Somogyi-Nelson method. This observation was in accordance with the literature, since it is known that the DNS method can yield ten-fold higher xylanase activity values than the Somogyi-Nelson method (Bailey *et al*, 1992). This commercial enzyme preparation gave higher activities with oats spelts xylan, indicating that this source of xylan was more readily hydrolysed than birchwood xylan.

Supernatants from glucose-grown flask cultures of the recombinant *S. cerevisiae* Y294 strain with significantly different xylanase activities (1250, 890, 620 and 480 nkat.ml^{-1}), as determined with the DNS assay, were chosen for the comparative study (Figure 2.2). The highest xylanase activities were recorded using the DNS method with oats spelts xylan as substrate, whereas significantly lower but comparable results were obtained with centrifuged and uncentrifuged birchwood xylan. However, the Somogyi-Nelson method, using centrifuged birchwood and oats spelt xylan as respective substrates, gave xylanase activities of 24 and 74-fold lower than the DNS method (Figure 2.2). Clearly, these differences in assay values were immensely greater than those found with Catazyme HS-10.

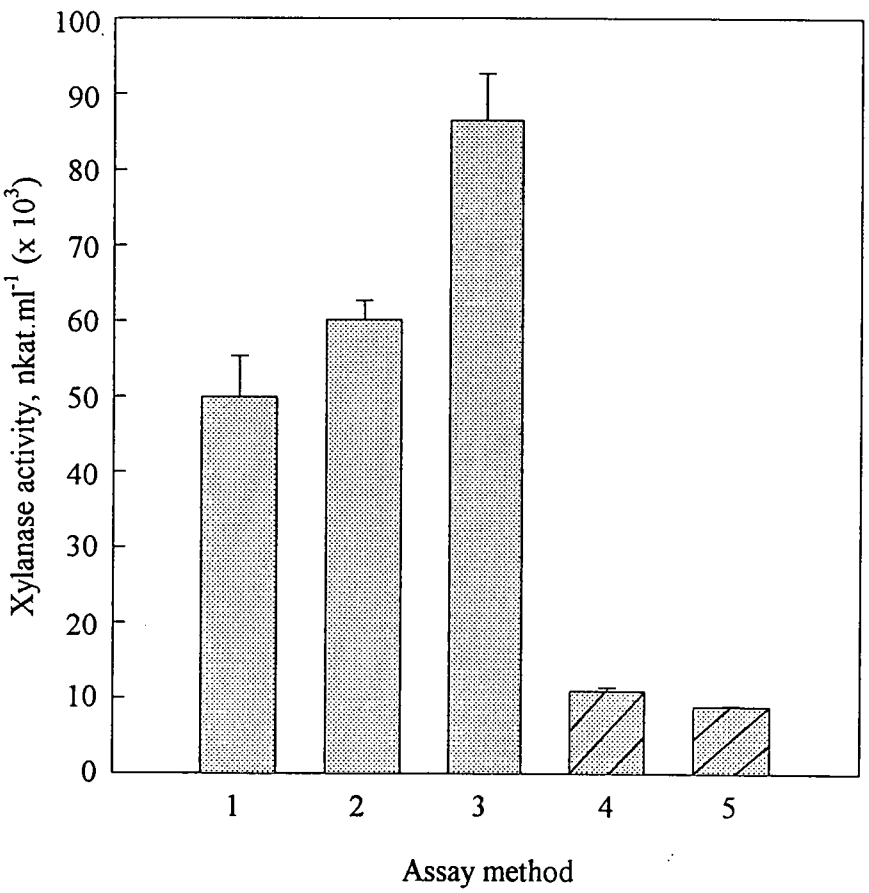


Figure 2.1. A comparison of the DNS and Somogyi-Nelson assays for xylanase activity of Cartazyme HS-10. Column 1, DNS assay with birchwood xylan; column 2, DNS assay with centrifuged birchwood xylan; column 3, DNS assay with oats spelts xylan; column 4, Somogyi-Nelson assay with centrifuged birchwood xylan and column 5, Somogyi-Nelson assay with centrifuged oats spelts xylan. The standard deviations of the mean are indicated by the error bars.

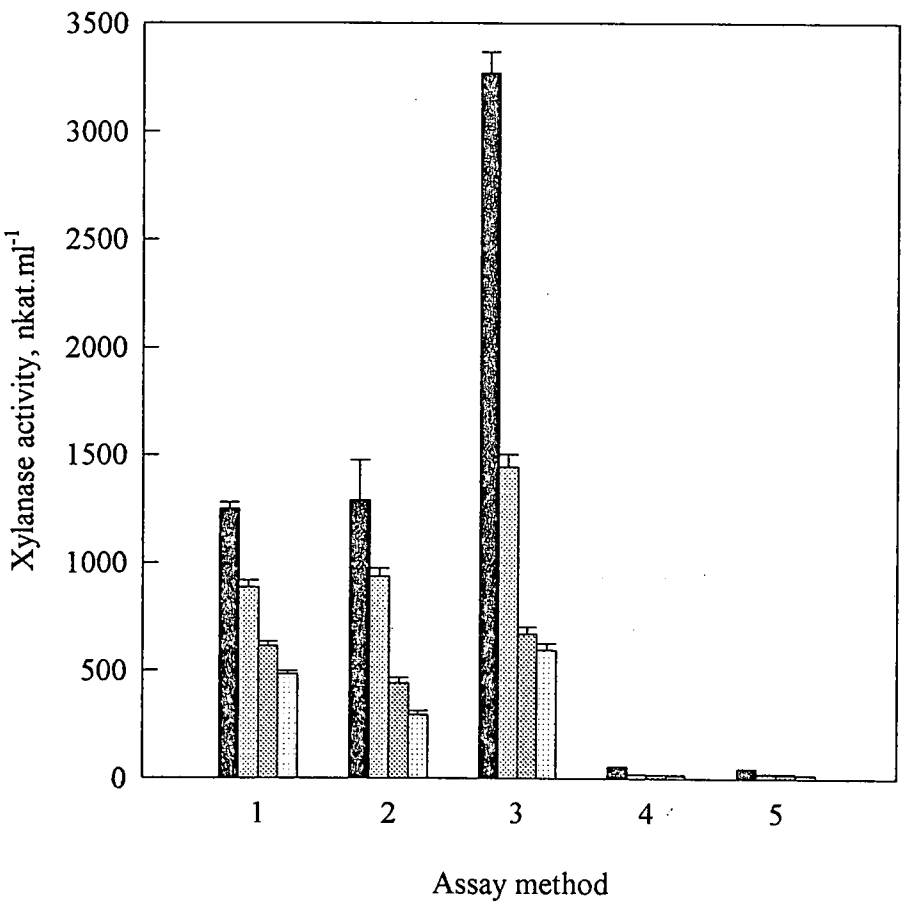


Figure 2.2. A comparison of the DNS and Somogyi-Nelson assays on four supernatants (of decreasing xylanase acivity) of a culture of a recombinant *S. cerevisiae* strain. Group 1, DNS assay with birchwood xylan as substrate; group 2, DNS assay with centrifuged birchwood xylan; group 3, DNS assay with oats spelts xylan; group 4, Somogyi-Nelson assay with centrifuged birchwood xylan and group 5, Somogyi-Nelson assay with centrifuged oats spelts xylan. The error bars indicate the standard deviations of the mean.

A possible explanation for this apparent discrepancy may lie in the fact that crude enzyme preparations from xylanolytic organisms (and commercial enzyme preparations) possess several other accessory xylanolytic enzymes that aid xylan degradation. The products of endo-β-1,4-xylanase action, namely substituted and unsubstituted xylo-oligosaccharides of varying degrees of depolymerisation, were possibly further degraded by the synergistic action of xylanases and the accessory enzymes. However, the recombinant yeast had no ancillary xylanolytic activity to enhance xylan depolymerisation; therefore, the main hydrolysis products were xylobiose, xylotriose and higher xylo-oligomers (Figure 2.3.). From Figure 2.3 it is evident that the concentrations of these xylo-oligomers were much higher in the recombinant xylanase reaction mixture.

It is conceivable that with the DNS assay, these xylo-oligomers were partially hydrolysed during the assay, thereby increasing the number of reducing groups, so that higher xylanase activities were obtained with the DNS assay than with the Somogyi-Nelson assay. A further reason why the DNS assay of the recombinant xylanase activity gave a much higher value than the Somogyi-Nelson assay, might be the colour-enhancing effect of an increased degree of polymerisation (DP) of the xylo-oligomers (Breuil and Saddler, 1985; Haltrich *et al.*, 1996; Khan, 1986). These results highlight the need for an accurate xylanase activity assay that provides information on the reaction products, as well as giving comparable results for crude xylanolytic enzyme mixtures and preparations containing only a single xylanase.

Although all the products and intermediates can be separated and assessed with HPAEC-PAD analysis, quantification on a molar basis remains problematic (Jeffries *et al.*, 1998). This is due to the fact that response factors for the majority of xylo-oligosaccharides, especially for the acidic xylo-oligosaccharides, are still unknown (Jeffries *et al.*, 1998).

In view of the fact that different levels of activity were more easily distinguished with the DNS assay and that this method is recommended by Bailey *et al.* (1992) for meaningful comparison of results obtained in other laboratories, it was chosen as the preferred assay procedure for the remainder of this thesis. Furthermore, in the majority of articles published after 1992, xylanase activities were assayed using the DNS method with birchwood xylan as substrate.

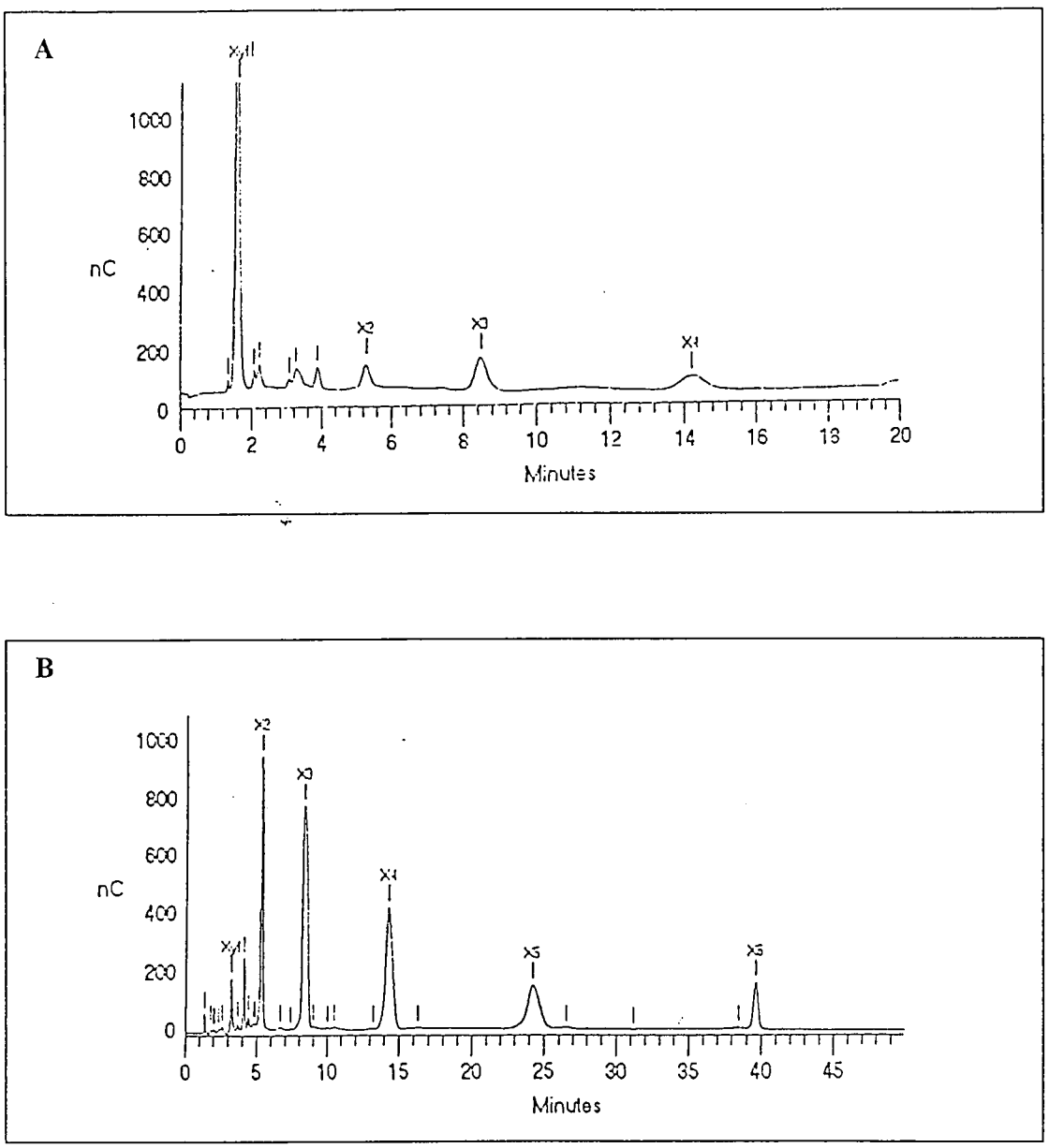


Figure 2.3. The main hydrolysis products given by a 1000-fold dilution of Cartazyme HS-10 (A) and the xylanase produced by the recombinant *S. cerevisiae* Y294 strain (B) after 5 min of incubation with 1 % (w/v) birchwood xylan as determined by HPAEC-PAD analysis. Xylose (Xyl1) and xylobiose (X2) were identified using standards. The peaks marked X3, X4, X5 and X6 are probably xylotriose, xylotetraose, xylopentaose and xylohexsaose.

2.5. References

Bailey, M.J. (1988). A note on the use of dinitrosalicylic acid for determining the products of enzymatic reactions. *Appl Microbiol Biotechnol* **29**, 494-496.

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

Breuil, C. & Saddler, J.N. (1985). Comparison of the 3,5-dinitrosalicylic acid and Nelson-Somogyi methods of assaying for reducing sugars and determining cellulase activity. *Enzyme Mirob Technol* 7, 327-332.

Ghose, T.K. & Bisaria, V.S. (1987). Measurement of hemicellulase acivities. Part1: xylanases. *Pure Appl Chem* **59**, 1739-1752.

Jeffries, T.W., Yang, V.W. & Davis, M.W. (1998). Comparative study of xylanase kinetics using dinitrosalicylic, arsenomolybdate, and ion chromatographic assays. *Appl Biochem Biotechnol* **70-72**, 257-265.

Khan, A.W. (1986). Assay of xylanase and xylosidase activities in bacterial and fungal cultures. *Enzyme Microb Technol* **8**, 373-377.

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.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426-428.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem* **153**, 375-380.

Sengupta, S., Khowala, S. & Goswami, P.K. (1987). Assay of endo-β-D-xylanase activity with a soluble *O*-(carboxymethyl) derivative of larch-wood D-xylan. *Carbohydr Res* **168**, 156-161.

Somogyi, M. (1952). Notes on sugar determination. *J Biol Chem* **195**, 19-23.

Wang, P. & Broda, P. (1992). Stable defined substrate for turbidometric assay of endoxylanases. *Appl Environ Microbiol* **58**, 3433-3436.

CHAPTER 3

THE EFFECT OF MEDIUM COMPOSITION AND CARBON SOURCES ON XYLANASE PRODUCTION IN BATCH CULTURE

3.1. Abstract

The effect of different carbon sources on the production of endo-β-1,4-xylanase by a recombinant strain of *Saccharomyces cerevisiae* Y294 was evaluated. Expression of the heterologous xylanase gene by this yeast was under control of a promoter-terminator expression cassette derived from the alcohol dehydrogenase II gene (*ADH2*), which is mainly regulated through carbon catabolite repression. The highest specific rate of xylanase production (20.8 nkat .mg biomass^{-1}.h^{-1}) and highest activity (1 590 nkat.ml^{-1}) were recorded with glucose as carbon source. Galactose was slightly inferior to glucose in sustaining xylanase production. The inclusion of fructose or sucrose in the culture medium resulted in low xylanase production rates and activity levels. Contrary to what was expected, this recombinant yeast strain was unable to utilise maltose or glycerol as carbon source. The use of ethanol as carbon source, which is classically used for derepression of *ADH2*, also resulted in poor xylanase production.

3.2. Introduction

La Grange *et al.* (1996) cloned and expressed *XYN2* (encoding an endo-β-xylanase, Xyn2, of *Trichoderma reesei*) in *Saccharomyces cerevisiae*. Expression of *XYN2* was obtained with multi-copy plasmids harbouring a promoter-terminator expression cassette derived from the alcohol dehydrogenase II gene (*ADH2*) (La Grange *et al.*, 1996). The selection system used was based on that described by Loison *et al.* (1986), thus facilitating β-xylanase production in complex growth media without risking loss of the episomal plasmid (La Grange *et al.*, 1996).

Fructose and mannose also exert glucose repression of a number of genes, but to a lesser extent than glucose, however (Gancedo, 1992; Klein *et al.*, 1998). After uptake, the above sugars are phosphorylated. This phosphorylation-step is carried out by hexokinase PI, PII (EC 2.7.1.1) and glucokinase, encoded by *HXK1*, *HXK2* and *GLK1*, respectively (Klein *et al.*, 1998). Glucokinase (EC 2.7.1.2) is specific for the phosphorylation of glucose and mannose. Sucrose is hydrolysed extracellularly into glucose and fructose by invertase (EC 3.2.1.26) , encoded by *SUC2* (Klein *et al.*, 1998; Meijer *et al.*, 1996). By contrast, maltose is taken up via maltose permease and then intracellularly hydrolysed by maltase (EC 3.2.1.20) into two glucose units (Klein *et al.*, 1998). Maltose permease is encoded by

MALT and maltase by *MALS*. The uptake of galactose is facilitated by galactose permease, encoded by *GAL2*. Once inside the cell, galactose is phosphorylated by galactokinase (EC 2.1.7.6), encoded by *GAL1* (Klein *et al.*, 1998). Galactose-1-phosphate is then further converted, first to glucose-1-phosphate and then to glucose-6-phosphate by the enzymes of the Leloir pathway, encoded by *GAL7*, *GAL10* and *GAL5* (Klein *et al.*, 1998). *GAL5* encodes for phosphoglucomutase (EC 5.4.2.2). A synopsis of the metabolism of the carbon sources used in this chapter is given in Figure 3.1, which is relevant to the proposed mechanism of glucose repression of gene expression.

Currently, it is envisaged that the 'signal' for glucose repression is produced at or above the level of glucose-6-phosphate (Gancedo, 1992). The observations that a mutant strain defective in hexokinase isozyme 2 (Hxk2) was unable to exhibit glucose repression of invertase (*SUC2*), that galactose and maltose were unable to cause glucose repression in this strain and that a glucose analogue, 2-deoxyglucose, which can be phosphorylated after uptake but not metabolised, caused glucose repression of *SUC2*, supports this view (Meijer *et al.*, 1996; 1998). In view of this, it has been suggested that the generation of the initial signal is caused either by the extra- or intracellular glucose concentration or by an increased carbon flux through the metabolic pathways of the yeast (Meijer *et al.*, 1998).

The main aim of the investigation reported here was to evaluate the effect of different carbon sources on xylanase production by a recombinant strain of *S. cerevisiae* Y294 in batch culture. For the subsequent experiments it was essential to determine the effect of the medium composition on the production of heterologous xylanase by this recombinant yeast strain.

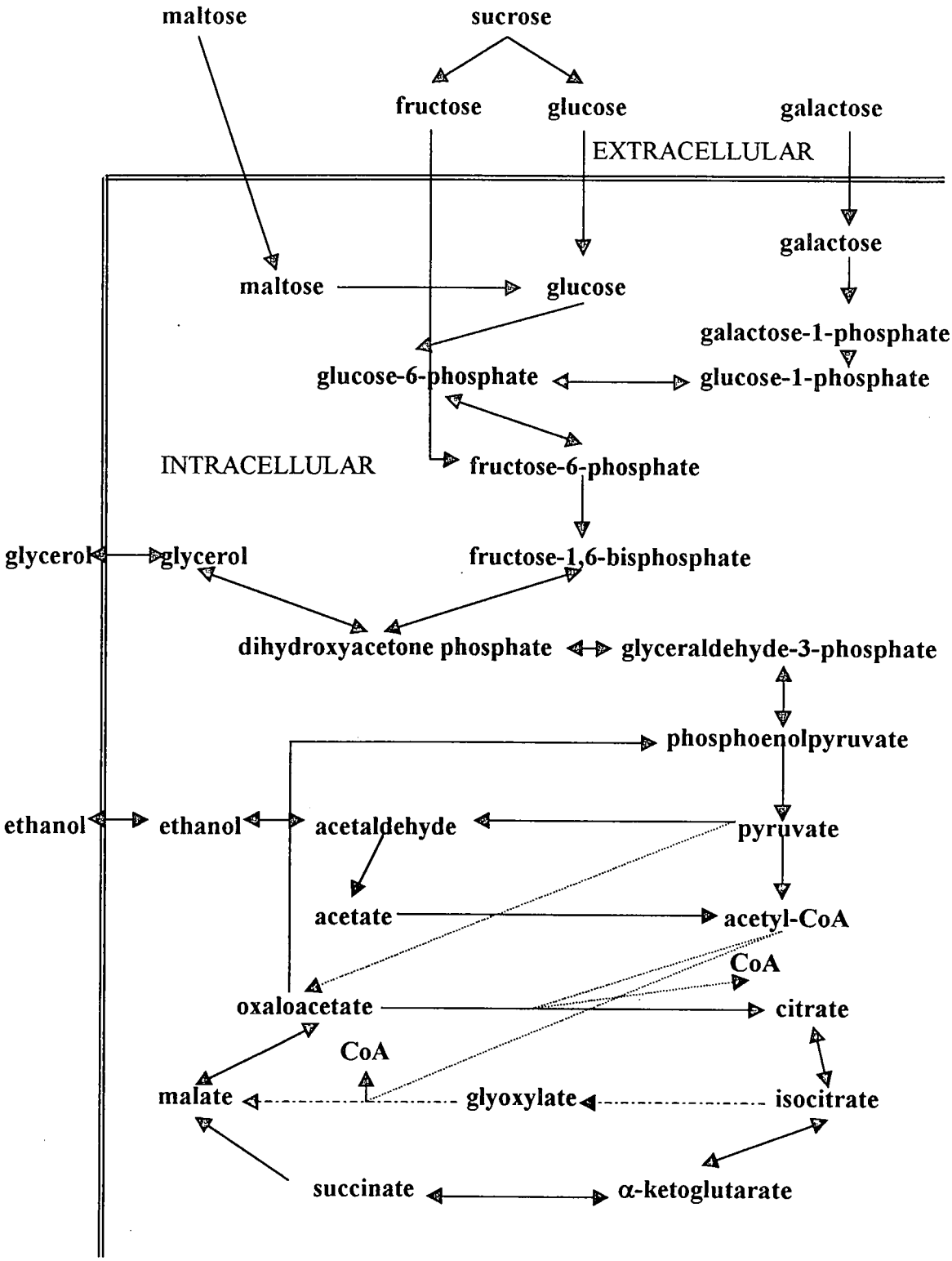


Figure 3.1. A synopsis of the pathways involved in the catabolism of maltose, sucrose, glucose, galactose, fructose, ethanol and glycerol (adapted from Klein *et al.*, 1998).

3.3. Materials and Methods

3.3.1 Yeast strains

The xylanase-producing *S. cerevisiae* Y294 strain was provided by Prof. W.H. van Zyl, Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa and maintained as described in Chapter 2.

3.3.2. Inoculum and medium

Five different media were tested for their ability to sustain xylanase production in shake flask culture (Table 3.1). The yeast nitrogen base medium was prepared according to the manufacturer's recommendations. All shake flask cultivations were carried out at an initial pH of 5.5. The carbon source for each medium was sterilised separately; sugars by autoclaving and ethanol by membrane filtration, and added aseptically to the above sterile basal media. This sterile broth was inoculated with a 10 % (v/v) inoculum prepared as described in Chapter 2.

Table 3.1. A summary of the different media used in this chapter.

	Concentration of medium components, ($g.l^{-1}$)				
Medium component	Medium A	Medium B	Medium C	Medium D	Medium E
Yeast extract[a]	3	10	2	3	-
Oxoid special peptone[b]	-	20	4	0	-
Citric acid	0.25	-	-	0.25	-
$(NH_4)_2SO_4$	6	-	-	-	-
Urea	-	-	-	2.7	-
KH_2PO_4	10	-	2	10	-
$MgSO_4.7H_2O$	0.8	-	0.4	0.8	-
$CaCl.2H_2O$	0.05	-	0.02	0.05	-
Yeast nitrogen base, (YNB)[c]	-	-	-	-	6.7
Trace element solution[d]	1 ml	1 ml	1 ml	1 ml	-

[a], Biolab diagnostics Ltd, Midrand, South Africa.
[b], Oxoid, Basingstoke, Hampshire, England.
[c], Difco Laboratories, Detroit, MI, USA.
[d], du Preez and van der Walt (1983).

3.3.3. Cultivation conditions

Aerobic flask cultivations were carried out in 1 litre Erlenmeyer flasks fitted with cotton wool plugs, containing 200 ml of the appropriate medium. These were incubated at 30 °C on a rotary shaker at 200 $r.min^{-1}$. Cultivations were also carried out in a 2-litre Multigen F-2000 bioreactor (New Brunswick Scientific, Edison, NJ, USA) equipped with an exhaust gas condensor (reflux cooler), a pH electrode (Mettler Toledo, Halstead, Great Britain) and a polarographic oxygen electrode (Ingold AG, Urdorf, Switzerland), using a 1000 ml working volume. These cultivations were conducted at 30 °C with the pH controlled at pH 5.5 (± 0.1) by automatic titration with 3 M KOH. The dissolved oxygen tension was maintained above 30 % of saturation using an aeration rate of 1000 $ml.min^{-1}$ and manual adjustment of the stirrer speed between 400 and 800 $r.min^{-1}$.

3.3.4. Analyses

Culture turbidity was monitored with a Klett-Summerson colorimeter (Klett Mfg Co. Inc., Philadelphia, PA, USA) fitted with a no. 64 red filter and converted to dry biomass using a standard curve. Whole broth samples were collected on ice, centrifuged at 3000 $r.min^{-1}$ for 5 min, washed twice with distilled water and dried at 105 °C overnight prior to gravimetric determination of the dry biomass concentration. Ethanol in the supernatants were determined with a Hewlett-Packard 5710A gas chromatograph equipped with a 3390A integrator (Hewlett-Packard, Atlanta, GA, USA) and fitted with a glass column (1.5 × 1.5 mm ID) packed with 80 to 100 mesh Porapak N (Waters Associates, Milford, MA, USA) and using 50 ml nitrogen carrier $gas.min^{-1}$ at an oven temperature of 165 °C. The remainder of the supernatant was preserved with sodium azide and stored at –20 °C until xylanase assays and glucose determinations were performed. The DNS method (Bailey *et al.*, 1992), using D-xylose (Sigma, St. Louis, MO, USA) as standard with a 1 % solution of birchwood xylan (Sigma) as substrate at pH 6 and 60 °C, was used for xylanase assays. One unit of activity (nkat) was defined as the amount of enzyme that released 1 nmol of xylose equivalents per second per ml of culture filtrate. Specific xylanase activity was expressed as $nkat.mg\ biomass^{-1}$. Sugar was determined with a Sugar Analyser I HPLC equipped with a refractive index detector and Sugar Pack 1^{TM} column (Waters Associates, Milford, MA, USA) operating at 85°C and with an eluent (degassed water) flow rate of 0.5 $ml.min^{-1}$.

3.4. Results and Discussion

Five different media, each with three different initial glucose concentrations, were evaluated for sustaining growth and xylanase production by the recombinant xylanase-producing *S. cerevisiae* strain (Figure 3.2). The highest xylanase activities and specific xylanase activities were obtained in complex medium B and medium E, respectively. Medium B was also the only medium that resulted in substantial assimilation of the ethanol formed during diauxic growth. In all the other media significant amounts of residual ethanol remained after 72 h of cultivation with either 10 or 20 g glucose.l^{-1}. High xylanase activities were also obtained in complex medium C and the yeast nitrogen base medium (E) with 10 g glucose.l^{-1}. Poor xylanase production was obtained in the urea-based medium (D). Medium B was chosen for subsequent investigation of xylanase production in bioreactor cultures, since the highest xylanase activities were obtained with this medium.

Seven different carbon sources were evaluated in 2-litre bioreactors in terms of their effect on xylanase production by the recombinant *S. cerevisiae* strain, using complex medium B (Figures 3.3. to 3.9 and Table 3.2). Monosaccharides (glucose, galactose, fructose), disaccharides (maltose, sucrose) and gluconeogenic carbon sources (ethanol, glycerol) were used. The highest xylanase activity (1 590 nkat.ml^{-1}) and specific rate of xylanase production (20.8 nkat.mg biomass^{-1}.h^{-1}) were found with 10 g glucose.l^{-1} as carbon source (Figure 3.3). The culture was characterised by a rapid fermentation of glucose, with a maximum specific growth rate of 0.42 h^{-1} in the first exponential growth phase, followed by the aerobic respiration of the accumulated ethanol during the second exponential growth phase. According to Beck and von Meyenburg (1968), specific growth rates of 0.12 to 0.18 h^{-1} are obtained during growth on ethanol, depending on the medium composition. Although a complex medium was used here, the maximum specific growth rate of the recombinant strain on ethanol was only 0.12 h^{-1}, probably due to the auxotrophic requirement of this yeast for histidine and tryptophan. Xylanase production was initiated upon depletion of glucose and coincided with the utilisation of the ethanol. This indicated that *ADH2*-regulated xylanase production was, as expected, repressed in the presence of glucose.

