

***ADH2* REGULATION IN THE YEAST  
*Saccharomyces cerevisiae***

BY

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ALL THAT A MAN ACHIEVES AND ALL  
THAT HE FAILS TO ACHIEVE IS THE  
DIRECT RESULT OF HIS THOUGHT...

James Allen

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# CHAPTER 1

## INTRODUCTION

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*ADH2* belong to a large family of enzymes that catalyze the reversible transfer of hydrogen to the carbonyl group of aldehydes or related compounds. *ADH2* encode for an enzyme, which is essential during ethanol utilization by *Saccharomyces cerevisiae*, this enzyme catalyses the initial step during ethanol assimilation by reducing ethanol into acetaldehyde. The alcohol dehydrogenase (ADH) systems of various organisms e.g. yeast; fungi and human have been the subject of thorough analyses on the molecular structure and the mode of catalyses. The *S. cerevisiae* system is of exceptional attractiveness because of its genetic accessibility, available molecular techniques and ease in cultivation. The regulatory system governing the expression of *ADH2* has been exploited as a model system for the analysis of regulation of glucose sensitive genes.

The synthesis of many enzymes in *S. cerevisiae* is repressed when the cells are grown in a glucose based medium and derepressed when the cells are grown in medium containing a nonfermentable carbon source such as ethanol (Polakis and Bartley, 1965). The *ADH2* gene of *S. cerevisiae* is not expressed when the cells are grown in medium containing glucose as a carbon source and derepressed over a 100-fold when the cells are shifted to a nonfermentable carbon source (Ciriacy, 1975). The absence of a fermentable carbon source signals yeast to activate the expression of genes controlling aerobic metabolism of alternative carbon sources.

The repression *ADH2* expression is not mediated by a repressor but primarily due to the absence of positive activation (Shuster *et al.*, 1986). Numerous transcription factors allow the cell to generate energy and metabolites from non-glucose carbon sources such as ethanol. Adr1p and Cat8p are the two

transcription activators required for the activation of the expression of *ADH2* (Walther and Schüller, 2001), these transcription factors are not active during growth on glucose. Two cis-acting upstream activating sequences (UASs) have been described for the *ADH2* gene. Both sequences confer glucose-regulated transcriptional regulation on this gene (Beier and Young, 1982; Shuster *et al.*, 1986; Beier *et al.*, 1985; Yu *et al.*, 1989). Adr1p binds to the UAS1 (Cook *et al.*, 1994) in the *ADH2* promoter and Cat8p (Hedges *et al.*, 1995) binds to the UAS2. Both Adr1p and Cat8p are active in the presence of Snf1p, a yeast homolog of AMP-activated protein kinase (Dombek *et al.*, 1999). Glucose exerts its gene repression on *ADH2* through the regulating the Snf1p activity. Snf1p is activated when the glucose level is low or absent in the medium (Jiang and Carlson, 1996). Snf1p regulates the expression of *CAT8* and the transcription activation function of its encoded enzyme (Cat8p) and Snf1p regulates chromatin binding by Adr1p (Young *et al.*, 2002).

*Saccharomyces cerevisiae* has been used in the expression of heterologous genes. Regulated promoters are more frequently used because their activity can be controlled during the fermentation process. Due to being under glucose repression, the *ADH2* promoter has been employed in regulated overexpression of heterologous genes in *S. cerevisiae* (Price *et al.*, 1990). For example, TBV25H, a malaria transmission blocking vaccine candidate was expressed from *S. cerevisiae* under the control of this promoter (Noronha *et al.*, 1998).

The expression of *ADH2* was recently found to be negatively affected by ethanol (du Preez *et al.*, 2001), these results were unexpected as ethanol is the substrate of Adh2p and this enzyme has been classified as the major alcohol dehydrogenase isozymes involved in the conversion of ethanol to acetaldehyde during ethanol assimilation. It would therefore be expected that the expression of *ADH2* would be induced by ethanol or if not induced, at least not be repressed by ethanol. Ethanol repression might thus have negative implications during the expression of heterologous genes using the *ADH2* promoter.



Because these results were obtained using an episomal multicopy plasmid with a promoter terminator expression cassette derived from the *S. cerevisiae ADH2* gene, these findings may not apply to the natural *ADH2* promoter. Due to the extensive sequence identity between *ADH1* and *ADH2* (95% identity) it is difficult to directly measure the *ADH2* mRNA through northern blot analysis without cross binding of an *ADH2* probe on *ADH1* mRNA (Sierkstra *et al.*, 1992).

It therefore became the aim of this study to determine the effect of ethanol on the genomic copy of *ADH2* in *S. cerevisiae*. *E. coli LacZ*, encoding  $\beta$ -galactosidase, was used as a reporter gene to construct an expression cassette for the study of the expression profile of *ADH2* under different growth conditions. The effect of ethanol was determined by cultivating *S. cerevisiae* containing an integrated copy of the expression cassette into three different ethanol concentrations: 5, 20 and 30 g ethanol l<sup>-1</sup>. Glucose repression was studied through cultivation on 2 g glucose l<sup>-1</sup>.

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# CHAPTER 2

## LITERATURE REVIEW

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### 1. INTRODUCTION

*Saccharomyces cerevisiae* (bakers' yeast) is widely used in industry for biomass or heterologous protein production. *Saccharomyces cerevisiae* belongs to a group of facultative anaerobic yeasts. These microorganisms will ferment hexose sugars, like glucose and fructose, under both aerobic and anaerobic growth conditions. *S. cerevisiae* displays the Crabtree effect where, even when oxygen may be present, NADH generated during glycolysis is mainly oxidized by fermentation rather than by respiration (Walker, 1998). The Crabtree effect probably results from a multiplicity of factors, including the mode of sugar transport and the regulation of enzyme activities involved in respiration and alcoholic fermentation. In aerobic batch cultures of this yeast typically about 70% of the available glucose is fermented to ethanol and CO<sub>2</sub>, 20% is incorporated into biomass, 8% is used in glycerol production and only 2% will yield CO<sub>2</sub> and H<sub>2</sub>O via the oxidative phosphorylation inside the mitochondria (Hohmann and Mager, 1997). Accordingly glycolytic flux is high and O<sub>2</sub> consumption is low (Ruis and Schüller, 1995; Mager and de Kruijff 1995).

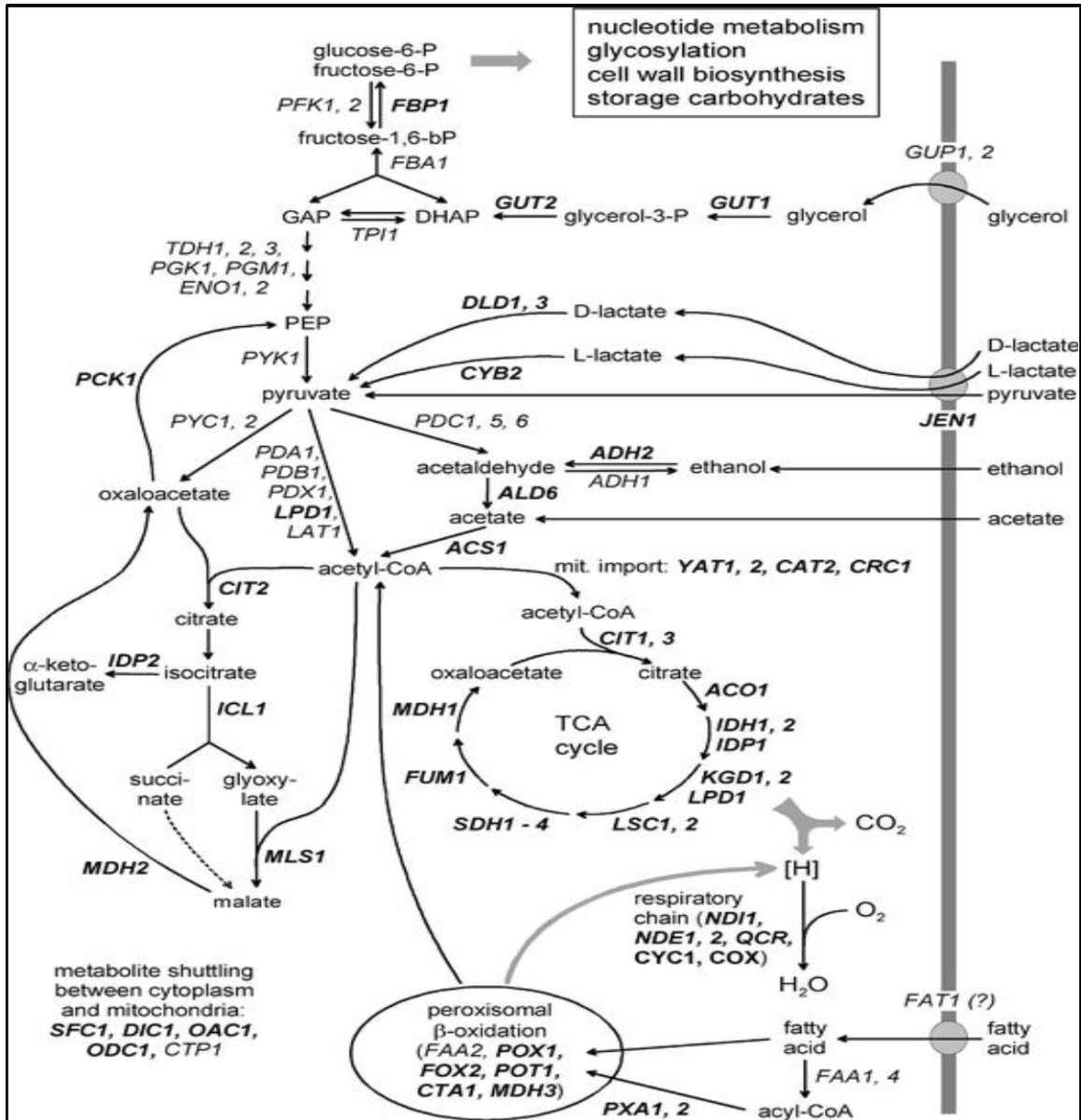
*Saccharomyces* sp. will utilize a wide variety of compounds as carbon and energy sources. Glucose and fructose enter immediately into the glycolytic pathway where ATP is obtained from substrate phosphorylation and sugars are converted into pyruvate and then ethanol and CO<sub>2</sub>. As stated above, although only a small fraction of the fermentable carbon source is initially completely metabolized and much less ATP is produced than during respiratory growth, yeasts prefer fermentative growth. This may seem wasteful; however, since the ethanol as well as the acetate produced during fermentation will be metabolized

in the post-diauxic growth phase, as soon as the fermentable sugar is exhausted, nearly all of the available carbon source will be used eventually. In addition, and perhaps more importantly, the production of high ethanol concentrations inhibits the growth of most other microorganisms allowing *S. cerevisiae* to eventually dominate in spontaneous fermentation. This success in fermentative growth and utilization of the produced ethanol is due to the alcohol dehydrogenase (ADH) isozymes available in the yeast.

Alcohol dehydrogenase isozymes of *S. cerevisiae* are involved in the fermentative growth of the yeast and ethanol assimilation. Adh1p and Adh2p isozymes (major isozymes) function in ethanol production during fermentative growth and ethanol assimilation respectively. There are however, other isozymes which have been identified to function as Adh1p, namely Adh3p or as Adh2p, namely Adh5p. The function of Adh1p during fermentative glucose assimilation is to reduce acetaldehyde into ethanol and this reaction is the final step in the production of ethanol during fermentative growth. The initial reaction during ethanol assimilation is catalyzed by Adh2p, encoded by a glucose repressible *ADH2*. Nonfermentable carbon sources such as ethanol and acetate are metabolized via respiration in the TCA cycle with ATP being produced by oxidative phosphorylation (Finley and Chau, 1991). Adh2p oxidises ethanol into acetaldehyde which then is converted into acetate. This acetate can either be used in the TCA cycle or enter the gluconeogenic cycle.

Yeast batch cultures grown on glucose show several well-defined growth phases due to multiple levels of metabolic regulation by the available carbon source. In the first phase characterized by rapid growth, glucose is fermented with concomitant repression of genes required for respiratory growth. When glucose is exhausted the culture enters a short adaptive lag-phase known as diauxic shift. During this phase the glucose-repressed genes become derepressed and the culture adopts its metabolism for the subsequent utilization of ethanol and other by-products of fermentation (Maeda *et al.*, 1994). The promoters of these glucose

repressible genes e.g. *ADH2* are widely employed in the expression of heterologous proteins.



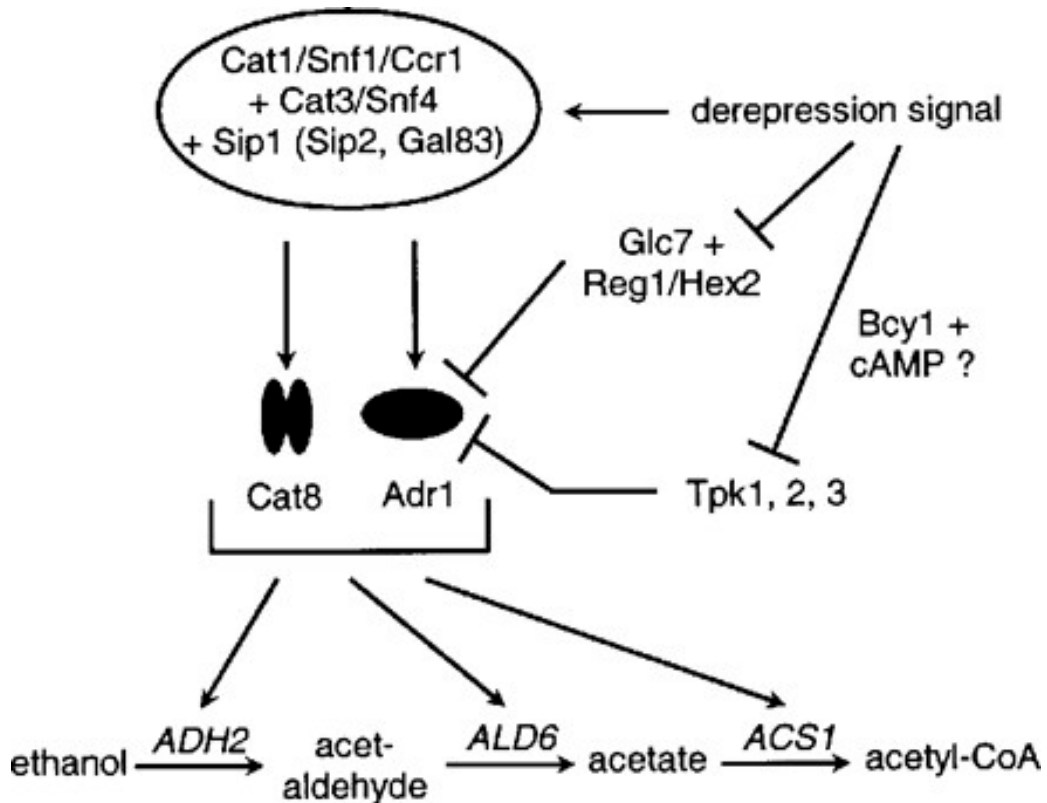
**FIGURE 1.** Genetics of oxidative and gluconeogenic metabolism in the yeast *S. cerevisiae*. Structural genes known to be transcriptionally regulated when glucose is used as sole carbon source are depicted in bold (Schüller, 2003).

## 2. ETHANOL METABOLISM

*Saccharomyces cerevisiae* utilizes ethanol that is produced during glucose assimilation as a carbon source when glucose is depleted. Availability of high glucose concentrations causes this production of ethanol under aerobic growth conditions. Fermentation occurs even when oxygen is available due to crab tree effect. The production of ethanol by *S. cerevisiae* during fermentative growth involves a two-step reaction after the ultimate product of glycolysis (pyruvate) has been formed. During fermentative sugar metabolism, pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase and subsequently reduced to ethanol by an alcohol dehydrogenase enzyme. The initial step in fermentation is catalyzed by pyruvate decarboxylase encoded by *PDC1*; the expression of this gene is induced by glucose (Butler and MacConnell, 1988). Pdc1p decarboxylates pyruvate into acetaldehyde, the penultimate product of fermentation. Adh1p catalyzes the second and final step where ethanol is produced; this enzyme reduces acetaldehyde into ethanol (van den Berg *et al.*, 1998) with *ADH1* expression being induced by glucose.

The ethanol that is produced during fermentation by the yeast is utilized after the depletion of glucose. During ethanol assimilation, ethanol is converted to acetyl-CoA. Three enzymes are involved in this pathway; alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS) are required for the production of acetyl-CoA, which is subsequently used for mitochondrial oxidation and cytoplasmic gluconeogenesis. Adh2p, which oxidises ethanol into acetaldehyde, catalyzes the initial step, Ald6 catalyzes the second step by converting acetaldehyde into acetate and the final step is catalyzed by Acs1p, which then converts acetate into acetyl-CoA (Fig. 1). The expression of these three genes is under glucose regulation. In addition, during ethanol assimilation the expression of these three genes is dependent on both Cat8p and Adr1p transcription activators (Walther and Schüller, 2001) (Fig. 2).





**FIGURE 2.** Regulatory pathway leading to transcriptional co-activation of *ADH2*, *ALD6* and *ACS1* by Cat8 and Adr1 (Walther and Schüller, 2001).

*ADH2* has been known as a major isozyme involved in ethanol assimilation, however van den Berg *et al.* (1998) indicated that *ADH2* expression is not derepressed on ethanol growth instead ethanol appears to repress *ADH2* expression and this expression is derepressed by acetate. Du Preez *et al.* (2001) have also observed that a high ethanol concentration represses the expression of this gene.

### 3. ALCOHOL DEHYDROGENASES (ADH) OF *S. CEREVISIAE*

When *S. cerevisiae* is grown on glucose almost all of the sugar is fermentatively used, regardless of the availability of oxygen, and a specific ADH isozyme serves to regenerate the glycolytic  $\text{NAD}^+$  by the reduction of acetaldehyde to ethanol.

After depletion of the fermentable sugar, rapid oxidation of the accumulated ethanol occurs, again by the action of a specific ADH isozyme. The alcohol dehydrogenase isozymes of *S. cerevisiae* are responsible for the interconversion of acetaldehyde and ethanol. This reaction is the final step during alcohol fermentation in yeast and the initial step in the metabolism of ethanol in a wide variety of organisms (Walker, 1998).

When grown on fermentable carbon sources such as glucose, yeast expresses an isozyme (Adh1p) which preferentially catalyses the conversion of the penultimate intermediate of the fermentative pathway, acetaldehyde to ethanol. *ADH1* was first isolated and characterized by Racker (1955) and studied by Lutstorf and Megnet (1968). Adh1p (cytoplasmic isozyme), Adh3p (mitochondrial isozyme) and Adh4p (cytoplasmic isozyme) convert acetaldehyde into ethanol. *ADH5* encodes for Adh5p, which is involved in the oxidation of ethanol to acetaldehyde like Adh2p. Sfa1p is a long chain alcohol dehydrogenase and it oxidizes ethanol and long chain alcohols. Adh6p and Adh7p are the only alcohol dehydrogenases not involved in interconversion between acetaldehyde and ethanol.

### **3.1. ALCOHOL DEHYDROGENASE I**

The Adh1p isozyme is the classical fermentative enzyme (Racker, 1953), which serves to generate the glycol  $\text{NAD}^+$  by the reduction of acetaldehyde to ethanol. Adh1p is very similar to Adh2p (approximately 95% identity on nucleotide level). The expression of *ADH1* was initially thought to be constitutive, but later it was found to be induced five to 10-fold by glucose or other fermentative carbon source and has also been shown to be repressed by growth on non-fermentative carbon sources (Denis *et al.*, 1983). This change in expression is correlated with an increase in *ADH1* mRNA level. The change in expression may be due to the negative role of Snf1p in the expression of this gene when glucose is depleted

(Young *et al.*, 2003). This was confirmed by the presence of higher levels of *ADH1* mRNA in the *snf1* mutant than in the wild type.

The molecular basis of *ADH1* expression and its regulation is not precisely known. High-level expression depends on a conserved sequence, designated UAS<sub>RPG</sub>, found at various glycolytic genes and ribosomal protein genes. Deletion of this element reduces *ADH1* expression 20-fold without changing its regulatory pattern (Tornow and Santangelo, 1990). The UAS<sub>RPG</sub> box at position -664 binds the general transcription activator Rap1; this ternary complex is crucial for *ADH1* transcription activation (Tornow and Santangelo, 1990; Tornow *et al.*, 1993).