Galactose was the second most efficient carbon source for xylanase production (17.9 nkat.mg biomass^{-1}.h^{-1}) and the second highest extracellular xylanase activity

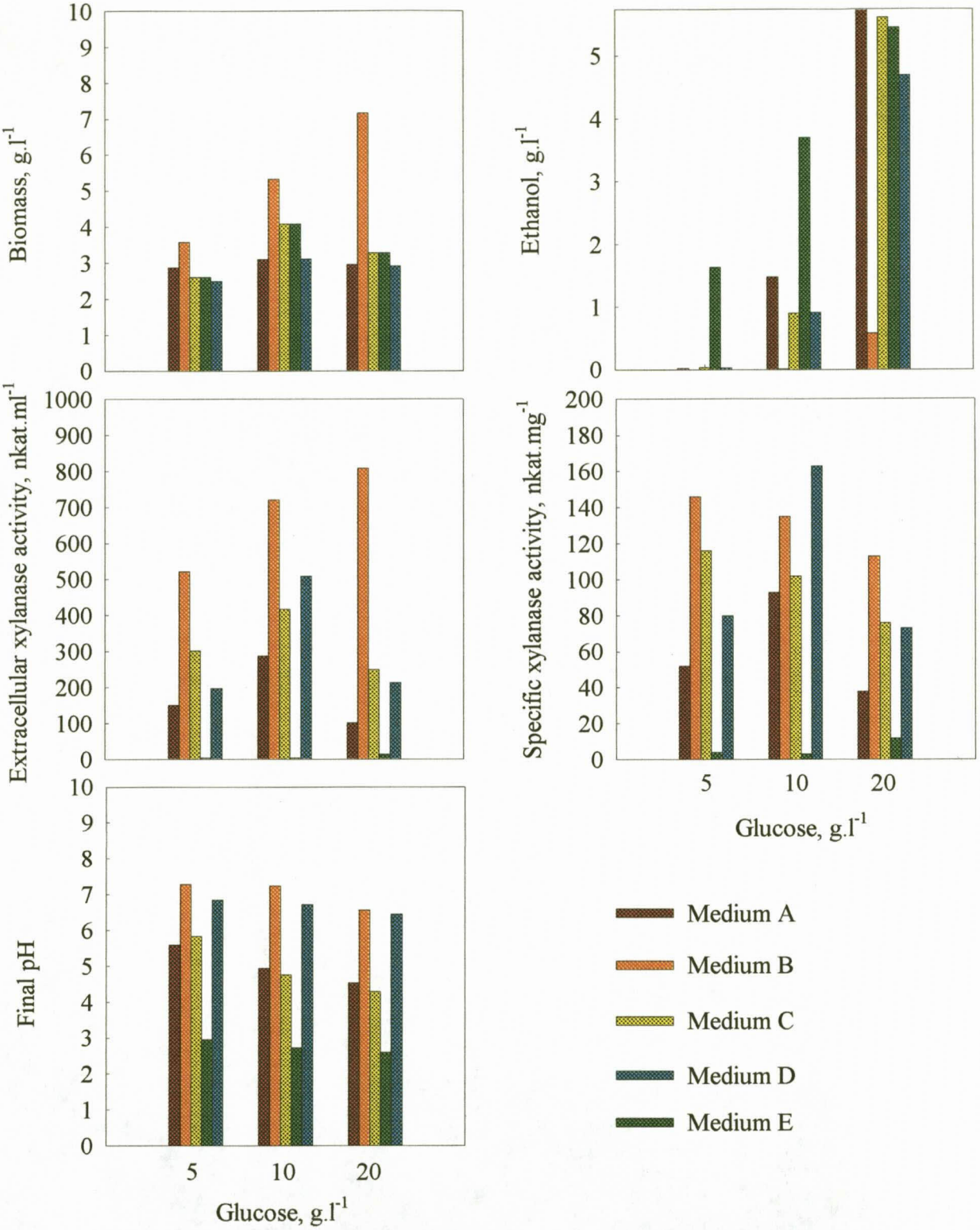


Figure 3.2. Culture parameters after 72 h growth of the recombinant *S. cerevisiae* strain in shake flasks using five different media with 5, 10, and 20 g glucose.l^{-1}, respectively, as initial carbon source. Mean values of independent duplicate experiments are given.

(1 455 nkat.ml^{-1}) was obtained using this carbon source (Table 3.2; Figure 3.4). Maximum xylanase activity was reached at 60 h of cultivation. Maximum specific growth rates of 0.39 and 0.10 h^{-1}, respectively, were obtained during growth on galactose and the resulting ethanol. Again xylanase production coincided with the depletion of the fermentable sugar (galactose) from the medium. La Grange *et al.* (1996) reported a maximum xylanase activity of 1 200 nkat.ml^{-1} after 80 h of shake flask cultivation in YPD medium with 8 g galactose.l^{-1} as carbon source.

Growth of the recombinant *S. cerevisiae* strain in the complex medium with fructose or sucrose as respective carbon sources (Figures 3.5 and 3.6) resulted in lower specific rates of xylanase production, as well as in lower xylanase activities than obtained with glucose or galactose as carbon source (Table 3.2). By contrast, fructose was found to repress the expression of invertase (*SUC2*) to a lesser extent than glucose (Gancedo, 1992; Klein *et al.*, 1998). Only slight differences in the maximum specific growth rate on ethanol and the specific rate of ethanol assimilation were observed, regardless whether glucose, galactose, fructose or sucrose was the initial carbon source (Table 3.2).

This recombinant yeast was unable to metabolise maltose (Figure 3.7). The recombinant yeast probably lacked the genes encoding maltose permease and / or maltase, thus prohibiting growth on this disaccharide. La Grange *et al.* (1996) did not indicate that this recombinant yeast was deficient in either of these two genes. Despite the inability of the recombinant to utilise maltose, a low xylanase activity (296 nkat.ml^{-1}) was found due to growth on the other constituents of the complex medium. This result was unfortunate, because maltose does not cause carbon catabolite repression of invertase (Meijer *et al.*, 1998). Therefore, the use of maltose-limited continuous cultures as a tool to study carbon catabolite repression of *ADH2*-regulated xylanase production by this recombinant yeast was not possible.

The recombinant *S. cerevisiae* strain failed to grow in the complex medium with glycerol as the carbon source (Figure 3.8). Both glycerol and ethanol are gluconeogenic carbon sources and generate energy (ATP) solely through aerobic respiration. However, there is no evidence that failure of growth on glycerol resulted from an inability of this strain to release glucose repression of gluconeogenic enzymes (Cortassa and Aon, 1998) which is essential for glycerol assimilation, since it could grow on ethanol (Figure 3.9).

The recombinant grew poorly when 10 g ethanol.l^{-1} was used as the initial carbon source (Figure 3.9). Ethanol has frequently been used as carbon source in molecular studies to obtain *ADH2* in a derepressed state (Ciriacy, 1975; Denis, 1984; Denis and Audino, 1991). However, even in the total absence of a carbon source that can cause carbon catabolite repression, xylanase production was poor and also resulted in a low extracellular xylanase activity (Table 3.2). Despite using an exhaust gas condensor at 4 °C on the bioreactor, a significant amount of ethanol was lost through stripping from the medium by aeration. Therefore, the specific rate of ethanol consumption and yield coefficient for this carbon source could not be accurately determined.

Denis (1984) reported that expression of *ADH2* was more efficient during the ethanol assimilation phase of diauxic growth than during growth on ethanol as sole initial carbon source. The results presented here support this observation, since xylanase production was poor in the complex medium with ethanol as initial carbon source compared to glucose and galactose as carbon sources (Table 3.2). Contrary to literature, the substrates generally believed to cause no or little carbon catabolite repression (ethanol, glycerol, maltose, fructose and galactose) failed to result in better xylanase production than obtained with glucose.

The better xylanase production during diauxic growth with glucose as initial carbon source resulted not only from the high biomass concentration at the onset of xylanase production. Despite the fact that the specific rates of ethanol assimilation in the media containing glucose and ethanol as the respective initial carbon sources were similar (Table 3.2), the maximum specific rate of xylanase production during the diauxic ethanol assimilation phase was more than three-fold than with ethanol as sole initial carbon source. This suggests that regulation of the *ADH2* promoter is more complicated than indicated by the current literature.

Table 3.2. Growth parameters of a recombinant strain of *S. cerevisiae* Y294 in bioreactor cultures on different carbon sources. Mean values of independent duplicate experiments are given.

Parameter	Carbon source						
	Glucose	Galactose	Fructose	Sucrose	Maltose	Glycerol	Ethanol
Final dry biomass, $g.l^{-1}$	7.08	7.55	7.78	7.52	1.90	2.25	3.78
$Y_{x/s}$	0.15	0.17	0.18	0.18	ND	ND	ND
$\mu_{max\ sugar}$, h^{-1}	0.42	0.39	0.35	0.33	ND	ND	ND
$q_s^{max}{}_{sugar}$, $g.(g.h)^{-1}$	3.38	3.68	3.62	3.81	ND	ND	ND
Maximum observed ethanol concentration, $g.l^{-1}$	4.36	4.04	4.48	4.27	0.45	0.12	ND
$Y_{ethanol/s}$	0.44	0.36	0.44	0.42	ND	ND	ND
$q_p^{max}{}_{ethanol}$, $g.(g.h)^{-1}$	0.895	0.781	0.892	0.98	ND	ND	ND
$\mu_{max\ ethanol}$, h^{-1}	0.12	0.10	0.12	0.09	ND	ND	0.11
$q_s^{max}{}_{ethanol}$, $g.(g.h)^{-1}$	0.119	0.110	0.113	0.114	ND	ND	0.121
Extracellular xylanase activity, $nkat.ml^{-1}$	1590	1455	805	759	296	257	438
$q_p^{max}{}_{xylanase}$, $nkat.(mg.h)^{-1}$	20.75	17.85	14.34	13.83	3.36	2.94	5.94

ND not determined.

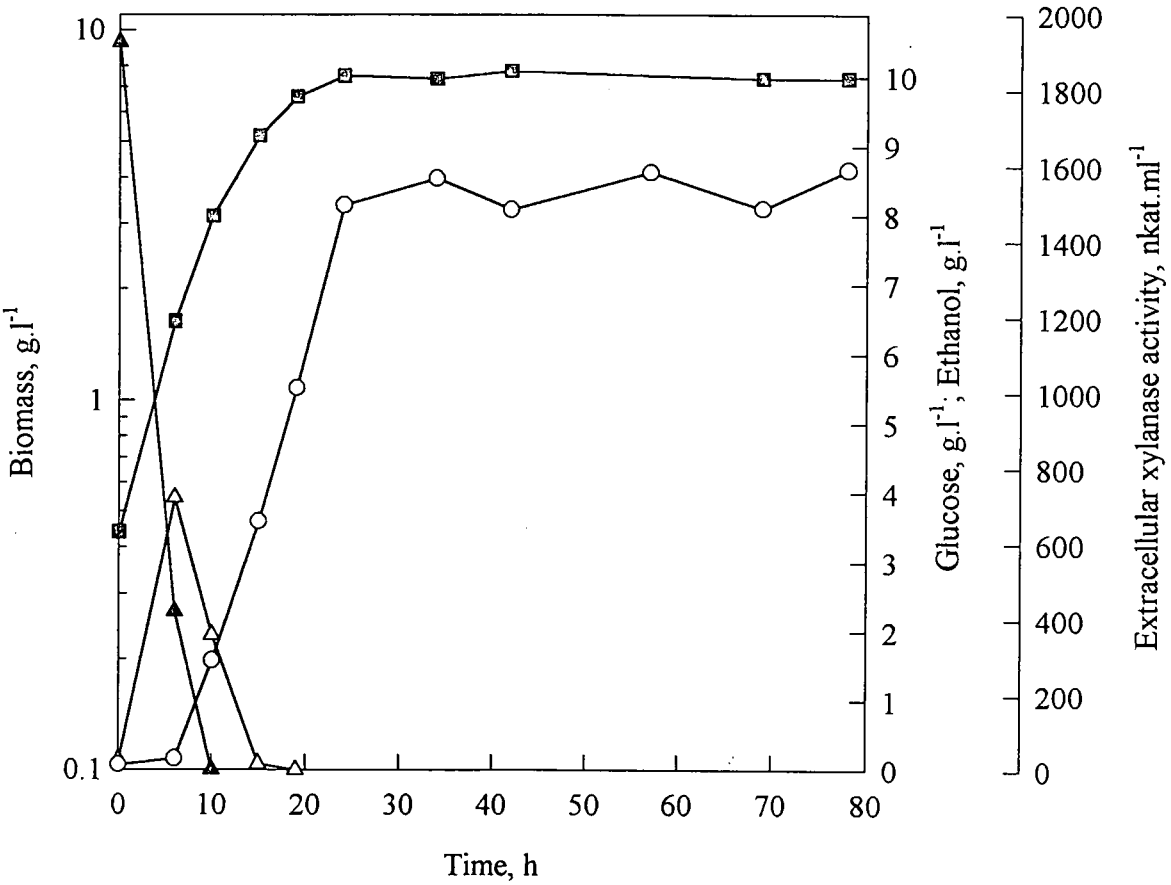


Figure 3.3. Typical bioreactor cultivation profile of the recombinant *S. cerevisiae* strain grown in complex medium B with 10 g glucose.l^{-1} as the initial carbon source. Symbols: biomass (▣), glucose (▲), ethanol (△) and extracellular xylanase activity (○).

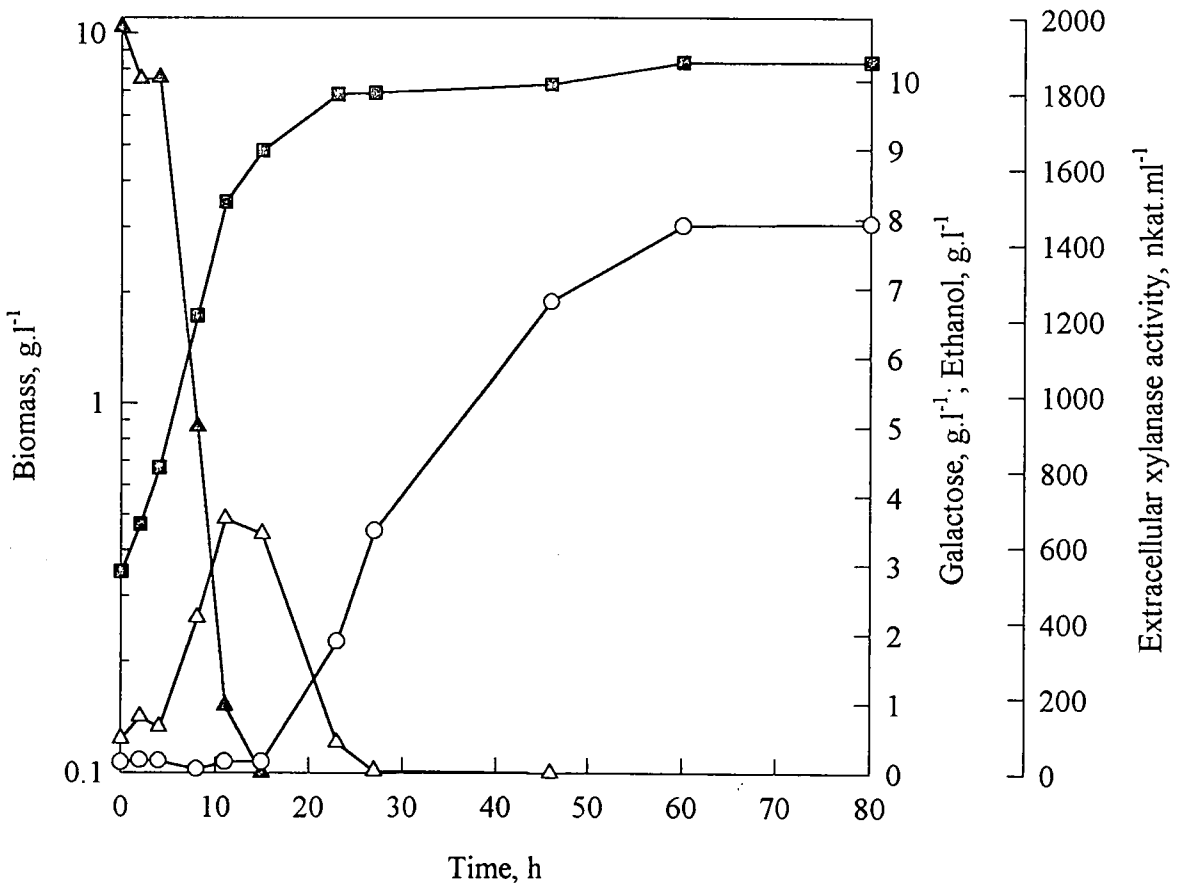


Figure 3.4. Typical bioreactor cultivation profile of the recombinant *S. cerevisiae* strain grown in complex medium B with 10 g galactose.l^{-1} as the initial carbon source. Symbols: biomass (■), galactose (▲), ethanol (△) and extracellular xylanase activity (○).

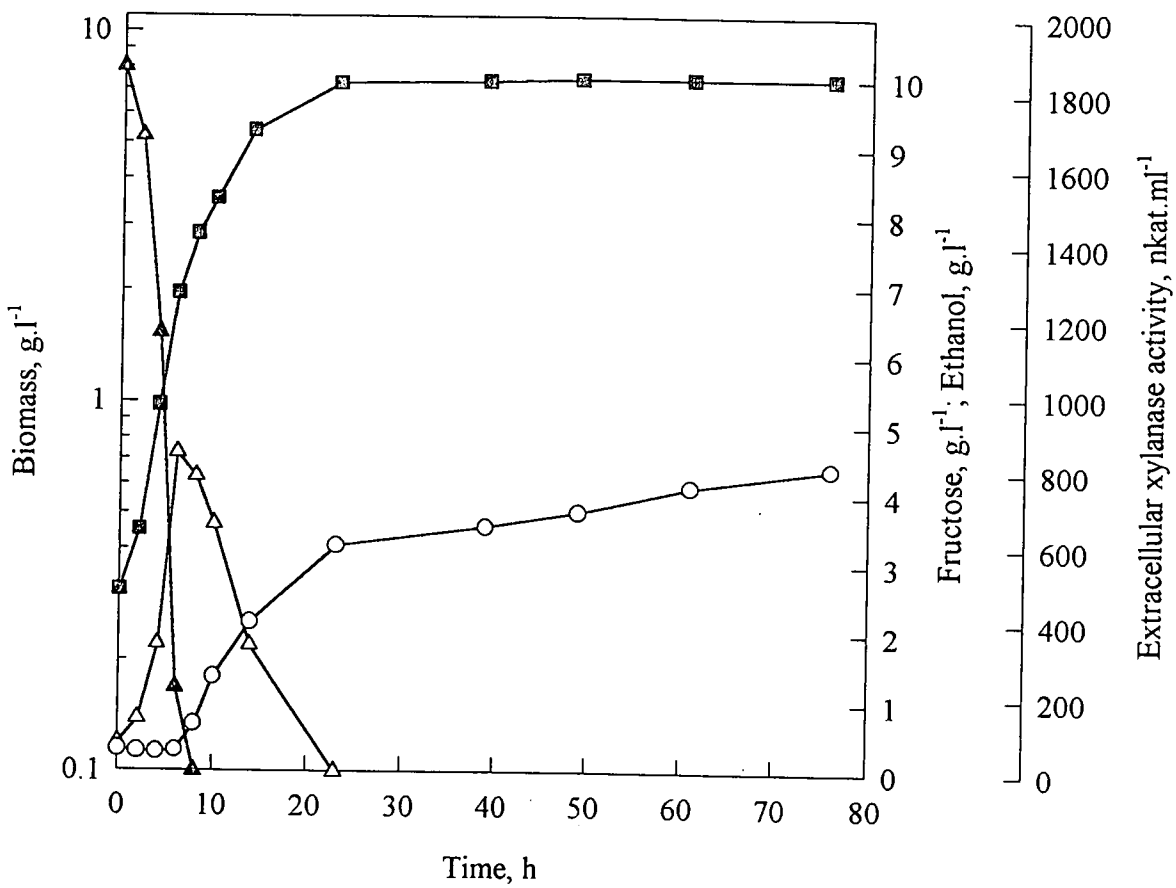


Figure 3.5. Typical bioreactor cultivation profile of the recombinant *S. cerevisiae* strain grown in complex medium B with 10 g fructose.l^{-1} as the initial carbon source. Symbols: biomass (■), fructose (▲), ethanol (△) and extracellular xylanase activity (○).

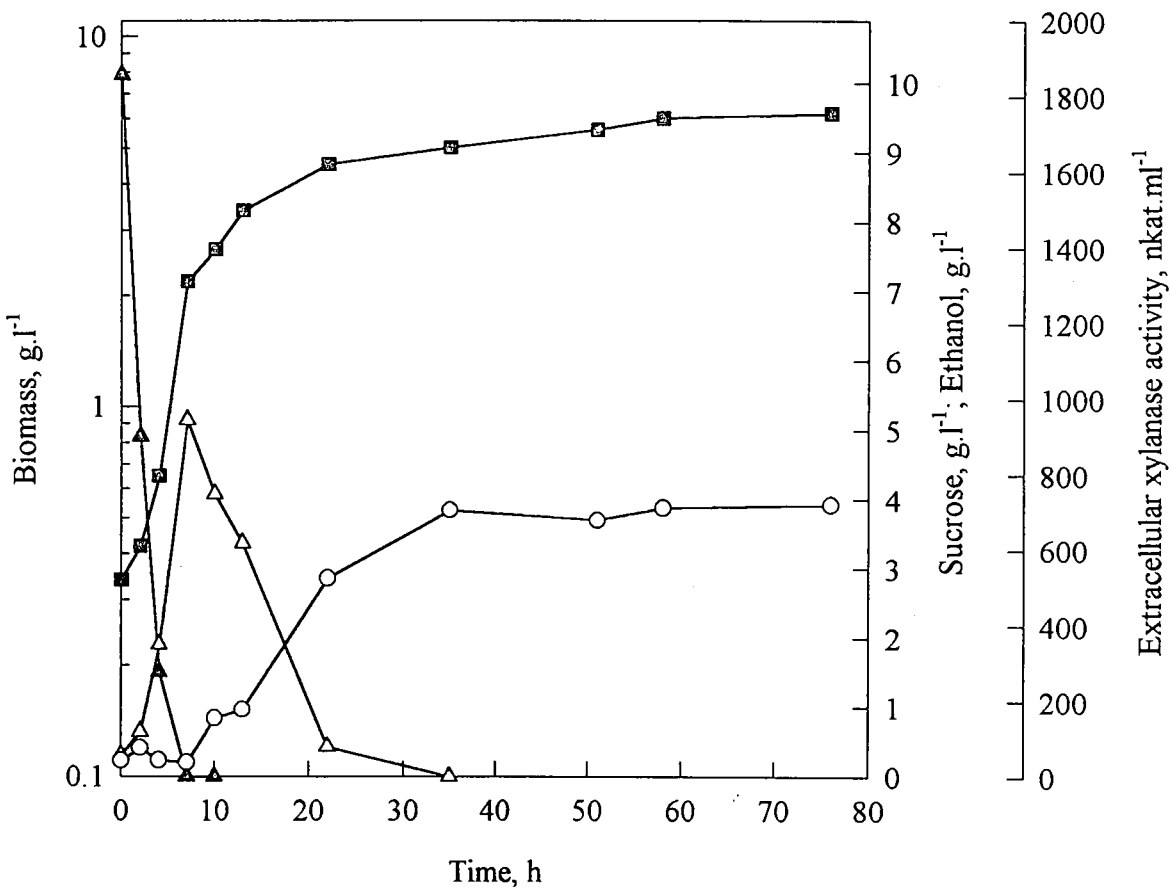


Figure 3.6. Typical bioreactor cultivation profile of the recombinant *S. cerevisiae* strain grown in complex medium B with 10 g sucrose.l^{-1} as the initial carbon source. Symbols: biomass (■), sucrose (▲), ethanol (△) and extracellular xylanase activity (○).

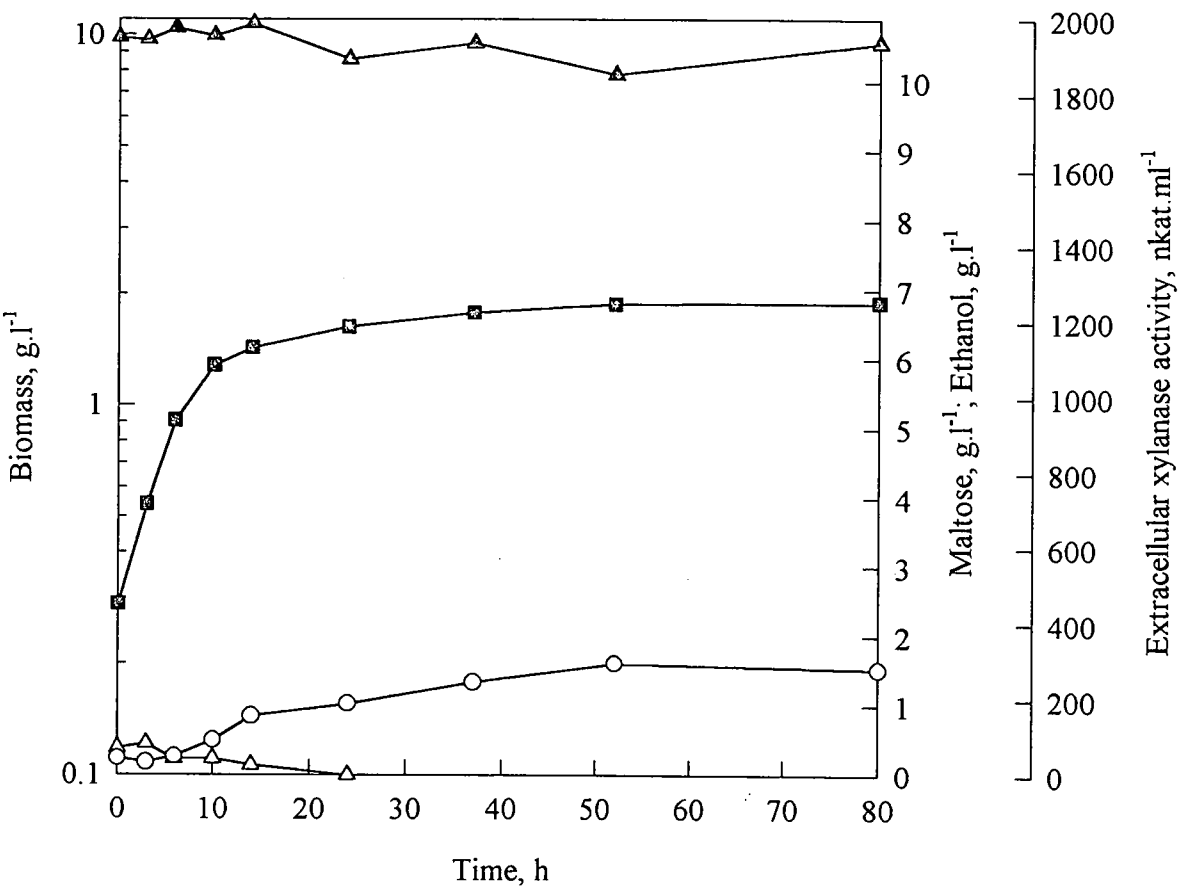


Figure 3.7. Typical bioreactor cultivation profile of the recombinant *S. cerevisiae* strain grown in complex medium B with 10 g maltose.l^{-1} as the initial carbon source. Symbols: biomass (■), maltose (▲), ethanol (△) and extracellular xylanase activity (○).