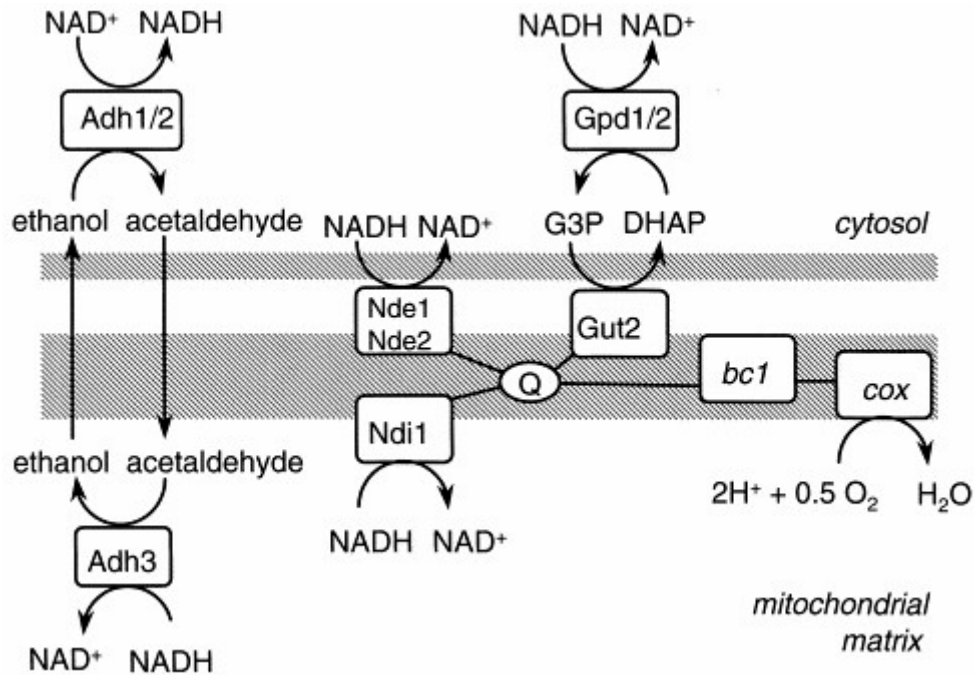
Adh1p is the second enzyme able to detoxify cellular formaldehyde, apparently via reduction to methanol and its action is weaker than that of Sfa1p. Due to its ability in mediating high expression levels, the *ADH1* promoter has been used widely in artificial overproduction of proteins in *S. cerevisiae* (Ammerer, 1983).

### 3.2. ALCOHOL DEHYDROGENASE III

*ADH3* is also known as the mitochondrial alcohol dehydrogenase, due to its activity being associated with this organelle. The Adh3p protein contains an N-terminal extension of twenty-eight residues that is required for its localization to the mitochondrial matrix, and this extension is cleaved away during the translocation process. Adh3p is among the isozymes that belong to a larger group of alcohol dehydrogenase that all contain coordinately bound zinc near its catalytic centers (Ciriacy, 1997) and it is involved in the reduction of acetaldehyde to ethanol (Fig. 3).

*ADH3* is induced by ethanol and repressed by glucose. *ADH3* is involved in the ethanol-acetaldehyde shuttle, consisting of mitochondrial and cytoplasmic isozymes of alcohol dehydrogenase. Since ethanol and acetaldehyde can diffuse

freely across biological membranes, the net results of the ethanol-acetaldehyde shuttle would be the exchange of NADH and H<sup>+</sup> for NAD<sup>+</sup> (Fig.3).



**FIGURE 3.** A scheme representing the respiratory chain of *S. cerevisiae*. Adh, alcohol dehydrogenase; *bc1*, *bc1* complex; cytochrome c oxidase; Gpd, soluble glycerol-3-phosphate dehydrogenase; Gut2, membrane-bound glycerol-3-phosphate dehydrogenase; Nde, external NADH dehydrogenase; Ndi1, internal NADH dehydrogenase; Q:ubiquinone. Note that the ethanol-acetaldehyde shuttle is reversible in principle (Bakker *et al.*, 2000).

This shuttle restores the mitochondrial redox balance under anaerobic conditions, where the NADH + H<sup>+</sup> must be transported to the cytosol, where it can be oxidized by the formation of glycerol or be oxidized by Nde1p and Nde2p (Bakker *et al.*, 2000). It can also compensate for Ndi1p by reoxidising the mitochondrial NADH + H<sup>+</sup>.

### 3.3. ALCOHOL DEHYDROGENASE IV

The *ADH4* gene, encoding ADH activity, is not homologous to the previously mentioned alcohol dehydrogenase genes. It is unrelated to *ADH1*, *ADH2*, or *ADH3* and its predicted amino acid sequence is most similar to the *ADH2* gene of the fermentative bacterium *Zymomonas mobilis*. It is activated by zinc and not by ferrous ions, like structurally similar alcohol dehydrogenase from *Zymomonas mobilis* (Drweke and Ciriacy, 1988). Adh4p reduces acetaldehyde to ethanol and is transcriptionally repressed under glucose and ethanol growth conditions (van den Berg *et al.*, 1998). *ADH4* expression is activated by Zap1p (zinc-responsive transcriptional activator) that regulates the genes involved in zinc uptake (Yuan, 2000). Transcription of *ADH4* is induced in low-zinc media and this transcriptional induction requires Zap1p. This induction suggests that Adh4p functions under these conditions (Yuan, 2000). The main purpose for the *ADH4* induction in low-zinc level is to compensate for a decrease zinc-dependent alcohol dehydrogenase activity; because the genes encoding zinc-dependent alcohol dehydrogenases (*ADH1*, *ADH2*, *ADH3* and *ADH5*) are repressed under zinc limiting conditions (Lyons *et al.*, 2000).

### 3.4. ALCOHOL DEHYDROGENASE V

Adh5p is a zinc dependent alcohol dehydrogenase similar to the other alcohol dehydrogenases that have been mentioned. It has a high homology on nucleotide level with *ADH1*, *ADH2* and *ADH3* as well as the *Zymomonas mobilis* *ADH2* gene (Ciriacy, 1997). Adh5p catalyzes the reaction where alcohol is converted to aldehyde or ketone e.g. where ethanol is oxidized to acetaldehyde. The transcription of this gene is constant in both glucose and ethanol growth conditions (van den Berg *et al.*, 1998).

### 3.5. GLUTATHIONE-DEPENDENT FORMALDEHYDE DEHYDROGENASE (*SFA1*)

A 3.7 kb DNA fragment of yeast chromosome IV has been sequenced and contains the *SFA1* gene which, when present on a multi-copy plasmid in *S. cerevisiae*, confers hyper-resistance to formaldehyde (FA). It belongs to the alcohol dehydrogenase class III (glutathione-dependent formaldehyde dehydrogenase) (Fernandez *et al.*, 1995). This regulated gene codes for a long chain alcohol dehydrogenase (glutathione-dependent formaldehyde dehydrogenase) of *S. cerevisiae*. Sfa1p oxidizes long chain alcohols and can oxidize formaldehyde in the presence of glutathione. Wehner *et al.* (1993) have shown that Sfa1p is non-essential, suggesting that other enzymes of general metabolism are sufficient to survive exposure to FA  $<10^{-3}$  M in this organism.

The predicted protein shows strong homologies to several mammalian alcohol dehydrogenases and contains a sequence characteristic of binding site for NAD<sup>+</sup>. Interestingly the protein has higher structural homology to several ADHs of man and rodents than to other known ADHs of *S. cerevisiae* (for example homology to *ADHI* is lower than 35% (Jörnvall, 1977)). *SFA1* requires (GSH) glutathione and NAD<sup>+</sup> for the oxidation of FA (Wehner *et al.*, 1993), while the oxidation of long-chain alcohols is independent of GSH and the enzyme activity improves with chain length. FA is not a substrate as such, but after spontaneous reaction with GSH to produce S-hydroxymethylglutathione (Mason and Sanders, 1986) yields the best substrate for the *SFA1*-encoded enzyme. Since S-hydroxymethylglutathione is the substrate of the FA-degrading enzyme, the enzyme-catalyzed reaction is the oxidation of a terminal alcoholic hydroxyl group residing on a tripeptide. This might be considered an analogous configuration of a long-chain alcohol. *SFA1* is not only inducible by FA but also by ethanol and methylmethanesulphonate (MMS), two structurally chemicals and unlikely substrates (Wehner *et al.*, 1993). Sfa1p is active towards ethanol only at high substrate concentrations (Fernandez *et al.*, 1995). *SFA1* was successfully employed in constructing a formaldehyde resistance expression cassette. When

this cassette is introduced into yeast strains, they can resist up to 6 mM-formaldehyde (van den Berg and Steensma, 1997)

### 3.6. ALCOHOL DEHYDROGENASE VI

The YMR318C gene was recently characterized by Larroy *et al.* (2002) and due to its substrate specificity and sequence characteristics the gene was called *ADH6*. *ADH6* is a member of the medium-chain dehydrogenases/reductases (MDRs). MDRs constitute a wide protein family with many different activities including the cinnamyl alcohol dehydrogenase to which *ADH6* belongs. *ADH6* encodes for an NADP(H)-dependent alcohol dehydrogenase (Larroy *et al.*, 2002) and it has been classified as a member of the cinnamyl alcohol dehydrogenase family. Adh6p is active towards both aromatic and aliphatic aldehyde and both linear and branched-chain primary alcohols as substrates.

Adh6p is strongly induced in yeast when galactose is used as a sole carbon source as compared to when glucose is the carbon source (Larroy *et al.*, 2003). The expression of this gene seems to be repressed by glycerol (Larroy *et al.*, 2003). The major function of Adh6p is the reduction of aldehyde rather than the oxidation of alcohols (Larroy *et al.*, 2002). This enzyme is capable of significantly eliminating veratraldehyde in *S. cerevisiae*, which is toxic to the cells. Reduction of this aldehyde to the corresponding alcohol is part of the lignin biodegradation process (Reiser *et al.*, 1994). Since veratraldehyde is a compound in the ligninolysis pathway, Adh6p may give the yeast the opportunity to live in ligninolytic environments where products derived from lignin biodegradation may be available. The other potential function of Adh6p may be in the biosynthesis of fusel alcohols during amino acid assimilation (Larroy *et al.*, 2002). Fusel alcohols confer major organoleptic properties to alcohol beverages during fermentation. The best substrates for Adh6p are pentanal and veratraldehyde, ethanol is a very poor substrate of this enzyme (Larroy *et al.*, 2003). Unlike alcohol

dehydrogenases previously mentioned in this literature, *ADH6* is not involved in the interconversion of ethanol and acetaldehyde.

### 3.7. ALCOHOL DEHYDROGENASE VII

YCR105W gene was also recently characterized by Larroy *et al.* (2003) and it also encodes for a NADP (H) dependent alcohol dehydrogenase. This gene, designated *ADH7*, is also a member of the MDRs superfamily. Adh7p is 64% identical to Adh6p and its substrate specificity is similar to that of Adh6p. Adh7p has the highest catalytic efficiencies for the reduction reaction rather than for the oxidative reactions, suggesting that the enzyme would act as an aldehyde reductase rather than an alcohol dehydrogenase (Larroy *et al.*, 2003). The close structural and functional similarities between Adh6p and Adh7p suggest common physiological roles for both enzymes. Adh6p and Adh7p are the only two members of the cinnamyl alcohol dehydrogenase family in yeast.

## 4. ALCOHOL DEHYDROGENASE II AND ITS REGULATION

The glucose repressible alcohol dehydrogenase (*ADH2*) from *S. cerevisiae* functions under non-fermentable growth conditions in the utilization of ethanol as carbon source. Yeast grown in media containing a fermentable carbon source such as glucose, repress the expression of *ADH2* and yeast grown on non-fermentable carbon source such as ethanol, derepress the level of Adh2p activity several hundred fold (Lutstorf and Megnet, 1968). The function of Adh2p in the cell is to oxidize ethanol formed during fermentation to acetaldehyde, which can then be metabolized via the tricarboxylic acid cycle in the mitochondria and also serves as an intermediate in gluconeogenesis (Young and Pilgrim, 1985). *ADH2* is not induced by ethanol as sometimes reported (Ciriacy, 1997). Adh2p has gained a specific attraction as its formation is under the control of carbon catabolite repression, which is considered as a central regulatory system in the control of many enzymes involved in carbon metabolism. Genetic analysis of

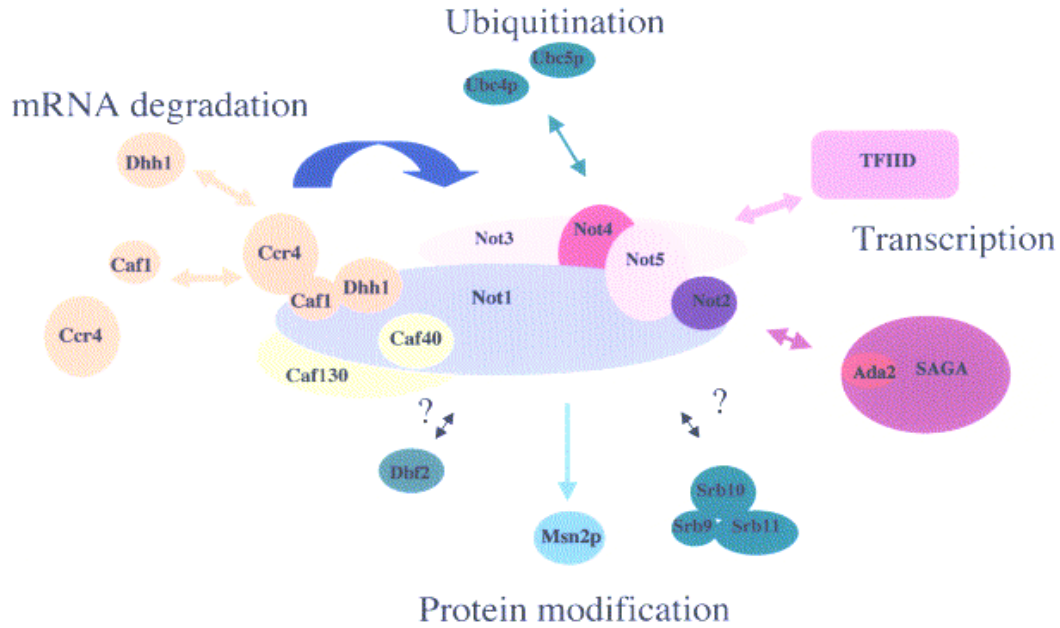


*ADH2* regulation has identified several genes that encode trans-acting factors, which are required for full derepression of *ADH2* (Ciriacy, 1975; Ciriacy, 1977; Denis, 1984; Taguchi and Young, 1987), some of these such as *CCR1* (*SNF1*) (Ciriacy, 1977), *CCR2*, *CCR3* and *CCR4* also affect other glucose repressible genes.

Both positive regulatory genes, *ADR1*, *CCR1-3* (Ciriacy, 1975), *CCR4* (Denis, 1984), *CAT8* (Walther and Schüller, 2001) and *ADR6* (Taguchi and Young, 1987) and the negative regulatory genes *ADR4* (Ciriacy, 1975), *CRE1* and *CRE2* (Denis, 1984) are involved in *ADH2* regulation. Each of these will be discussed extensively in the following sections.

#### **4.1. CCR4-NOT**

The CCR4-NOT complex is one of several large groups of proteins involved in transcription (Liu *et al.*, 1998). This complex affects the expression of many genes including *ADH2*, which are required for the assimilation of non-fermentable carbon sources (Denis, 1984; Denis and Malvar, 1990). It consists of at least 2 complexes, 1.9 and 1.0 MDa in sizes that are distinct from other large, transcriptionally important groups of proteins such as SNF/SWI complex. CCR4-NOT complexes contains Ccr4p, Caf1p (Pop2p), the five NOT proteins and two other proteins. Not1p appears to be the core component of the complex. Caf1p binds to the central region of Not1p and links Ccr4p to the rest of the Not proteins. The C terminus of Not1p in turn contacts Not2p, Not4p and Not5p (Liu *et al.*, 1998), whereas Caf1p is required for Ccr4p to associate with the 1.0 MDa complex (Liu *et al.*, 1998; Bai *et al.*, 1999), since Ccr4p cannot associate with the 1.9 MDa complex in the absence of Caf1p (Fig. 4).



**FIGURE 4.** Schematic representation of the Ccr-Not complex and the interaction with the Srb proteins. The Ccr-Not complex interacts with many different proteins such as proteins, as Dhh1p, TFIIID, and the Ubc4 and Ubc5 E2 enzymes, contributing to mRNA degradation, transcription initiation and ubiquitination respectively (Collart, 2003). In the case of *ADH2* expression, Ccr-Not complex may be involved in transcription initiation.

Two additional factors (Caf4p and Caf16p), which physically interact with the CCR4-NOT complex, were identified by Liu *et al.* (2001). Caf4p interacted in the two-hybrid assay with Ccr4p and Not1p whereas Caf16p interacted only with Ccr4p.

These two factors (Caf4p and Caf16p) were found to interact with Srb9p, Srb10p and Srb11p, components of the RNA polymerase II holoenzyme (Song *et al.*, 1996) during *ADH2* derepression (Liu *et al.*, 2001). Defects in the Srb9, 10 and 11 proteins affect *ADH2* expression in a manner similar to that observed for defects in Ccr4p complex suggesting that the physical interactions between these protein groups represents shared regulatory interactions.

Three possible models may explain the interactions between the CCR4-NOT complex and Srb9-11 proteins: In the first model the SRB proteins may stabilize, recruit or influence CCR4-NOT function. In the second model the CCR4-NOT proteins may regulate the function of Srb9-11 proteins and in the third model the physical connections between the CCR4-NOT complex and Srb9-11 derive from their proximity at the promoter in shared regulatory events.

Ccr4p acts at a post chromatin remodeling step in affecting *ADH2* derepression (Verdone *et al.*, 1997). Evidence has been found that implicate the CCR4-NOT proteins in playing an important role in the control of transcriptional initiation (Denis and Malvar, 1990). In addition, the CCR4-NOT proteins exhibit multiple contacts to proteins playing important roles in controlling initiation (Liu *et al.*, 2001). Ccr4p was found not to affect the degradation rate of *ADH2* mRNA or elongation through the *ADH2* gene, indicating that the effects of Ccr4 on *ADH2* expression must be at the level of initiation of transcription, in addition, the CCR4-NOT proteins exhibit multiple contacts to proteins such as TFIID, Ada2p and Srb9-11p playing important roles in controlling initiation (Benson *et al.*, 1998; Liu *et al.*, 2001). Denis *et al.* (2001) provided genetic evidence that the CCR4-NOT complex is involved in regulating transcriptional elongation. Defects in transcriptional elongation factors such as TFIIS in yeast elicit 6-azauracil (6AU) and mycophenolic sensitive phenotypes (Archambault *et al.*, 1992). They observed that a deletion of *CCR4*, or any other component of the CCR4-NOT complex, gives rise to a 6AU sensitive phenotype and that *ccr4* deletion or *NOT1* overexpression suppresses a *spt5-242* defect that has been shown to be suppressed by the slowing of elongation. CCR4-NOT1 complex could aid in setting up processive polymerases at the promoter and thereby generate more active or increased numbers of elongating polymerases.

Ccr4p activity is blocked by *CRE1* and *CRE2* genes, which are allelic to Spt10p and Spt6p respectively. *SPT10* and *SPT6* are two genes, which are believed to

be involved in chromatin maintenance. Mutations in *SPT10* and *SPT6* allow for *ADH2* transcription following growth on glucose (Denis, 1984). *SPT10* and *SPT6* genes have been identified as playing important transcriptional roles in several systems. The mechanism in which Spt10p and Spt6p affects Ccr4p activity was examined by Denis (1984) and it was found that Spt10p and Spt6p did not control *CCR4* mRNA or protein expression nor did Spt10p and Spt6p co-immunoprecipitate with Ccr4p. This suggested that Spt10p and Spt6p negatively regulates the transcription of *ADH2* by acting through a factor that requires Ccr4p function, but do not regulate *CCR4* expression, control its activity, physically interact with it or affect its binding to other factors. *CCR4* and its negative effectors Spt10p and Spt6p do not require the upstream activation sequences for their function and the derepression activity is independent of *ADR1*.

#### **4.2. *ADR6***

*ADR6* was initially identified in a screen of mutants able to decrease Ty-activated *ADH2* expression and it was shown to be required for *ADH2* expression when glycerol was used as a derepressing carbon source (Taguchi and Young, 1987). *ADR6*, encoding a polypeptide of 1314 amino acids (O'Hara *et al.*, 1988), is a transcription factor whose site of action is unknown. This factor may mediate *ADH2* expression through binding to a sequence downstream of the *ADH2* upstream activating sequence (UAS). *ADR6* was found to be allelic to *SWI1* by Peterson and Herskowitz (1992) a component of the SWI/SNF chromatin remodeling complex required for the transcription of a subset of genes in *S. cerevisiae* (Cairns *et al.*, 1994). This gene was also found to be allelic to *GAM3*, a gene required for *STAI* and *ADH2* transcription as well and for initiation of meiosis (Yoshimoto *et al.* 1992).

The *ADH2* sequence expression requirements for *ADR6* and *ADR1* action appear to differ, suggesting independent modes of action for these two elements. *ADR1* gene product function in the regulation of *ADH2* transcription initiation and

the *ADR6* gene product may act at some subsequent step but prior to the translation of *ADH2* mRNA. It was demonstrated that Swi/Snf complexes could cause ATP-dependent disruption of nucleosome structure and could increase the binding of transcription factors to their sites on nucleosomal templates (Kingston and Narlikar, 1999), suggesting the role of *ADR6/SWI1* in *ADH2* derepression at this level. However, observations obtained by Di Mauro *et al.* (2000) proved that *ADR6/SWI1* is not required for nucleosome modification and transcription during *ADH2* derepression, leaving the role of *ADR6/SWI1* in *ADH2* derepression to the possible roles suggested Taguchi and Young (1987) that *ADR6/SWI1* may be involved in facilitation of *ADH2* RNA transcription processing or maturation of *ADH2* mRNA or stabilization of *ADH2* mRNA.

### **4.3. *ADR7, 8 AND 9***

*ADR7, ADR8* and *ADR9* were identified by Karnitz *et al.* (1992) and they affect *ADH2* expression under both repressing and derepressing growth conditions. Strains containing mutations in *ADR7, ADR8* and *ADR9*, unlike wild type strains, allowed *ADH2* expression in the absence of *ADR1*. These regulatory genes do not function through any known cis-acting element and do not affect *ADH2* mRNA or protein stability. Possibly *ADR7, ADR8* and *ADR9* may play roles in analogous glucose mediated repressor pathway for *ADH2*.

#### 4.4. *ADR1*

The Adr1p regulatory protein is a transcriptional activator of *ADH2* and is required for a dramatic 500-fold increase in *ADH2* activity. Genetic analysis of *ADH2* regulation revealed that derepression of *ADH2* activity and mRNA accumulation requires the product of an unlinked regulatory locus encoded by *ADR1*.

*ADR1* was identified by Ciriacy (1975) to be a possible *ADH2* positive regulatory gene. Some recessive mutations of *ADR1* prevent *ADH2* derepression (Ciriacy, 1975) and other semi-dominant mutations at *ADR1* (*adr1-5<sup>c</sup>*) allow partially constitutive synthesis of *ADH2* (Ciriacy, 1979). Nucleotide sequence analysis has shown that *ADR1* encode for a protein of 1323 amino acids, of which the amino terminal 302 amino acids are sufficient to stimulate *ADH2* transcription (Hartshorne *et al.*, 1986). Adr1p is a large transcriptional factor containing a complex DNA binding domain consisting of C<sub>2</sub>H<sub>2</sub> zinc fingers which are essential for DNA binding (Blumberg *et al.*, 1987) and a proximal accessory region (PAR). A nuclear localization signal is located near the amino terminus and four transcription activating domains (TADs) have been identified by deletions and gene fusions to LexA (Cook *et al.*, 1994) and *GAL4* (Young *et al.*, 1998). In vitro binding studies as well as in vivo co-immunoprecipitation suggests that these TADs interact with TFIIB, TFIID, Ada2p (transcription co-activator) and Gcn5p acetyltransferase (Chiang *et al.*, 1996; Komarnitsky *et al.*, 1998) showing the recruitment of transcription initiation complex (pol II complex) and chromatin remodeling by Adr1p to the *ADH2* promoter. Denis *et al.* (1981) proved that Adr1p does not act on the Adh2p protein but is involved in the expression of *ADH2*. Initial increase in functional *ADH2* mRNA levels after derepression was shown to follow the same kinetics in the strain containing *adr1* and *adr1-5<sup>c</sup>* alleles.

*ADR1* mediates *ADH2* expression through a 22 base pair inverted repeat dyad sequence (Shuster *et al.*, 1986; Yu *et al.*, 1989); an upstream activator sequence

(UAS1) located 230 base pairs from the transcription initiation site (Beier *et al.*, 1985). Activation of *ADH2* expression from UAS1 requires the binding of two Adr1p monomers (Thukral *et al.*, 1991). Each half of the inverted repeat is an independent functional binding site for one monomer of Adr1p. The central region of UAS1 provides non-base specific contacts for Adr1p and may prevent steric interference between the two Adr1p monomers bound in close proximity to one another (Cheng *et al.*, 1994).

The activity of the Adr1p protein is regulated in a carbon source dependent manner in strains, which do not regulate *ADR1* mRNA levels (Blumberg *et al.*, 1988). Glucose regulation of *ADR1* function appears to occur principally at the posttranscriptional level since *ADR1* mRNA do not differ between glucose and ethanol grown cells. Adr1p is inactive as a transcriptional factor in the presence of glucose, although the intact protein is present and DNA binding appears normal (Taylor and Young, 1990). The activity of Adr1p is regulated positively by the Snf1 complex and negatively by cAMP-dependent protein kinase and the Reg1-Glc7 complex.

Under derepressing conditions Adr1p primes the assembly of the pre-initiation complex and at the same time the disruption of the two nucleosomes (-1 & +1) protecting the TATA box and the RNA initiation sites (Verdone *et al.*, 1997). Two distinct structural alterations occur during chromatin remodeling by Adr1p under derepressing conditions (Di Mauro *et al.*, 2000). The first consists of a localized modification induced by the Adr1p DNA binding domain at the level of the nucleosome -1, this cleavage site contains the TATA box. It is located halfway between UAS1 where Adr1p binds to the TATA box. The second type of structural alteration, which is induced when the activation domain is present, is more stable and involves both nucleosome -1 and nucleosome +1 containing the RNA initiation site. This second structural alteration leads to a fully derepressed *ADH2* promoter, which is functionally active. Two functions can thus be attributed to Adr1p:

- 1) Adr1p reconfigures nucleosomes in the immediate vicinity of its binding site allowing the basal promoter elements to assume the most appropriate structure for the subsequent activation.
- 2) Adr1p recruits the transcription machinery through its activation domain allowing mRNA accumulation.

Adr1p contributes to the transcriptional derepression of *ADH2* synergistically with Cat8p (Walther and Schüller, 2001)

## 4.5 *CAT8*

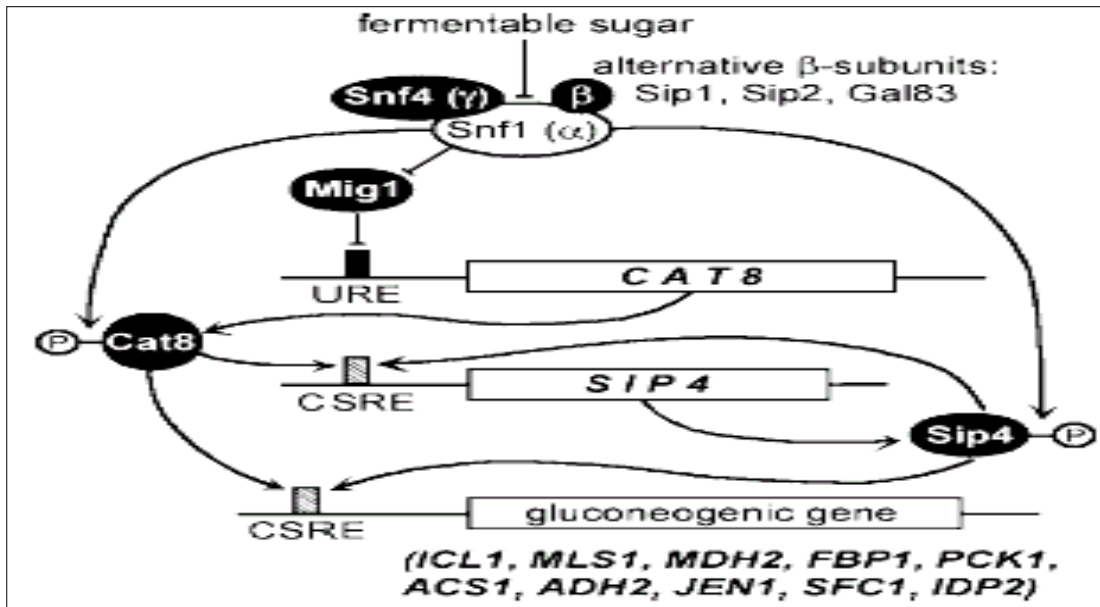
Cat8 is required for the activation of gluconeogenic genes through a carbon source-responsive element (CSRE) present in their promoters (Hedges *et al.*, 1995). The carbon source-responsive element functions as an activating promoter motif of gluconeogenic genes in *S. cerevisiae*. The *CAT8* gene encodes a transcription factor with a binuclear zinc cluster domain at its N terminus and a C terminal transcription activation domain. Expression of *CAT8* as well as transcription activation by Cat8p is affected by carbon source (Rahner *et al.*, 1996).

Before *CAT8* could be associated with the derepression of *ADH2* the UAS2 was identified in *ADH2* and it was found to have the CSRE required for Cat8p dependent derepression. The deletion of either UAS1 or *ADR1* allowed a substantial (but not complete) derepression of *ADH2*, arguing for an additional UAS element and trans-activator. This led to the discovery of the UAS2 by Yu *et al.* (1989). The deletion of *ADH2* sequences 5' to UAS1 identified a GC rich 20 bp sequence referred to as UAS2. Its activity as a single copy was glucose regulated, Adr1p independent and comparable to that of a single copy of UAS1. The two UAS elements acted synergistically to activate expression 20-fold as compared with the activation measured with either element alone (Yu *et al.*, 1989). These results suggested that neither *ADR1* nor the putative UAS2 binding factor alone can effectively activate *ADH2* transcription.



UAS2 was suggested to be a binding site for a protein that interacts with Adr1p bound to UAS1 (Donoviel *et al.*, 1995) and DNA binding data suggested that Adr1p might bind to this site. However a functional CSRE, which specifically interacts with Cat8p, was identified within UAS2 (Walther and Schüller, 2001). Gel retardation experiments showed that Cat8p and the related zinc cluster protein Sip4p differ with respect to binding affinity to CSRE sequence elements, providing the evidence that Cat8p is the dominating regulator acting via UAS2. The Cat8p activator contributes to the transcriptional derepression of *ADH2* synergistically with Adr1p and together they mediate more than 98% of total *ADH2* derepression.

Cat8p activity is in turn regulated at the expression, as well as the transcription activating levels. Mig1p, a DNA binding zinc finger protein, represses the expression of *CAT8*. Mig1p recruits the Ssn6/Cyc8-Tup1 complex, which repress transcription (Treitel and Carlson, 1995). The activator function of Cat8p is inhibited by glucose and is dependent on Snf1p. A functional Snf1p complex is required for Cat8p function (Randez-Gil *et al.*, 1997). The Snf1p complex regulates the activating function of Cat8p and is required for the phosphorylation of Cat8p in derepressed state (Fig. 5).



**FIGURE 5.** Derepression of gluconeogenic structural genes by carbon source-responsive element (CSRE)-binding factors, Cat8p and Sip4p. A dual function of the Snf1 protein kinase complex is proposed: deactivation of the Mig1p repressor allows biosynthesis of Cat8p and post-translational modification converts Cat8p into a transcriptional activator which subsequently stimulates expression of *SIP4*. Cat8p and Sip4p both contribute to derepression of gluconeogenic structural genes, with Cat8p being the more important activator. The activator of *CAT8* transcription in the absence of Mig1p is unknown. It is also unclear whether Cat8p and Sip4p are directly phosphorylated by Snf1p. URE Upstream regulatory element (Schüller, 2003).

#### 4.6 CYCLIC AMP-DEPENDENT PROTEIN KINASE (CAPK)

The cyclic AMP (cAMP) signal transduction pathway plays an important role in triggering changes in gene expression that is responsible for long-term adaptation to fluctuations in environmental conditions. The penultimate effector of

this pathway, cAMP-dependent protein kinase (cAPK), can influence the level of gene expression either positively or negatively thereby controlling transcription. In *S. cerevisiae*, growth on glucose induces a cAMP signal that initiates a cascade of protein phosphorylation by cAPK (Matsumoto *et al.*, 1982), resulting in increased glycolysis and inactivation of several enzymes involved in gluconeogenesis. cAPK also regulate the expression of *ADH2* by phosphorylating its transcription factor Adr1p. Adr1p is a serine phosphoprotein whose activity appears to be negatively regulated by cAPK-dependent phosphorylation. Genetic evidence suggests that Adr1p is partially inactivated by cAMP-dependent protein kinase (Cherry *et al.*, 1989).

Three different genes *TPK1*, *TPK2* and *TPK3* encode the cAPK catalytic subunits in yeast. The activation of the cAPK phosphorylation system under glucose growth conditions appear to be mediated by a glucose-dependent signal transmitted through the adenyl cyclase effectors *CDC25*, *RAS1* and *RAS2* (Mbonyi *et al.*, 1988; Munder and Küntzel, 1989). Yeast contains only one regulatory cAPK regulatory subunit encoded by the *BCY1* gene (Toda *et al.*, 1987). *ADH2* expression is inhibited in cells containing a deletion of the *BCY1* gene, and this inhibition can be restored by mutations in the *TPK* genes (Cherry *et al.*, 1989; Denis *et al.*, 1992). This suggests that unregulated cAPK activity inhibits an important step required for the activation of *ADH2* expression.

It was originally postulated that the dominant *adr1<sup>C</sup>* mutations causing enhanced *ADH2* transcription under repressed conditions (Ciriacy, 1979), affected the cyclic AMP-dependent protein kinase phosphorylation site at ser-230 of *ADR1*, thereby inhibiting the inactivation of Adr1p (Cherry *et al.*, 1989). However Denis *et al.* (1992) provided evidence which indicates that this protein kinase inhibits *ADR1* function by a mechanism that is independent of ser-230. Dominant mutations in *ADR1* (*adr1<sup>C</sup>*) which allow glucose-insensitive *ADH2* transcription (Denis *et al.*, 1992) has been identified as amino acid substitutions between amino acid 227 and 239 of *ADR1*. It was suggested that cAPK inhibits *ADH2* expression by

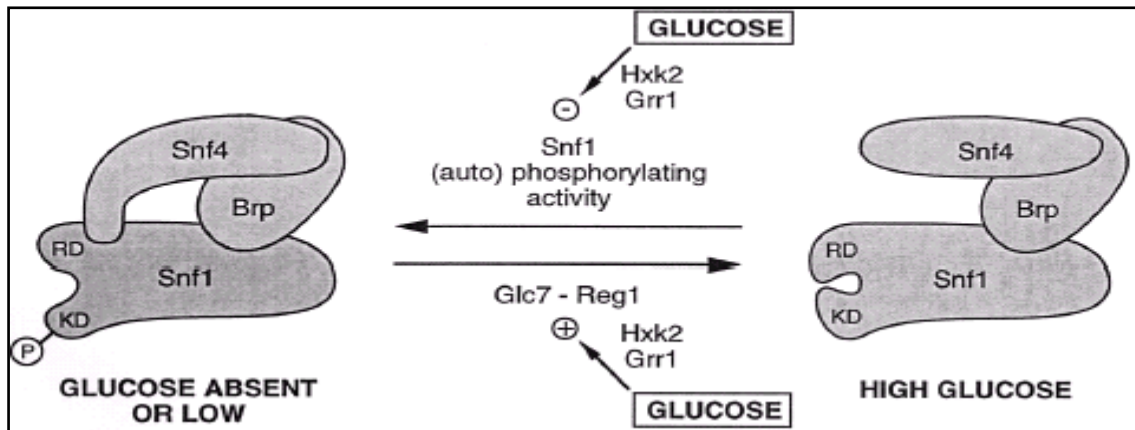
phosphorylating either Adr1p or another protein required for Adr1p activity. The phosphorylation of Adr1p does not affect the binding of this transcription factor to the UAS1 (Taylor and Young, 1990) suggesting that during glucose repression, cAMP-dependent phosphorylation of Adr1p may inhibit its ability to interact with general transcription factors or RNA polymerase II. Dombek and Young (1997) suggested that cAPK may also inhibit *ADH2* expression by decreasing the expression of *ADR1* and indicated that cAPK acts through UAS1 in decreasing the abundance of Adr1. These observations suggests that the negative control of *ADH2* expression by cAPK is exerted through several mechanisms, one being the inhibition of *ADR1* transcription and the other being the direct phosphorylation of Adr1p.

#### **4.7 *SNF1/CCR1/CAT1***

*CCR1* is a cAMP-independent protein kinase involved in *ADH2* regulation. It was found to be allelic to *SNF1* (Denis, 1984) and *CAT1* (Ciriacy, 1977) which encodes for a yeast protein kinase required for the transcription of several glucose-repressed genes including *ADH2* (Denis, 1987). Snf1p protein kinase phosphorylates and controls factors required for *ADH2* regulation and was thought to act independently of *ADR1* (Denis and Audino, 1991). Genetic analysis indicates that *SNF1/CCR1/CAT1* is required for Adr1p activation of *ADH2* expression.

A combination of genetic, two-hybrid and co-immunoprecipitation experiments indicated that Snf1p is complexed with Snf4p and one member of the Sip/Gal83 class proteins (Celenza *et al.*, 1989; Yang *et al.*, 1994). The interactions between Snf1 and Snf4 appear to be carbon source regulated (Jiang and Carlson, 1996). In repressed cells the N-terminal kinase domain of Snf1p appears to interact with its C-terminal regulatory domain, which is thought to inhibit kinase activity. Upon depletion of glucose from growth medium, Snf4p binds to the kinase, displacing

the regulatory domain and thereby freeing the Snf1p kinase domain from auto inhibition. This scenario is indicated in the Fig. 6 (Jiang and Carlson, 1996).



**FIGURE 6.** Model for the regulation of the Snf1 complex by glucose. The bridging protein (Brp) between Snf1p and Snf4p can be Gal83p, Sip1p, Sip2p or some other, as yet, unidentified protein. Glucose affects the interaction between the catalytic domain (KD) and the regulatory domain (RD) of Snf1p, presumably by inhibiting the (auto)phosphorylation of Snf1p and/or activating its dephosphorylation. Glucose may act at the level of the corresponding kinase and phosphatase but may also alter the conformation of Snf1p or even Brp, making Snf1p a worse or better substrate for the corresponding enzyme. Hxk2p and Grr1p are required for transmitting the glucose signal (Jiang and Carlson, 1996).

The Snf1 kinase complex is inactivated by Reg1-Glc7 phosphatase complex. Protein phosphatase type 1 (PP1) plays a key role in regulating a variety of processes in eukaryotic cells (Bollens and Stalmans, 1992) and the gene coding for the *S. cerevisiae* homologue of PP1 is *GLC7*. Glc7p is required for the appropriate regulation of a number of cellular processes, including glycogen biosynthesis, translation, cell cycle progression, chromosome segregation, meiosis and sporulation as well as repression of many glucose-regulated genes

(Stark, 1996). Reg1p, a regulatory subunit that affects glucose repression, growth and glycogen accumulation (Entian and Zimmermann, 1980), confers substrate specificity for Glc7p. Reg1p interacts with the kinase domain of Snf1p, altering protein-protein interactions within the kinase complex (Ludin *et al.*, 1998). Two-hybrid experiments have suggested that Reg1p interacts weakly with the kinase domain of Snf1p in repressed cells and strongly in derepressed cells. Once bound, Glc7p dephosphorylates Snf1p thereby releasing Snf4p from the kinase regulatory domain and returning the complex to an autoinhibited state. The Reg1-Glc7 phosphatase complex is part of the cytoplasmic machinery for resetting Snf1p kinase activity to a basal level implying that the activated form of Snf1p complex rapidly cycles between the nucleus and the cytoplasm (Dombek *et al.*, 1999).

The Snf1 kinase complex relieves *ADH2* derepression by activating Adr1p transcription factor. The regulated Adr1p binding to the *ADH2* promoter requires the Snf1p kinase complex and under repressing conditions Adr1p is non-functional due to the autoinhibition of the Snf1p kinase complex exerted by the Reg1-Glc7 phosphatase complex (Young *et al.*, 2002). Snf1p kinase complex regulates chromatin binding by Adr1p and it was recently found to activate Adr1p-dependent *ADH2* derepression by allowing Adr1p to bind to UAS1 and does not enhance the activation potential of Adr1p (Young *et al.*, 2002). Therefore the role of Snf1p in *ADH2* derepression is to modify chromatin to allow Adr1p to bind to the *ADH2* UAS1. Snf1p also regulates Cat8p expression and its transcription activation function.

#### **4.8 cAMP-INDEPENDENT PROTEIN KINASE (*SCH9*)**

*SCH9* encodes for a cAMP-independent protein kinase and it was initially proposed to be involved in *ADH2* repression. It was postulated that Sch9 protein kinase could functionally substitute for cAPK (Cameron *et al.*, 1988). Denis and Audino (1991) provided evidence that Sch9p is required for *ADH2* derepression

and not *ADH2* repression. Increased expression of *SCH9* had no effect in the expression of *ADH2* and strains carrying a *sch9* disruption displayed a three to ten-fold decrease in Adh2p activity when compared to their isogenic parental strains. Sch9p does not act through *ADR1* to derepress *ADH2* expression (Denis and Audino, 1991) but appears to act in a pathway separate from that and not parallel to that involving cAPK. Since Sch9 does not act through UAS1 it may control factors that act through other activation sequences or through factors that control the general transcriptional machinery.