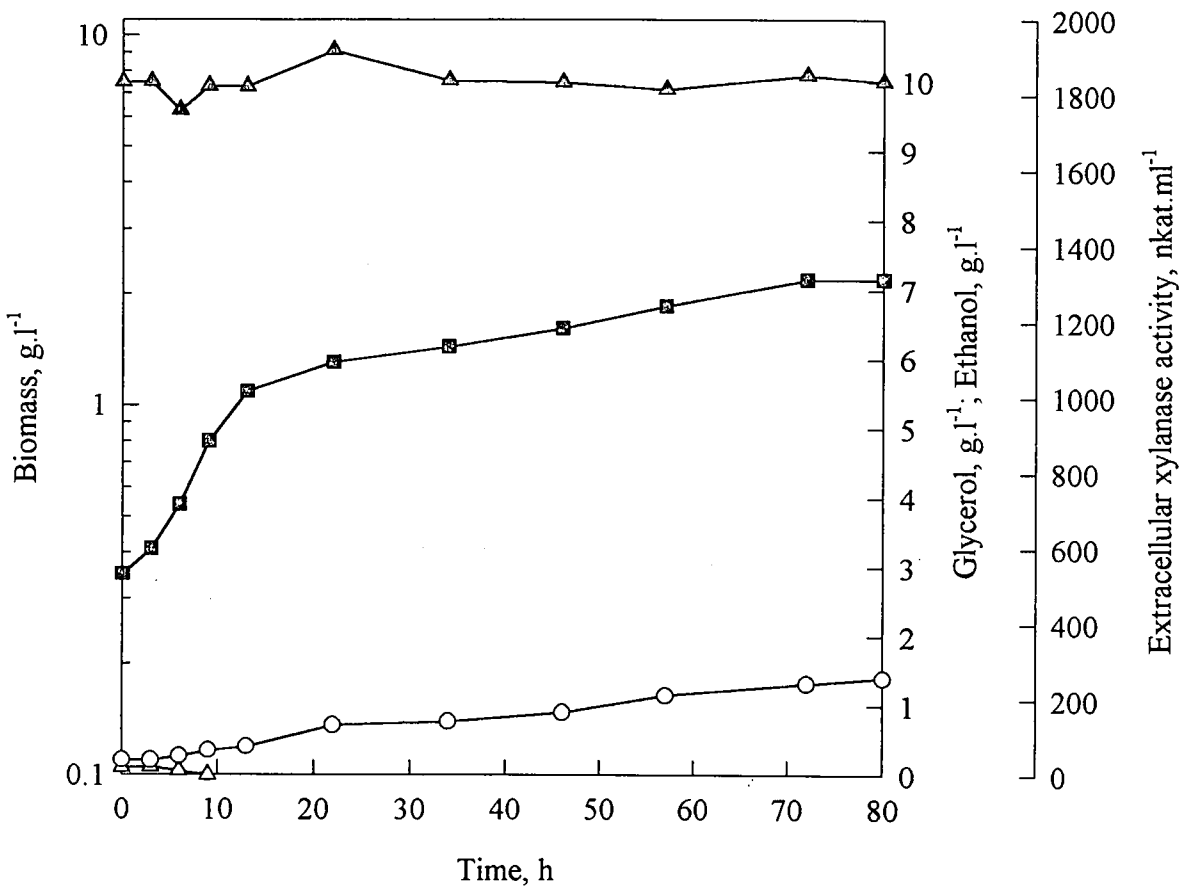


Figure 3.8. Typical bioreactor cultivation profile of the recombinant *S. cerevisiae* strain grown in complex medium B with 10 g glycerol.l^{-1} as the initial carbon source. Symbols: biomass (■), glycerol (▲), ethanol (△) and extracellular xylanase activity (○).

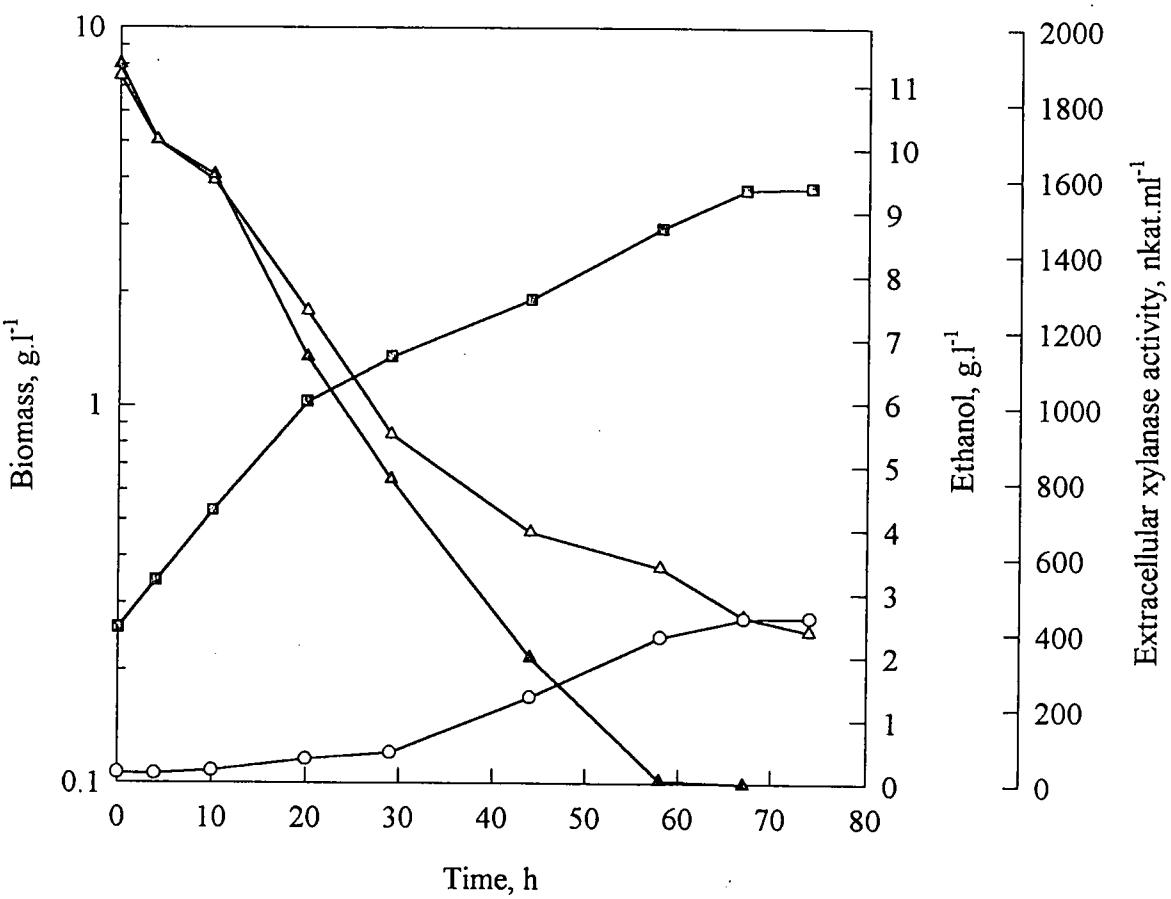


Figure 3.9. Typical bioreactor cultivation profile of the recombinant *S. cerevisiae* strain grown in complex medium B with 10 g ethanol.l^{-1} as the initial carbon source. Symbols: biomass (■), ethanol (▲), extracellular xylanase activity (○) and decrease in ethanol concentration in a sterile culture vessel due to evaporation (△).

3.5. Nomenclature

$Y_{x/s}$	Biomass yield coefficient
$\mu_{max\ sugar}$, h^{-1}	Maximum specific growth rate on carbon source
$q_s^{max}{}_{sugar}$, $g.g^{-1}.h^{-1}$	Maximum specific rate of sugar assimilation
$Y_{ethanol/s}$	Ethanol yield coefficient
$q_p^{max}{}_{ethanol}$, $g.(g.h)^{-1}$	Maximum specific rate of ethanol production
$\mu_{max\ ethanol}$, h^{-1}	Maximum specific growth rate on ethanol
$q_s^{max}{}_{ethanol}$, $g.(g.h)^{-1}$	Maximum specific rate of ethanol assimilation
$q_p^{max}{}_{xylanase}$, $nkat.(mg.h)^{-1}$	Maximum specific rate of xylanase production per mg of dry biomass

3.6. References

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

Beck, C & von Meyenburg, H.K. (1968). Enzyme pattern and aerobic growth of *Saccharomyces cerevisiae* under various degrees of glucose limitation. *J Bacteriol* **96**, 479-486.

Ciriacy, M. (1975). Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae.* II. Two loci controlling synthesis of the glucose-repressible ADH II. *Mol Gen Genet* **138,** 157-164.

Cortassa, S. & Aon, M.A. (1998). The onset of fermentative metabolism in continuous culture depends on the carbon catabolite properties of *Saccharomyces cerevisiae*. *Enzyme Microb Technol* **22**, 705-712.

Denis, C.L. (1984). Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II. *Genetics* **108,** 833-844.

Denis, C.L. & Audino, D.C. (1991). The CCR1 (SNF1) and SCH9 protein kinases act independently of cAMP-dependent protein kinase and the transcriptional activator ADR1 in controlling yeast *ADH2* expression. *Mol Gen Genet* **229,** 395-399.

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

Gancedo, J.M. (1992). Carbon catabolite repression in yeast. *Eur J Biochem* **206**, 297-313.

Klein, C.J.L., Olsson, L., & Nielsen, J. (1998). Glucose control in *Saccharomyces cerevisiae*: the role of *MIG1* in metabolic functions. *Microbiol* **144**, 13-24.

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.

Meijer, M.M.C., Boonstra, J., Verkleij, A.J. & Verrips, C.T. (1996). Kinetic analysis of hexose uptake in *Saccharomyces cerevisiae* cultivated in continuous culture. *Biochim et Biophys Acta* **1277**, 209-216.

Meijer, M.M.C., Boonstra, J., Verkleij, A.J. & Verrips, C.T. (1998). Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than the glucose flux. *J Biol Chem* **273,** 24102-24107.

CHAPTER 4

INFLUENCE OF HOST STRAIN ON XYLANASE PRODUCTION IN CONTINUOUS CULTURE BY THREE RECOMBINANT STRAINS OF *SACCHAROMYCES CEREVISIAE*

An abridged version of this chapter has been prepared for submission to Applied Microbiology and Biotechnology.

4.1. Abstract

The influence of glucose concentration, glucose flux and growth rate on xylanase production by a recombinant strain of *Saccharomyces cerevisiae* Y294, transformed with a *Trichoderma reesei* xylanase, was investigated in carbon-limited continuous culture and compared with results obtained with *S. cerevisiae* strains H158 and CEN.PK110-6C under similar cultivation conditions. Xylanase production by strains Y294, H158 and CEN.PK110-6C was markedly reduced at glucose flux values greater than 0.98, 1.44 and 1.68 mmol glucose .g biomass^{-1}.h^{-1}, respectively, whereas aerobic ethanol production occurred only at higher glucose flux values. With strains Y294 and H158, maximum xylanase activities were found at dilution rates of 0.17 and 0.20 h^{-1}, respectively. Despite the obvious strain differences, with all three yeast strains the glucose flux appeared to contribute to a greater extent towards the regulation of xylanase production than did the steady-state residual glucose concentration in the culture. *S. cerevisiae* CEN.PK110-6C differed from strains Y294 and H158 in that xylanase production firstly decreased above a certain glucose flux and virtually ceased only at a respective higher glucose flux value, while with strains Y294 and H158 xylanase production virtually ceased upon increasing the glucose flux above a certain critical value. The *ADH2* promoter in *S. cerevisiae* CEN.PK110-6C was thus more tolerant towards an increase in glucose flux than in strains Y294 and H158.

4.2. Introduction

The regulation of *ADH2* has been extensively studied in order to elucidate the molecular mechanisms controlling carbon catabolite repression (Ciriacy, 1997). *ADH2* is expressed only in the absence or depletion of a fermentable hexose sugar from the medium (Ciriacy, 1975; Denis *et al.*, 1981; Denis and Malvar, 1990) and is not induced by ethanol (Ciriacy, 1997) as has been occasionally reported (La Grange *et al.*, 1996; Romanos *et al.*, 1992; Tøttrup and Carlsen, 1989).

Despite the wealth of knowledge that exists regarding the molecular mechanisms of *ADH2* regulation (see Chapter 1), there have been few investigations of the influence of yeast physiology on *ADH2* regulation. In addition, most of the work to date on the molecular mechanisms of *ADH2* regulation was done under ill-defined physiological conditions, using

shake flask cultures under either repressed or derepressed conditions (Sierkstra *et al.*, 1992a). A batch culture is always in a transient state; therefore, the effect of growth rate on gene expression and product formation is often ignored (Romanos *et al.*, 1992). Furthermore, it is virtually impossible to accurately determine the effect of glucose flux on gene regulation and product formation in batch cultures. The above-mentioned problems can be overcome by using chemostat cultures that facilitate experiments with cells in a well-defined physiological state.

The mere presence of glucose is incapable of causing glucose repression, suggesting that a minimum threshold concentration of extracellular or intracellular glucose or a metabolite thereof is involved with the generation of the initial signal (Meijer *et al.*, 1998; Sierkstra *et al.*, 1992b; 1994). However, the metabolic carbon flux determines the *in vivo* metabolite concentrations, which could constitute the signal mediating glucose repression. *S. cerevisiae* grown in glucose and nitrogen-limited continuous cultures, respectively, exhibited an inverse relationship between growth rate and transcription of *ADH1 / ADH2* and phosphoglucomutase (*GAL5*) (Postma *et al.*, 1989; Sierkstra *et al.*, 1994). Sierkstra *et al.* (1994) concluded that the contribution of growth rate to the regulation of *ADH2* was at least as important as regulation by glucose repression. Recently, Meijer *et al.* (1998) concluded that the glucose-repressible invertase gene (*SUC2*) of *S. cerevisiae* in nitrogen-limited continuous culture was regulated by the external glucose concentration and that no relationship existed between glucose repression and the glucose flux. In the above experiments glucose repression of *SUC2* was initiated at extracellular glucose concentrations of between 2.5 and 3.2 g glucose.l^{-1} at every dilution rate used.

The determination of physiological parameters influencing the regulation of *ADH2* is of interest, since this promoter has been used in the production of heterologous proteins (Buckholz and Gleeson, 1991; Chiruvolu, *et al.*, 1996; Cousens *et al.*, 1987). In this chapter the influence of glucose concentration, glucose flux and growth rate on xylanase production in continuous culture by three different recombinant *S. cerevisiae* strains is reported. The three *S. cerevisiae* strains discussed in this chapter were each transformed with a xylanase (*xyn2*) of *T. reesei* as described by La Grange *et al.* (1996). Data for *Saccharomyces cerevisiae* strains H158 and CEN.PK110-6C were supplied by Fredrik Wahlbom (Lund University, Lund, Sweden) and included for comparative purposes.

4.3. Materials and Methods

4.3.1. Yeast strains

S. cerevisiae Y294 *(α leu2-3,112 ura3,-52 his3 trp1-289)*, *S. cerevisiae* Y294 (pDLG1; *bla URA3 ADH2*$_{PT}$) and the recombinant *S. cerevisiae* Y294 strain (pDLG5; *bla URA3 ADH2*$_{P}$-*XYN2-ADH2*$_{T}$) were provided by W.H. van Zyl, Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa. *S. cerevisiae* H158 and *S. cerevisiae* CEN.PK110-6C strains, each containing *XYN2* from *T. reesei*, were provided by B. Hahn-Hägerdal, Department of Applied Microbiology, Lund University, Lund, Sweden. In all three strains the same promoter-terminator expression cassette derived from the alcohol dehydrogenase II gene (*ADH2*) governed the expression of *XYN2* (La Grange *et al.* 1996). The yeast strains were maintained as described in Chapter 2.

4.3.2. Inoculum and medium

Basal medium C was used in continuous culture experiments to ensure oxygen transfer rates within the capacity of the bioreactor and to save on nutrient cost. Medium C contained (per litre): 2 g yeast extract, 4 g peptone, 2 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.5 ml trace element solution (du Preez and van der Walt, 1983) and 0.5 ml Silfoamex CF antifoam (SA silicones, Boksburg, South Africa) or Dow Corning 1520 silicone antifoam (Dow Corning, Seneffe, Belgium). A glucose solution in distilled water was separately autoclaved and added aseptically to the sterile basal medium. The sterile broth was inoculated with a 10 % (v/v) inoculum prepared as described in Chapter 2.

4.3.3. Cultivation conditions

Cultivations were carried out in a 2-litre Multigen F-2000 bioreactor (New Brunswick Scientific, Edison, NJ, USA) equipped with an exhaust gas condensor, a pH electrode (Mettler Toledo, Halstead, Great Britain) and a polarographic oxygen electrode (Ingold AG, Urdorf, Switzerland). Silicone rubber tubing with peristaltic pumps (Watson Marlow Ltd, Cornwall, England) were used for the medium feed and level control by withdrawal of excess culture to maintain a constant working volume. All cultivations were carried out at 30 °C with the pH controlled at pH 5.5 (± 0.1) by automatic titration with 3 M KOH. The dissolved oxygen

tension was maintained above 30 % of saturation using aeration at 700 to 1000 ml.min^{-1} and manual adjustment of the stirrer speed between 400 and 800 r.min^{-1}. Samples were taken directly from the culture at steady state, usually after three to five residence times, except when shifts from the respiratory region to the respiro-fermentative region were carried out, in which case at least eight residence times were allowed before sampling. Steady state conditions were defined as occurring when culture turbidity did not vary by more than 7 % for at least three consecutive residence times with no upward or downward trend.

4.3.4. Analyses

The biomass concentration, ethanol concentration and extracellular xylanase activity of each sample were determined as described in Chapter 3. One unit of activity (nkat) was defined as the amount of enzyme that released 1 nmol of xylose equivalents per second per ml of culture filtrate. Specific xylanase activity was expressed as nkat.mg biomass^{-1}. The glucose concentration of the feed medium and the continuous culture supernatants were determined as in Chapter 3.

Samples containing less than 1 g glucose.l^{-1} were analysed with a D-glucose bioanalysis kit (Cat. No. 716251, Boehringer Mannheim, Mannheim, Germany) with a detection limit of 4 mg glucose.l^{-1}. For these assays, 20 ml samples were rapidly inactivated by aspirating into 30-ml sample bottles containing 500 µl ice-cold 5 M HCl and immediately put on ice. The pH of the supernatants was adjusted to neutrality with 5 M KOH and stored at –20 °C after centrifugation until enzymatic determination of D-glucose were performed. The dilution factor of each sample was incorporated in the subsequent calculations. YPD agar plates containing (per litre): 10 g yeast extract, 20 g peptone, 2 g 4-O-methyl-D-glucurono-xylan-remazol brilliant blue R (RBB)-xylan (Sigma, St. Louis, MO, USA) and 10 g glucose were used to determine the percentage of xylanase-producing colonies (La Grange *et al.*, 1996).

The number of generations in continuous culture was calculated as

$$n = \frac{t}{\ln 2.D^{-1}}$$

where n is the generation number, t the time and D the dilution rate. The maintenance energy coefficient, m, was calculated as

$$m = q_s - \frac{\mu}{Y_G}$$

where q_s is the specific rate of glucose assimilation ($g.g^{-1}.h^{-1}$), μ the specific growth rate and Y_G the "true" biomass yield coefficient (g biomass produced .g glucose assimilated^{-1}). The p-values of the maintenance coefficients were determined using the t-test in Sigma Plot® for Windows 4.01.

4.4. Results and Discussion

Figure 4.1 depicts a steady-state culture of the recombinant *S. cerevisiae* Y294 strain for 100 generations at a dilution rate (D) of 0.18 h^{-1}, using a feed glucose concentration of 2.1 to 2.4 g glucose.l^{-1}. The extracellular xylanase activity and dry biomass concentration remained relatively constant, with no upward or downward trend, for the full duration of this experiment. High biomass concentrations were observed using the above glucose feed concentration and the graphical method indicates that the complex medium without glucose sustained 0.93 g biomass.l^{-1} (Figure 4.2). The percentage of xylanase-producing cells remained at 100 % for 100 generations, indicating that no plasmid-cured cells arose (Figure 4.1). This observation was similar to that of Marquet *et al.* (1987), who used a *ura3fur1* double mutant of *S. cerevisiae* for heterologous human α_1-antitrypsin (α_1-AT) production. The only difference with regard to heterologous xylanase expression in *S. cerevisiae* Y294 (La Grange *et al.*, 1996) was that Marquet *et al.* (1987) used a constitutive phosphoglycerate kinase (*PGK*) promotor in the *URA3*-bearing plasmids for expression of α_1-AT. In continuous culture at a constant dilution rate, their recombinant *S. cerevisiae* strain stably expressed α_1-AT for at least 150 generations without the appearance of plasmid-cured cells. They also showed that between dilution rates of 0.1 to 0.32 h^{-1}, the plasmid copy number remained constant, regardless of the dilution rate used. The plasmid copy number, although not determined, probably also remained constant for the duration of the experiment depicted in Figure 4.1, since no decrease in extracellular xylanase activity was observed.

The parental *S. cerevisiae* Y294 strain (*α leu2-3,112 ura3,-52 his3 trp1-289*), *S. cerevisiae* Y294 harbouring the episomal plasmid, but without *XYN2* (pDLG1; *bla URA3 ADH2*$_{PT}$) and

S. cerevisiae Y294 transformed with the episomal plasmid containing *XYN2* (pDLG5; *bla URA3 ADH2*$_P$-*XYN2*-*ADH2*$_T$) were compared to evaluate the effect of the protein burden on heterologous xylanase production (Figure 4.3). The specific glucose uptake rate was plotted against dilution rate so that the maintenance energy coefficient (m) was given by the intercept with the Y-axis, calculated using linear regression analysis. The parental *S. cerevisiae* Y294 strain (not containing plasmids) had the highest m-value of 0.153 $mmol.g^{-1}.h^{-1}$, possibly because this strain was auxotrophic for a number of amino acids. The strain harbouring the episomal plasmid without *XYN2* exhibited a slightly lower m–value (0.068 $mmol.g^{-1}.h^{-1}$) than the recombinant xylanase-producing *S. cerevisiae* Y294 strain (0.079 $mmol.g^{-1}.h^{-1}$).

The maintenance and propagation of a heterologous plasmid requires energy, which is obtained from the cellular resources of the host (Bhattacharya and Dubey, 1995). The cellular energy resources are further stressed upon induction of the promoter to obtain high-level expression of the cloned gene. Measurement of the maintenance energy coefficient (m) has been found to be a useful measure of metabolic burden due to high-level expression of foreign genes (Bhattacharya and Dubey, 1995; Fieschko and Humphrey, 1984).

Statistical analysis of the data depicted in Figure 4.3 yielded p-values of less than 0.05 ($p < 0.05$), thus indicating that the differences in m-values were significant. Thus, expression of heterologous xylanase by the recombinant strain exerted a minimal, but significant, protein burden on the host.

Figure 4.4 depicts the effect of increased glucose concentration in the feed on growth and xylanase production by three different recombinant *S. cerevisiae* strains in steady-state cultures at a dilution rate of 0.17 h^{-1}. With all three yeast strains, the extracellular xylanase activity was maximal at low glucose feed concentrations (less than 28 mmol glucose $.l^{-1}$). Xylanase production by *S. cerevisae* Y294 and H158 virtually ceased when the glucose concentration in the feed increased above 12 and 18 $mmol.l^{-1}$, respectively, whereas with strain CEN.PK110-6C the xylanase activity peaked at a feed glucose concentration of 27 mmol glucose $.l^{-1}$. According to chemostat theory, at a constant dilution rate under carbon-limited conditions, the steady-state residual glucose concentration should not increase with an increase in the glucose concentration in the feed. The culture of strain Y294 remained carbon-limited up to a glucose feed concentration of 32 mmol glucose.l^{-1} (Figure 4.5). No detectable

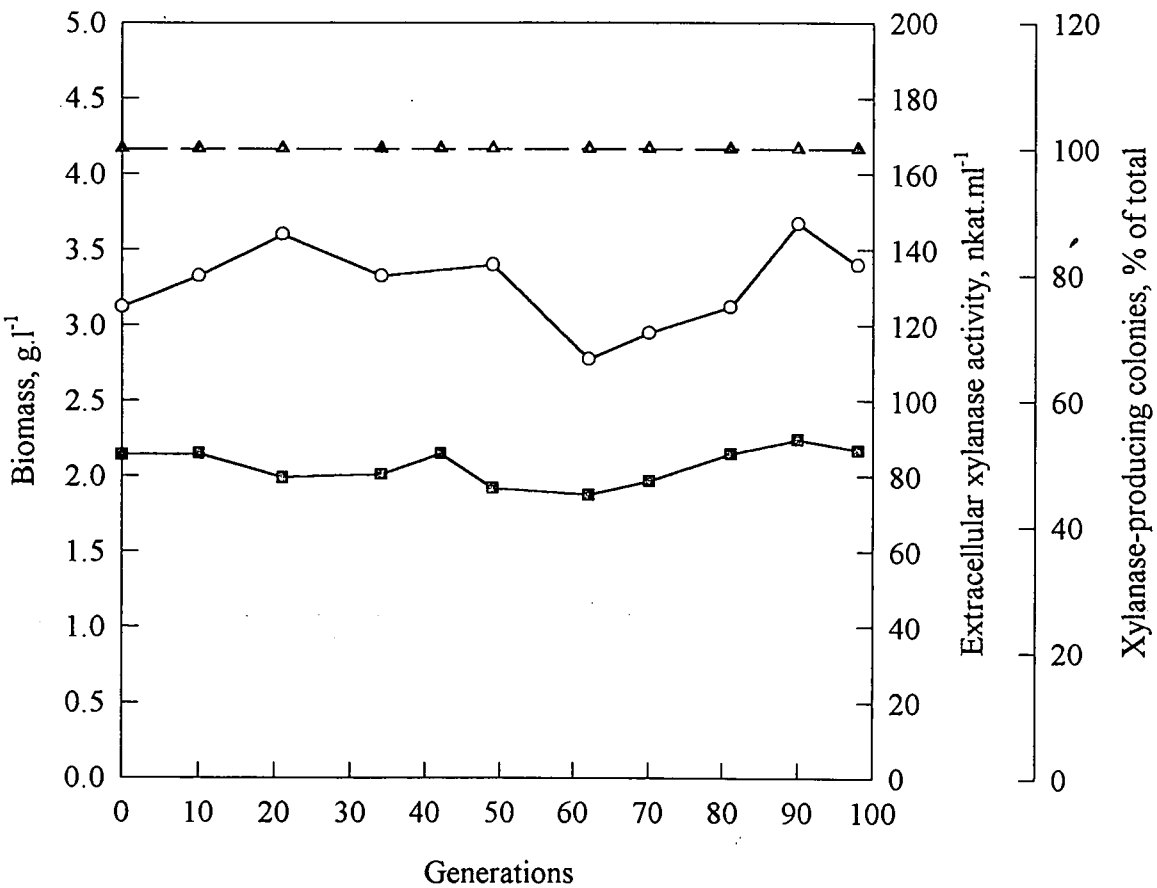


Figure 4.1. A steady-state continuous culture of recombinant *S. cerevisiae* Y294 monitored for 100 generations at a dilution rate of 0.18 h^{-1}, using a feed glucose concentration of 2.1 to 2.4 $g.l^{-1}$. Symbols: biomass (■), extracellular xylanase activity (○) and percentage xylanase-producing colonies (▲).

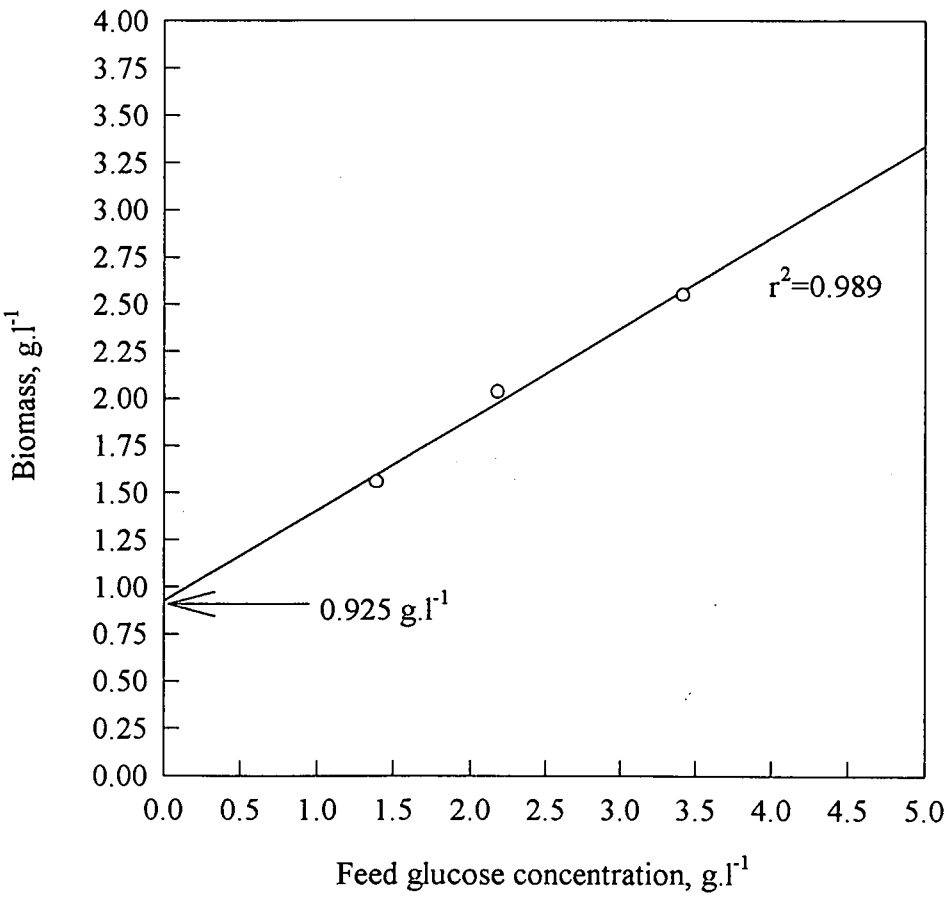


Figure 4.2. The steady-state biomass concentration of the recombinant *S. cerevisiae* Y294 strain as a function of the glucose concentration in medium C at a dilution rate of 0.17 h^{-1}. The correlation coefficient is indicated by r^2.