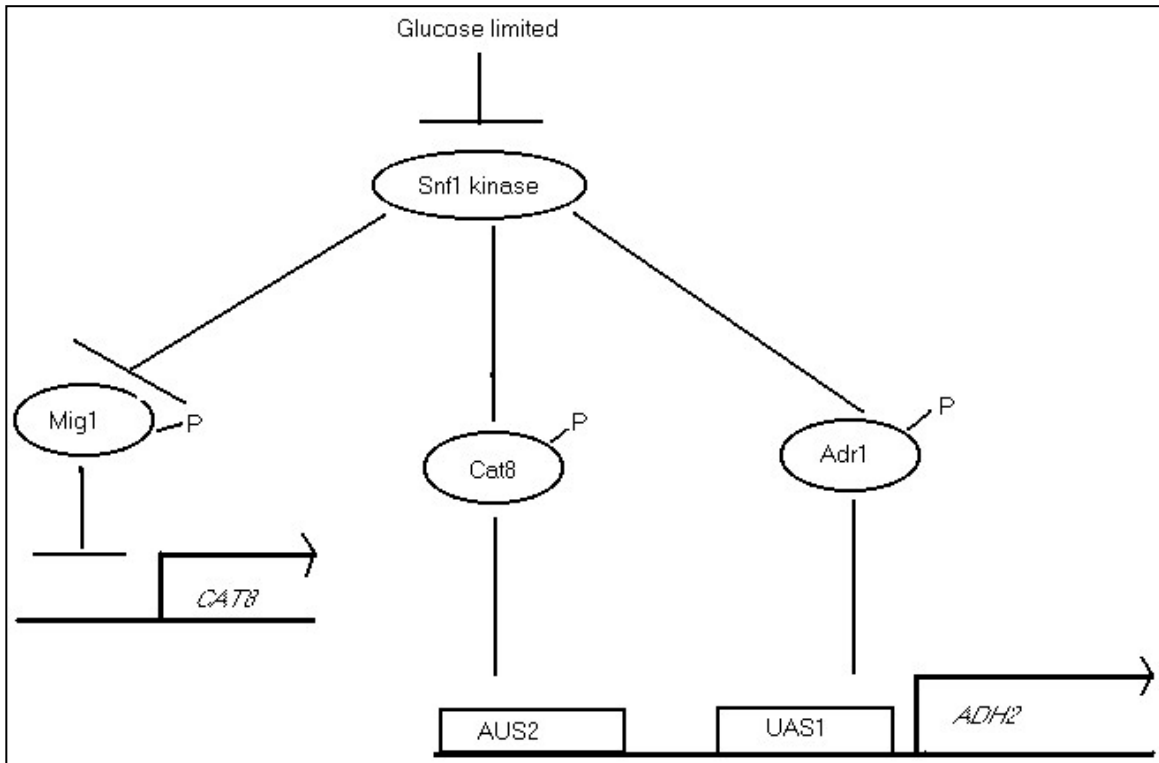
## 5. ALCOHOL DEHYDROGENASE II REGULATION: AN OVERVIEW

In the yeast *S. cerevisiae*, growth, metabolism and gene expression are highly regulated in response to carbon source availability. Glucose is the most abundant monosaccharide in nature and it is also the primary fuel for microorganisms. Yeast cells also prefer to use glucose as a carbon source. A major route by which glucose encourages its own use and stimulates fermentation is by regulating gene expression. When glucose is abundant the expression of a set of genes encoding glycolytic enzymes, glucose transporters, ribosomal proteins and other proteins is induced. At the same time the expression of a large set of genes is repressed (Gancedo, 1998; Vallari *et al.*, 1992). This set includes genes involved in utilization of alternative carbon sources (i.e. *ADH2* in ethanol utilization), gluconeogenesis, the tricarboxylic acid cycle, respiration and other processes (Hohmann and Mager, 1997). When glucose is limiting, an important aspect of the cell's regulatory response is the release of glucose repression and the expression of a large set of genes including *ADH2*. When the concentration of glucose drops below 0.2%, expression of these genes is activated or derepressed. For repressible genes like *ADH2*, this change in the rate of expression can be 200-fold or greater (Celenza *et al.*, 1989; Dombek *et al.* 1993; Griggs and Johnston, 1991). The repression of *ADH2* expression by glucose is not due to the availability/binding of a repressor to prevent transcription but it is due to the unavailability or lack of function of the transcription activators (Cat8p

and Adr1p). Although mutations in a variety of genes such as *CCR4*, *ADR6* or *CRE1* have an effect in the expression of *ADH2*, the corresponding proteins are not likely to be involved directly in the glucose signal (Gancedo, 1998).

The expression of *ADH2* under derepressing conditions is dependent on the two UASs (UAS1 and UAS2) located at the promoter. Two transcription activators, whose activity/expression is under glucose regulation binds to these UASs, therefore two major pathways appear to be involved in the glucose regulated *ADH2* expression. The first one is exerted through Adr1p, which binds to the AUS1 (Shuster *et al.*, 1986; Yu *et al.*, 1989); the second one is exerted through Cat8p, which binds to the UAS2 (Walther and Schüller, 2001). A number of genes have been identified as playing integral roles in glucose repression (Gancedo, 1998). Among these are *REG1*, *GLC7* and *SNF1*, the yeast homologue of the catabolic subunit of AMP-activated protein kinase (Hardie *et al.*, 1998). The Snf1p function is activated when glucose is limiting (Wilson *et al.*, 1996). Snf1 is essential for the regulatory response to glucose starvation in yeast (Celenza and Carlson, 1986) and is required for the transcription of glucose repressed genes when glucose is limiting. Both of these pathways are affected by the Snf1 protein kinase complex. Snf1p and Reg1-Glc7 a protein phosphatase1 and its regulatory subunits are the central components of the two pathways for glucose repression of *ADH2* expression. The pathway exerted through Cat8p is also affected by Mig1p, a transcriptional repressor; this means that *CAT8* is regulated at a level of transcription and protein function. In the first pathway, involving UAS1, Adr1p function is activated in the absence of glucose by Snf1p and the second pathway involves UAS2. Activation through UAS2 involves the activation of Cat8p through phosphorylation. Snf1p activates the expression of *CAT8* by inhibiting Mig1p through phosphorylation during glucose limitation (Treitel *et al.*, 1998). The derepression of *ADH2* is summarized in Fig. 7.





**FIGURE 7.** Model of the regulation of *ADH2* by glucose. When glucose is limiting, the Snf1 kinase activates Cat8 and Adr1, which in turn activate the expression of *ADH2*, and inhibits Mig1 repressor function leading to the expression of Cat8.

## 6. THE USE OF THE *ADH2* PROMOTER

*S. cerevisiae* is the most extensively studied non-mammalian eukaryote; its genetics, biochemistry, and life cycle are well known. Due to this it is an attractive host for the production of heterologous proteins. Expressed proteins can be specifically engineered for cytoplasmic localization or for extracellular export. In addition, yeasts are exceedingly well suited for large-scale fermentation for the production of large quantities of heterologous protein. Either constitutive or inducible promoters can be used for heterologous protein production depending on the type of the protein being produced. In principle, any promoter isolated from a yeast gene can be employed to achieve expression of a foreign coding

sequence in yeast. However, since promoter control is a primary way in which the expression of genes is controlled in yeast, yeast promoters are usually subjected to some form of regulation that will also affect any derivative heterologous constructions (Hadfield *et al.*, 1993).

Inducible expression cassettes are employed for the regulated production of heterologous proteins and the *ADH2* promoter has been used for this purpose due to its regulation through glucose repression. The *ADH2* promoter is both powerful and tightly regulated and is therefore very useful in regulated overexpression of heterologous genes in *S. cerevisiae* (Price *et al.*, 1990). In order to maintain repression of *ADH2*, cells must be grown in excess glucose until induction, which is effected by changing to the fresh medium containing a non-fermentable carbon source e.g. ethanol. Alternatively, *ADH2* can be induced by culturing initially in a lower concentration of glucose which is gradually depleted (Romanos *et al.*, 1992).

Since the *ADH2* promoter is repressed over 100-fold by glucose, it can be used for efficient expression of toxic proteins e.g. insulin-like growth factor (IGF-1). *ADH2* promoter has been successfully used for the production of TBV25H, a malaria transmission blocking vaccine candidate (Noronha *et al.*, 1998). This production was feasible only due to the properties of *ADH2* promoter, as previously available fermentation procedures for the production of this protein have been unsatisfactory mainly because of irreproducibility. A 260 bp region 5' to the initiation site, which contains two UASs that are sufficient for full promoter activity and regulation, has been used in efficient expression vectors.

Hybrid glycolytic *ADH2* promoters have been constructed by using the *ADH2* UAS containing 22bp dyad 320bp upstream of the GAP TATA element, and were able to achieve tightly regulated production of peroxide dismutase (50D)-proinsulin fusion (Romanos *et al.*, 1992).

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## CHAPTER3

### THE CONSTRUCTION OF THE EXPRESSION CASSETTE

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#### 1. ABSTRACT

*ADH2* encodes for the main alcohol dehydrogenase isozyme that catalyses the conversion of ethanol to acetaldehyde during ethanol assimilation. Expression of this gene is known to be under glucose repression and is derepressed by growth on ethanol and other non-fermentable carbon sources. Results obtained by du Preez *et al.* (2001) showed, however, that this gene was repressed by growth on ethanol. These results were obtained using a multicopy expression vector with a promoter-terminator expression cassette derived from the *S. cerevisiae ADH2* gene. The aim of this study is the investigation of ethanol repression of *ADH2* in the genome of *S. cerevisiae*. To achieve this, an expression cassette using *LacZ* as a reporter gene was constructed using the YIp356R shuttle vector. A 1000 bp up and downstream region, flanking the open reading frame of *ADH2*, was fused to the 5'-end and 3'-end of the *LacZ* gene. Attempts to replace the *ADH2* ORF with the expression cassette were unsuccessful therefore the expression cassette was cloned into Ylplac211 and integrated into the *S. cerevisiae* genome.

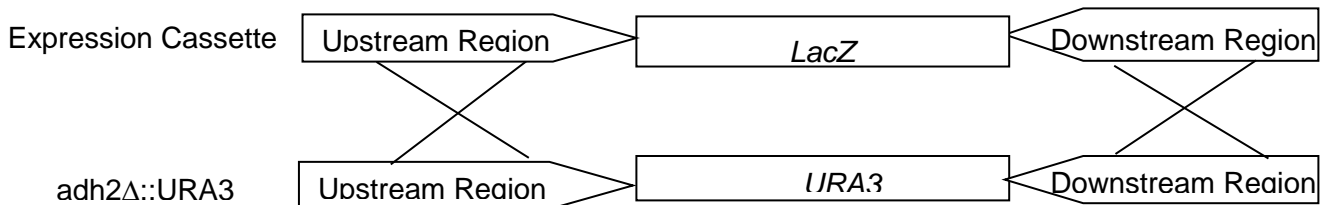
#### 2. INTRODUCTION

The *ADH2* gene encodes an enzyme which has been identified as the main alcohol dehydrogenase isozyme involved in ethanol assimilation (Ciriacy, 1975; Ciriacy, 1979; Lutstorf and Megnet, 1968). A study conducted by du Preez *et al.* (2001) revealed that the expression of *ADH2* is, however, repressed under high ethanol growth conditions. In this study an episomal multicopy plasmid with a promoter-terminator expression cassette derived from the *S. cerevisiae ADH2* gene was used and *ADH2* expression was studied using *XYN2* as a reporter gene.

Thus the results observed could be due to the presence of negative regulatory elements, such as downstream repression sequences in the expression cassette and the possibility of multicopy effects causing transcriptional misregulation.

Therefore it became a necessity that the effect of ethanol on the expression of this gene be studied in the native complete *ADH2* gene, however, because of high sequence similarity between the *ADH2* and *ADH1* genes it is difficult to discriminate between the mRNA sequences of these genes, rendering it virtually impossible to use northern blots to investigate glucose repression of *ADH2* without the interference of *ADH1* mRNA.

The aim of this study was the investigation of the effect of ethanol concentration on the expression of *ADH2* by constructing an expression cassette with *LacZ* as a reporter gene. To accomplish this, an expression cassette was constructed where the *LacZ* gene is flanked by the upstream and downstream regions of *ADH2*. This was followed by an attempt to replace the *ADH2* gene *in vivo* through homologous recombination. Due to the non-selective properties of *LacZ*, the donor cells used contained an *ADH2* deletion, where the coding part of this gene was replaced with *URA3* (*URA3::ADH2*) (Fig. 1).



**FIGURE 1.** Scheme representing homologous recombination between the expression cassette and *adh2Δ::URA3*. Attempts to replace *ADH2* ORF with the cassette were unsuccessful.

However, an attempt to replace the ORF of *ADH2* with the expression cassette was unsuccessful and a second approach was used where *S. cerevisiae* was transformed with an integrative plasmid (YIplac211) containing the expression cassette.

### 3. MATERIALS AND METHODS

#### 3.1. STRAINS USED

*Saccharomyces cerevisiae* W303 (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*); *Escherichia coli* Top 10 (Invitrogen, USA).

#### 3.2. ISOLATION OF *ESCHERICHIA COLI* GENOMIC DNA

Bacterial cells were grown overnight in 5 ml Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) for 12 h. The culture was transferred into a 1.5 ml microcentrifuge tube, centrifuged for 5 min at 13,000 rpm and 500  $\mu$ l Trizol (life technologies) added to the culture, mixed by pipetting up and down and centrifuged for 10 min at 13,000 rpm. The supernatant was transferred into a new microcentrifuge tube and DNA was precipitated by adding 0.5 ml 100% ethanol and incubated for 5 min at room temperature. DNA was spooled with a pipette tip and transferred to another tube, centrifuged briefly, washed twice with 70% ethanol, centrifuged for 5 min at full speed, dried and resuspended in TE containing 50  $\mu$ g RNase ml<sup>-1</sup>.

#### 3.3. ISOLATION OF *S. CEREVISIAE* GENOMIC DNA

Yeast cells were grown overnight in a 5 ml YEPD medium at 30 °C, centrifuged for 1 min at full speed and washed once with 1ml sterile water. The cells were resuspended in 500  $\mu$ l lysis buffer (100 mM Tris pH 8, 50 mM EDTA, 1% SDS), acid washed glass beads were added and then mixed for 2 min by vortex. The liquid phase was transferred to another tube, 275  $\mu$ l of 7 M ammonium acetate (pH 7.0) was added and the tube was incubated at 65 °C for 5 min then placed on ice for 5 min. Chloroform (500  $\mu$ l) was added, mixed by vortex and centrifuged for 2 min. The top phase of the supernatant was transferred to a new tube and DNA was precipitated with 1 ml isopropanol, incubated for 5 min at room temperature and

centrifuged for 5 min. The pellet was washed with 700  $\mu$ l 70% ethanol, centrifuged for 10 min at 14000 rpm at 4 °C, dried and resuspend in 50  $\mu$ l water.

### 3.4. AMPLIFICATION OF THE DOWNSTREAM AND UPSTREAM REGION (FLANKING REGIONS) OF *ADH2*, *LACZ*, UPSTREAM+*LACZ*, DOWNSTREAM+*LACZ* AND THE EXPRESSION CASSETTE

**TABLE 1.** Primers used in this study

PCR Product	Forward Orientation	Reverse Orientation
<i>ADH2</i>	ADH2-1F 5'-CGG ATC AAC TGG CAC CAT CT-3'	ADH2-1R 5'-TTT TCT CCC TCA CTG CAT CCC-3
Upstream Region	ADH2-4F 5'-ACT GCA GTT CCG GAT GAG GTT CCA G-3'	ADH2-2R 5'-TTG AGT TTC TGG TCT AGA CAT TGT G-3'
Downstream Region	ADH2-2F 5'-AGT AGG TAC CAT CTT TAG ATG ACA GTG-3'	ADH2-4R 5'-AGT CGA CTT TTC TCC CTC ACT GCA TCC-3'
<i>LacZ</i>	LACZ-4F 5'-ATC TAG AAC CAT GAT TAC GGA TTC ACT G-3'	LACZ-4R 5'-GGT ACC TTA TTT TTG ACA CCA GAC CAA C-3'
<i>LacZ</i> +Upstream Region	ADH2-4F 5'-ACT GCA GTT CCG GAT GAG GTT CCA G-3'	LACZ-4R 5'-GGT ACC TTA TTT TTG ACA CCA GAC CAA C-3'
Expression construct	ADH2-4F 5'-ACT GCA GTT CCG GAT GAG GTT CCA G-3'	ADH2-1R 5'-TTT TCT CCC TCA CTG CAT CCC-3

The general method followed for amplification is as follows. The amplification was performed in a total volume of 50  $\mu$ l. The following reagents were added: dNTPs (final concentration of 0.2 mM), reverse primer (final concentration of 2 pmol), forward primer (final concentration of 2pmol), genomic/plasmid DNA, depending on the PCR product (final concentration of 500 ng  $\mu$ l<sup>-1</sup>), 10X PCR buffer and sterile water up to a final volume of 50  $\mu$ l. All the reagents were added in a 0.2 ml PCR tube and incubated for 5 min at 94 °C before adding the enzyme mix (Taq polymerase) or Expand Long Template DNA polymerase mix (for PCR products with  $\geq$  3000 bp band sizes) to a final concentration of 0.02 U  $\mu$ l<sup>-1</sup> (Roche).

The polymerase chain reaction was performed under the following cycling programs for 30 cycles:

Amplification of *ADH2*:

Denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec and extension at 68 °C for 2.5 min repeated 30 times followed by a final step of 10 min at 68 °C to complete elongation.

Amplification of the flanking regions of *ADH2*:

Denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 1 min.

*LacZ* amplification:

Denaturation at 94°C for 30 sec, annealing at 57 °C for 30 sec and extension at 68 °C for 2.5 min.

Upstream region+*LacZ*:

Denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 68 °C for 2.5 min.

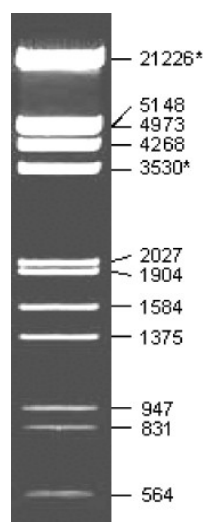
Amplification of the expression cassette:

Denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 68° C for 4 min.

Confirmation of transformation by PCR:

Denaturation at 94 °C for 30sec, annealing at 59 °C for 30 sec and extension at 68 °C for 2.5 min.

Confirmation of the correct PCR product was achieved through restriction digest (to confirm the restriction sites included in the PCR product) and by subjecting the DNA to gel electrophoresis and visualisation under UV radiation. A 0.1% ethidium bromide stained 1% agarose gel was prepared by dissolving 0.5 g agarose gel through boiling in 50 ml TAE buffer (50X stock solution: 24.2 g Tris base, 57.1 ml glacial acetic acid, 37.2 g Na<sub>2</sub>EDTA.2H<sub>2</sub>O, up to 1 liter. pH 8.0). With gel electrophoresis band sizes were compared with  $\lambda$ DNA digested with *Eco*RI and *Hind*III ( $\lambda$ III). The profile of this molecular weight marker is given in Fig. 2.



**FIGURE 2.** Profile of molecular weight marker  $\lambda$ III with corresponding sizes.

### 3.5. CLONING OF PCR PRODUCTS, CONSTRUCTION OF THE EXPRESSION CONSTRUCT AND TRANSFORMATION

Three plasmid vectors were used for cloning of PCR products: pGem-T-Easy was used to clone all PCR products, YIp356R shuttle vector (Myers *et al.*, 1986) to clone the upstream region and downstream region of *ADH2* for expression cassette construction and YIplac211 to clone the expression cassette followed by transformation into yeast. The expression cassette was constructed by digesting the flanking regions of *ADH2* with *Xba*I/*Sa*I and *Nco*I/*Nsi*I restriction enzyme combination for cloning the upstream region and downstream region, respectively, into YIp356R.

DNA ligation was performed as follows. The plasmids and desired DNA fragments were added together in a 1:3 molar concentration together with ligation buffer to a final working solution of 1 X (from a 10 X concentrated stock solution supplied by the manufacturers). 1  $\mu$ l T4 DNA ligase (final concentration of 0.2 U ml<sup>-1</sup>) was added and sterile distilled water up to a final volume of 20  $\mu$ l. This was incubated at 14-16 °C overnight.

The vectors were transformed into Top 10 *E.coli* competent cells. Competent cells were prepared according to the method of Inoue *et al.* (1990). Bacterial transformation was performed as follows:

Plasmid DNA was added to 80  $\mu$ l competent *E.coli* cells, placed on ice for 30 min followed by 40 sec at 42 °C and then for 2 min on ice. 800  $\mu$ l LB media (containing 100  $\mu$ l of 1 M glucose per 5 ml media) was added. This was incubated at 37 °C for 1 hour, and centrifuged for 2 min at 4000 rpm. The supernatant was removed and 100  $\mu$ l LB was added to the cell pellet. The cells were plated on LB plates containing Ampicillin, X-gal and IPTG (15 g agar to 1 liter of LB, allowed the medium to cool to 50 °C before adding Ampicillin to a final concentration of 100  $\mu$ g ml<sup>-1</sup>, 2 ml of 100 mM IPTG l<sup>-1</sup> and 400  $\mu$ l X-gal l<sup>-1</sup>) and incubated overnight at 37 °C. White colonies were inoculated into 5 ml LB medium containing Ampicillin (100  $\mu$ g ml<sup>-1</sup> medium), and incubated overnight at 37 °C shaker.

Small scale plasmid isolations were performed by taking 1 ml of the transformed *E. coli* cells grown overnight and centrifuged at 13000 rpm for 2 min. The pellet was resuspended in 200  $\mu$ l STET buffer (16 g sucrose, 10 ml Triton X-100, 10 ml 1M Tris-HCl pH8 and 20 ml 0.5M EDTA pH 8 dissolved in 200 ml distilled water, autoclaved and stored at 4 °C), after the addition of 4  $\mu$ l lysozyme the tubes were placed in boiling water bath for 60 sec followed by incubation on ice for 10 min and centrifuged for 10-15 min (13000-15000 rpm). The pellet was removed with a toothpick and 200  $\mu$ l Isopropanol was added to the supernatant. This was left for 10 min at -20 °C, centrifuged for 10 min at ~15000 rpm. The pellets were dried for 10-15 min and resuspended in 50  $\mu$ l TE + RNase (50  $\mu$ g ml<sup>-1</sup>). Screening of the



correct clones were performed through restriction digest with relevant restriction enzymes or PCR and assessed by gel electrophoresis. YIplac211, containing the complete expression cassette, was linearised by digesting with *Stu*I followed by transformation into *S. cerevisiae*.