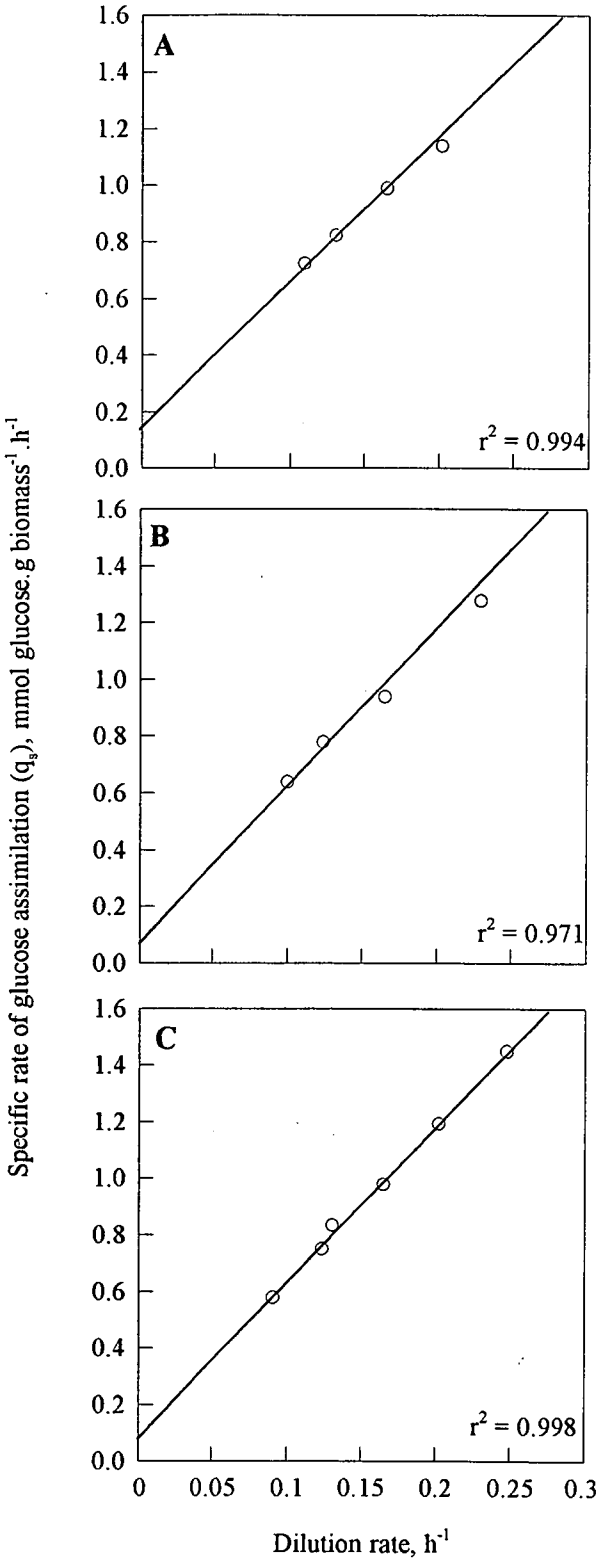


Figure 4.3. The steady-state specific rate of glucose assimilation (q_s) as a function of dilution rate in aerobic continuous cultures of **(A)** the parental *S. cerevisae* Y294 strain (*α leu2-3, 112 ura3,-52 his3 trp1-289*), **(B)** *S. cerevisiae* Y294 (pDLG1; *bla URA3 ADH2*$_{PT}$) and **(C)** the recombinant *S. cerevisiae* Y294 strain (pDLG5; *bla URA3 ADH2*$_P$*-XYN2-ADH2*$_T$). The correlation coefficients are indicated by r^2.

increase in the residual glucose concentration (which remained below 0.02 mmol.l^{-1}), which would have indicated a secondary nutrient limitation, was observed with strains Y294 and H158 at feed glucose concentrations of less than 48 and 28 mmol.l^{-1}, respectively (Figure 4.4). Ethanol only appeared in the cultures of these strains when the feed glucose concentration was increased to above 19.8 and 28 mmol.l^{-1}, respectively (Figure 4.4). Xylanase production by strain CEN.PK110-6C gradually decreased upon increasing the feed glucose concentration above 30.5 mmol.l^{-1} and ceased only above a feed glucose concentration of 111 mmol.l^{-1} (Figure 4.4). In addition, its xylanase production decreased before ethanol accumulated in the medium, which occurred only at feed glucose concentrations above 66.7 mmol.l^{-1} (Figure 4.4). Similar to strains Y294 and H158, xylanase production by strain CEN.PK110-6C decreased before a detectable increase in residual glucose concentration was observed (above a feed concentration of 66.7 mmol glucose.l^{-1}) (Figure 4.4).

A plot of the specific rate of xylanase production (q_p) against the specific rate of glucose uptake (q_s) revealed that xylanase production by strains Y294 and H158 was greatly reduced when the glucose flux exceeded 0.98 and 1.17 mmol glucose .g biomass^{-1}.h^{-1}, respectively (Figure 4.6). With strain CEN.PK110-6C, xylanase production decreased when the glucose flux reached 1.68 mmol glucose.g biomass^{-1}.h^{-1} and ceased at a flux of 2.51 mmol glucose. g biomass^{-1}.h^{-1} (Figure 4.6). In all three strains, therefore, glucose flux appeared to be largely responsible for carbon catabolite repression of xylanase production because the above data indicated that xylanase production decreased while the steady-state residual glucose concentration in the culture still was very low with no detectable increase.

Figure 4.7 shows the effect of the specific growth rate, which is numerically equivalent to the dilution rate, on xylanase production in chemostat cultures. The highest extracellular xylanase activity and specific xylanase activity values of strains Y294 and H158 were recorded at dilution rates of 0.1 h^{-1} and 0.13 h^{-1}, respectively. The biomass concentrations remained relatively constant, but with strain Y294 it decreased in the respiratory region (above a dilution rate of 0.29 h^{-1}), as a result of aerobic ethanol production (Figure 4.7). Strain H158 was not grown at high dilution rates and no data for strain CEN.PK110-6C was available. Xylanase activity in cultures of strains Y294 and H158 peaked at respective specific growth rates of 0.1 and 0.13 h^{-1}. It should be realised that an increase in dilution rate not only resulted in an increase in the specific growth rate, but also in the specific rate of glucose uptake (Figure 4.8)

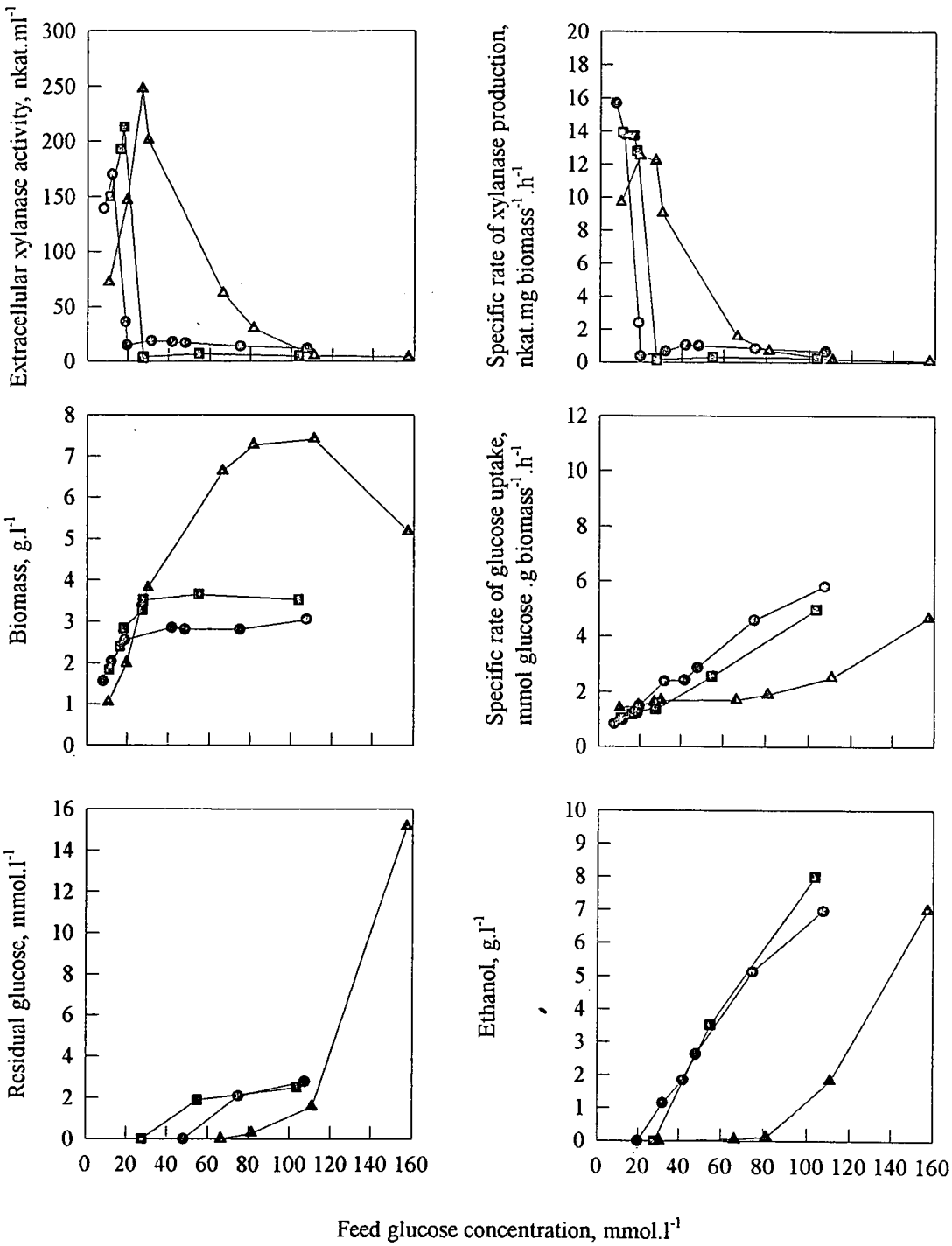


Figure 4.4. Steady-state values of xylanase activity, specific xylanase activity, biomass concentration, specific rate of glucose uptake, residual glucose concentration and ethanol concentration obtained with rcombinant strains of *S. cerevisiae* Y294 (●), *S. cerevisiae* H158 (■) and *S. cerevisiae* CEN.PK110-6C (▲) at a dilution rate of 0.17 h^{-1} in a aerobic chemostat culture using different glucose feed concentrations.

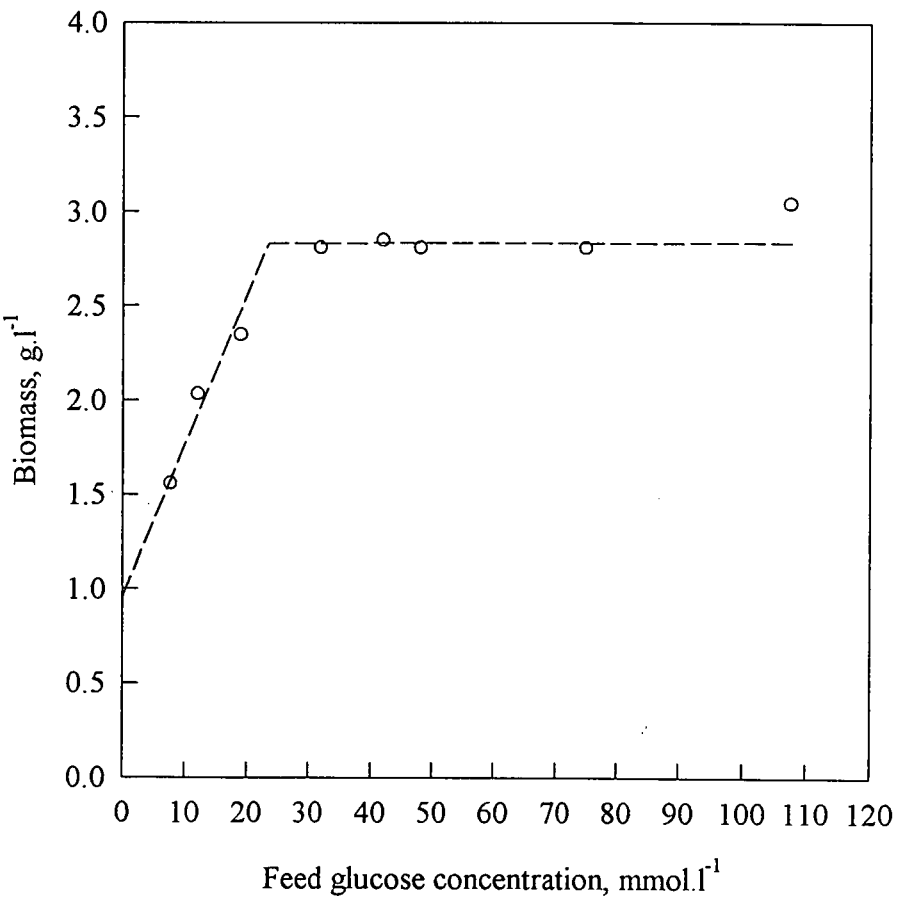


Figure 4.5. The steady-state biomass concentration of the recombinant *S. cerevisiae* Y294 strain as a function of the glucose concentration in medium C at a dilution rate of 0.17 h^{-1}.

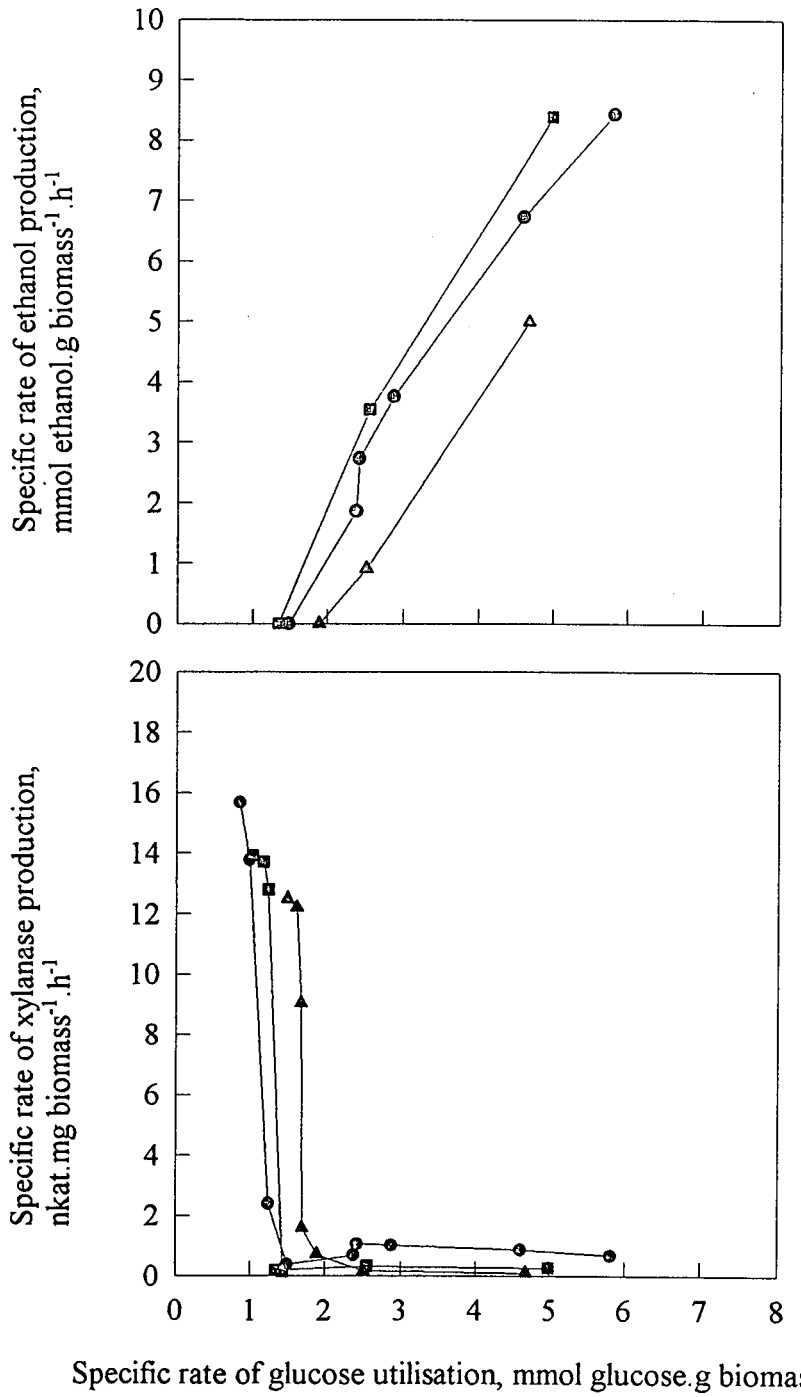


Figure 4.6. The specific rates of ethanol production and xylanase production as a function of the specific rate of glucose uptake by *S. cerevisiae* Y294 (●), *S. cerevisiae* H158 (■) and *S. cerevisiae* CEN.PK110-6C (▲) plotted using data from Figure 4.4.

and residual glucose concentration. Thus, the data shown in figure 4.7 cannot distinguish between the effects of glucose concentration, glucose flux and growth rate. However, from Figure 4.8 it is evident that xylanase production by both strains Y294 and H158 was severely reduced at a specific rate of glucose uptake of about 1.72 mmol glucose.g biomass^{-1}.h^{-1}. It is not implausible that glucose repression of enzyme production could be affected by the glucose flux as well as by the growth rate.

In the above experiments, glucose flux was manipulated in two ways : through changing the glucose feed concentration at a constant dilution rate (Figure 4.4) and through changing the dilution rate, using a constant feed glucose concentration (Figure 4.7). With all three of the recombinant yeast strains, a severe decrease in xylanase production occurred before the onset of aerobic alcohol production due to the Crabtree effect (Postma *et al.*, 1989), confirming the results of Sierkstra *et al.* (1992b) that glucose repression of *ADH2* and aerobic ethanol production were not related events. The above results suggest that carbon catabolite repression was initiated by physiological parameters such as growth rate and glucose flux (specific rate of glucose uptake, q_s).

Xylanase production by strains Y294 and H158 virtually ceased when q_s exceeded 0.98 and 1.44 mmol glucose.g biomass^{-1}.h^{-1}, respectively. Xylanase production by strain CEN.PK110-6C was noticeably reduced only when q_s exceeded 1.68 mmol glucose.g biomass^{-1}.h^{-1} and ceased completely when q_s reached 2.51 mmol glucose.g biomass^{-1}.h^{-1}. It can be concluded that mainly the glucose flux rather than the glucose concentration regulated the expression of the *ADH2*-mediated heterologous xylanase gene, since xylanase production was reduced in all three strains before an increase in the residual glucose concentration or ethanol production was detected (Figure 4.4). It is possible that the regulation of glucose-repressible genes may differ with regard to the gene concerned. In the elegant study of Meijer *et al.* (1998) the regulation of *SUC2* in *S. cerevisiae* SU32 was clearly shown to be regulated by the glucose concentration (see Chapter 1.3.4.4. Glucose metabolism and carbon catabolite repression in *Saccharomyces cerevisiae*). Our data and that of Sierkstra *et al.* (1994) suggest regulation of *ADH2* to be dependent on the growth rate and glucose flux. However, this apparent discrepancy may be the result of different regulation mechanisms for the regulation of genes involved in the utilisation of alternate carbon sources as compared to glycolytic genes involved in the catabolism of glucose.

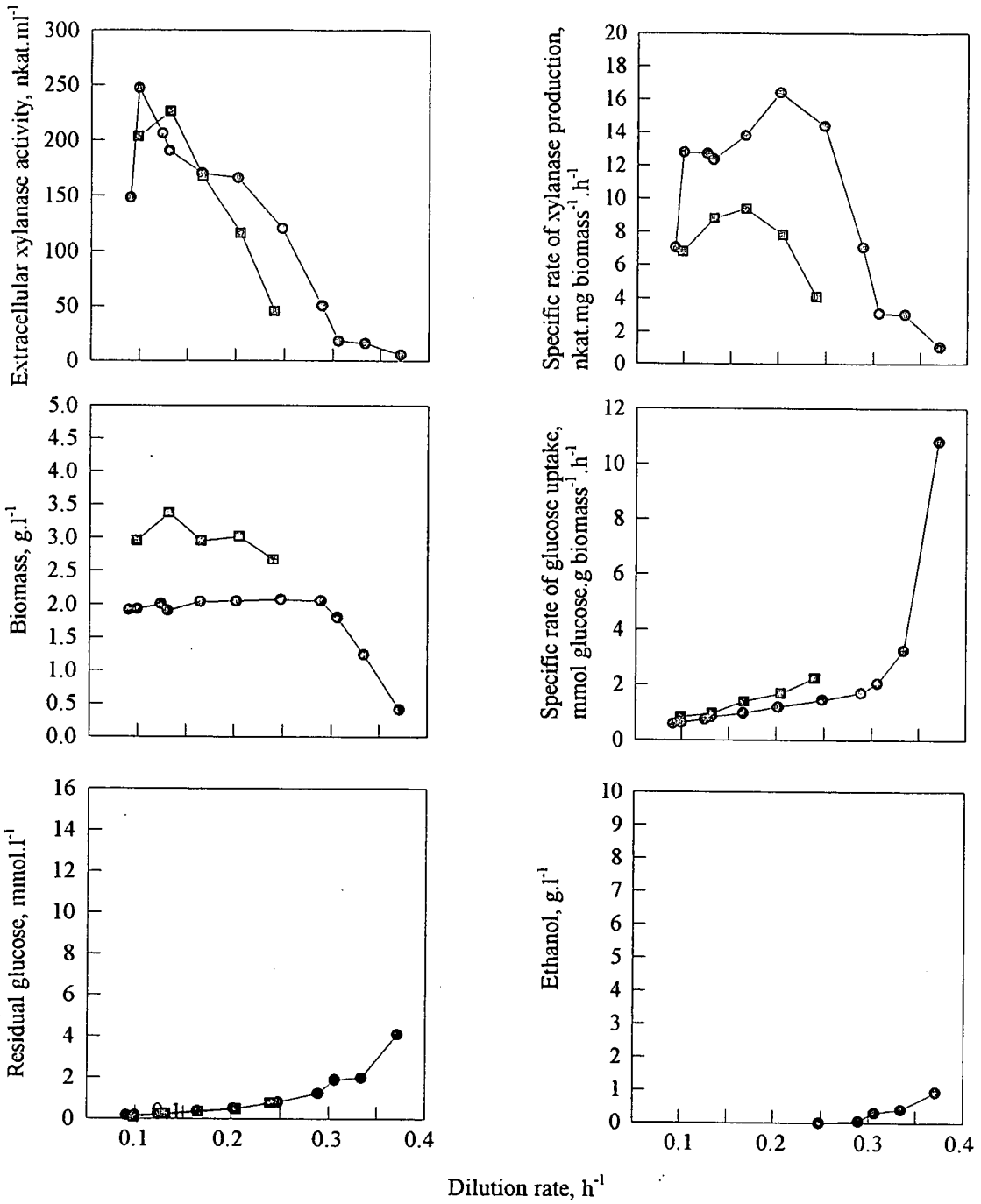


Figure 4.7. Steady-state values of xylanase activity, specific xylanase activity, biomass concentration, specific rate of glucose uptake, residual glucose concentration and ethanol concentration obtained with recombinant strains *S. cerevisiae* Y294 (●) and *S. cerevisiae* H158 (■) as a function of the dilution rate in a carbon-limited aerobic chemostat culture, using respective glucose feed concentrations of 2.18 $g.l^{-1}$ and 4.5 $g.l^{-1}$ for each strain.

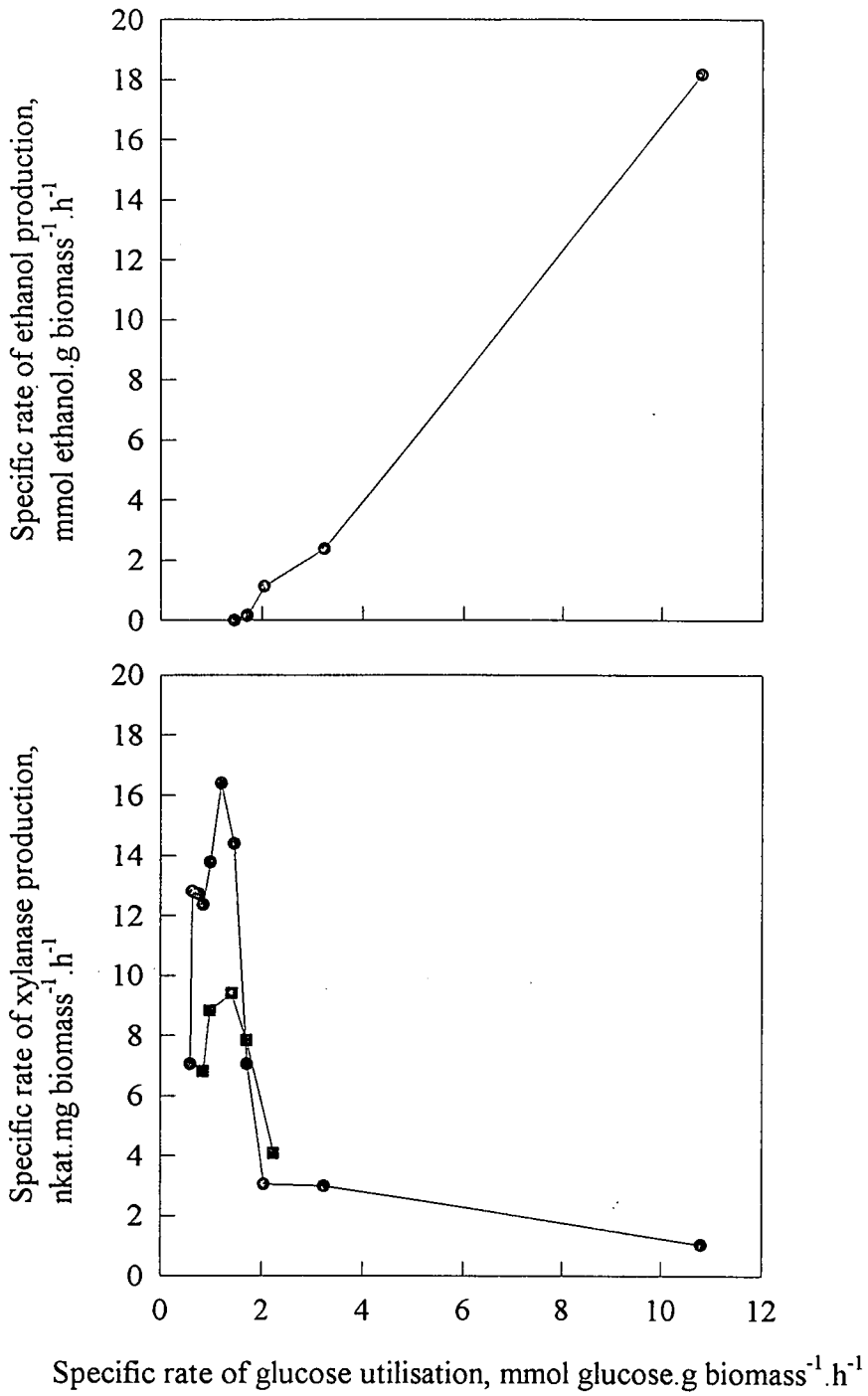


Figure 4.8. The specific rates of ethanol and xylanase production as a function of the specific rate of glucose uptake by *S. cerevisiae* Y294 (●) and *S. cerevisiae* H158 (■) plotted using data from Figure 4.7.

Despite the obvious strain differences, with all three yeast strains the glucose flux appeared to contribute to a greater extent towards the regulation of xylanase production than did the steady-state residual glucose concentration in the culture. *S. cerevisiae* CEN.PK110-6C differed from strains Y294 and H158 in that xylanase production firstly decreased above a certain glucose flux and virtually ceased only at a respective higher glucose flux value, while with strains Y294 and H158 xylanase production virtually ceased upon increasing the glucose flux above a certain critical value. The *ADH2* promoter in *S. cerevisiae* CEN.PK110-6C was thus more tolerant towards an increase in glucose flux than in strains Y294 and H158.

4.5. Nomenclature

D, h^{-1}	Dilution rate
s, $mmol.l^{-1}$	Residual glucose concentration
q_s, mmol glucose .g $biomass^{-1}.h^{-1}$	Specific rate of glucose assimilation (glucose flux)
q_p, nkat. mg $biomass^{-1}.h^{-1}$	Specific rate of enzyme (xylanase) production
μ, h^{-1}	Specific growth rate

4.6. References

Bhattacharya, S.K. & Dubey, A.K. (1995). Metabolic burden as reflected by the maintenance coefficient of recombinant *Escherichia coli* overexpressing target gene. *Biotechnol Lett* **17**, 1155-1160.

Buckholz, R.G. & Gleeson, A.G. (1991). Yeast systems for the commercial production of heterologous proteins. *Bio/Technology* **9**, 1067-1072.

Chiruvolu, V., Stratton, J.M., Ott, T.L., Bazer, F.W. & Meagher, M.M. (1996). Effect of medium composition on growth, plasmid stability and ovine interferon-τ production in *Saccharomyces cerevisiae*. *J Ferment Bioeng* **82**, 565-569.

Ciriacy, M. (1975). Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae.* II. Two loci controlling synthesis of the glucose-repressible ADH II. *Mol Gen Genet* **138,** 157-164.

Ciriacy, M. (1997). Alcohol dehydrogenases. In Zimmermann, F.K. & Entian, K.-D. (Eds), *Yeast sugar metabolism : Biochemistry, genetics, biotechnology and applications.* Technomic Publishing Co. Inc., Lancaster, U.S.A. pp 213-223.

Cousens, L.S., Shuster, J.R., Gallegos, C., Ku, L., Stempien, M.M., Urdea, M.S., Sanchez-Pescador, R., Taylor, A. & Tekamp-Olson, P. (1987). High level expression of proinsulin in the yeast, *Saccharomyces cerevisiae. Gene* **61,** 265-275.

Denis, C.L., Ciriacy, M. & Young, E.T. (1981). A positive regulatory gene is required for accumulation of the functional messenger RNA for the glucose-repressible alcohol dehydrogenase from *Saccharomyces cerevisiae. J Mol Biol* **148,** 355-368.

Denis, C.L. & Malvar, T. (1990). The *CCR4* gene from *Saccharomyces cerevisiae* is required for both nonfermentative and *spt*-mediated gene expression. *Genetics* **124,** 283-291.

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.

Fieschko, J. & Humphrey, A.E. (1984). Statistical analysis in the estimation of maintenance and true growth yield coefficients. *Biotechnol Bioeng* **26**, 394-396.

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.

Meijer, M.M.C., Boonstra, J., Verkleij, A.J. & Verrips, C.T. (1998). Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than the glucose flux. *J Biol Chem* **273,** 24102-24107.

Postma, E., Verduyn, C., Scheffers, A. & van Dijken, J.P. (1989). Enzymatic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Sacharomyces cerevisiae*. *Appl Environ Microbiol* **55**, 468-477.

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

Sierkstra, L.N., Nouwen, N.P., Verbakel, J.M.A. & Verrips, C.T. (1992a). Analysis of glucose repression in *Saccharomyces cerevisiae* by pulsing glucose to a galactose-limited continuous culture. *Yeast* **8**, 1077-1087.

Sierkstra, L.N., ter Schure, E.G., Verbakel, J.M.A. & Verrips, C.T. (1994). A nitrogen-limited, glucose-repressed, continuous culture of *Saccharomyces cerevisiae*. *Microbiol* **140**, 593-599.

Sierkstra, L.N., Verbakel, J.M.A. & Verrips C.T. (1992b). Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**, 2559-2566.

Tøttrup, H.V. & Carlsen, S. (1989). A process for the production of human proinsulin in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **35**, 339-348.

CHAPTER 5

THE EFFECT OF ETHANOL CONCENTRATION ON XYLANASE PRODUCTION IN BATCH AND CONTINUOUS CULTURE

An abridged version of this chapter has been prepared for submission to Yeast.

5.1. Abstract

In batch culture, the volumetric and specific rates of xylanase production by a recombinant strain of *Saccharomyces cerevisiae* Y294 were severely decreased by the presence of 22 g ethanol.l^{-1} (478 mmol ethanol.l^{-1}) as compared to production in the presence of 4 g ethanol.l^{-1} (87 mmol ethanol.l^{-1}). The effect of ethanol on xylanase production was further investigated under defined physiological conditions by pulsing ethanol into an aerobic glucose-limited continuous culture at steady state. Xylanase production was transcriptionally inhibited by the ethanol pulse, as indicated by Northern blots of the *XYN2* mRNA over time. Repression of xylanase production was clearly evident within the first hour following the ethanol pulse. Ethanol inhibited transcription of xylanase in a similar fashion as in the case of a glucose pulse. To ensure that this phenomenon was not strain specific, these results were verified using another xylanase-producing strain, *Saccharomyces cerevisiae* CEN.PK110-6C, which had been transformed with the same expression cassette. This finding adds a new dimension to the regulation of alchohol dehydrogenase II (*ADH2*).

5.2. Introduction

The molecular mechanisms governing the expression of the alcohol dehydrogenase II gene (*ADH2*) have been extensively studied and documented (Ciriacy, 1975; Ciriacy, 1997; Denis, 1984; Denis *et al.*, 1984; Denis and Malvar, 1990; Gancedo, 1998; Lutstorf and Megnet, 1968; Russell *et al.*, 1983; Saliola *et al.*, 1990). *ADH2* is expressed only in the absence or depletion of a fermentable hexose sugar and is not induced by ethanol (Ciriacy, 1997) as occasionally reported. Sierkstra *et al.* (1992a, b) pointed out that the majority of studies conducted to elucidate the mechanisms involved in carbon catabolite repression was done with mutant strains grown in shake flasks under ill-defined physiological conditions. Although a wealth of knowledge on the molecular regulation of *ADH2* has been obtained this way, the physiological aspects of *ADH2* regulation have often been ignored.

In this chapter the regulation of the *ADH2* promoter was investigated by using *S. cerevisiae* transformed with the *XYN2* gene from *Trichoderma reesei*, with this xylanase gene under control of the *ADH2* promoter. Thus, using *XYN2* as a reporter gene, the effect of ethanol on the *ADH2* promoter was investigated at both the physiological and molecular level in batch and continuous cultures.

5.3. Materials and Methods

5.3.1. Yeast strains

The recombinant *S. cerevisiae* Y294 and *S. cerevisiae* CEN.PK110-6C strains, each containing *XYN2* from *T. reesei*, were respectively provided by W.H. van Zyl, Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa and B. Hahn-Hägerdal, Department of Applied Microbiology, Lund University, Lund, Sweden. In both yeast strains, the same promoter-terminator expression cassette derived from the alcohol dehydrogenase II gene (*ADH2*), governed expression of *XYN2* (La Grange *et al.* 1996).

5.3.2. Inoculum and medium

Medium B was used for batch cultures and medium C for continuous cultures as described in Chapters 3 and 4. The carbon source for each medium was sterilised separately; glucose by autoclaving and ethanol by membrane filtration, and added aseptically to the above sterile basal media. The sterile culture broth was inoculated with a 10 % (v/v) inoculum prepared as described in Chapter 2.

5.3.3. Cultivation conditions

Batch cultivations were carried out as described in Chapter 3 and continuous cultivations as described in Chapter 4. Ethanol pulses were done directly into the culture vessel after a steady state had been reached in continuous carbon-limited cultures.

5.3.4. Analyses

The biomass concentration, ethanol concentration and extracellular xylanase activity of each sample were determined as described in Chapter 3. The glucose concentration of the feed medium and in the continuous culture supernatants was determined as in Chapters 3 and 4. Samples of 10 ml were withdrawn directly from the culture vessel, centrifuged and the sediment used for determination of the intracellular xylanase activity by breaking the cells with glass beads (425 to 600 μm diameter) through vortexing at high speed in a 10 ml test tube after addition of 1 ml 20 mM imidazol buffer (pH 7). Each vortexing step lasted 1 min,

followed by cooling on ice for 1 min and the cycle was repeated four times. The extracellular ethanol concentration and xylanase activity were monitored over time, following a pulse with ethanol. The theoretical washout rates of ethanol and xylanase activity were calculated using the equation

$$P_t = P_o.e^{-Dt}$$

where P_t was the ethanol concentration or xylanase activity at time t, P_o the ethanol concentration or xylanase activity at the time of the ethanol pulse and D the dilution rate. Total RNA was isolated according to the method of Köhrer and Domdey (1990), electrophoresed in formaldehyde agarose gels and blotted onto Magnacharge fiters (Micron Separations Inc., Westboro, MA, USA). The bound RNA was hybridised to the ^{32}P-labelled probes (DLG1L, 5'-GCATGAATTCGCCAAACCTGAACAACCC-3' and DLG1R, 5'-GCATAGATCTCCCTTTAGCTGACGGTGA-3') as used by La Grange *et al.* (1996) and ^{32}P-labelled *ACT1* (used as internal standard), using the High Prime labelling kit of Boehringer Mannheim (Boehringer Mannheim GmbH, Mannheim, Germany) according to the instructions of the manufacturer.

5.4. Results and Discussion

As expected, xylanase production by the recombinant *S. cerevisiae* Y294 strain was subject to glucose repression. The xylanase activity increased only close to glucose depletion (Figures 5.1 and 5.2). The highest specific rate of xylanase production (20.75 nkat.mg biomass^{-1}.h^{-1}) was recorded with 10 g glucose.l^{-1} as carbon source (Figure 5.1, Table 5.1). Xylanase production coincided with ethanol assimilation, but continued for some hours after ethanol depletion and virtually ceased after 25 h.

In medium B with 50 g glucose.l^{-1}, the xylanase production period was extended up to a cultivation time of 60 h due to the increased amount of ethanol produced (Figure 5.2). However, despite the higher biomass concentration reached, the extracellular xylanase activity was similar to that obtained with 10 g glucose.l^{-1} as initial carbon source and was produced at significantly lower specific and volumetric productivities (Table 5.1). Growing the yeast under glucose-derepressed conditions on ethanol failed to improve the xylanase production (Figure 5.3). The volumetric rate of xylanase production in medium B with 10 g ethanol.l^{-1}

(220 mmol ethanol.l^{-1}) as carbon source was initially slow, but after 29 h an increase in the volumetric rate of xylanase production was observed (Figure 5.3). At this point the ethanol concentration in the medium was 5.5 g.l^{-1} (120 mmol.l^{-1}).

The pulse addition of 18 g ethanol.l^{-1} (390 mmol ethanol.l^{-1}) to a batch culture with an initial glucose concentration of 10 g.l^{-1} at respective cultivation times of 6 h (Figure 5.4) and 2 h (Figure 5.5) exerted an insignificant effect on the specific growth rate and did not decrease the specific rate of ethanol assimilation (Table 5.1). However, in both cases the xylanase production profile resembled that of Figure 5.2, but with even lower xylanase productivity and final activity values. This suggested that the higher ethanol concentration, rather than the higher initial glucose concentration, impaired xylanase production. In addition, exogeneously added ethanol inhibited xylanase production more severely (Figures 5.5 and 5.6) than ethanol produced by fermentation during the course of growth on glucose (Figure 5.3). This observation was contradictory to the observation that exogeneously added ethanol was less toxic than endogenously produced ethanol (Casey and Ingledew, 1985; Hoppe and Hansford, 1982; van Uden, 1985;). The amount of ethanol able to cause a decrease in growth rate is highly dependent on the yeast strain and medium composition (Peres and Laluce, 1998) and values ranging from 10 to 35 g ethanol.l^{-1} (217 to 760 mmol ethanol.l^{-1}) are cited in the literature (Casey and Ingledew, 1985; Luong, 1985;). In the complex medium used here, ethanol concentrations up to 22 g.l^{-1} (478 mmol.l^{-1}) did not affect the maximum specific growth rate of *S. cerevisiae* Y294 (Table 5.1), and were, therefore, unlikely to have an effect on xylanase production by inhibiting growth rate.

To determine the effect of ethanol concentration on xylanase activity, the DNS assay was performed with 10, 20 and 30 g ethanol.l^{-1} (220, 434 and 650 mmol ethanol.l^{-1}) in the assay mixture (Table 5.2). No inhibition of xylanase activity was observed in the presence of up to 30 g ethanol.l^{-1} (650 mmol ethanol.l^{-1}). The standard deviation of activities determined in the presence of the three ethanol concentrations was only 2.63 % (Table 5.2). These results ruled out the possibility that the ethanol interfered with the xylanase assays. It therefore appeared that ethanol might affect xylanase production on the level of transcription or translation.

The above phenomenon was further investigated by using pulse additions of ethanol to aerobic carbon-limited continuous cultures. A dilution rate of 0.17 h^{-1} and feed glucose concentration

Table 5.1. Growth parameters of the recombinant *S. cerevisiae* Y294 strain grown on 10 and 50 g glucose.l^{-1}, respectively, and with ethanol supplementation as indicated. The mean values of independent duplicate experiments are given.

	Glucose concentration			
Parameter	10	50	10 + Ethanol[1]	10 + Ethanol[2]
Final dry biomass, $g.l^{-1}$	7.078	16.543	9.066	9.819
$Y_{x/s}$	0.152	0.165	0.137	0.165
μ_{max}, h^{-1}	0.424	0.424	0.417	0.430
q_s^{max}, $g.(g.h)^{-1}$	4.372	4.676	4.084	4.489
Maximum ethanol concentration, $g.l^{-1}$	4.358	22.346	22.68	22.40
$Y_{ethanol/s}$	0.443	0.422	ND	N.D.
$q_p^{max}{}_{ethanol}$, $g.(g.h)^{-1}$	0.895	1.055	1.077	0.886
$\mu_{max\ ethanol}$, h^{-1}	0.120	0.114	0.126	0.117
$q_s^{max}{}_{ethanol}$, $g\ (g.h)^{-1}$	0.119	0.136	0.162	0.131
Extracellular xylanase activity, $nkat.ml^{-1}$	1590	1580	981	988
$q_p^{max}{}_{xylanase}$, $nkat.(mg.h)^{-1}$	20.75	9.017	8.493	10.583
$Q_p^{overall}$, $nkat.(l.h)^{-1}$	76.50	28.345	16.565	14.951

[1] ethanol pulse at 6 h, [2] ethanol pulse at 2 h.

ND Not determined

Table 5.2. The effect of ethanol concentration on xylanase activity assays. The mean values of independent duplicate xylanase assays are indicated.

Initial ethanol concentration, $g.l^{-1}$	Final ethanol concentration[1], $g.l^{-1}$	Xylanase activity, $nkat.ml^{-1}$
0	0	932
10.45	10.38	886
19.49	18.17	937
30.04	24.71	934

[1]The ethanol concentration remaining in the test mixture after incubation in a water bath at 60 °C for 5 min.

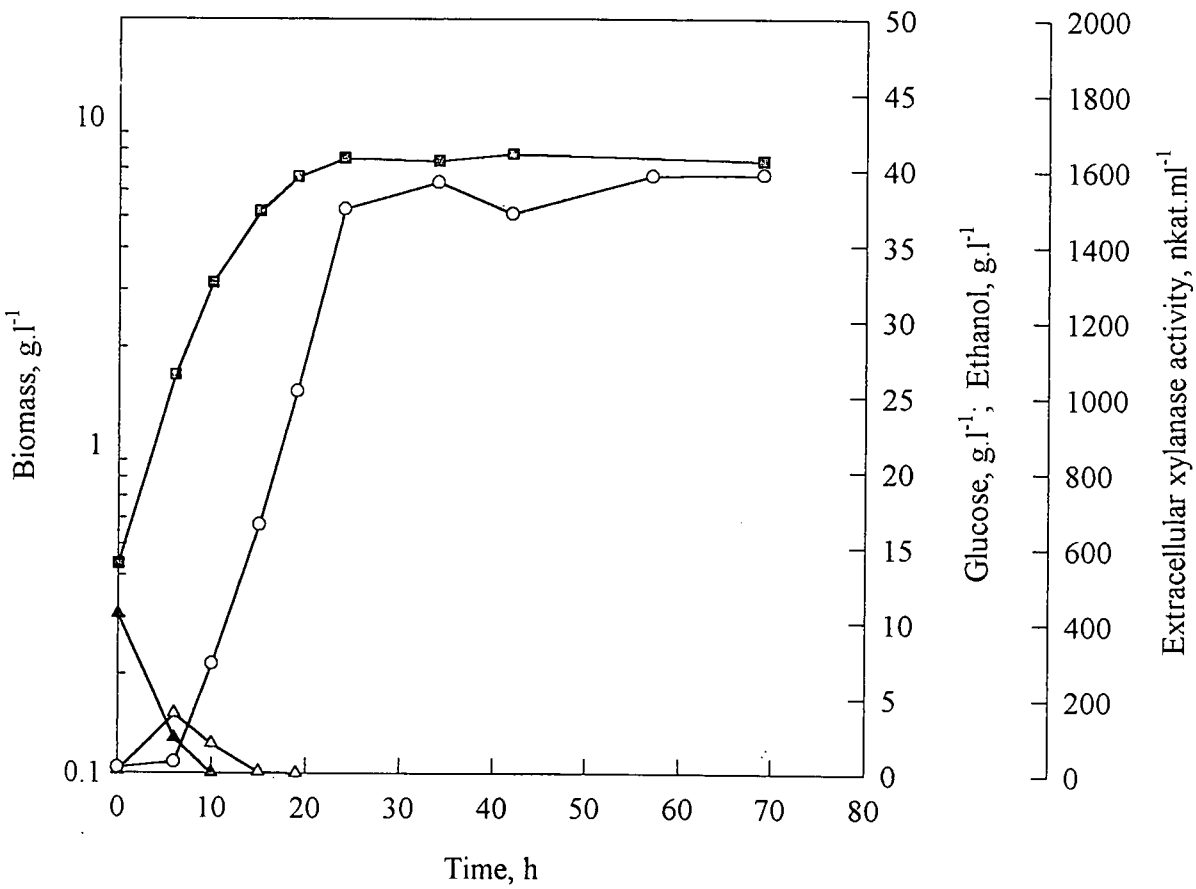


Figure 5.1. Batch cultivation profile of recombinant *S. cerevisiae* Y294 in medium B with 10 g glucose.l^{-1} as carbon source at pH 5.5 and 30 °C. Symbols : biomass (▣), ethanol concentration (△), glucose concentration (▲) and extracellular xylanase activity (○).

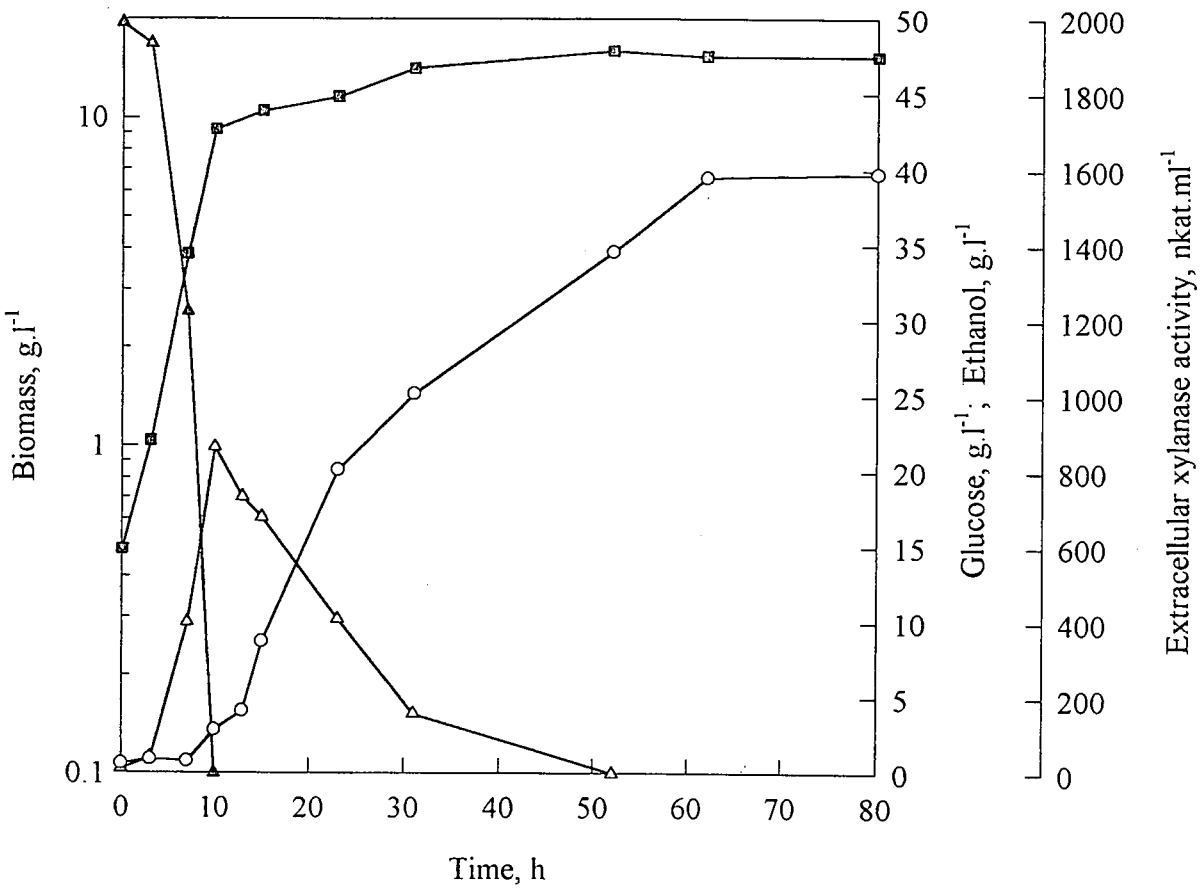


Figure 5.2. Batch cultivation profile of recombinant *S. cerevisiae* Y294 in medium B with 50 g glucose.l^{-1} as carbon source at pH 5.5 and 30 °C. Symbols : biomass (■), ethanol concentration (△), glucose concentration (▲) and extracellular xylanase activity (○).

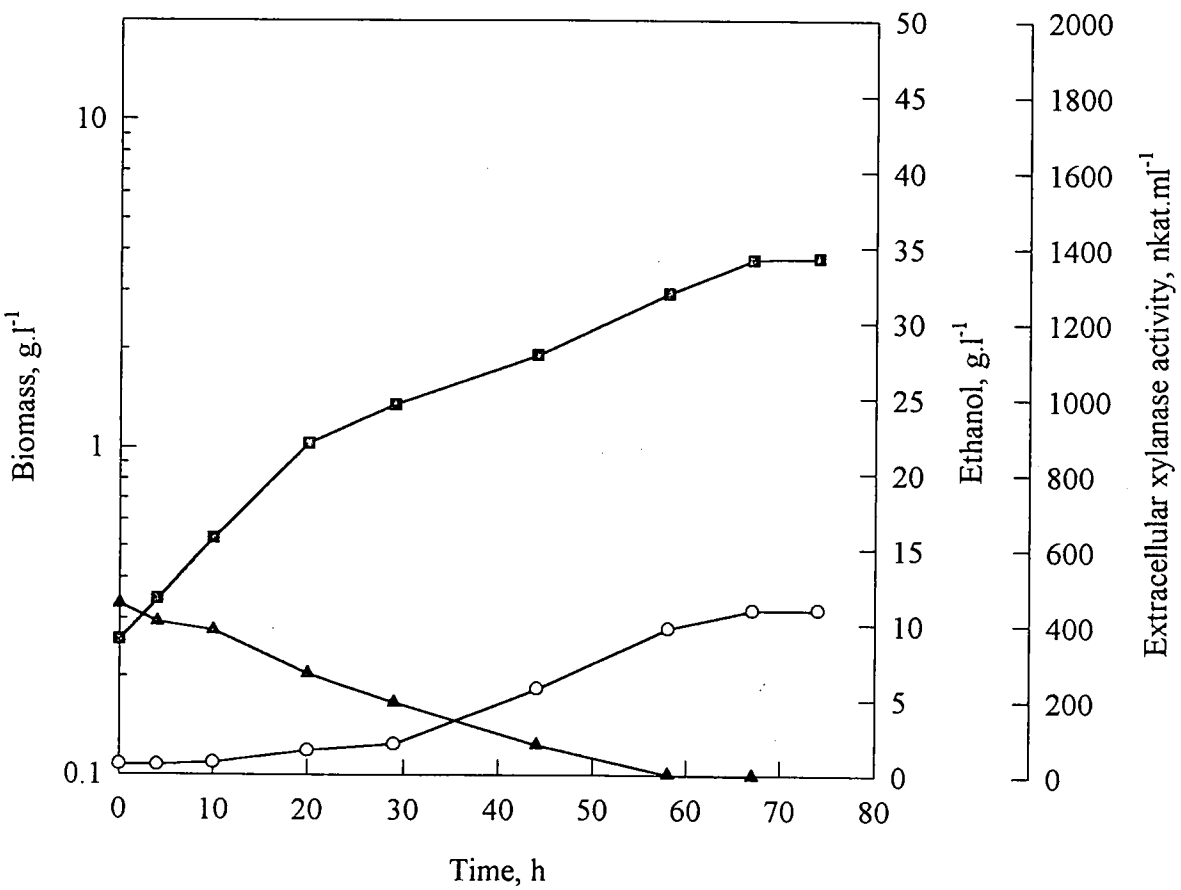


Figure 5.3. Batch cultivation profile of recombinant *S. cerevisiae* Y294 in medium B with 10 g ethanol.l^{-1} as carbon source at pH 5.5 and 30 °C. Symbols : biomass (■), ethanol concentration (▲) and extracellular xylanase activity (○).

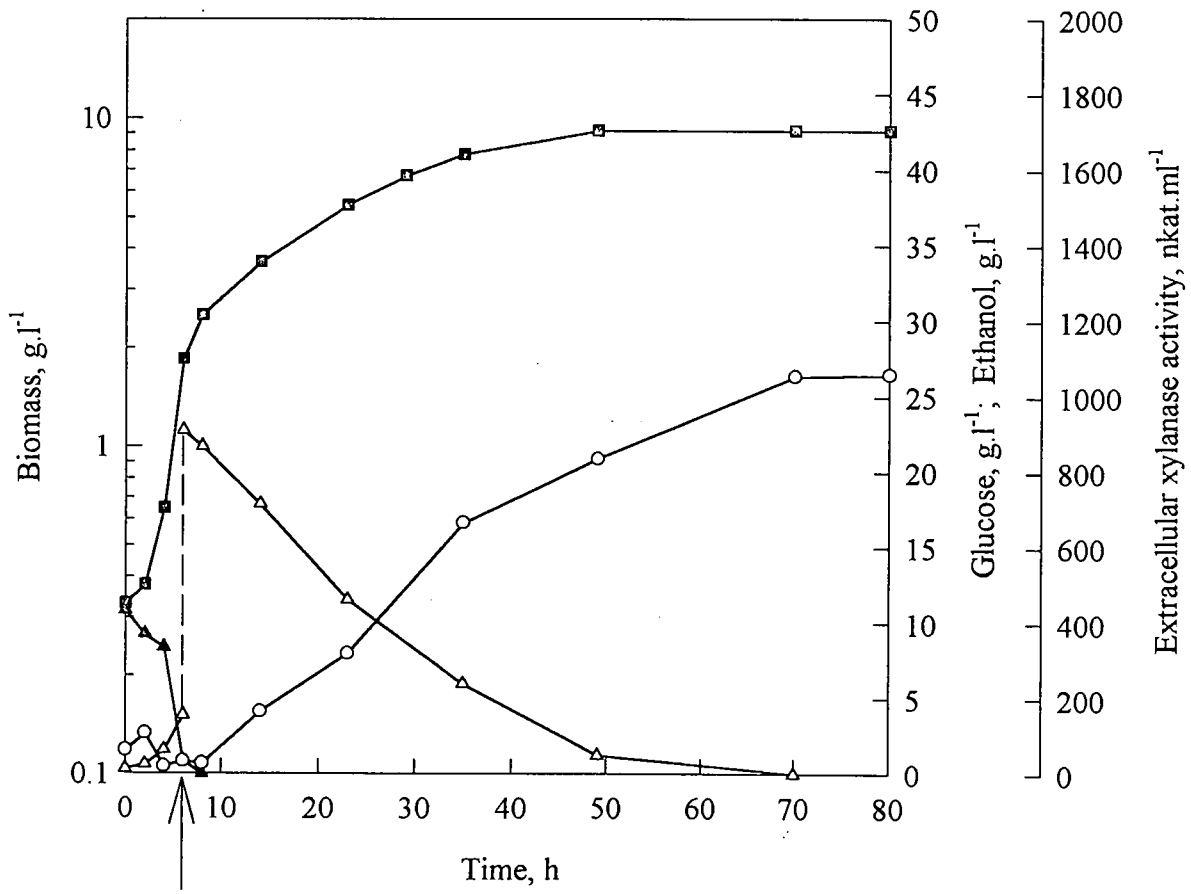


Figure 5.4. Batch cultivation profile of recombinant *S. cerevisiae* Y294 in medium B with 10 g glucose.l^{-1} as initial carbon source, with an additional 18 g ethanol.l^{-1} added after 6 h, at pH 5.5 and 30 °C. Symbols : biomass (■), ethanol concentration (△), glucose concentration (▲) and extracellular xylanase activity (○).