Yeast transformation was performed as follows: The cells were inoculated in 5 ml YEPD medium (20 g peptone l<sup>-1</sup>, 10 g yeast extract l<sup>-1</sup> and 20 g glucose l<sup>-1</sup>) and incubated overnight at 30 °C. The cells suspension was transferred into microcentrifuge tubes and centrifuged at 4000 rpm for 2 min at room temperature and the supernatant was discarded. The cells were washed in 10 ml LiOAc/TE solution (0.1 M LiOAc, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA), centrifuged at 4000 rpm for 2 min at room temperature and the supernatant was discarded. The pellet was resuspended in 1 ml LiOAc/TE solution and mixed carefully by hand. Plasmid DNA (5-10 µl) together with 5 µl sheared salmon sperm DNA (to increase the efficiency of transformation) was added to 100 µl of cell suspension followed by incubation for 5 min at room temperature. 280 µl of PEG-4000 solution (50% PEG in LiOAc/TE solution) was added to the cell suspension and incubated for 45 min in a 30 °C waterbath. DMSO (40 µl) was added and mixed by inverting the tube 4-6 times. Heat shock was performed by placing the cell suspension in a 42 °C waterbath for 5 min followed by centrifugation at 4000 rpm for 2 min. The supernatant was discarded and the cells were washed by resuspending in 1 ml sterile dH<sub>2</sub>O and centrifuged at 4000 rpm for 2 min. The supernatant was discarded, the cell were resuspended in 0.5 ml sterile dH<sub>2</sub>O and 100 µl was plated on -URA selective medium (6.7g yeast nitrogen base l<sup>-1</sup> without amino acids, 15 g agar l<sup>-1</sup>, 1 pellet NaOH, 20 g glucose l<sup>-1</sup> and 100 ml of 10X amino acid dropout solution without L-Uracil (300 mg L-Isoleucine, 1500 mg L-Valine, 200 mg L-Adenine hemisulfate salt, 200 mg L-Arginine HCL, 300 mg L-Lysine, 200 mg L-Methionine, 500 mg L-Phenylalanine, 2000 mg L-Threonine, 300 mg L-Tyrosine, 200 mg L-Histidine HCL monohydrate, 1000 mg L-Leucine and 200 mg L-Tryptophan per L) and incubated for 3-5 days at 30 °C. For 5-Fluorotic acid (FOA) selection, plates were prepared by media supplemented with 0.1-0.2% 5-FOA and 10 mg Uracil l<sup>-1</sup>.

### 3.6. DETECTION OF B-GALACTOSIDASE ACTIVITY FROM POSITIVE COLONIES

Yeast colonies, obtained from the transformation with YIplac211 plasmid containing the expression construct, were streaked on plates, which derepresses the *ADH2* promoter thus enabling the detection of  $\beta$ -galactosidase activity resulting in positive colonies turning blue. Ethanol and galactose were used as carbon sources [6.7 g/l yeast nitrogen base without amino acids, 100 ml of 10X dropout solution without L-Uracil, 20 g galactose l<sup>-1</sup> or 30 ml ethanol l<sup>-1</sup>, pH 7.0]. After autoclaving, 1M KH<sub>2</sub>PO<sub>4</sub> (pH 7) to a final concentration of 50 mM (50 ml l<sup>-1</sup>) and 2 ml l<sup>-1</sup> of X-Gal (of a 20 mg ml<sup>-1</sup> solution in DMFA) was added before pouring the plates. After streaking out the plates were incubated at 30 °C for 2 days or until the blue colour appeared. The positive colonies were selected for preliminary  $\beta$ -galactosidase assay.

### 3.7. $\beta$ -GALACTOSIDASE ASSAY

The method of Rose and Botstein (1983) was used for  $\beta$ -galactosidase determinations. The culture was prepared by inoculating 100  $\mu$ l of the preinoculum (yeast cells inoculated in YEPD broth and incubated for 12 h at 30 °C) into YEP media containing three different carbon sources to compare the expression of  $\beta$ -galactosidase under both repressing (glucose) and derepressing (galactose and ethanol) growth conditions. Cells were grown until an OD<sub>595</sub> 1-1.5 was reached, harvested by centrifugation and the supernatant was discarded. The cells were washed with 2 ml ice cold buffer Z (16.1 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O l<sup>-1</sup>, 5.5 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O l<sup>-1</sup>, 0.75 g KCl l<sup>-1</sup> and 0.246 g MgSO<sub>4</sub>·7H<sub>2</sub>O l<sup>-1</sup>; pH 7.0), pelleted and resuspended in the same buffer. Cold glass beads were added up to the level just below the meniscus of the liquid and 12.5  $\mu$ l of 40 mM phenylmethylsulfonyl fluoride (PMSF) was added, vortexed twice for 1 min with cooling on ice in between followed by the addition of 0.5 ml cold buffer Z. The liquid phase was transferred to a new microcentrifuge tube and centrifuged at 14 000 x g for 15 min at 4°C. The supernatant was transferred to another microcentrifuge tube for enzymatic

determinations. For the assay o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) was used as a substrate for  $\beta$ -galactosidase enzyme. 100  $\mu$ l of crude extract was added to 900  $\mu$ l of Z buffer and incubated for 5 min at 28 °C. The reaction was started through addition of 200  $\mu$ l ONPG and incubated at 28 °C for 10 min. The reaction was stopped by adding 0.5 ml  $\text{Na}_2\text{CO}_3$  and the OD was measured at  $A_{420}$ . The Miller units were calculated using the following formula:

$$\text{Units/ml} = \frac{(\Delta A_{420}/\text{min}) (V_r) (D)}{0.0045 \times \text{extract volume} \times \text{time}}$$

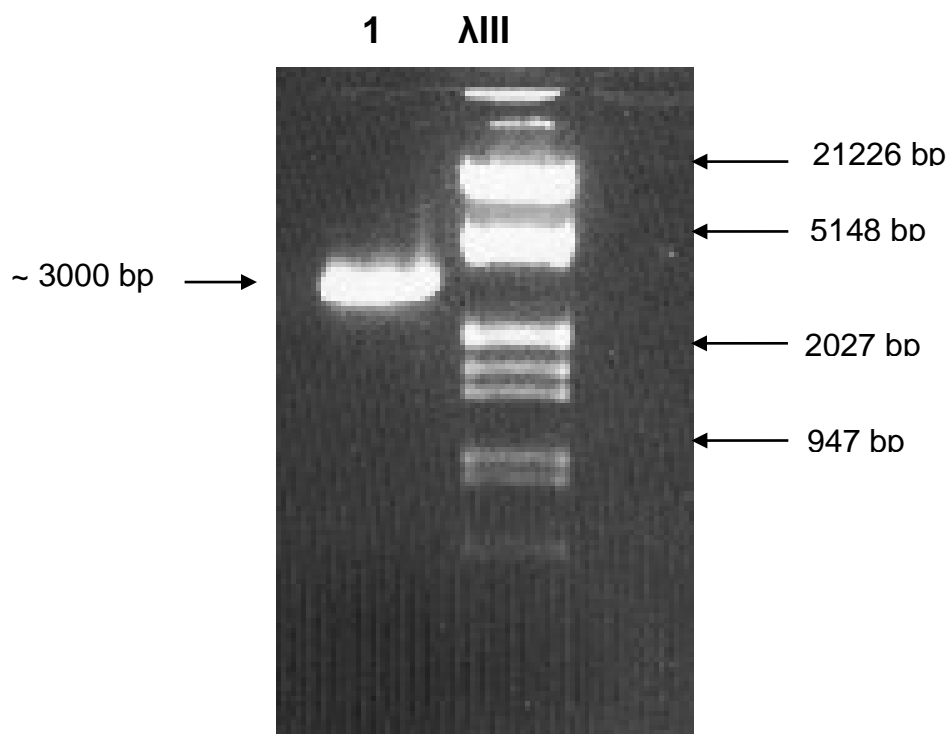
## 4. RESULTS AND DISCUSSIONS

### 4.1. CONSTRUCTION OF THE EXPRESSION CASSETTE

The gene encoding  $\beta$ -galactosidase (*LacZ*) of *E. coli* has been widely used as a reporter gene in many different prokaryotic and eukaryotic organisms. In particular, this gene has proven useful for studying gene expression in the yeast *S. cerevisiae*.  $\beta$ -galactosidase enzyme catalyzes the hydrolysis of lactose into glucose and galactose and many beta-D-galactopyranosides. Advantages in using the *LacZ* gene include sensitive biochemical assays to detect  $\beta$ -galactosidase activity (Ausubel *et al.* 1987) and an associated blue-colour colony phenotype when cells are grown in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal) (Kudla *et al.* 1988).  $\beta$ -galactosidase cleaves X-gal into galactose resulting in the formation of a blue insoluble product. In order to study the expression of *ADH2* in *S. cerevisiae*, an expression cassette was constructed using the *E. coli LacZ* gene as a reporter gene. Detection of the blue-colour phenotype on colonies of *S. cerevisiae* is dependent on the pH of the growth medium, with optimal conditions at pH 7.0 (Rose *et al.* 1990). The blue colonies obtained after yeast transformation confirmed that the cassette was successfully constructed.

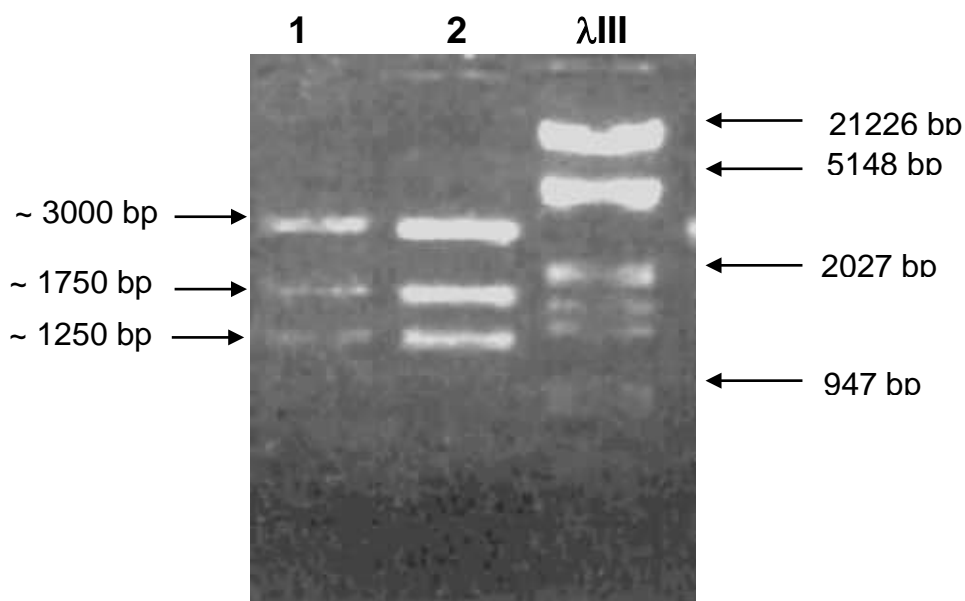
### 4.2. AMPLIFICATION OF THE *ADH2* FLANKING REGIONS AND *LACZ* GENE

To construct the expression cassette, the genomic DNA of *S. cerevisiae* (W303) was isolated and used as a template for the amplification of the *ADH2*. This was carried out by amplifying the ORF with ~1000 bp on either side yielding a product of approximately 3000 bp (Fig. 2). To confirm successful amplification the PCR product was subjected to gel electrophoresis and a single band was observed (Fig. 3).



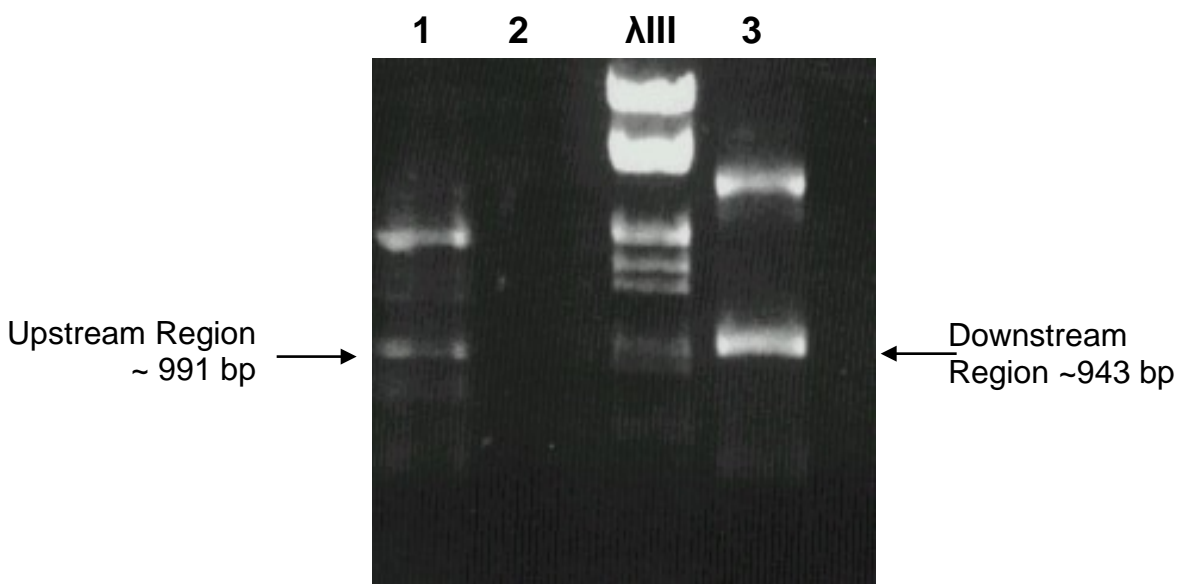
**FIGURE 3.** PCR product after amplification of *ADH2* together with ~1000 bp region up – and downstream from the open reading frame, lane 1 and λIII molecular weight marker, lane 2.

The PCR product was cloned into a pGEM-T-Easy vector and transformed into competent *E. coli* cells. Small scale plasmid isolation were performed on white colonies and digested with *HindIII/EcoRI* restriction enzymes to confirm whether the *ADH2* was successfully cloned. The expected band sizes of approximately ~3000 bp, ~1750 bp and ~1250 bp were obtained (Fig. 4).



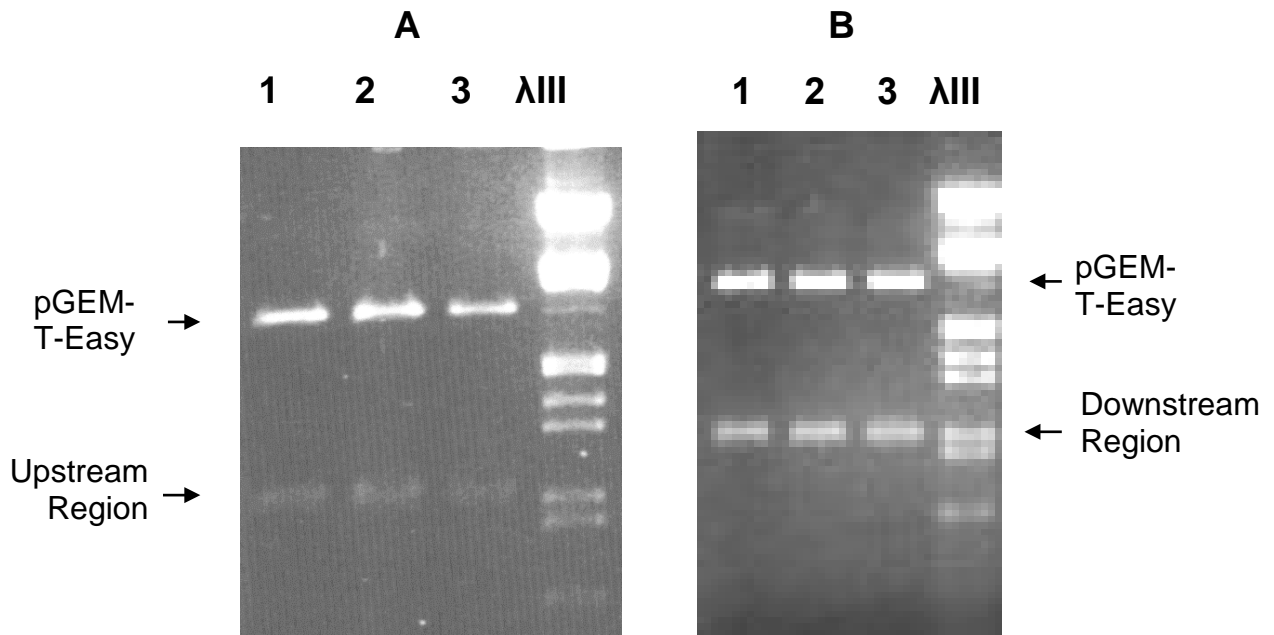
**FIGURE 4.** Lane 1 and 2 represents the expected bands from *ADH2* in pGEM-T-Easy after digestion with *EcoRI* and *HindIII*. Lane 3 contains  $\lambda$ III DNA marker

The pGEM-T-Easy plasmid containing the complete *ADH2* gene together with the up and downstream regions was used as a template to amplify the upstream and downstream regions separately after numerous attempts to clone the *LacZ* between the flanking in the pGEM-T-Easy plasmid were unsuccessful. The primers used for the amplification of the upstream region were designed in such a way that they incorporated a *PstI* restriction site at the 5' end and an *XbaI* site at the 3' end and those used for the amplification of the downstream region incorporated an *Acc65I* site at the 5' end and a *SaII* site at the 3' end to facilitate cloning. The expected bands of ~991 bp and ~943 bp of both the upstream and downstream regions respectively were obtained (Fig. 5).



**FIGURE 5.** Lane 1 and 3 represents the expected bands after amplification of the upstream and downstream regions of *ADH2*. Lane 2 contains  $\lambda$ III DNA marker.

Due to non specific amplicons obtained during amplification, the PCR product with the correct size were isolated from the gel and purified using GFX™ PCR DNA and Gel Band purification kit (Amersham) followed by cloning into pGEM-T-Easy. The cloned pGEM-T-Easy plasmid was transformed into Top 10 competent *E.coli* cells. White colonies were selected for plasmid isolation and purified plasmid DNA was digested with *EcoRI* for confirmation of the correct insert. The expected bands of ~3000 bp representing the plasmid backbone and ~991 bp (upstream region) and ~943 bp (downstream region) were observed for both upstream and downstream region cloned in pGEM-T-Easy (Fig. 6 A & B respectively).



**FIGURE 6.** Lanes 1 to 3 in both A and B represents the correct band profile expected after cloning the upstream and downstream region. in pGEM-T-Easy. **A-** upstream region and **B-**downstream region.

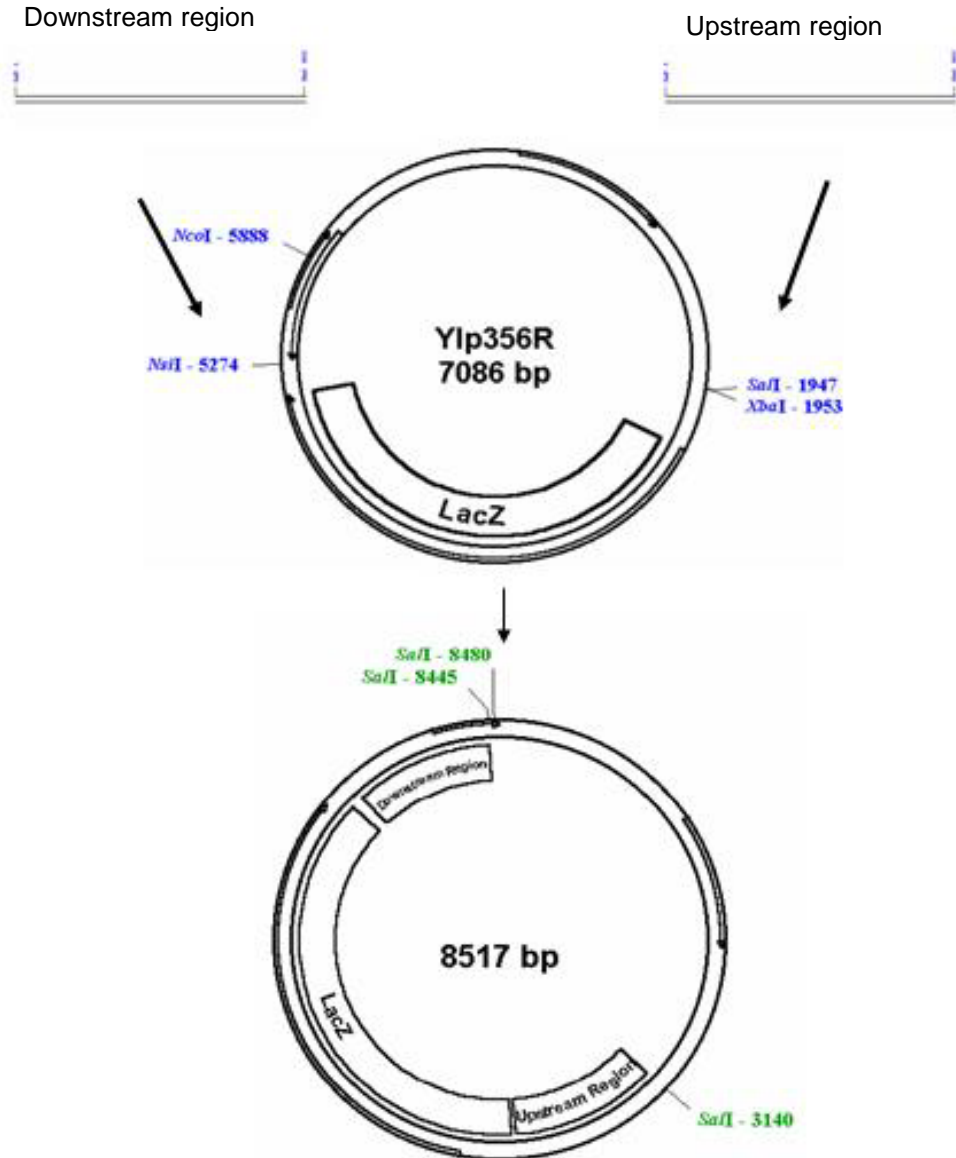
The *LacZ* reporter gene was amplified from wild type *E.coli* strain using primers, which incorporated an *Xba*I restriction site and an *Acc65*I restriction site at the 3' end and 5' end of the gene respectively to facilitate ligation of *LacZ* with the *ADH2* flanking region. Numerous approaches were employed to directly ligate the upstream and downstream regions of *ADH2* with the *LacZ* gene amplified from *E. coli* without any success. An alternative approach was attempted using a shuttle vector (YIp356R) generally used for expression studies to construct the expression cassette.