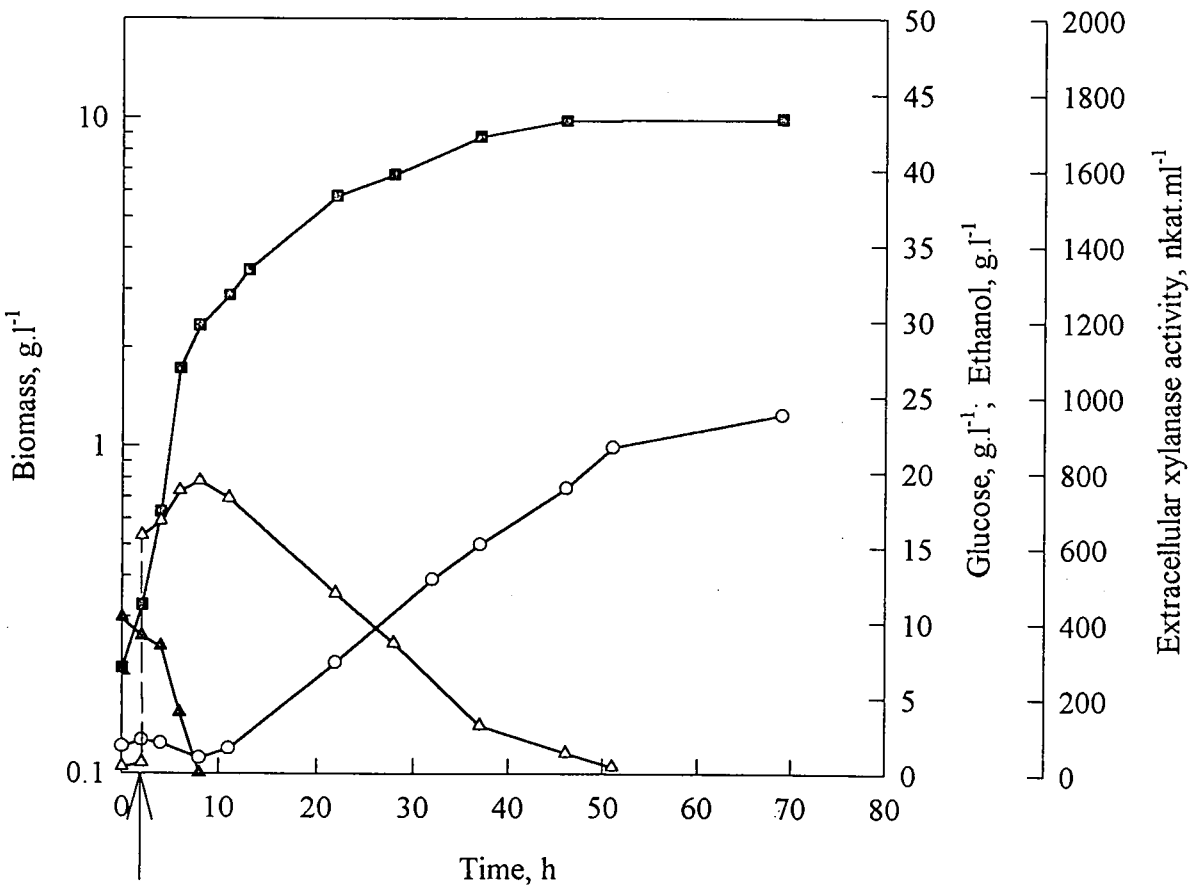


Figure 5.5. Batch cultivation profile of recombinant *S. cerevisiae* Y294 in medium B with 10 g glucose.l^{-1} as initial carbon source, with an additional 18 g ethanol.l^{-1} added after 2 h, at pH 5.5 and 30 °C. Symbols : biomass (■), ethanol concentration (△), glucose concentration (▲) and extracellular xylanase activity (○).

of 2.05 g glucose.l^{-1} was used, since this was the optimum growth rate and glucose feed concentration for xylanase production by *S. cerevisiae* Y294 (see Chapter 4). The steady-state extracellular xylanase activity was 124 (± 2) nkat.ml^{-1}. In determining the intracellular xylanase activity, it was shown that four cycles of vortexing yielded the maximum enzyme activity (Figure 5.6). Subsequently, ethanol pulses giving 27.25, 18.62 and 8.62 g ethanol.l culture^{-1}(590, 400 and 190 mmol ethanol.l culture^{-1}), respectively, were administered. After each ethanol pulse, the xylanase activity decreased within one hour and reached 50 to 60 nkat.ml^{-1} within 5 h (Figures 5.7, 5.8 and 5.9). Intracellular and extracellular xylanase activities decreased simultaneously, thus ruling out the possibility that xylanase excretion had been hampered due to an altered cell membrane permeability.

The reduction in measured enzyme activities after an ethanol pulse was found to correlate well with *XYN2* mRNA-levels, thus providing evidence that xylanase production was transcriptionally inhibited by ethanol (Figures 5.7, 5.8 and 5.9). The biomass concentration also transiently increased from 2 g.l^{-1} to 2.75 to 3 g.l^{-1} after each pulse. This transient increase in biomass was possibly the result of decreased heterologous xylanase production, which resulted in a higher biomass yield. Even at this low glucose feed concentration the yeast did not utilise the exogeneously added ethanol as indicated by fitting the experimental ethanol concentrations in the culture to the theoretical wash-out curves (Figures 5.10, 5.11 and 5.12). The wash-out curves constructed for extracellular xylanase activity showed that xylanase activity decreased immediately after an ethanol pulse and only started increasing again after 5 to 8 h when the ethanol concentration had decreased to below 6 g.l^{-1} (130 mmol.l^{-1}) (Figures 5.10, 5.11 and 5.12). During the first 5 h after an ethanol pulse, extracellular xylanase activity followed the theoretical values quite closely, which indicated that the production of xylanase (transcription) either ceased or was severely reduced almost immediately.

A pulse with 5 g glucose.l^{-1} (28 mmol glucose.l^{-1}) to an aerobic carbon-limited continuous culture of *S. cerevisiae* Y294 gave a remarkably similar response (Figure 5.13) to that obtained with ethanol pulses. The steady-state value for xylanase activity at time zero was 176 nkat.ml^{-1}. Within 1 h the extracellular xylanase activity was reduced to 85 nkat.ml^{-1} and reached a minimum of 57 nkat.ml^{-1} at 6 h. The only apparent difference between the responses obtained with the ethanol pulses and the glucose pulse was that with the latter, the biomass started to increase within 1 h.

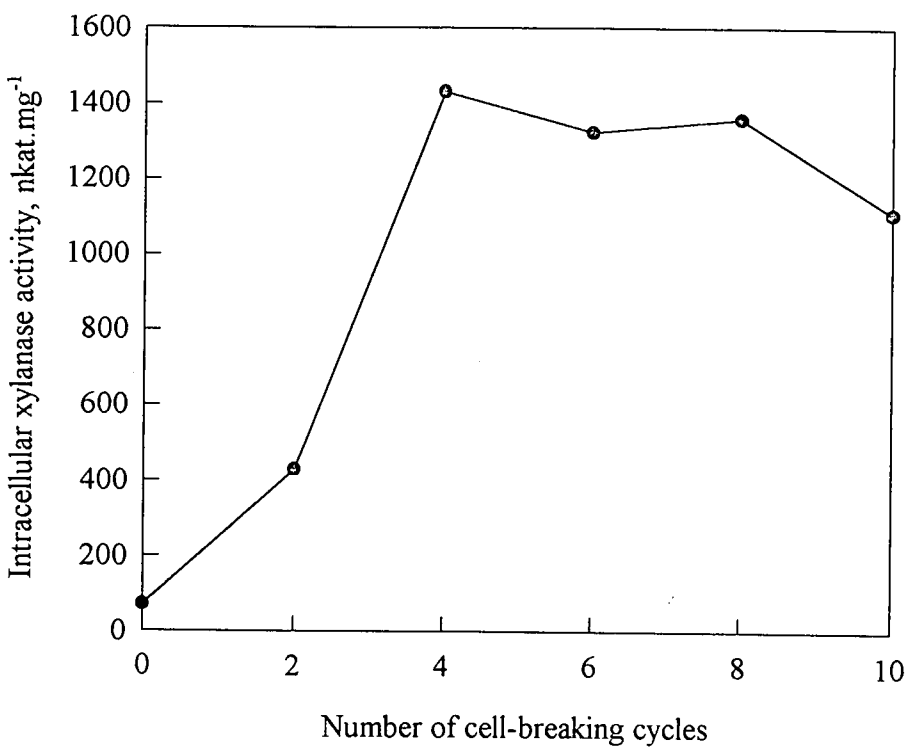


Figure 5.6. Intracellular xylanase activity determined as a function of the number of cell breaking cycles.

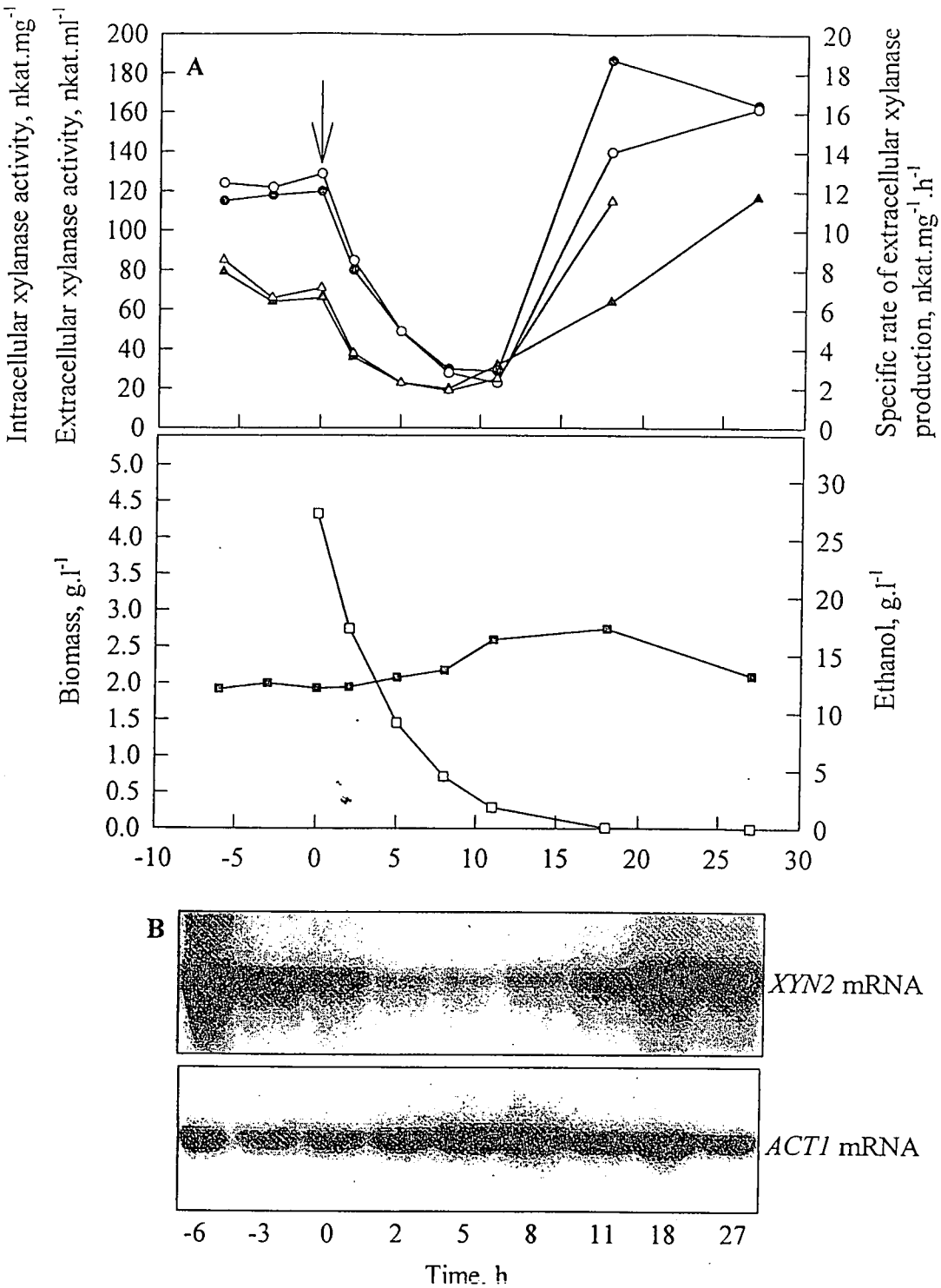


Figure 5.7. **A)** The effect of a pulse addition at time zero (indicated by arrow) of 30 g ethanol.l^{-1} (concentration in culture) to an aerobic carbon-limited continuous culture of *S. cerevisiae* Y294 at a dilution rate of 0.17 h^{-1}, using a glucose feed concentration of 2.05 g.l^{-1} at pH 5.5 and 30 °C. Symbols : extracellular xylanase activity (○), intracellular xylanase activity (●), specific rate of extracellular xylanase production (△), specific rate of intracellular xylanase production (▲), biomass (■) and ethanol concentration (□). **B)** Northern blot analysis of *XYN2* mRNA and *ACT1* mRNA (used as internal standard) following the ethanol pulse.

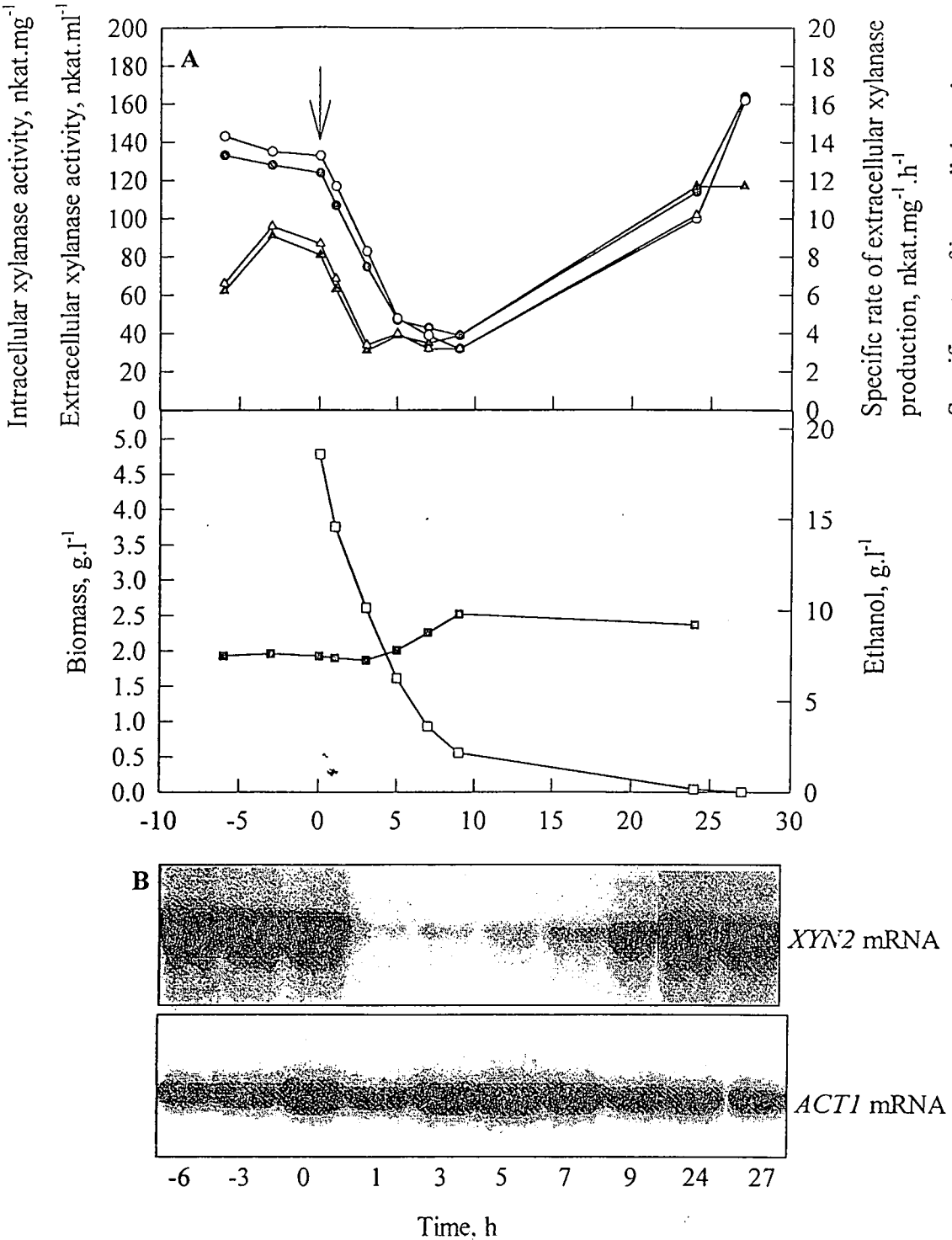


Figure 5.8. **A)** The effect of a pulse addition (at time zero, indicated by arrow) of 20 g ethanol.l^{-1} (concentration in culture) to an aerobic carbon-limited continuous culture of *S. cerevisiae* Y294 at a dilution rate of 0.17 h^{-1}, using a glucose feed concentration of 2.05 g.l^{-1} at pH 5.5 and 30 °C. Symbols : extracellular xylanase activity (○), intracellular xylanase activity (●), specific rate of extracellular xylanase production (△), specific rate of intracellular xylanase production (▲), biomass (■) and ethanol concentration (□). **B)** Northern blot analysis of *XYN2* mRNA and *ACT1* mRNA (used as internal standard) following the ethanol pulse.

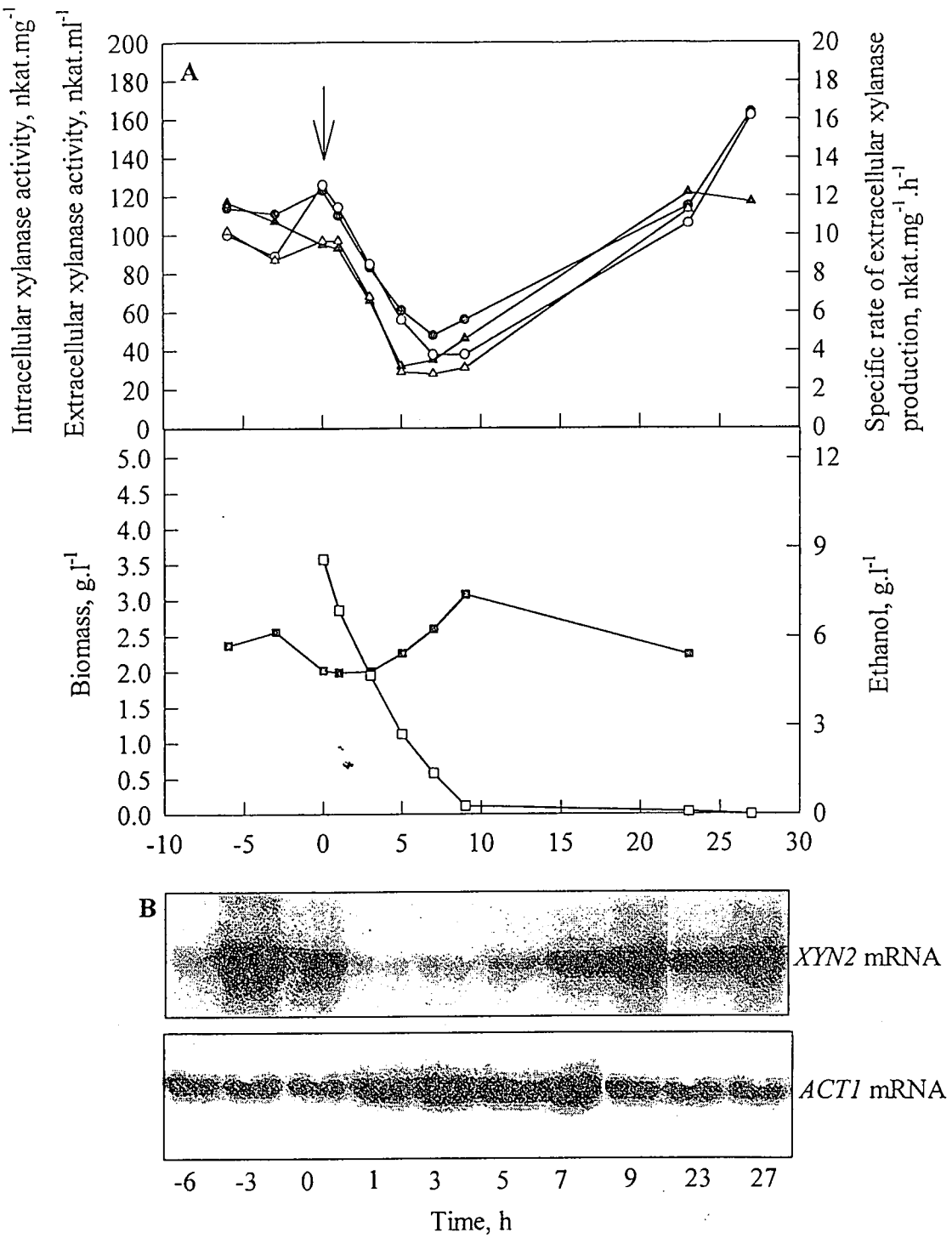


Figure 5.9. **A)** The effect of a pulse addition (at time zero, indicated by arrow) of 10 g ethanol.l^{-1} (concentration in culture) to an aerobic carbon-limited continuous culture of *S. cerevisiae* Y294 at a dilution rate of 0.17 h^{-1}, using a glucose feed concentration of 2.05 g.l^{-1} at pH 5.5 and 30 °C. Symbols : extracellular xylanase activity (○), intracellular xylanase activity (●), specific rate of extracellular xylanase production (△), specific rate of intracellular xylanase production (▲), biomass (■) and ethanol concentration (□). **B)** Northern blot analysis of *XYN2* mRNA and *ACT1* mRNA (used as internal standard) following the ethanol pulse.

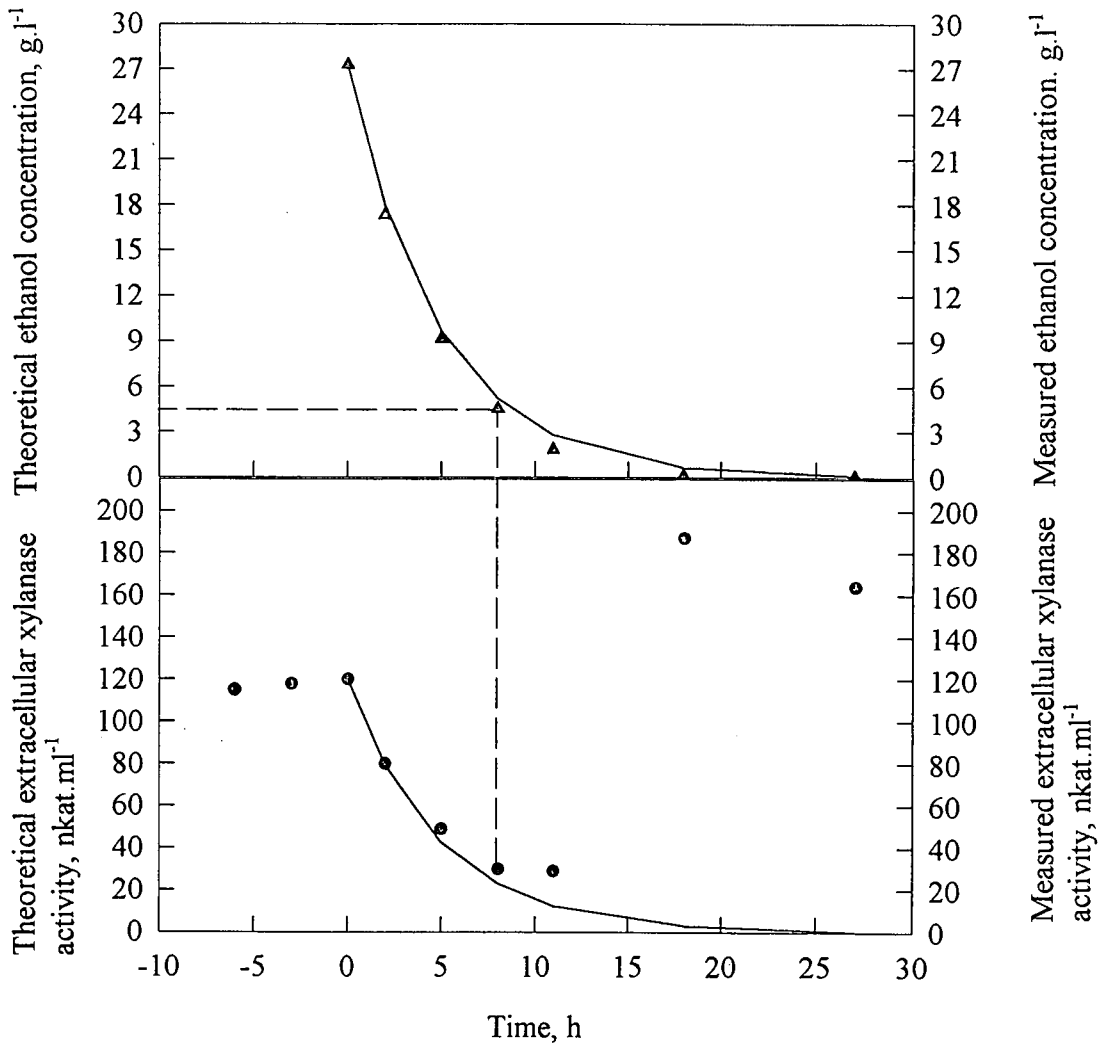


Figure 5.10. Experimental (symbols) and theoretical values (lines) of ethanol concentration (▲) and extracellular xylanase activity (●) following a pulse with 30 g ethanol.l^{-1} as constructed using values from figure 5.7. The broken lines correlate the points at which xylanase production resumed with the ethanol concentration in the culture.

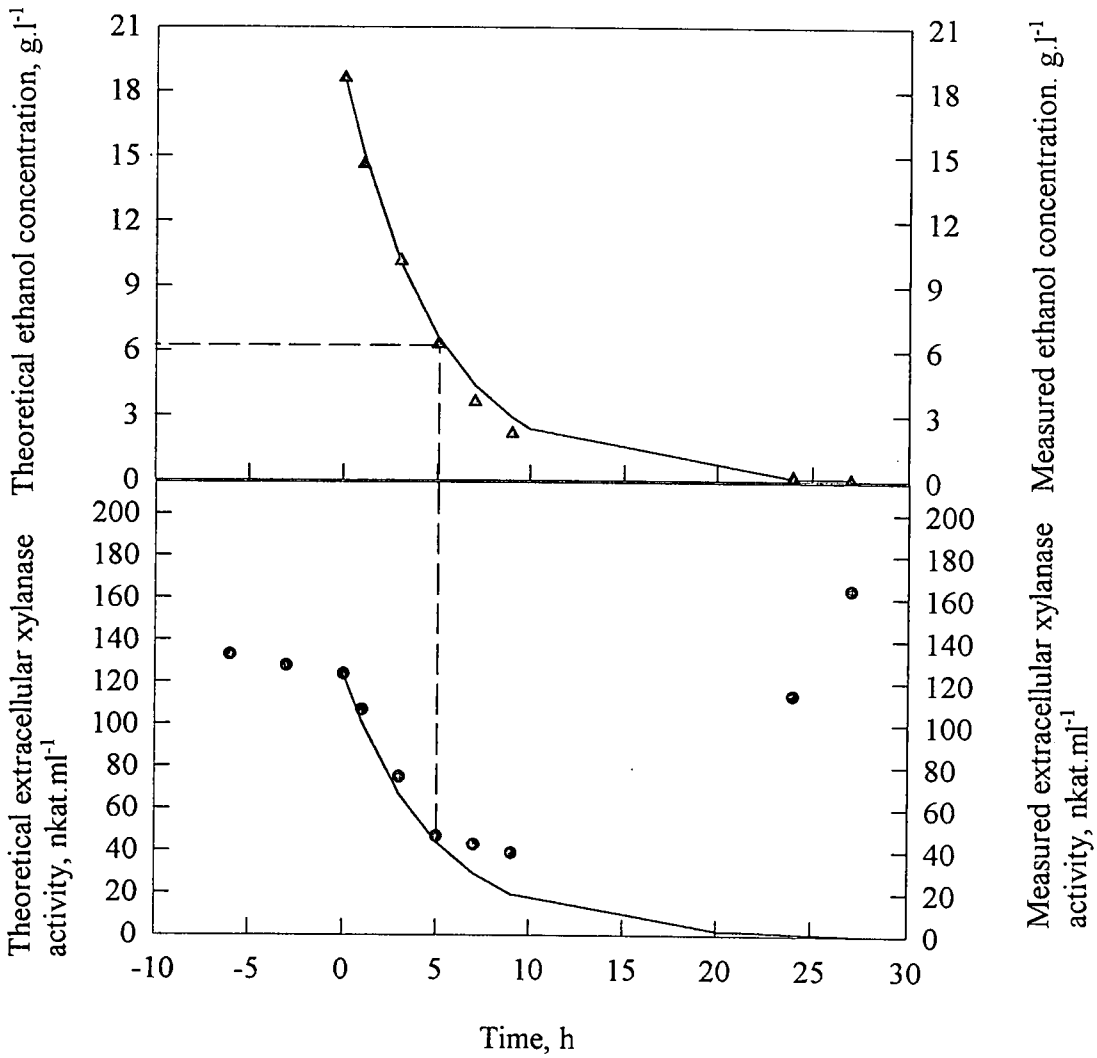


Figure 5.11. Experimental (symbols) and theoretical values (lines) of ethanol concentration (▲) and extracellular xylanase activity (●) following a pulse with 20 g ethanol.l^{-1} as constructed using values from figure 5.8. The broken lines correlate the points at which xylanase production resumed with the ethanol concentration in the culture.