### 4.3. CONSTRUCTION OF THE EXPRESSION CASSETTE USING YIp356R

There are a number of shuttle vectors, which can be used for gene expression studies in yeasts. Most of these expression vectors utilise the initiation codon of the gene under study. It was therefore important to select a shuttle vector, which will enable in frame cloning of the upstream region of *ADH2* with the reporter gene. YIp356R shuttle vector (Myers *et al.*, 1986) was selected because when the

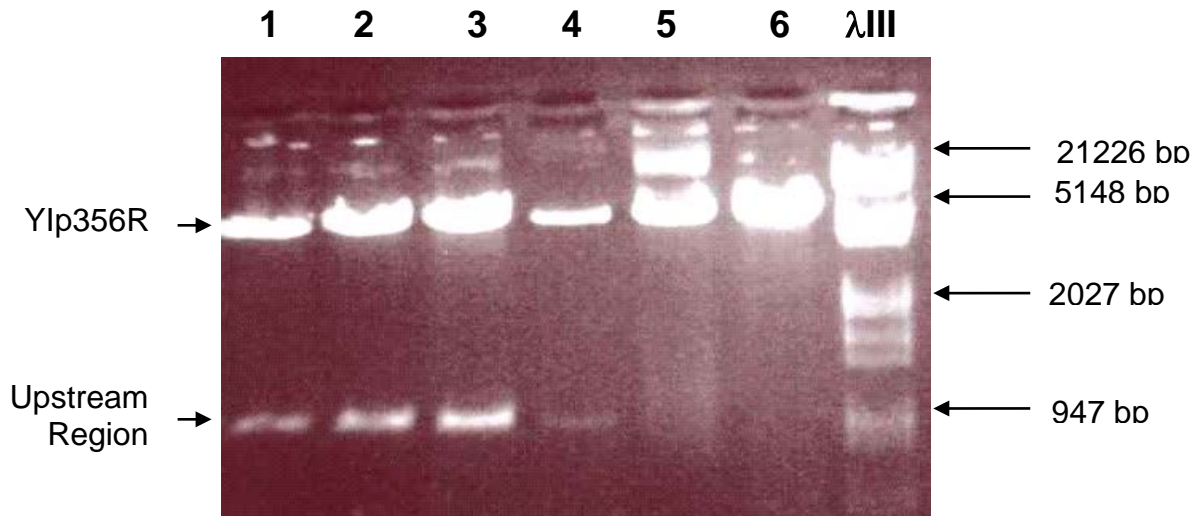


upstream region of *ADH2* is fused to the 5' end of the *LacZ* gene in the shuttle vector using the incorporated *XbaI* restriction site, the correct reading frame of *LacZ* is maintained (Fig. 7).



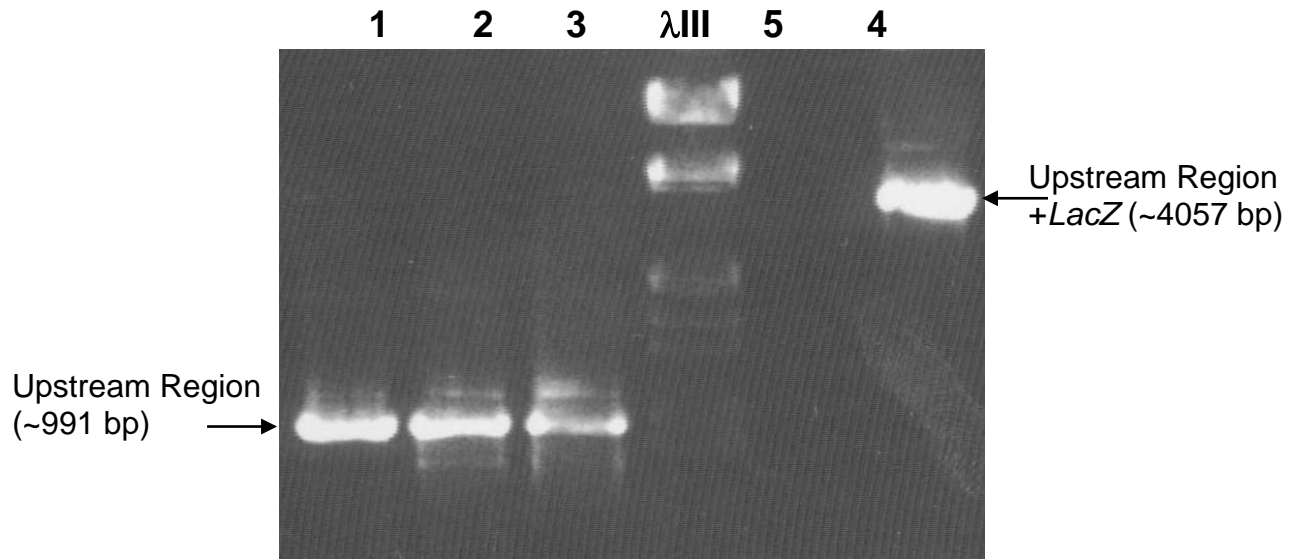
**FIGURE 7.** Construction of the expression cassette from the YIP356R shuttle vector. The *LacZ* gene in this plasmid is not functional in both *E. coli* and *S. cerevisiae*, unless when a functional promoter is cloned at the 5' end of this gene in the plasmid. The expression cassette was constructed through restriction enzyme digestion with relevant restriction enzyme to clone the upstream and downstream region.

To achieve this, pGEM-T-Easy containing the upstream region and the YIp356R shuttle vector were both digested with *Xba*I/*Sa*I. The digestion products were subjected to gel electrophoresis for selection of appropriate bands [~ 991 bp (Upstream region) and ~7086 bp (YIp356R plasmid with *LacZ*)]. The bands were isolated, purified, and ligated overnight followed by transformation into competent *E.coli* cells. Colonies were selected for plasmid isolation and confirmation was done by digesting the plasmid with *Bam*HI/*Hind*III and the correct bands were observed (~7086 bp plasmid and ~991bp upstream region) (Fig. 8).



**FIGURE 8.** Confirmation of cloning of the upstream region at the 5' end of *LacZ* in YIp356R shuttle vector by digestion with *Bam*HI/*Hind*III. Lane 1-3 represents the correct band profile.

Additional confirmation was obtained through amplification of the *ADH2* upstream region and the upstream region fused to *LacZ* gene using YIp356R shuttle vector with the cloned upstream region as a template. The expected bands were observed (~991 bp & ~4057 bp) (Fig. 9).

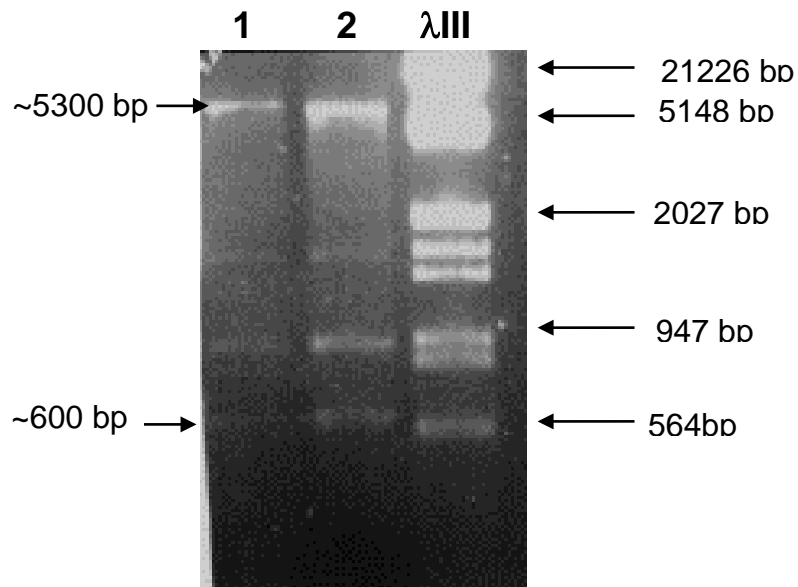


**FIGURE 9.** Confirmation of cloning of the upstream region at the 5' end of *LacZ* in YIp356R shuttle vector by amplification of the upstream region (Lane 1 to 3) and upstream region fused to *LacZ* (Lane 4).

Before the downstream region of *ADH2* was cloned in the YIp356R, containing the upstream region fused to *LacZ*, this shuttle vector was transformed into *S. cerevisiae* to confirm that the cloning of the *ADH2* upstream region was in frame with the *LacZ* gene. The expression of *LacZ* gene in the yeast was qualitatively determined by detecting  $\beta$ -galactosidase presence using the X-Gal agarose overlay assay method. The colonies obtained were streaked out on a medium that allows for the detection of  $\beta$ -galactosidase activity, the media contains a non-fermentable carbon source that allows the derepression of the *ADH2* promoter (ethanol and galactose were used) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal), which is a substrate for  $\beta$ -galactosidase. Therefore colonies with the functional *LacZ* expression construct should be blue in colour. The blue colour appeared after 2 h confirming that the upstream region was incorporated in frame with the *LacZ* resulting in the expression of  $\beta$ -galactosidase.

To complete the expression construct, the downstream region of *ADH2* was cloned in the shuttle vector already containing the upstream region at the 3' end of *LacZ*.

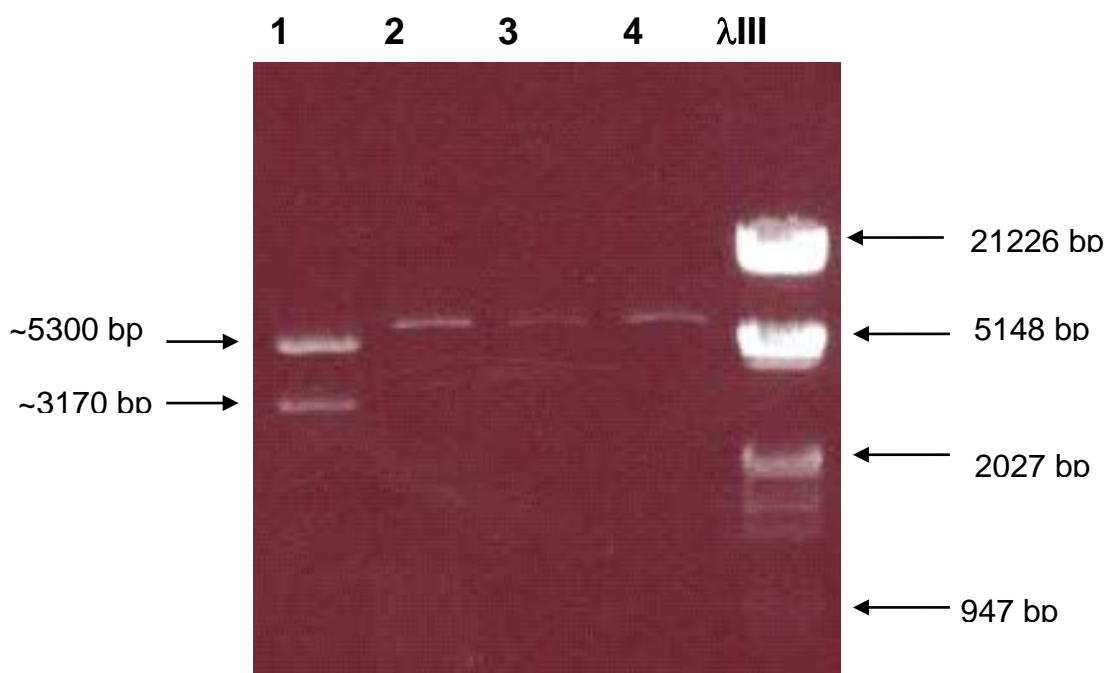
To clone the downstream region in the shuttle vector both the shuttle vector and the pGEM-T-Easy containing the downstream region were digested with *NcoI*/*NsiI*. Two bands were expected from the pGEM-T-Easy digestion and two bands (~600 bp & ~6500 bp) from the shuttle vector. However the results obtained from the digestion of YIp356R with the upstream region showed additional bands indicating partial digestion occurred (Fig. 10).



**FIGURE 10.** Band profile obtained after the digestion of YIp356R+Upstream Region with *NcoI*/*NsiI*. Arrows indicate the expected bands after digestion.

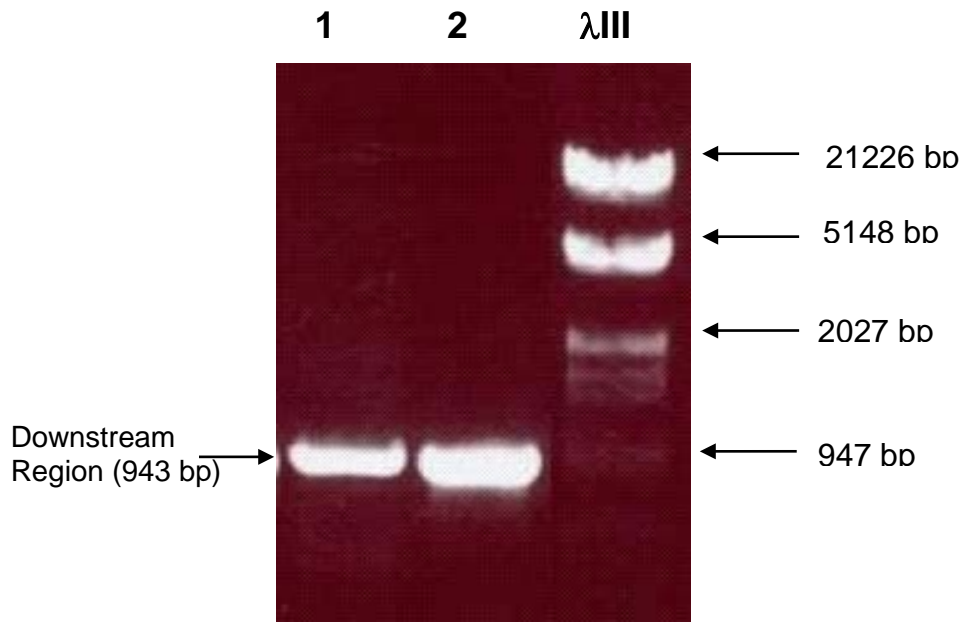
The digestion was repeated and the same profile was observed. To overcome this problem the band with the expected size of the YIp356R vector containing the upstream region fused to the *LacZ* gene (~6500 bp) was isolated from the gel. The 1000 bp fragment consisting of the downstream was ligated into this linearised fragment. Ligation was performed and the ligation product was transformed into Top 10 *E.coli* competent cells. Since the positive clones expressed *LacZ*, blue colonies were selected for plasmid isolation. The plasmid was digested with *SaI* to select for positive clones. *SaI* was selected for confirmation and the presence of

two bands (~5300 bp and ~3170 bp) was an indication that the downstream region was fused to the 5'-side of *LacZ* (Fig. 11).

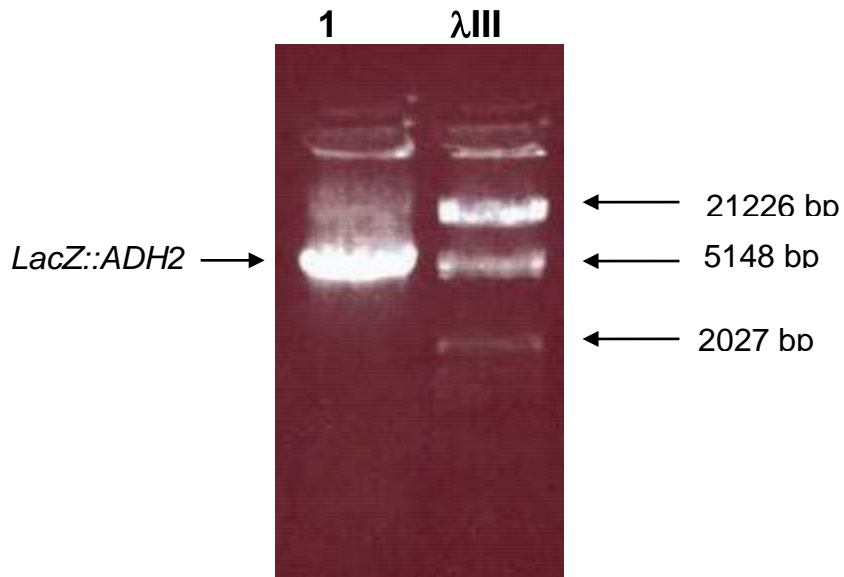


**FIGURE 11.** Confirmation of the cloning of downstream region at the 3'end of *LacZ* in YIp356R shuttle vector. Lane 1 represents the expected band profile.

To confirm that the complete construct was obtained, a PCR was performed to firstly, detect the downstream region (Fig. 12) and secondly, the *LacZ* gene flanked by the *ADH2* up- and downstream regions (Fig. 13).

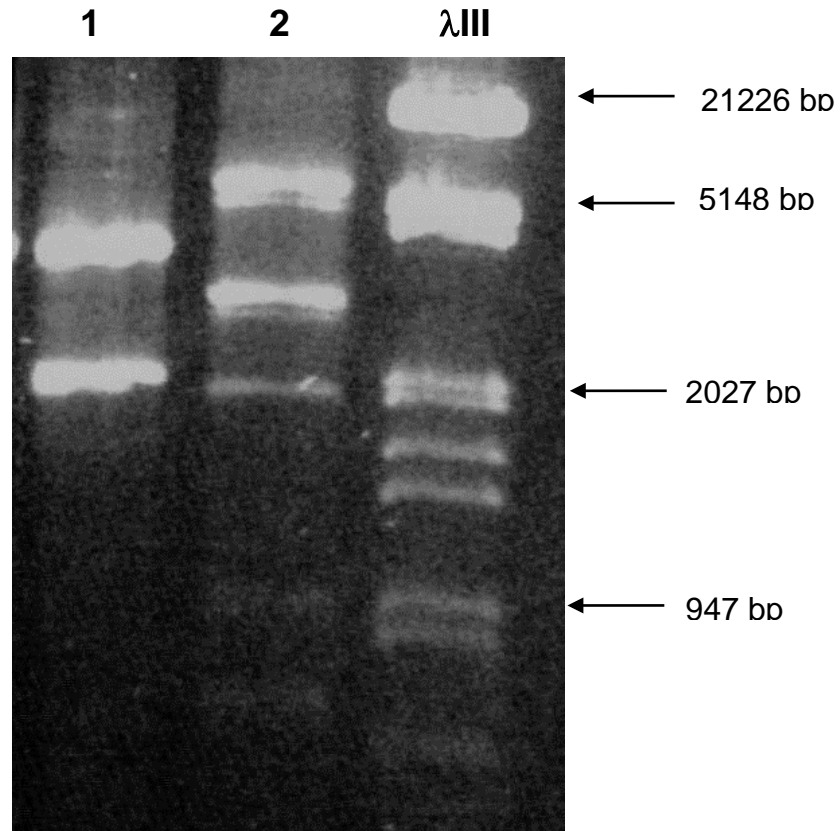


**FIGURE 12.** Amplification of the downstream region from YIp356R after ligation of this region to the 3'-side of the LacZ gene. Lane 1-2 represents the amplified downstream region from two positive clones.



**FIGURE 13.** Amplification of the complete expression cassette containing the *LacZ::ADH2* from YIp356R. Lane 1 represents the correct PCR product and lane 2 represents the molecular marker.

To further confirm that the PCR product obtained when the complete construct was amplified was correct, this amplicon was subjected to restriction analysis. The PCR product was digested with *EcoRV* and *SacI*. The expected bands were observed in both digestions (~3067bp & ~2159bp for *EcoRV* digestion and ~296 bp, ~2007 bp & ~2340 bp for *SacI* digestion) (Fig.14) confirming the complete construction of the expression cassette.

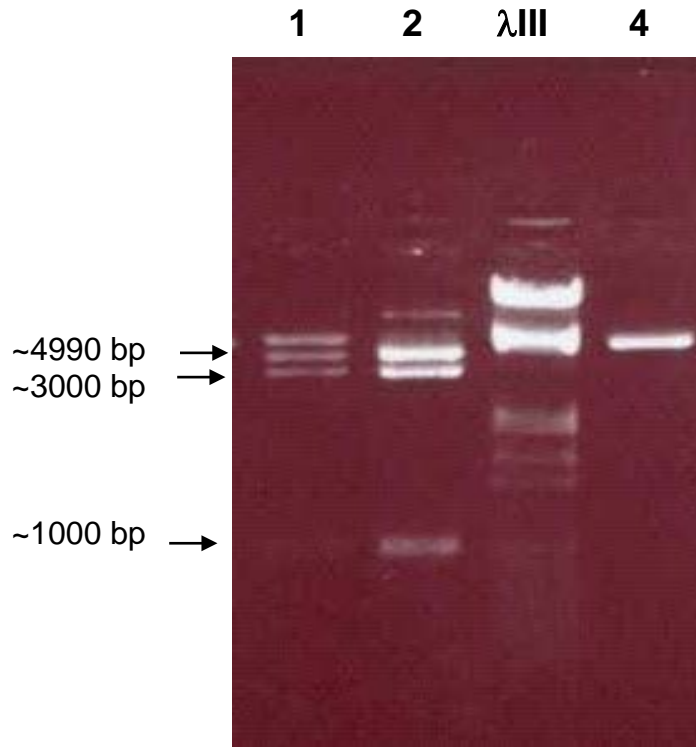


**FIGURE 14.** Digestion of the *LacZ::ADH2* PCR product with with *EcoRV* (Lane 1) and *SacI* (Lane 2) The top band in lane 2 is the PCR product.

#### 4.4. CONFIRMATION OF THE EXPRESSION CASSETTE

Before using the *LacZ* expression cassette to replace the *URA3::ADH2* in the *Saccharomyces cerevisiae* genome, the expression construct was cloned into YEplac112 (Gietz and Sugino, 1988), an episomal plasmid shuttle vector with *TRP1* as an auxotrophic marker. This was done to ensure that the *LacZ*

expression construct would be functional when transformed into yeast. The plasmid was digested with *SmaI* and a PCR product containing the *LacZ* flanked by the *ADH2* up and downstream regions was ligated to the linearised plasmid and transformed into Top 10 *E.coli* competent cells. The plasmid isolated was digested with *EcoRI* for confirmation. Four bands expected (~4990 bp, ~3000 bp & 2X ~1000 bp) were obtained (Fig.15).

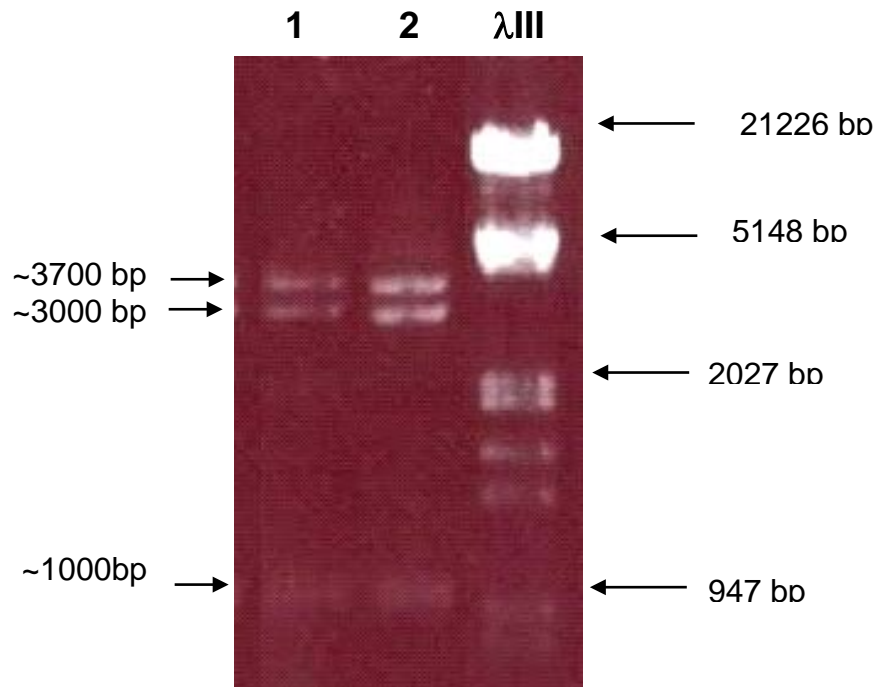


**FIGURE 15.** Digestion of YEplac112 containing *LacZ::ADH2* with *EcoRI*. Lane 2 represents the correct band profile.

The positive plasmid was transformed into *S. cerevisiae* (W303) and grown on dropout media without tryptophan for selection of transformed colonies. The colonies obtained were streaked on a medium that allows for the detection of  $\beta$ -galactosidase activity. However, after several attempts  $\beta$ -galactosidase activity could not be detected. To confirm that the colonies obtained from the yeast transformation contained the plasmid, the colonies were plated out on different dropout media and incubated for 4 days. The following dropout media was used: - URA, -LEU and -TRP plates. The only colonies, which grew, were the ones in -



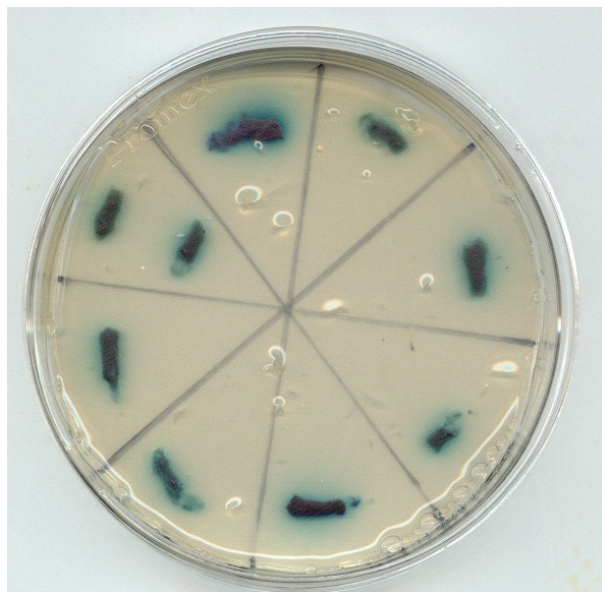
TRP plates. This confirmed that the transformation was successful but there was something that prevented the expression of the *LacZ* gene. Since it was confirmed that the upstream region, when ligated to the *LacZ* in the YIp356R leads to the expression of the gene it was then concluded that the lack of expression was due to the YEplac112 that was used for cloning the expression construct. It was thus decided to clone the expression construct in another shuttle vector, YIp1ac211 (Gietz and Sugino, 1988), an integrating plasmid with *URA3* as an auxotrophic marker. To clone the expression construct, the plasmid was linearised by digesting with *SmaI* followed by the ligation of the complete expression construct into this vector. After transformation into *E. coli* Top10 cells, plasmid DNA was isolated and digested with *EcoRI* and the expected bands were observed (~3790 bp, ~3000 bp & 2X ~1000 bp) (Fig.16).



**FIGURE 16.** Digestion of YIp1ac211 cloned with *LacZ::ADH2* with *EcoRI*. Lanes 1 and 2 represent the correct banding profile.

The YIp1ac211 plasmid containing the expression cassette was linearised (to allow integration into the yeast genomic DNA at the *URA3* locus) and transformed into *S. cerevisiae* followed by plating on  $URA^-$  plates for selection of positive clones. The

colonies obtained were streaked out on X-gal plates, which allows for the detection of  $\beta$ -galactosidase activity. The detection of a blue colour in all the transformants screened confirmed that the expression construct was successfully constructed and integrated into the genome of *S. cerevisiae* (Fig.17).



**FIGURE 17.** Growth of transformants on media containing X-gal. Blue color is indicative of active expression of  $\beta$ -galactosidase confirming the expression of this enzyme through regulation of the *ADH2* promoter.

#### **4.5. PRELIMINARY B-GALACTOSIDASE ACTIVITY ASSAY FROM THE TRANSFORMED YEAST**

The positive colonies were selected for preliminary assay of  $\beta$ -galactosidase activity. The enzyme assay was done under repressed and derepressed state of the *ADH2* promoter. The enzyme assay was performed by selecting five colonies and was grown in media containing three different types of carbon sources. For the enzyme assay under repressed conditions, YEPD medium was used since it contains glucose as a carbon source. For the assay under derepressed conditions ethanol and galactose were used as carbon sources. The results obtained confirmed that the expression of *LacZ* is under glucose repression, the lowest

specific activity was obtained during growth on glucose after 14 h of growth and the lowest specific activity after 37 h of growth were obtained during growth on galactose, in both sampling times the highest activity was obtained during growth on ethanol (Table 2).

**Table 2.** Enzyme activity after growth under repressing and derepressing growth conditions

<b>Carbon Source</b>	<b>Specific Enzyme Activity (U/mg) (14h)</b>	<b>Specific Enzyme Activity (U/mg) (37h)</b>
20 g glucose l <sup>-1</sup>	78.6	504
20 g galactose l <sup>-1</sup>	361.4	414.5
30 g ethanol l <sup>-1</sup>	1679.6	2120.8

After this confirmation, the expression cassette in Ylp356R shuttle vector was amplified and this product was transformed into *S. cerevisiae* (W303). The host strain used, contained a deletion of *ADH2* where the ORF of *ADH2* is replaced with the *URA3* gene (*adh2Δ::URA3*). The reasoning behind this was as follows: the expression cassette (*ADH2::LacZ*) gene needs to be targeted through homologous recombination to replace the native *ADH2* gene; however, this construct does not contain any selective properties to enable the selection of the recombination event. To circumvent this problem, a strain containing an *adh2Δ::URA3* was used as host. Therefore, if the recombination event is successful this strain will revert to a uracil negative auxotroph. For selection purposes, 5-FOA was included in the media on which the transformed cells were plated. If 5-FOA is present, the gene product of *URA3* (orotidine-5'-phosphate decarboxylase) converts 5-FOA to a toxic product that kills cells containing an active *URA3* gene. Therefore the cells without the *LacZ::ADH2* will not form colonies and the colonies obtained will be those with the expression construct. However transformations also yielded colonies on negative control plates, indicating that the level of 5-FOA toxicity was too low. Therefore the

concentration of 5-FOA in the medium was increased to 2 g l<sup>-1</sup> which lead to no colonies obtained on the negative control plate. The colonies obtained on the positive transformation plates were plated out on X-Gal plates for selection, however, no blue colonies were obtained. Further confirmation was obtained through amplification of a section of the *LacZ::ADH2* cassette from the genomic DNA isolated from positive transformants. In all the colonies tested no PCR product could be obtained. After numerous attempts without any success it was clear that it is not possible to direct the expression cassette to the *ADH2* locus.

Because the expression cassette is functional in *S. cerevisiae*, seeing that  $\beta$ -galactosidase activity was obtained when the expression cassette was transformed using the integrative plasmid YIplac211, it was concluded that the problem is not with the cassette itself but rather with the targeting of this construct to the *ADH2* locus. Furthermore, the construction of the deletion of *ADH2* was also extremely difficult and it was only successful after numerous attempts (O. De Smidt, personal communication), which indicated that the replacement of the deletion cassette with the expression cassette would also be problematic, as was proved in this case.

As an alternative approach the YIplac211 containing the expression cassette integrated in the genome of *S. cerevisiae* (used to test the functionality of the expression cassette, see section 4.4), was used to study the expression of *ADH2* under different growth conditions.

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## CHAPTER 4

### INVESTIGATION OF $\beta$ -GALACTOSIDASE PRODUCTION UNDER THE CONTROL OF *ADH2*

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#### 1. ABSTRACT

To study the effect of ethanol on the expression of *ADH2*, *Saccharomyces cerevisiae* transformed with an integrative plasmid containing the *ADH2::LacZ* fusion, was grown on 20 g glucose l<sup>-1</sup> as well as on three different ethanol concentrations (5, 20 and 30 g l<sup>-1</sup>).  $\beta$ -Galactosidase activity was measured as an indicator of *ADH2* expression. The highest  $\beta$ -galactosidase specific activity was obtained during growth on 20 g ethanol l<sup>-1</sup> (3 643 U mg<sup>-1</sup>) and the lowest during growth on 5 g ethanol l<sup>-1</sup> (2 533 U mg<sup>-1</sup>). At ethanol concentrations above 24 g l<sup>-1</sup> low  $\beta$ -galactosidase activity were obtained that might have been due to the low growth rate of the yeast on 30 g ethanol l<sup>-1</sup>. The data suggested that ethanol do not repress the expression of *ADH2*. These results are contrary to previous findings (du Preez *et al.*, 2001) that ethanol at high concentrations repressed *ADH2* expression.

#### 2. INTRODUCTION

The growth, metabolism and gene expression of *Saccharomyces cerevisiae* are highly regulated in response to carbon source availability (Carlson, 1998). This yeast prefers rapidly fermentable sugars namely glucose, mannose and fructose but it can also adapt to growth on non-fermentable carbon sources such as ethanol. *S. cerevisiae* has evolved a regulatory mechanism to cope with widely fluctuating levels of glucose. During the assimilation of glucose, various genes involved in the assimilation of alternative carbon sources are repressed including

*ADH2*, and are only derepressed during growth on a non-fermentable carbon source (Maeda *et al.*, 1994). Ethanol, the by-product of fermentation, is re-introduced into metabolism via its oxidation and the subsequent biosynthesis of acetyl-CoA. Adh2p, an alcohol dehydrogenase isozyme encoded by *ADH2*, is the major isozyme involved in the assimilation of ethanol (Lutstorf and Megnet, 1968; Ciriacy, 1979).

Because *ADH2* has an inducible promoter, this promoter has been widely used for the production of heterologous proteins (Hadfield *et al.*, 1993). Inducible expression cassettes are employed for the regulated production of heterologous proteins. Since the *ADH2* promoter is repressed over 100-fold by glucose, it can be used for the efficient expression of toxic proteins, such as the insulin-like growth factor (IGF-1) (Brierley, 1998). To maintain repression of *ADH2*, cells must be grown in the presence of excess glucose until induction, which is effected by changing to fresh medium containing a non-fermentable carbon source such as ethanol. Alternatively, *ADH2* can be induced by culturing initially on a low concentration of glucose that is gradually depleted (Romanos *et al.*, 1992). Since this promoter is often utilized for heterologous protein production, it is necessary to understand its regulation, especially the effect of ethanol on the expression of *ADH2*.

*ADH2* transcription is known to be derepressed during growth on ethanol (Ciriacy, 1979), however du Preez *et al.* (2001) showed that 17.9 g ethanol l<sup>-1</sup> repressed the expression of *ADH2* in batch cultures and that derepression occurred below 2.3 g ethanol l<sup>-1</sup> in continuous cultures. Van den Berg *et al.* (1998) also reported that *ADH2* expression was partially repressed when the yeast grew in the presence of ethanol. These authors performed a glucose pulse into a glucose-limited chemostat culture and analysed mRNA levels at different phases distinguished by the carbon source detectable during the growth. During growth on glucose, ethanol and acetate were produced and mRNA levels were analysed during the presence of glucose followed by ethanol, ethanol/acetate



and acetate in the medium. These authors found that ethanol had to disappear completely from the culture to obtain full transcription of *ADH2* when the yeast was growing on acetate. This suggested that Adh3p, a mitochondrial alcohol dehydrogenase, might be an important isozyme catalyzing the initial step (ethanol conversion to acetaldehyde) during ethanol assimilation, since the transcription of *ADH3* was elevated during growth on ethanol (Ciriacy, 1997; Bakker *et al.*, 2000).

*Saccharomyces cerevisiae* W303, transformed with YIplac211 (Gietz and Sugino, 1988), an integrative plasmid containing the *ADH2::LacZ* expression cassette, was grown on 20 g glucose l<sup>-1</sup> and three different ethanol concentrations (5, 20 and 30 g l<sup>-1</sup>) as sole carbon sources. Whereas the results of du Preez *et al.* (2001) were obtained using a multicopy plasmid, in the work reported here an integrative plasmid was used to avoid transcriptional misregulation that might result from multicopy effects.

### 3. MATERIALS AND METHODS

#### 3.1 YEAST STRAIN

*Saccharomyces cerevisiae* W303-1A strain (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*) transformed with the Ylplac211 vector containing a *LacZ::ADH2* expression cassette, prepared as in chapter 3 and preserved in 50% glycerol at  $-80^{\circ}\text{C}$  was used.

#### 3.2 INOCULUM AND MEDIUM COMPOSITION

Two yeast colonies from URA<sup>-</sup> selective plates were randomly selected to perform all the experiments in duplicate. To prepare the preculture, each was inoculated in a test tube containing 5 ml of a chemically defined medium. The medium contained the following per litre: C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>.H<sub>2</sub>O, 0.5 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 g; MgSO<sub>4</sub>.H<sub>2</sub>O, 0.4 g; CaCl<sub>2</sub>, 0.015 g; KH<sub>2</sub>PO<sub>4</sub>, 5 g; 10 ml trace elements, 10 ml vitamin solution, 10 ml of a 10 X amino acids solution, 1.6 ml leucine solution, 1 ml TRP/HIS solution, glucose (20 g l<sup>-1</sup>) or ethanol (5, 20, 30 g l<sup>-1</sup>) as a carbon source and adjusted pH to 5.5. To maintain selection pressure, uracil was not added to the medium. The test tubes were then incubated at 30°C for 12 h before being used to inoculate a 100 ml shake flask with 20 ml of the same media to an OD<sub>600</sub> of 0.1-0.2. The flasks were incubated for 10 h to inoculate the glucose containing medium. The medium with ethanol as a carbon source was inoculated after 15 h of incubation to ensure that all the glucose was depleted.

The composition of the 10 X trace element solution was as follows: FeSO<sub>4</sub>.7H<sub>2</sub>O, 35 g; MnSO<sub>4</sub>.H<sub>2</sub>O, 7 g; ZnSO<sub>4</sub>.7H<sub>2</sub>O, 11 g; CuSO<sub>4</sub>.5H<sub>2</sub>O, 1 g; CoCl<sub>2</sub>, 2 g; NaMoO<sub>4</sub>.2H<sub>2</sub>O, 1.3 g; H<sub>3</sub>BO<sub>3</sub>, 2 g; KI, 0.35 g; Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, 0.5 g; dissolved in 950 ml distilled water and 50 ml concentrated HCl. The composition of the vitamin solution was as follows: biotin, 0.005 g; calcium pantothenate, 0.1 g; folic acid,

0.005 g; niacin, 0.1 g; p-aminobenzoic acid, 0.02 g; pyridoxine HCl, 0.1 g; riboflavin, 0.1 g; thiamine HCl, 0.1 g; inositol, 2.5 g; dissolved in 20 ml of 1 M NaOH and distilled water up to 1 l.

To prepare the 10 X amino acid solution, 0.5 g of each of the following amino acids was dissolved in a 100 ml distilled water; arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. A 1000 X TRP/HIS stock were prepared by dissolving tryptophan (2.5 g) and histidine (2.5 g) in 50 ml distilled water. Cysteine and leucine were prepared separately, 1.25 g of cysteine was dissolved in 25 ml of 1 N HCl, 4 g of leucine was dissolved in 50 ml distilled water. NaOH pearls were used to dissolve the amino acids in water with a resulting pH of between 10-11.

### 3.3. ANALYTICAL METHODS

Samples for assays were taken at 2 to 10 h intervals, depending on the carbon source and growth phase of the yeast. The growth of the yeast cells was followed by measuring the optical density at 600 nm with an Eppendorf biophotometer (Eppendorf AG, Germany). The dry biomass of 10 ml samples from the late exponential phase were gravimetrically determined after centrifugation at 5 000 rpm for 5 min at 4 °C, washed twice with distilled water and dried at 105 °C for 24 h. Optical densities were converted to cell dry weight according to a standard curve. The maximum specific growth rate was determined from the slope of the exponential phase of the growth curve.

The culture supernatant was used for glucose and ethanol analysis. Glucose concentration was determined with a Sugar Analyser 1 HPLC equipped with a refractive index detector and a Sugar Pack 1 column (Waters Associates, Milford, MA, USA) operating at 85 °C with a deionised water flow rate of 0.5 ml min<sup>-1</sup>.

## CHAPTER 4

Ethanol was determined with a Hewlett-Packard 6 890 A gas chromatograph using 2 ml hydrogen carrier gas  $\text{min}^{-1}$  at an oven temperature of  $70^{\circ}\text{C}$  for 2 min followed by temperature programming at  $10^{\circ}\text{C min}^{-1}$  to  $130^{\circ}\text{C}$  and held for 1 min.