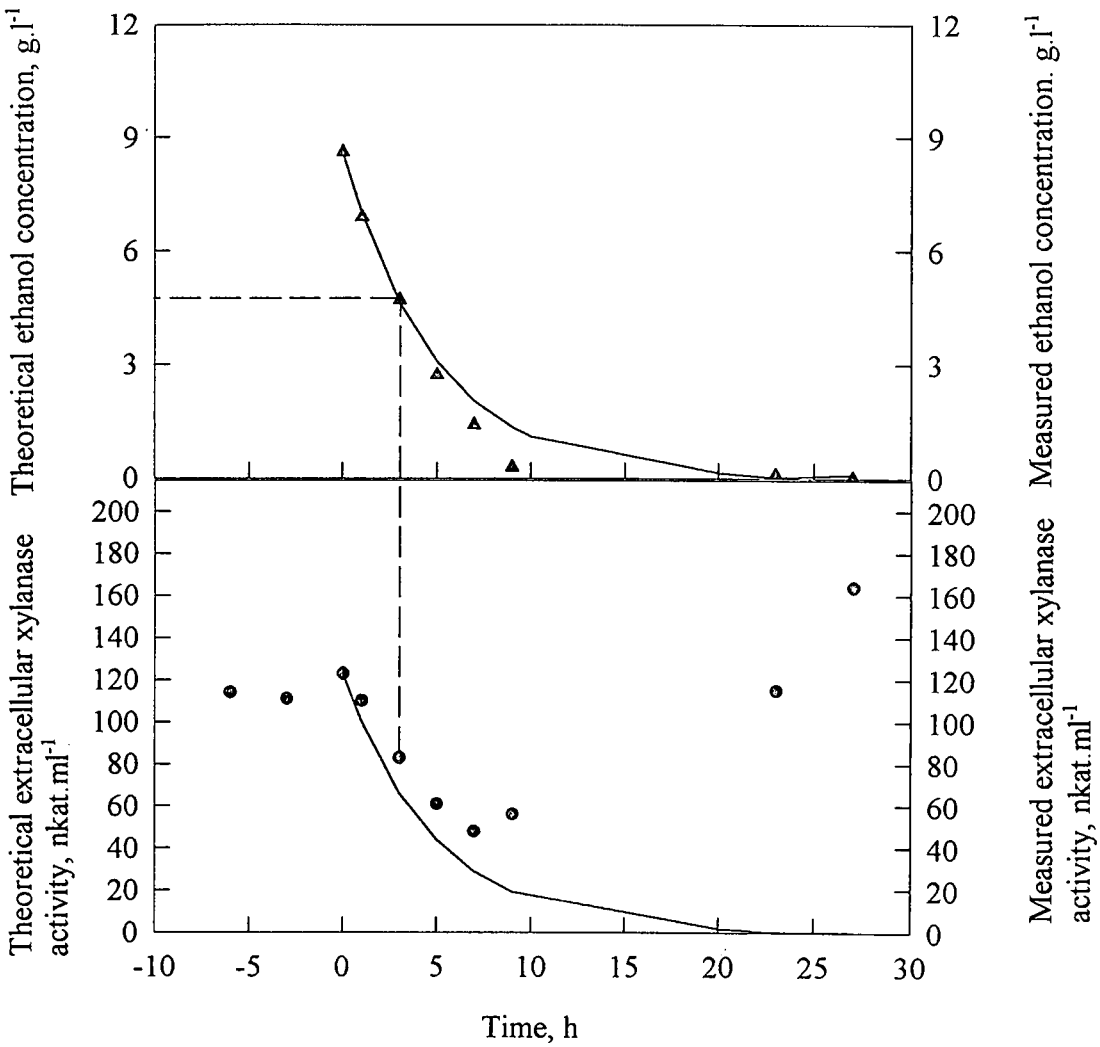


Figure 5.12. Experimental (symbols) and theoretical values (lines) of ethanol concentration (▲) and extracellular xylanase activity (●) following a pulse with 10 g ethanol.l^{-1} as constructed using values from figure 5.9. The broken lines correlate the points at which xylanase production resumed with the ethanol concentration in the culture.

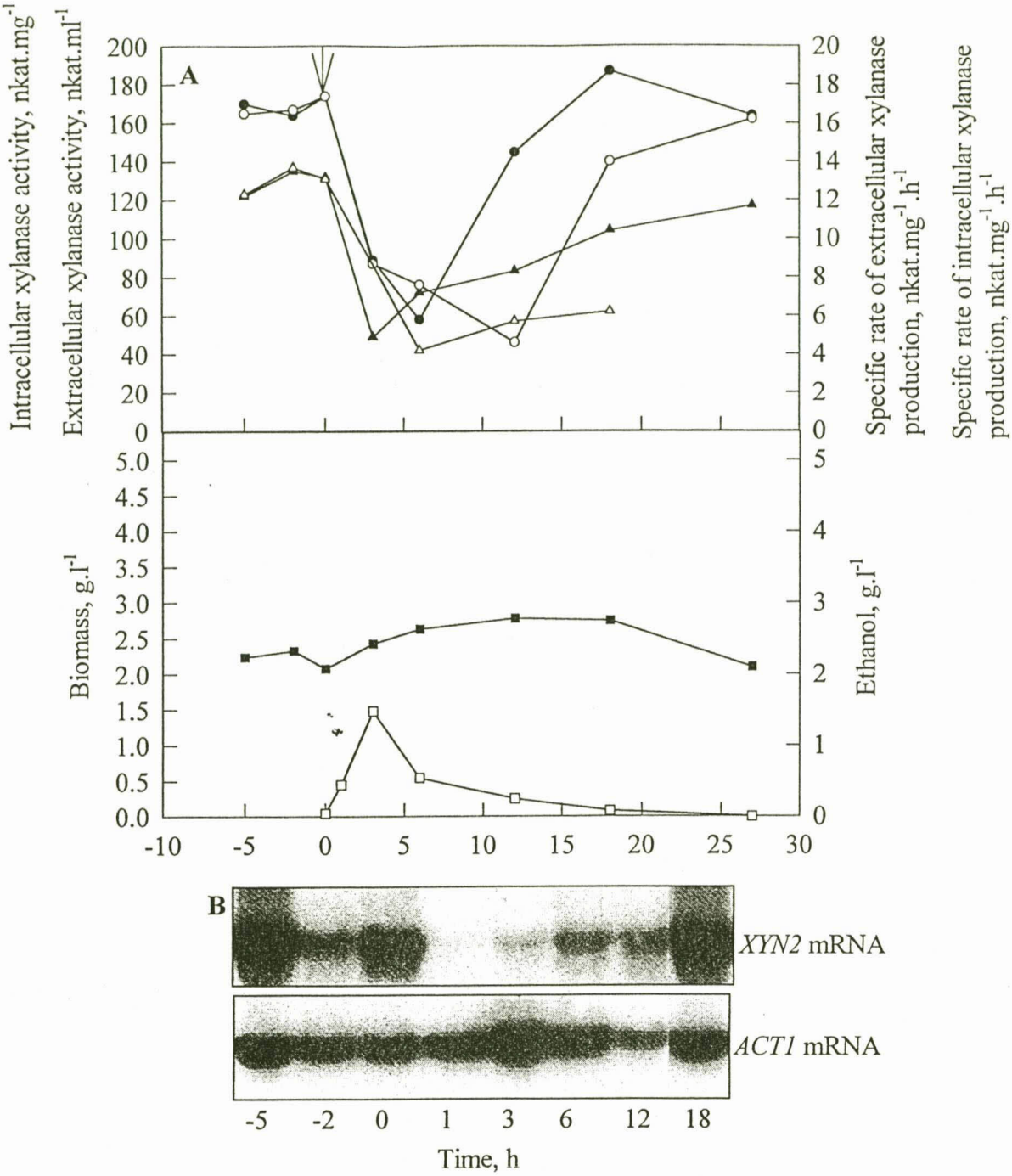


Figure 5.13. **A)** The effect of a pulse addition at time zero (indicated by arrow) of 5 g glucose.l^{-1} (concentration in culture) to an aerobic carbon-limited continuous culture of *S. cerevisiae* Y294 at a dilution rate of 0.17 h^{-1}, using a glucose feed concentration of 2.05 g.l^{-1} at pH 5.5 and 30 °C. Symbols : extracellular xylanase activity (○), intracellular xylanase activity (●), specific rate of extracellular xylanase production (△), specific rate of intracellular xylanase production (▲), biomass (■) and ethanol concentration (□). **B)** Northern blot analysis of *XYN2* mRNA and *ACT1* mRNA (used as internal standard) following the glucose pulse.

A pulse experiment with 30 g ethanol.l^{-1} (650 mmol ethanol.l^{-1}) was also performed with an aerobic carbon-limited continuous culture of a recombinant xylanase-producing *S. cerevisiae* CEN.PK110-6C strain (Figure 5.14). The same promoter-terminator expression cassette derived from *ADH2* regulated xylanase production by this strain. The extracellular activity decreased from 300 nkat.ml^{-1} to 200 nkat.ml^{-1} within 2 h and reached a minimum of 85 nkat.ml^{-1} at 10 h after the ethanol pulse. The decline in extracellular activity and the increase in biomass (from 4 to 5 g.l^{-1}) at 5 h was quite similar to what was observed with *S. cerevisiae* Y294. However, the two strains differed with regard to the ability of *S. cerevisiae* CEN.PK110-6C to assimilate ethanol (Figure 5.15). The theoretical wash-out curve for extracellular xylanase activity was always slightly below the measured values, which indicated that ethanol did not shut-down xylanase production as completely as in *S. cerevisiae* Y294. Excretion of the xylanase enzyme was found to be insufficient in both strains Y294 and CEN.PK110-6C, since significant xylanase activity resided intracellularly (Figures 5.7, 5.8, 5.9, 5.13, 5.14).

In batch cultures an increased glucose concentration, therefore also an increased fermentation ethanol concentration, resulted in a decrease in the specific and volumetric rates of xylanase production. In carbon-limited continuous cultures the pulse addition of ethanol in the range of 8.62 to 27.25 g.l^{-1} (190 to 590 mmol.l^{-1}) severely reduced the rate of transcription of xylanase. Transcription appeared to resume only when ethanol concentrations decreased to below 6 g.l^{-1} (130 mmol.l^{-1}). Both recombinant yeast strains exhibited a similar response towards an ethanol pulse: xylanase production was severely retarded (strain CEN.PK110-6C) or virtually ceased (strain Y294). A glucose pulse elicited a similar response. It is evident from literature that the role of ethanol in *ADH2* transcription has gone unnoticed. Up to date, there are no reports on the effect of ethanol concentration on *ADH2* transcription, which warrants further investigation into this aspect of regulation. Apart from the physiological significance of this finding, it has practical implications as well for the production of heterologous proteins under control of the *ADH2* promoter in *S. cerevisiae*. In aerobic batch culture with glucose as carbon feedstock, a substantial amount of the sugar is converted to fermentation ethanol due to the Crabtree effect (Sonnleitner and Käppeli, 1986). However, with heterologous protein production under control of the *ADH2* promotor, ethanol production would be detrimental to high levels of heterologous protein production. This would dictate the use of fed-batch or continuous culture systems to minimise ethanol accumulation in the culture through control of the sugar concentration in the culture.

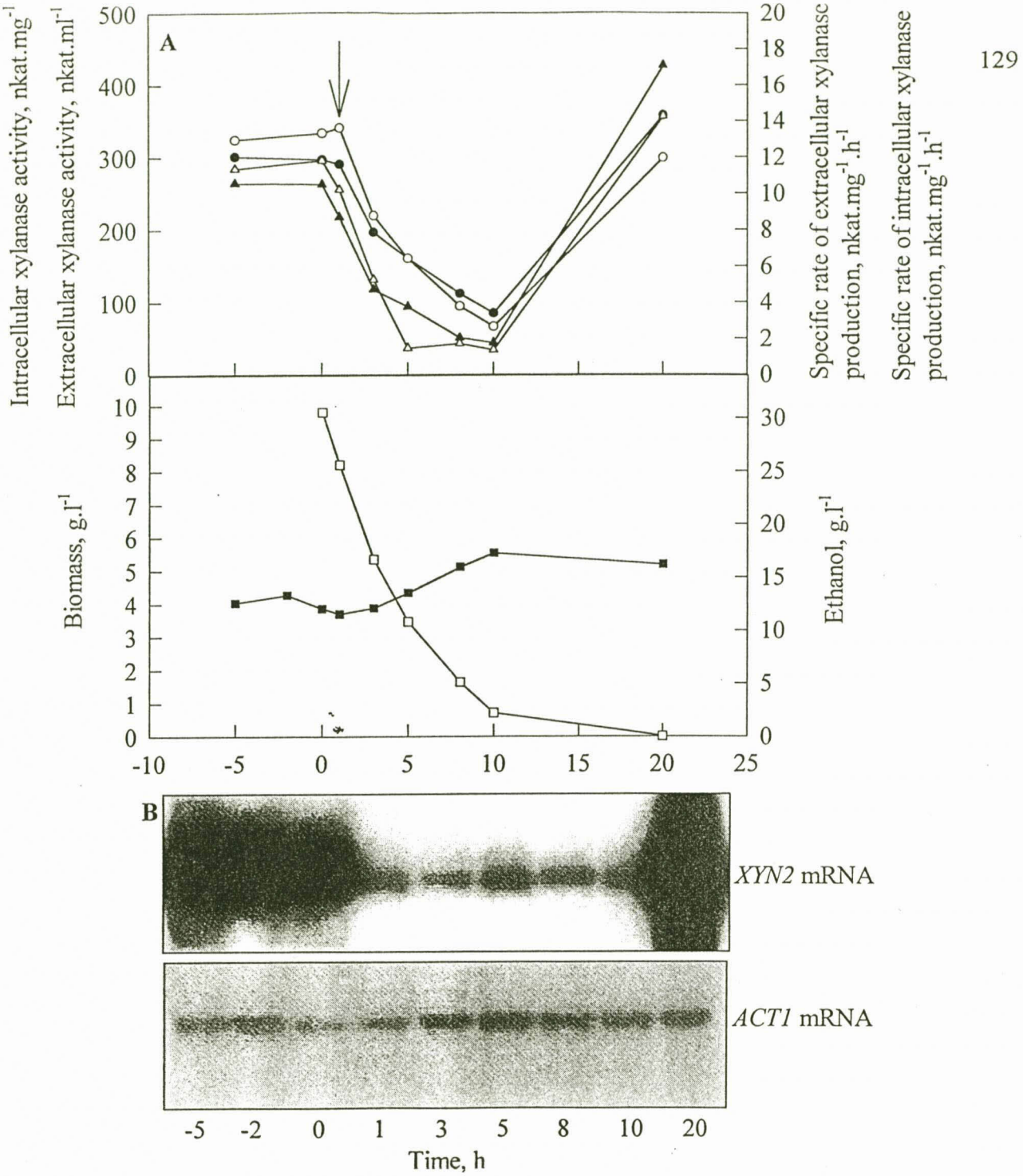


Figure 5.14. **A)** The effect of a pulse addition at time zero (indicated by arrow) of 30 g ethanol.l^{-1} (concentration in culture) to an aerobic carbon-limited continuous culture of *S. cerevisiae* CEN.PK110-6C at a dilution rate of 0.174 h^{-1}, using a glucose feed concentration of 5 g.l^{-1} at pH 5.5 and 30 °C. Symbols : extracellular xylanase activity (○), intracellular xylanase activity (●), specific rate of extracellular xylanase production (△), specific rate of intracellular xylanase production (▲), biomass (■) and ethanol concentration (□). **B)** Northern blot analysis of *XYN2* mRNA and *ACT1* mRNA (used as internal standard) following the ethanol pulse.

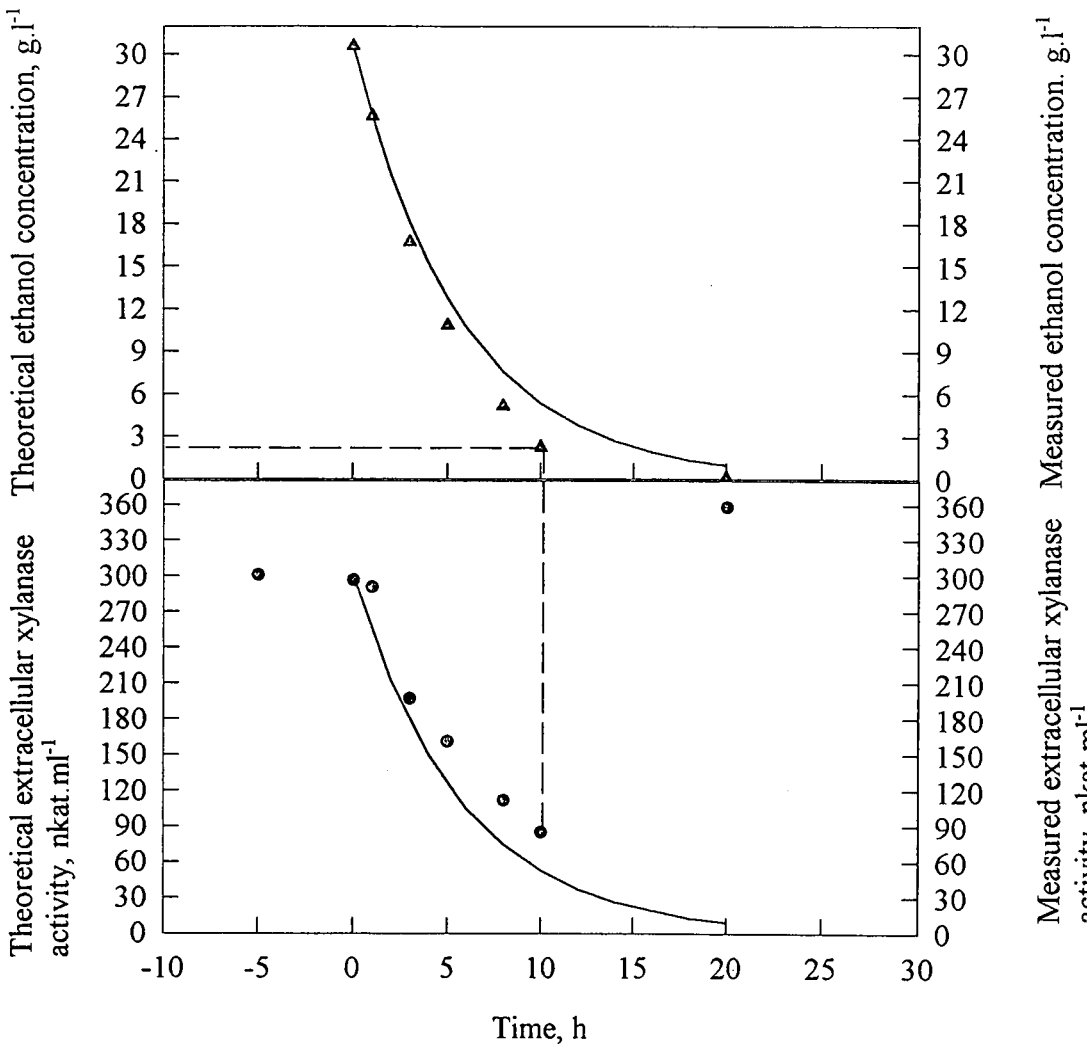


Figure 5.15. Experimental (symbols) and theoretical values (lines) of ethanol concentration (▲) and extracellular xylanase activity (●) following a pulse with 30 g ethanol.l^{-1} as constructed using values from figure 5.14. The broken lines correlate the points at which xylanase production resumed with the ethanol concentration in the culture.

It was also observed that the recombinant *S. cerevisiae* Y294 and CEN.PK110-6C strains did not secrete the heterologous xylanase efficiently, since significant enzyme titers were found intracellularly. The retention of heterologous proteins in the ER, Golgi-apparatus or cell wall is one of the main problems encountered when expressing heterologous proteins in *S. cerevisiae* (Sudbery, 1996).

Ethanol pulses administered to glucose-limited continuous cultures of *S. cerevisiae* Y294 resulted in repression of *ADH2*-regulated xylanase production. Therefore, it remains to be determined whether repression of *ADH2*-regulated xylanase production was caused by ethanol concentration or by ethanol flux.

5.5. Nomenclature

Batch cultivations

$Y_{x/s}$	Biomass yield coefficient
μ_{max}, h^{-1}	Maximum specific growth rate on carbon source
q_s^{max}, $g.g^{-1}.h^{-1}$	Maximum specific rate of glucose assimilation
$Y_{ethanol/s}$	Ethanol yield coefficient
$q_p^{max}{}_{ethanol}$, $g.(g.h)^{-1}$	Maximum specific rate of ethanol production
$\mu_{max\ ethanol}$, h^{-1}	Maximum specific growth rate on ethanol
$q_s^{max}{}_{ethanol}$, $g.(g.h)^{-1}$	Maximum specific rate of ethanol assimilation
$q_p^{max}{}_{xylanase}$, $nkat.(mg.h)^{-1}$	Maximum specific rate of xylanase production

5.6. References

Casey, G.P. & Ingledew, W.M. (1985). Re-evaluation of alcohol synthesis and tolerance in brewer's yeast. *ASBC J* **43**, 75-83.

Ciriacy, M. (1975). Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae.* II. Two loci controlling synthesis of the glucose-repressible ADH II. *Mol Gen Genet* **138,** 157-164.

Ciriacy, M. (1997). Alcohol dehydrogenases. In Zimmermann, F.K. & Entian, K.-D. (Eds), *Yeast sugar metabolism : Biochemistry, genetics, biotechnology and applications.* Technomic Publishing Co., Inc., Lancaster, U.S.A. pp 213-223.

Denis, C.L. (1984). Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II. *Genetics* **108,** 833-844.

Denis, C.L., Ciriacy, M. & Young, E.T. (1981). A positive regulatory gene is required for accumulation of the functional messenger RNA for the glucose-repressible alcohol dehydrogenase from *Saccharomyces cerevisiae. J Mol Biol* **148,** 355-368.

Denis, C.L. & Malvar, T. (1990). The *CCR4* gene from *Saccharomyces cerevisiae* is required for both nonfermentative and *spt*-mediated gene expression. *Genetics* **124,** 283-291.

Gancedo, J.M. (1998). Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* **62**, 334-361.

Hoppe, G.K. & Hansford, G.S. (1982). Ethanol inhibition of continuous anaerobic yeast growth. *Biotechnol Lett* **4**, 39-44.

Köhrer, K. & Domdey, H. (1990). Preparation of high molecular weight RNA. *Methods Enzymol* **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.

Luong, J.H.T. (1985). Kinetics of alcohol inhibition in alcohol fermentation. *Biotechnol Bioeng* **27**, 280-285.

Lutstorf, U. & Megnet, R. (1968). Multiple forms of alcohol dehydrogenase in *Saccharomyces cerevisiae. Arch Biochem Biophys* **126**, 933-944.

Peres, M.F.S. & Laluce, C. (1998). Ethanol tolerance of thermotolerant yeasts cultivated on mixtures of sucrose and ethanol. *J Ferment Bioeng* **85**, 388-397.

Russell, D.W., Smith, M., Williamson, V.M. & Young, E.T. (1982). Nucleotide sequence of the yeast alcohol dehydrogenase II gene. *J Biol Chem* **258,** 2674-2682.

Saliola, M., Shuster, J.R. & Falcone, C. (1990) The alcohol dehydrogenase system in the yeast, *Kluyveromyces lactis. Yeast* **6,** 193-204.

Sierkstra, L.N., Nouwen, N.P., Verbakel, J.M.A. & Verrips, C.T. (1992a). Analysis of glucose repression in *Saccharomyces cerevisiae* by pulsing glucose to a galactose-limited continuous culture. *Yeast* **8**, 1077-1087.

Sierkstra, L.N., Verbakel, J.M.A. & Verrips C.T. (1992b). Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**, 2559-2566.

Sonnleitner, B. & Käppeli, O. (1986). Growth of *Saccharomyces cerevisiae* is controlled by its limited respiratory capacity: Formulation and verification of a hypothesis. *Biotechnol Bioeng* **28**, 927-937.

Sudbery, P.E. (1996). The expression of recombinant proteins in yeasts. *Curr Opinion Biotechnol* 7, 517-524.

Van Uden, N. (1985). Ethanol toxicity and ethanol tolerance in yeasts. In Tsao, T. & Jovanovich, H.B. (Eds), *Annual reports on fermentation processes*. Academic Press, Inc., Vol 8, pp. 11-43.

CHAPTER 6

XYLANASE PRODUCTION IN FED-BATCH CULTURE

6.1. Abstract

Endo-β-1,4-xylanase production by recombinant *Saccharomyces cerevisiae* Y294 (pDLG1; *bla URA3 ADH2*$_{PT}$) was investigated in fed-batch cultures. The maximum specific rate of xylanase production was 9.9 nkat.mg^{-1}.h^{-1} in a fed-batch culture with a constant glucose feed rate. A fed-batch culture with an exponentially increasing feed rate facilitated a high maximum specific rate of xylanase production (52 nkat.mg biomass^{-1}.h^{-1}), which corresponded to a specific growth rate of 0.1 h^{-1}. The maximum extracellular xylanase activity obtained was 1520 nkat.ml^{-1}, similar to activity values obtained in batch cultures. However, the maximum specific rate of xylanase production was increased 2.5-fold as compared to batch cultures. The importance of the specific growth rate on xylanase production was demonstrated, with a high specific rate of xylanase production occurring only within a narrow range of growth rates, despite a very low residual glucose concentration.

6.2. Introduction

Little information is available on the use of the alcohol dehydrogenase II (*ADH2*) promoter for heterologous gene expression in continuous and fed-batch cultures. However, several articles have been published on the use of *ADH2* / *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) hybrid promoters (Chiruvolu *et al.*, 1996; Cousens *et al.*, 1987; Tøttrup and Carlsen, 1989).

Cousens *et al.* (1987) described the production of human pro-insulin of up to 100 mg.l^{-1} by *S. cerevisiae*, using a regulated hybrid promoter (*ADH2 / GAPDH*) consisting of the regulatory region of *ADH2* fused to the 3' region of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Similarly, Chiruvolu *et al.* (1996) descibed the production of heterologous ovine interferon-τ in *S. cerevisiae*, using an *ADH2 / GAPDH* hybrid promoter in fed-batch cultures. The recombinant yeast was grown under glucose limitation to reduce ethanol accumulation, so as to maximise the biomass yield. During the production period, ethanol was continuously fed to the bioreactor at a rate of 1 g.l^{-1}.h^{-1}.

Tøttrup and Carlsen (1989) developed a fed-batch cultivation process for the production of SOD-pro-insulin, also using the *ADH2 / GAPDH* hybrid promoter. In these experiments, a

constant, empirically-determined glucose feed was used, which resulted in a glucose excess during most of the growth phase with a low residual glucose concentration (20 $mg.l^{-1}$) during the later stages as the cell density increased. Upon changing from growth on glucose to an ethanol feed, the concentration of SOD-pro-insulin increased from 1.2 $g.l^{-1}$ to 1.6 $g.l^{-1}$. These authors concluded that the relatively small increase (25%) in product concentration was due to partial repression of the promoter by the residual glucose (20 $mg.l^{-1}$) present in the medium.

In this chapter, results on fed-batch experiments conducted with the recombinant *S. cerevisiae* Y294 strain in a 15-litre bioreactor are reported. Two different feeding regimens were used to minimise repression of the *ADH2* promoter by glucose and ethanol.

6.3. Materials and Methods

6.3.1. Yeast strains

The recombinant xylanase-producing *S. cerevisiae* Y294 strain (pDLG5; *bla URA3 ADH2*$_P$-*XYN2-ADH2*$_T$) used in the previous chapters was maintained as described in Chapter 2.

6.3.2. Inoculum and medium

Medium B was used in fed-batch cultures contained (per litre): 10 g yeast extract, 20 g peptone, 0.5 ml of a trace element solution (du Preez and van der Walt, 1983) and 0.5 ml Dow Corning 1520 silicone antifoam (Dow Corning, Seneffe, Belgium). A glucose solution was autoclaved separately and added aseptically to the basal medium after sterilisation. The sterile culture broth was inoculated with a 250 ml inoculum prepared as described in Chapter 2.

6.3.3. Cultivation conditions

Fed-batch cultivations were carried out in a 15-litre Biostat C bioreactor (B. Braun Biotech International, Melsungen, Germany) equipped with an exhaust gas condensor, a pH electrode (Mettler Toledo, Halstead, Great Britain) and a polarographic oxygen electrode (Mettler Toledo). A peristaltic pump (Watson Marlow Ltd, Cornwall, England) with silicone rubber

tubing was used for the glucose feed. Cultivations were carried out at 30 °C with the pH controlled at pH 5.5 (± 0.1) by automatic titration with 3 M KOH and 3 M H_2SO_4. The dissolved oxygen tension was maintained above 20 % of saturation using aeration at 3.14 standard litres.min^{-1} and a stirrer speed of 500 r.min^{-1}. Two fed-batch experiments were performed, first by using a constant glucose feed rate and secondly, by using a exponentially increasing feed rate to facilitate a constant growth rate (μ). For the exponentially increasing feed rate, the feed rate at any specific time [F(t)] was calculated using the following equation (Chung *et al.*, 1997):

$$F(t) = \frac{\mu . x_0 . V_0 . e^{\mu t}}{Y_{x/s}(s_{in} - s)}$$

where F(t) was the feed rate at time t (l.h^{-1}), μ the specific growth rate (h^{-1}), x_0 the biomass concentration at the time the feed was started (g.l^{-1}), V_0 the initial reactor volume (litres), $Y_{x/s}$ the biomass yield coefficient (g biomass.g glucose assimilated^{-1}), s_{in} the glucose concentration in the feed (g.l^{-1}) and s the residual glucose concentration in the culture (g.l^{-1}). The calculations for adjusting the feed rate were performed using a Microsoft Excel™ (Microsoft Corporation, Redmond, WA, USA) spreadsheet (Appendix A).

6.3.4. Analyses

The biomass concentration, ethanol concentration and extracellular xylanase activity of each sample were determined as described in Chapters 3 and 4. One unit of activity (nkat) was defined as the amount of enzyme that released 1 nmol of xylose equivalents per second per ml of culture filtrate. Specific rate of xylanase production was expressed as nkat.mg biomass^{-1}h^{-1}.

6.4. Results and Discussion

Considering the results obtained with regard to the influence of glucose flux (q_s), growth rate and ethanol concentration on xylanase production, several parameters were chosen for the operation of fed-batch cultures (Table 6.1). Firstly, it had been established that xylanase production was optimal at a specific growth rate of 0.1 h^{-1}, corresponding to a specific rate of glucose assimilation of 0.98 mmol glucose.g biomass^{-1}.h^{-1} (Chapter 4). Secondly, ethanol concentrations at or higher than 5.5 g.l^{-1} transcriptionally repressed *ADH2*-regulated xylanase production similar to repression caused by glucose (Chapter 5).

Figure 6.1 depicts a fed-batch culture of the recombinant *S. cerevisiae* Y294 strain with 10 g glucose.l^{-1} as initial carbon source. A feed consisting of 400 g glucose.l^{-1} was initiated at 12 h and kept constant at 51 ml.h^{-1} until 47 h of cultivation time. Upon initiation of the constant feed the specific growth rate (μ) gradually decreased from 0.38 to 0.005 h^{-1} as the biomass concentration increased. The extracellular xylanase activity at 12 h (at the start of the constant glucose feed) was 126 nkat.ml^{-1} and reached 660 nkat.ml^{-1} at 47 h. The highest specific rate of xylanase production (15 nkat .mg^{-1}.h^{-1}) occurred at 11 h, an hour before the glucose feed was initiated. The residual glucose concentration (s) at this point was 87 mg.l^{-1}.

Table 6.1. Parameters chosen for fed-batch cultivations.

Parameter	Constant glucose feed	Exponentially increasing glucose feed
Initial carbon source	10 g glucose.l^{-1}	5 g glucose.l^{-1}
Inoculum size	250 ml	250 ml
Initial volume (V_0)	8 l	8 l
Glucose feed concentration	400 g glucose.l^{-1}	200 g glucose.l^{-1}
Criterion for start of feed	Initial glucose depleted	Initial glucose depleted
Air flow rate	3.14 slpm	3.14 slpm
Growth rate (μ)	0.38 to 0.005 h^{-1}	0.1 h^{-1}
$Y_{x/s}$	0.45	0.45
Maximum fermentation ethanol concentration	5.5 g.l^{-1}	5.5 g.l^{-1}

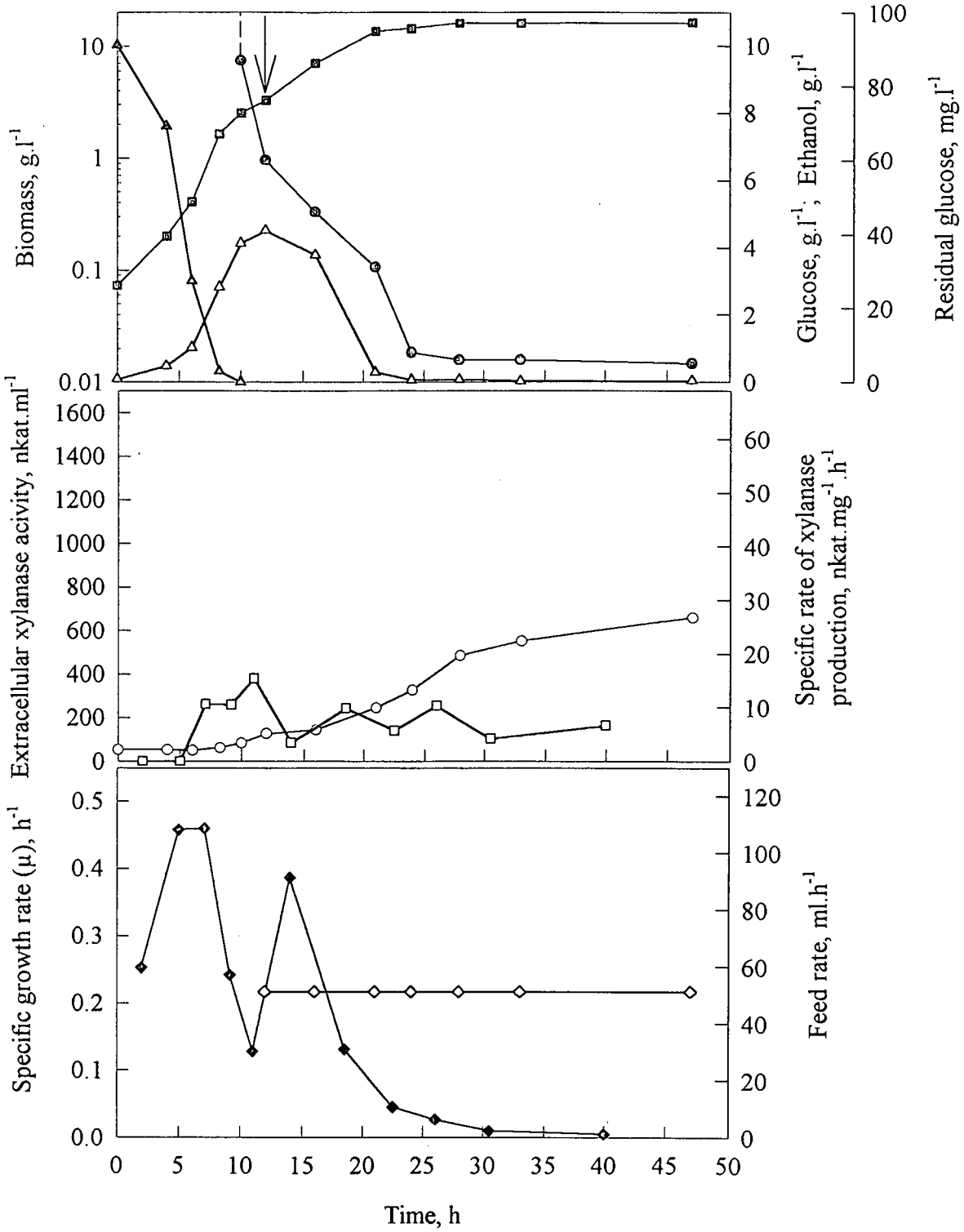


Figure 6.1. Fed-batch cultivation profile of the recombinant *S. cerevisiae* Y294 in medium B with 10 g glucose.l^{-1} as initial carbon source at pH 5.5 and 30°C. A constant glucose feed consisting of 400 g glucose.l^{-1} was initiated at 12 h (indicated by arrow). Symbols: biomass (■), glucose on g.l^{-1} scale (▲), ethanol (△), glucose on mg.l^{-1} scale (◉), extracellular xylanase activity (○), specific rate of xylanase production (□), specific growth rate (◆) and feed rate (◇).

During the constant glucose feed a low specific rate of xylanase production in the range of 9.9 to 3.5 nkat.mg^{-1}.h^{-1} was obtained (Figure 6.1). However, during the constant glucose feed the maximum residual glucose concentration was only 30 mg.l^{-1}. Therefore, it appeared that the residual glucose concentration was not the causative agent of the low specific rate of xylanase production, but rather the lack of control of the growth rate.

A second fed-batch experiment was conducted with 5 g glucose.l^{-1} as initial carbon source with an exponentially increasing feed rate, initiated upon depletion of the initial glucose at 9.16 h (Figure 6.2). The feed contained 200 g glucose.l^{-1}. During the first 5 h after starting the feed, the feed rate was adjusted to maintain a constant specific growth rate (μ) of 0.21 h^{-1}. At a specific growth rate of 0.21 h^{-1}, the specific rate of xylanase production was negligible (2 nkat.mg biomass^{-1}.h^{-1}) and the residual glucose concentration was 68 mg.l^{-1}. The feed rate was adjusted at 16 h to obtain a specific growth rate of 0.1 h^{-1}, resulting in an increase of the specific rate of xylanase production to 52 nkat.mg biomass^{-1}.h^{-1}. The residual glucose concentration during this period was 31 mg.l^{-1}. Concomitantly, the extracellular xylanase activity increased from 108 nkat.ml^{-1} at 16 h to 1340 nkat.ml^{-1} at 20 h (Figure 6.2). At a cultivation time of 20 h, control of the specific growth rate was lost and ethanol accumulated to 1.02 g.l^{-1} in the culture broth at 25 h. The decline in the specific growth rate after 20 h resulted in a decrease in the specific rate of xylanase production to 0.97 nkat.mg biomass^{-1}.h^{-1} as well as in the residual glucose concentration (Figure 6.2). At a cultivation time of 23 h the glucose feed was kept constant at 60 ml.h^{-1}. The only satisfactory explanation for the loss of growth rate control after 20 h of cultivation, was that an incorrect biomass yield coefficient for glucose ($Y_{x/s}$) was used. The biomass yield coefficient used was as calculated in Chapter 4, but a different medium (Medium B) was used for the fed-batch experiments. Medium B had higher yeast extract and peptone concentrations than Medium C used in Chapter 4, to ensure that there is no other nutrient limitation except for the carbon source.

Although the extracellular xylanase activity obtained during a fed-batch culture with an exponentially increasing feed rate was similar to that obtained in batch culture with 10 g glucose.l^{-1} as initial carbon source (1600 nkat.ml^{-1}), the maximum specific rate of xylanase production was improved from 20.75 to 52 nkat.mg biomass^{-1}.h^{-1}. This 2.5-fold increase in the specific rate of xylanase production could be attributed to control of the specific growth rate at 0.1 h^{-1} for several hours, which was the optimum specific growth rate for xylanase

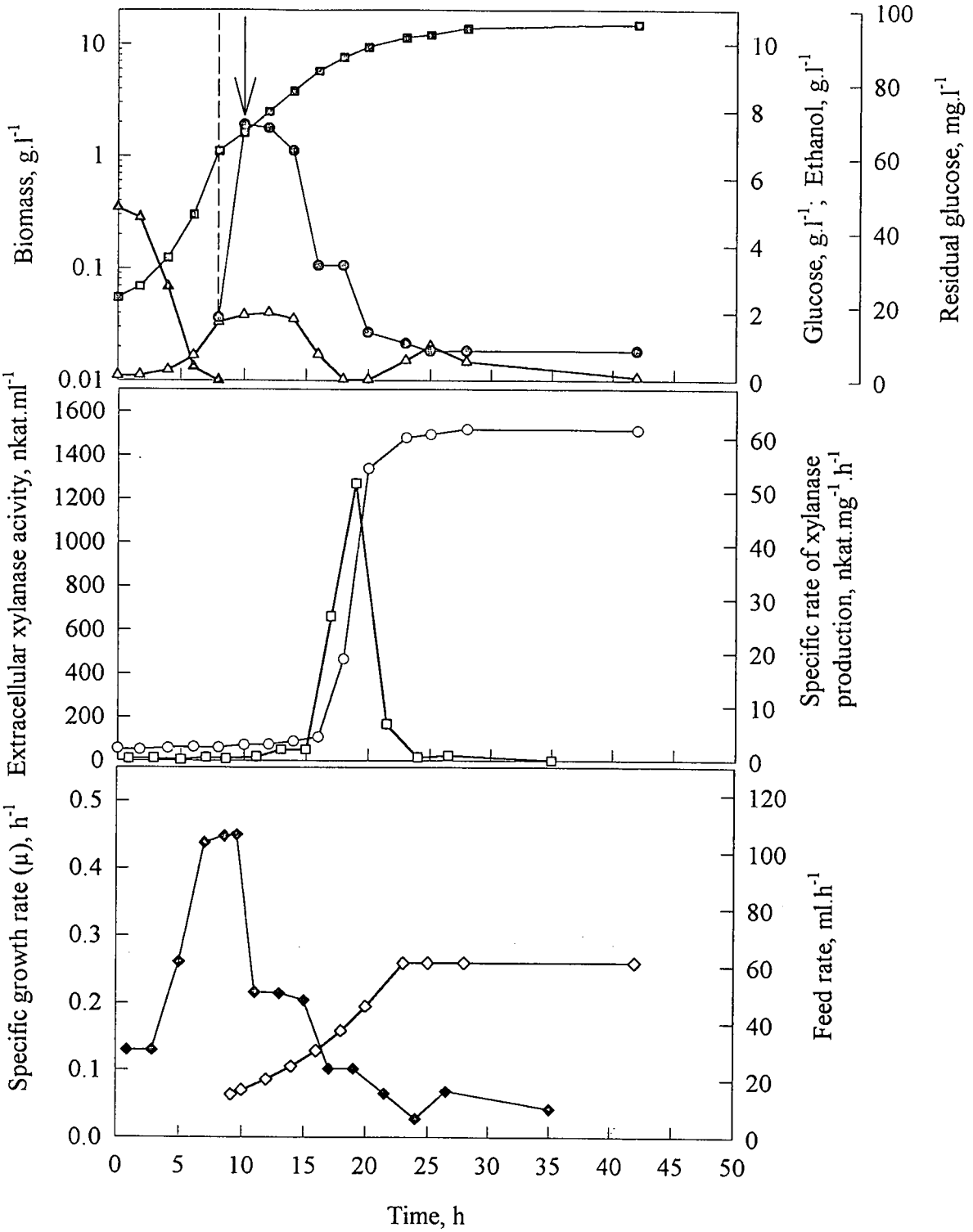


Figure 6.2. Fed-batch cultivation profile of the recombinant *S. cerevisiae* Y294 in medium B with 5 g glucose.l^{-1} as initial carbon source at pH 5.5 and 30°C. An exponentially increasing glucose feed, consisting of 200 g glucose.l^{-1}, was initiated at 9.16 h (indicated by arrow). Symbols: biomass (▣), glucose on g.l^{-1} scale (▲), glucose on mg.l^{-1} scale (❷), ethanol (△), extracellular xylanase activity (○), specific rate of xylanase production (□), specific growth rate (◆) and feed rate (◇).

production by this recombinant yeast strain. Chiruvolu *et al.* (1996) as well as Tøttrup and Carlsen (1989) made use of an ethanol feed for *ADH2* / *GAPDH*-regulated heterologous protein production and attributed the low product yield to partial repression of the promoter by the residual glucose (20 $mg.l^{-1}$) present in the medium. However, the above results suggest that *ADH2*-regulated heterologous protein production can be obtained in fed-batch cultures using glucose as the growth rate limiting carbon source and that residual glucose concentrations up to 87 $mg.l^{-1}$ did not cause repression of the *ADH2* promoter.

These data indicate that optimal xylanase production by this recombinant yeast strain can only be obtained within a narrow range of growth rates with a low residual glucose concentration and low ethanol concentration in fed-batch culture. Although these limited experiments failed to obtain an increased xylanase activity in the culture broth, the importance of growth rate and / or glucose flux control with respect to xylanase production was demonstrated. It was shown that the specific rate of xylanase production decreased even though the residual glucose concentration was very low and decreasing.

6.5. Nomenclature

μ, h^{-1}	Specific growth rate
q_s, mmol glucose .g biomass^{-1}.h^{-1}	Specific rate of glucose assimilation (glucose flux)
q_p, nkat. mg biomass^{-1}.h^{-1}	Specific rate of enzyme (xylanase) production
s, $mg.l^{-1}$	Residual glucose concentration
$Y_{x/s}$	Biomass yield coefficient, g biomass obtained per g glucose assimilated

6.6. References

Chiruvolu, V., Stratton, J.M., Ott, T.L., Bazer, F.W. & Meagher, M.M. (1996). Effect of medium composition on growth, plasmid stability and ovine interferon-τ production in *Saccharomyces cerevisiae*. *J Ferm Bioeng* **82**, 565-569.

Chung, B.H., Seo, D.J., Nam, S.W., Na, J.G. & Chang, Y.K. (1997). Optimization of feeding strategy for overproduction of human lipocortin-I in *Saccharomyces cerevisiae* controlled by the *GAL10* promoter. *J Ferm Bioeng* **84**, 466-470.

Cousens, L.S., Shuster, J.R., Gallegos, C., Ku, L., Stempien, M.M., Urdea, M.S., Sanchez-Pescador, R., Taylor, A. & Tekamp-Olson, P. (1987). High level expression of proinsulin in the yeast, *Saccharomyces cerevisiae. Gene* **61**, 265-275.

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

Tøttrup, H.V. & Carlsen, S. (1989). A process for the production of human proinsulin in *Saccharomyces cerevisiae. Biotechnol Bioeng* **35**, 339-348.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Transcriptional repression of *ADH2*-regulated xylanase production by the recombinant *S. cerevisiae* Y294 strain by ethanol at and above 5.5 g ethanol.l^{-1} (120 mmol ethanol.l^{-1}) was an unexpected result and at first glance seemed to be contradictory to the literature. The negative effect of ethanol was first observed in aerobic batch cultures of the recombinant yeast strain. When glucose-sensitive *S. cerevisiae* was grown in aerobic batch culture with glucose as carbon source, the glucose was consumed with concomitant ethanol production (due to the Crabtree effect) during the exponential growth phase. Upon depletion of the glucose, a secondary exponential growth phase (diauxic growth) was observed when the ethanol that had accumulated in the medium was metabolised. However, an increase in the initial glucose concentration, and thus an increase in the concentration of fermentation ethanol, led to both lower specific and overall rates of xylanase production by the recombinant *S. cerevisiae* Y294 strain in aerobic batch cultures (Chapter 5).

Tøttrup and Carlsen (1989) described the production of a superoxide dismutase-human proinsulin fusion product (SOD-PI) by a recombinant *S. cerevisiae* strain, using a regulated hybrid promoter (*ADH2 / GAPDH*) consisting of the regulatory region of *ADH2* fused to the 3' region of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Similar to the results obtained in this study, they found that the specific and overall rates of *ADH2 / GAPDH*-regulated SOD-PI production in this recombinant *S. cerevisiae* strain were impaired above glucose concentrations of 50 g.l^{-1} (Figure 7.1). They concluded that the decreased rates of specific and overall SOD-PI production were caused by the depletion of a critical medium component during the production period of SOD-PI (Tøttrup and Carlsen, 1989). However, at all the glucose concentrations tested, the product (SOD-PI) concentration was less in cultures with exogeneously added ethanol (Figure 7.1). Therefore, in the light of the findings presented in this thesis, it is suggested that the increased ethanol concentration, rather than a nutrient limitation, caused the decreased rates of specific and overall SOD-PI production observed by these authors.

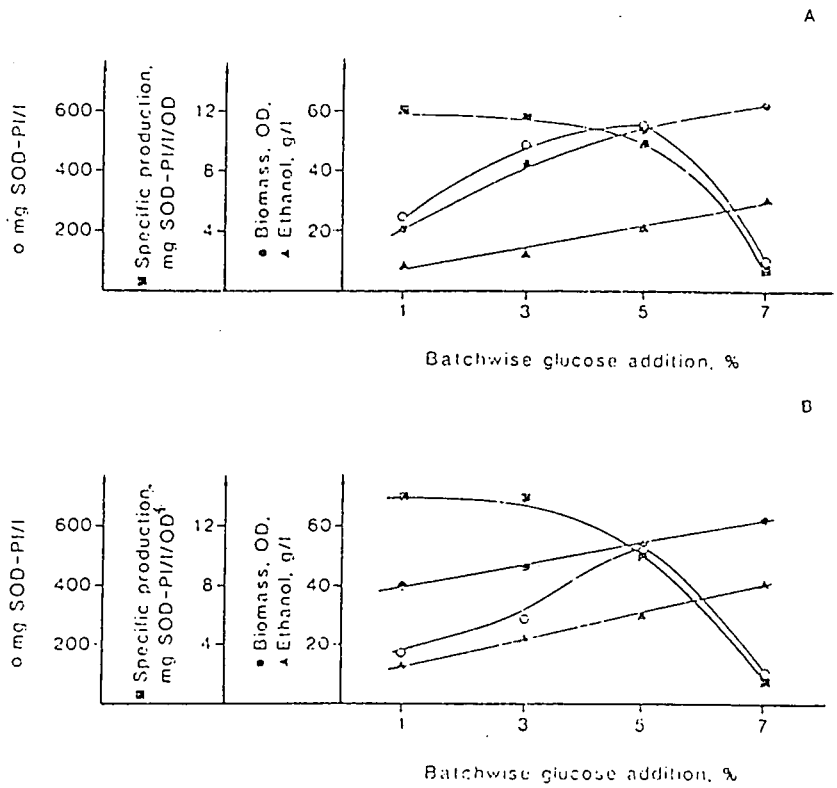


Figure 6. (A) Product formation in batch fermentations as function of amount of glucose added batchwise. (B) Product formation as a function of added amount of glucose in batch fermentations which have been supplemented with 1% ethanol before glucose was depleted from the medium. Symbols as in Figure 5.

Figure 7.1. **A)** SOD-PI production by a recombinant *S. cerevisiae* strain as a function of the initial glucose concentration in aerobic batch cultures. **B)** SOD-PI production by a recombinant *S. cerevisiae* strain as a function of the initial glucose concentration in aerobic batch cultures, with an additional 10 g ethanol.l^{-1} added before glucose was depleted from the medium (taken from Tøttrup and Carlsen, 1989).

Ethanol-containing media have predominantly been used in molecular studies that required *ADH2* in a derepressed state (Ciriacy, 1979; Denis, 1984; Shuster *et al.*, 1986; Vallari *et al.*, 1992). However in this thesis, it was shown that ethanol concentrations at or above 5.5 g ethanol.l^{-1} (120 mmol ethanol.l^{-1}) transcriptionally inhibited *ADH2*-regulated xylanase production by the recombinant *S. cerevisiae* Y294 strain (Chapter 5). Furthermore, the repression of transcription by ethanol was very similar to the repression caused by glucose. This finding was not strain specific, since ethanol repressed *ADH2*-regulated xylanase production also in another recombinant *S. cerevisiae* strain, namely *S. cerevisiae* CEN.PK110-6C. Xylanase production by strain Y294 did occur in aerobic batch cultures with ethanol as carbon source, but with low specific rates of xylanase production compared to xylanase production during diauxic growth with glucose as initial carbon source.

According to Beck and von Meyenburg (1968), specific growth rates of 0.12 to 0.18 h^{-1} are obtained during growth of *S. cerevisiae* on ethanol, depending on the medium composition. The recombinant *S. cerevisiae* Y294 exhibited a maximum specific growth rate of 0.1 to 0.12 h^{-1} in ethanol-containing media (Chaper 3). In carbon-limited chemostat cultures, the specific rate of xylanase production was maximal at dilution rates (which are numerically equivalent to the specific growth rate, μ) of 0.1 to 0.18 h^{-1}.

Therefore, it appeared that *ADH2*-regulated xylanase production by the recombinant *S. cerevisiae* Y294 was maximal at specific growth rates of 0.1 to 0.18 h^{-1} and that xylanase production was independent of whether glucose or ethanol was used as the carbon source. Sierkstra *et al.* (1994) concluded that the contribution of growth rate to the regulation of *ADH2* was at least as important as regulation by glucose repression. However, in view of the fact that ethanol also caused repression of xylanase production in the recombinant *S. cerevisiae* Y294, it is postulated that the specific growth rate, and therefore possibly also the carbon flux, were the main factors that influenced *ADH2*-regulated xylanase production.

The data presented here suggested that the glucose flux rather than the glucose concentration, caused glucose repression of xylanase production, supporting the findings of Sierkstra *et al.* (1994). Ethanol pulses administered to glucose-limited continuous cultures of *S. cerevisiae* Y294

resulted in repression of *ADH2*-regulated xylanase production, even though the exogeneously added ethanol was not metabolised. Therefore, it remains to be determined whether repression of *ADH2*-regulated xylanase production was caused by the extracellular ethanol concentration or by the carbon flux. Throughout this study complex media were used, but it is unlikely that this could have influenced the results obtained regarding the physiological parameters governing the expression of *ADH2*-regulated xylanase by the recombinant yeast strains, since similar complex media were used in studies of the molecular mechanisms regulating *ADH2* (see Chaper 1, Table 1.8). Clearly, the regulation of the *ADH2* promoter is more complex than apparent from the available literature.

It was also observed that the recombinant *S. cerevisiae* Y294 and CEN.PK110-6C strains did not secrete the heterologous xylanase efficiently, since significant enzyme titers were found intracellularly. The retention of heterologous proteins in the ER, Golgi-apparatus or cell wall is one of the main problems encountered when expressing heterologous proteins in *S. cerevisiae* (Sudbery, 1996). The production of *ADH2*-regulated heterologous xylanase in *S. cerevisiae* Y294 exhibited several negative characteristics: (1) tight regulation of the carbon flux was critical for optimal xylanase production and the margin for error was small, (2) the secretion efficiency of the heterologous xylanase was poor and (3) ethanol repression of *ADH2* added an additional facet to the already intricate regulation of *ADH2*. However, high plasmid stability during prolonged cultivations of recombinant *S. cerevisae* Y294, was a major advantage.

7.1. References

Beck, C & von Meyenburg, H.K. (1968). Enzyme pattern and aerobic growth of *Saccharomyces cerevisiae* under various degrees of glucose limitation. *J Bacteriol* **96**, 479-486.

Ciriacy, M. (1979). Isolation and characterization of further *cis*- and *trans*-acting regulatory elements involved in the synthesis of glucose-repressible alcohol dehydrogenase (ADHII) in *Saccharomyces cerevisiae*. *Mol Gen Genet* **176,** 427-431.

Denis, C.L. (1984). Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II. *Genetics* **108,** 833-844.

Sierkstra, L.N., ter Schure, E.G., Verbakel, J.M.A. & Verrips, C.T. (1994). A nitrogen-limited, glucose-repressed, continuous culture of *Saccharomyces cerevisiae*. *Microbiol* **140**, 593-599.

Shuster, J., Yu, J., Cox, D., Chan, R.V.L., Smith, M. & Young, E. (1986). *ADR1*-mediated regulation of *ADH2* requires an inverted repeat sequence. *Mol Cell Biol* **6,** 1894-1902.

Sudbery, P.E. (1996). The expression of recombinant proteins in yeasts. *Curr Opinion Biotechnol* 7, 517-524.

Tøttrup, H.V. & Carlsen, S. (1989). A process for the production of human proinsulin in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **35**, 339-348.

Vallari, R.C., Cook, W.C., Audino, D.C., Morgan, M.J., Jensen, D.E., Laudano, A.P. & Denis, C.L. (1992). Glucose repression of the yeast *ADH2* gene occurs through multiple mechanisms, including control of the protein synthesis of its transcriptional activator, ADR1. *Mol Cell Biol* **12,** 1663-1673.

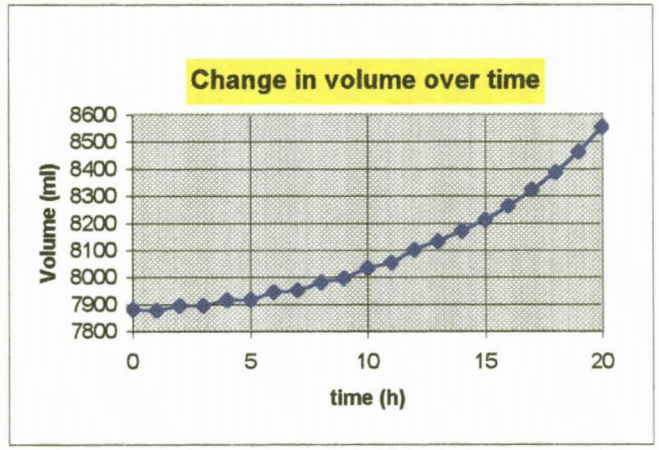
APPENDIX A

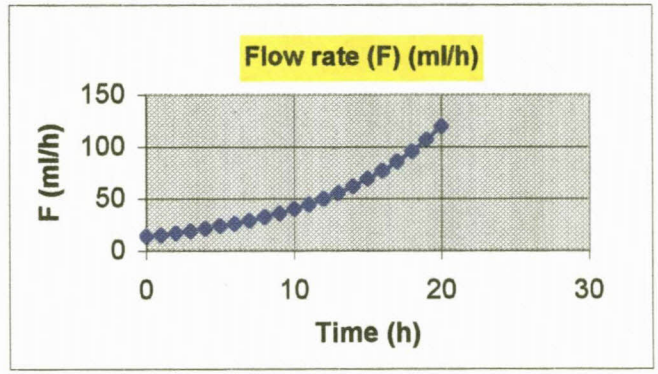
The spreadsheet used to calculate adjustments of the feed rate in a fed-batch culture with an exponentially increasing feed rate.

Time	Vo	F (ml/h)	xt (g)	qs, mmol/g	Volume	Sample volume
0	7885	15.02075	12.70274	1.312702	8000	20
1	7882.021	16.59423	14.03339	1.312702	7882.021	18
2	7898.615	18.37807	15.54195	1.312702	7898.615	0
3	7898.993	20.31188	17.17733	1.312702	7898.993	18
4	7919.305	22.50582	19.0327	1.312702	7919.305	0
5	7923.811	24.88693	21.04635	1.312702	7923.811	18
6	7948.698	27.5907	23.33287	1.312702	7948.698	0
7	7956.288	30.52155	25.81144	1.312702	7956.288	20
8	7986.81	33.86093	28.63548	1.312702	7986.81	0
9	8000.671	37.48706	31.70202	1.312702	8000.671	20
10	8038.158	41.62373	35.20032	1.312702	8038.158	0
11	8059.782	46.12509	39.00702	1.312702	8059.782	20
12	8105.907	51.26783	43.35613	1.312702	8105.907	0
13	8137.175	56.87828	48.10077	1.312702	8137.175	20
14	8174.053	63.14511	53.40049	1.312702	8174.053	20
15	8217.198	70.15449	59.32818	1.312702	8217.198	20
16	8267.352	78.00593	65.96798	1.312702	8267.352	20
17	8325.358	86.81476	73.41742	1.312702	8325.358	20
18	8392.173	96.71515	81.78997	1.312702	8392.173	20
19	8468.888	107.8638	91.21819	1.312702	8468.888	20
20	8556.752	120.4448	101.8576	1.312702	8556.752	20

Parameters used to determine F	
Xo	1.611
μ	0.1
Yx/s	0.5
So	200
Sr (mg/l)	30.8642

Total volume added	1066.197





EXPONENTIAL INCREASING FEED (CONSTANT μ)