The  $\beta$ -galactosidase activity was assayed according to the method of Rose and Botstein (1983), using 4 ml samples. Culture samples were harvested by centrifugation and washed with buffer Z (16.1 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O l}^{-1}$ ; 5.5 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O l}^{-1}$ ; 0.75 g  $\text{KCl l}^{-1}$  and 0.246 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O l}^{-1}$ , adjusted pH to 7.0) and stored at  $-20^{\circ}\text{C}$ . Before being assayed the samples were thawed and resuspended in 200  $\mu\text{l}$  ice cold buffer Z. Cell extracts were prepared by adding cold glass beads up to the level of the liquid suspension and 12.5  $\mu\text{l}$  PMSF (phenylmethylsulfonyl fluoride) before vortexing for 2 min at 1 min intervals on ice. Cell debris was removed by centrifugation and the supernatant was used as the cell extract. This extract and the ONPG (o-nitrophenyl-  $\beta$ -D-galactopyranoside) substrate were incubated for 15 min at  $28^{\circ}\text{C}$  and absorbance was measured at 420 nm using a spectrophotometer (Spectronic genesis<sup>TM</sup>, Miltonroy Co., USA). Protein concentrations were determined using the Bio-Rad protein reagent as indicated by the manufacturers (BioRad Laboratories, USA), based on the method of Bradford (1976) and measured at an absorbance of 595 nm. One unit of  $\beta$ -galactosidase activity is the amount of  $\beta$ -galactosidase that hydrolyses 1 nmol of ONPG to o-nitrophenol and D-galactose per min at  $28^{\circ}\text{C}$  at pH 7. The specific rate of enzyme production was determined from the slope of the curve of specific enzyme activity ( $\text{U protein mg}^{-1}$ ) vs time.

## 4. RESULTS

### 4.1 GROWTH PROFILE DURING GROWTH ON GLUCOSE AND ETHANOL AS RESPECTIVE CARBON SOURCES.

To study the effect of ethanol on the expression of *ADH2*, *S. cerevisiae* transformed with an *ADH2::LacZ* expression cassette was grown on ethanol concentrations of 5, 20, and 30 g l<sup>-1</sup>. In addition, glucose (20 g l<sup>-1</sup>) was used to investigate *ADH2* expression under repressed conditions. These experiments were performed in a chemically defined medium using two randomly selected recombinant *S. cerevisiae* W303 colonies and the presented results are the average obtained for the two transformants (Table 1). The cultivation parameters are shown in Table 1.

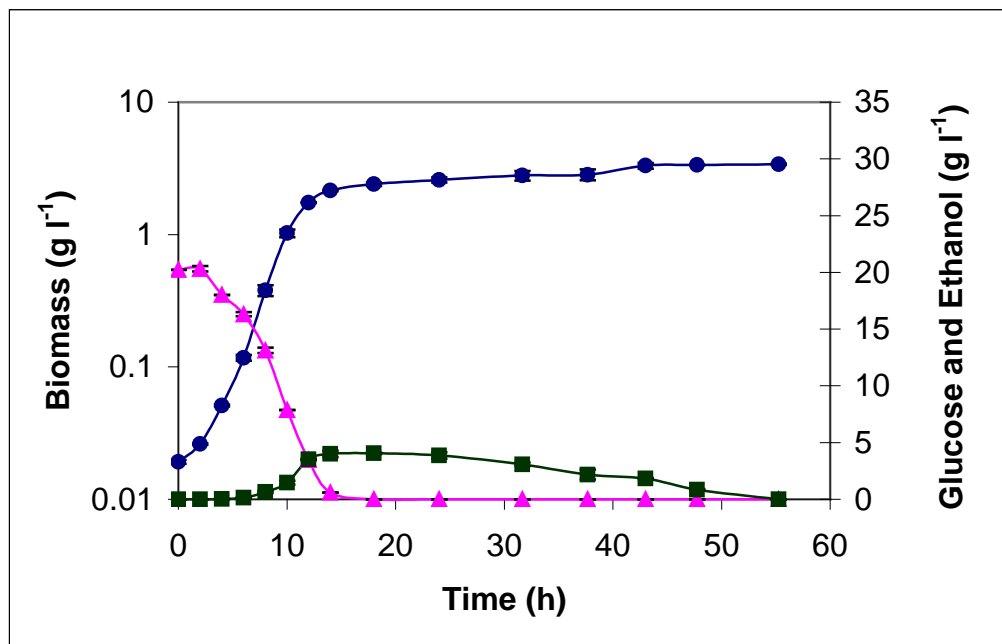
**Table 1.** Specific growth rate, final biomass produced and rate of  $\beta$ -galactosidase production during growth on glucose and different concentrations of ethanol as carbon source

Carbon Source (g l <sup>-1</sup> )	$\mu_{\max}$ (h <sup>-1</sup> )	Final Biomass (g l <sup>-1</sup> )	$Y_{X/S}$ (g g <sup>-1</sup> )	Enzyme Activity (U ml <sup>-1</sup> )	Specific Enzyme Activity (U mg <sup>-1</sup> )	$q_p^{\max}$ (U mg <sup>-1</sup> h <sup>-1</sup> )
Glucose, 20	0.47	3.4	0.17	947	532	46
Ethanol, 5	0.24	2.9	0.54	4911	2533	128
Ethanol, 20	0.18	2.7	0.24	6372	3643	294
Ethanol, 30	0.13	1.4	0.118	5919	3496	93

$\mu_{\max}$ , maximum specific growth rate;  $q_p^{\max}$ , maximum specific rate of  $\beta$ -galactosidase production;  $Y_{X/S}$ , biomass yield.

### 4.1.1 GROWTH UNDER REPRESSING CONDITIONS ON GLUCOSE

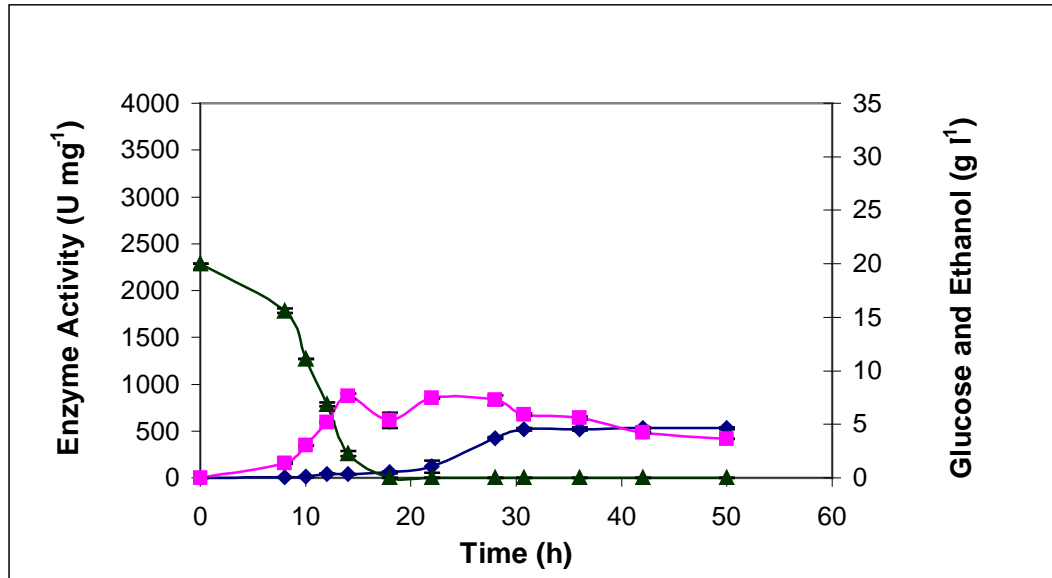
The yeast exhibited the highest growth rate ( $0.47 \text{ h}^{-1}$ ) and the highest final biomass concentration on glucose (Table 1). After 14 h the yeast reached stationary phase with concomitant depletion of the glucose in the medium. Ethanol production was evident after 8 h of growth and reached the maximum concentration after 18 h (Fig. 1). After 55 h of growth the ethanol was depleted, indicating the slow assimilation of ethanol in comparison to the rapid utilization of glucose.



**FIGURE 1.** Cultivation profile of the transformed yeast on  $20 \text{ g l}^{-1}$  glucose as carbon source. Symbols: Biomass (●), Glucose (▲) and ethanol (■)

An increase in the  $\beta$ -galactosidase activity was only observed when glucose was almost depleted (Fig. 2). This, together with the fact that the ethanol concentration after 14 h was about  $7 \text{ g l}^{-1}$ , suggested that glucose repressed the *ADH2* promoter. This is in agreement with the existing literature that the activity

of both Cat8p and Adr1p, the transcription activators of *ADH2*, was carbon source dependent (Walther and Schüller, 2001; Hedges *et al.*, 1995; Young *et al.*, 2002).



**FIGURE 2.**  $\beta$ -Galactosidase activity, glucose utilisation, ethanol production and utilisation by the yeast during growth on 20 g l<sup>-1</sup> glucose. Symbols: Enzyme activity ( $\blacklozenge$ ), ethanol concentration ( $\blacksquare$ ) and glucose concentration ( $\blacktriangle$ )

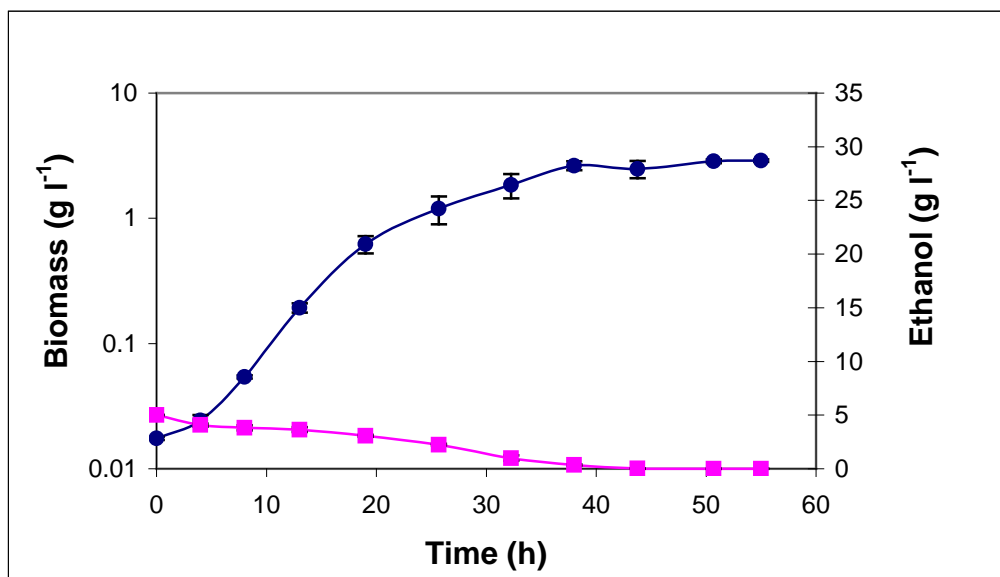
This steady increase of  $\beta$ -galactosidase activity shows that derepression of the *ADH2* promoter was due to activation of the *ADH2* transcription activators in the absence of glucose when ethanol is assimilated by the yeast (Young *et al.* 2002). The maximum specific enzyme activity obtained during this cultivation was 532 U protein mg<sup>-1</sup> (Table 1).  $\beta$ -Galactosidase production reached a maximum level after 30 h of growth with no decrease in activity during the following 20 h. During this time there was also a slow decrease in the ethanol concentration from a maximum of 7.2 g l<sup>-1</sup> to 3.6 g l<sup>-1</sup> after a 50 h cultivation period.

These results indicate that when the *ADH2* promoter is employed in the production of heterologous genes, it is beneficial to grow the yeast in glucose to

obtain maximum biomass production prior to the induction of the *ADH2* promoter using ethanol, so as to avoid the slow growth and low biomass production when ethanol is utilized.

#### 4.1.2 GROWTH UNDER DEREPRESSING CONDITIONS ON ETHANOL

To determine the effect of ethanol concentration on the expression of *ADH2*, the yeast was grown on different ethanol concentrations (5, 20 and 30 g l<sup>-1</sup>) as a sole carbon source. The growth rate (Table 1) and biomass produced was higher during growth on 5 g ethanol l<sup>-1</sup> in comparison to growth on either 20 or 30 g l<sup>-1</sup>. This growth rate was approximately two-fold less than on glucose. The ethanol in the medium was completely utilized after 40 h of cultivation (Fig. 3).

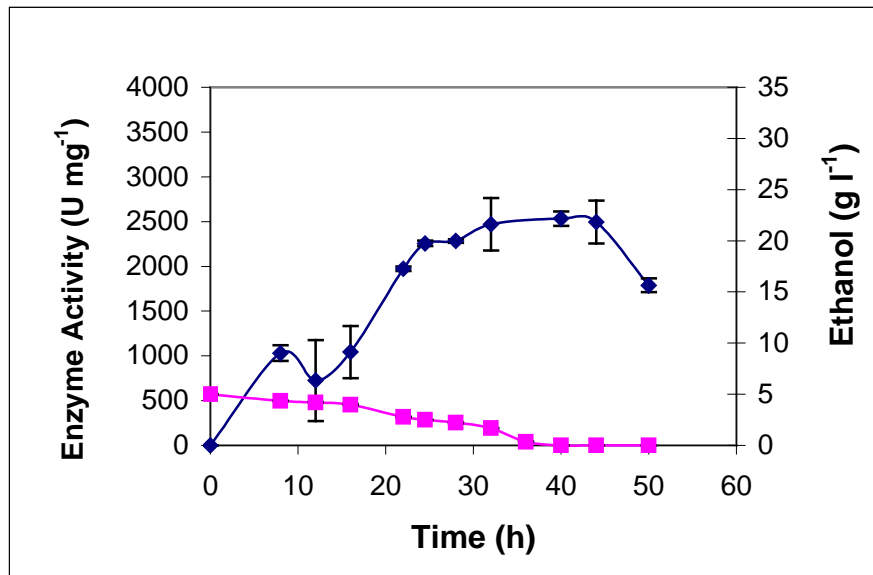


**FIGURE 3.** Cultivation profile of the transformed yeast on 5 g ethanol l<sup>-1</sup> as sole carbon source. Symbols: Biomass (●) and ethanol (■)

The  $\beta$ -galactosidase activity was expected to be high during this cultivation in comparison to growth on the other two ethanol concentrations, according to the



results obtained by du Preez *et al.* (2001), since high ethanol concentrations had a negative effect on the expression of *ADH2*. However, this was not the case as the lowest  $\beta$ -galactosidase activity was obtained during growth on 5 g ethanol l<sup>-1</sup>. A maximum specific activity of 2 533 U protein mg<sup>-1</sup> was obtained (Fig. 4). This maximum specific activity was higher to that observed during growth on glucose, thus confirming derepression of the *ADH2* promoter during growth on a non-fermentable carbon source such as ethanol.

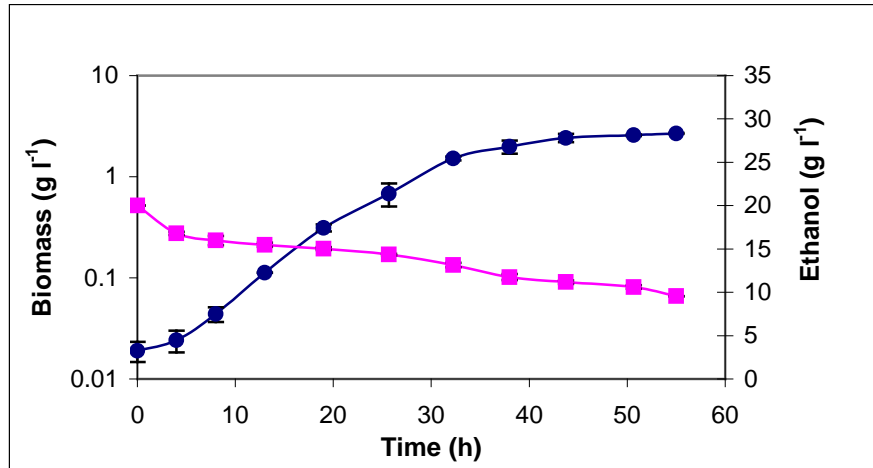


**FIGURE 4.**  $\beta$ -Galactosidase activity and ethanol utilization during growth on 5 g ethanol l<sup>-1</sup>. Symbols: Enzyme activity (◆) and ethanol concentration (■)

The enzyme production rate of 128 U mg<sup>-1</sup>h<sup>-1</sup> was observed, and it was higher than that observed during growth on glucose. The  $\beta$ -galactosidase production rate decreased after 24 h when the ethanol concentration decreased to below 2.5 g l<sup>-1</sup>.

The final biomass obtained during growth on 20 g ethanol l<sup>-1</sup> was similar to that obtained during growth on 5 g ethanol l<sup>-1</sup> (Table 1). However, the growth rate was

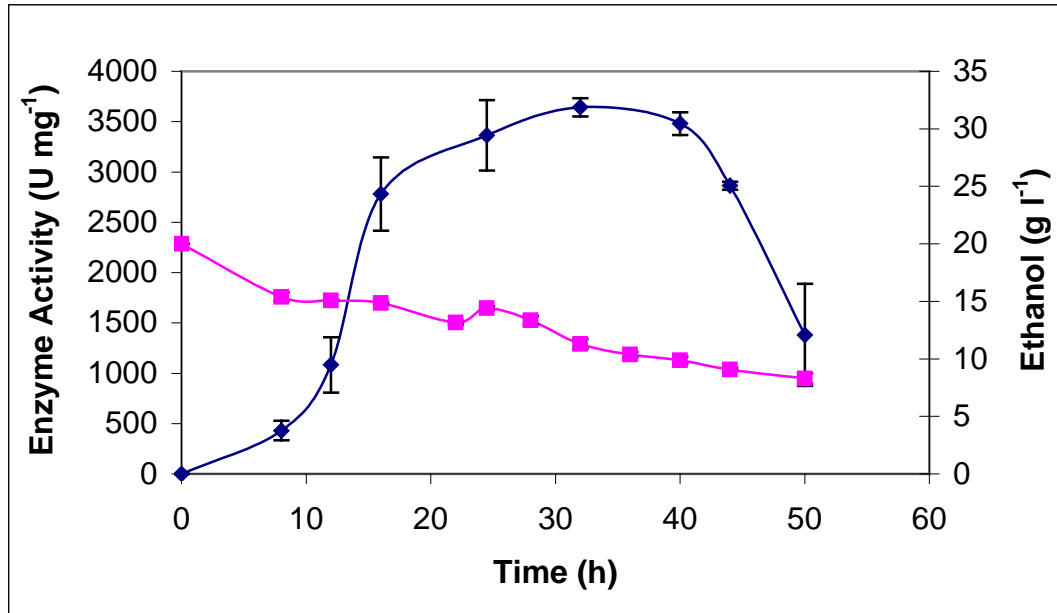
slower than during growth on 5 g ethanol l<sup>-1</sup> (Table 1), indicating that ethanol has negative effect on the growth of the yeast cells (Walker-Caprioglio *et al.*, 1985). Approximately 10 g ethanol l<sup>-1</sup> was utilized after 55 h of growth (Fig. 5). At a cultivation time of 55 h approximately 9.5 g ethanol l<sup>-1</sup> was still present (Fig.5).



**FIGURE 5.** Cultivation profile on 20 g ethanol l<sup>-1</sup>. Symbols: Biomass (●) and ethanol concentration (■)

These results showed that 50% (10 g ethanol l<sup>-1</sup>) of the ethanol provided was utilized during this cultivation to produce the amount of biomass similar to that produced during growth on 5 g ethanol l<sup>-1</sup>.

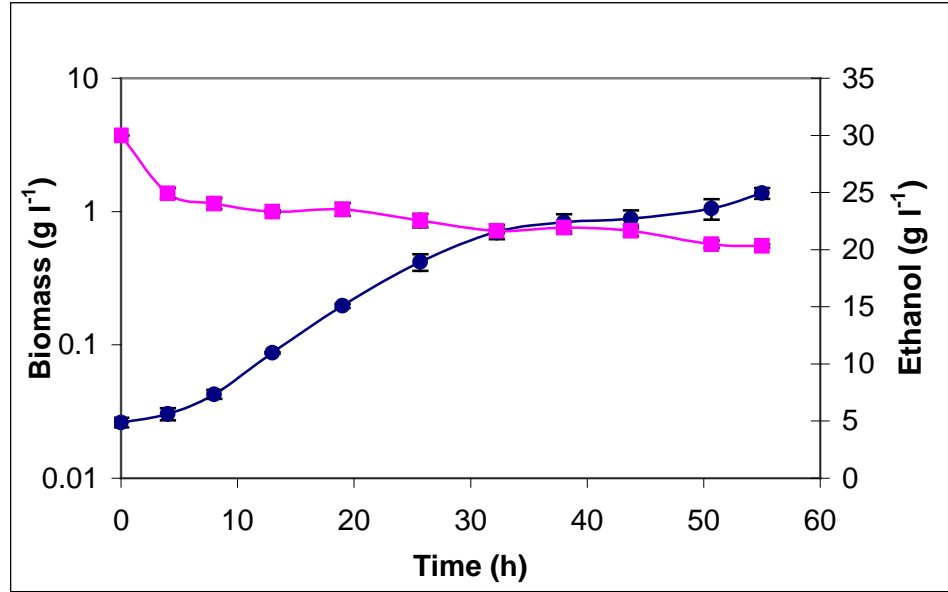
The  $\beta$ -galactosidase activity during this cultivation was higher than that obtained on 5 g ethanol l<sup>-1</sup>, with a maximum specific activity of 3 643 U protein mg<sup>-1</sup> (Fig. 6). The  $\beta$ -galactosidase production rate was also higher (Table 1). After 16 h a  $\beta$ -galactosidase specific activity of about 2 780 U protein mg<sup>-1</sup> was obtained as compared to 1 032 U protein mg<sup>-1</sup> obtained during growth on 5 g ethanol l<sup>-1</sup> after 16 h (Fig.6). This indicated that a decrease in the ethanol concentration during this cultivation was not necessary to obtain high expression levels. The  $\beta$ -galactosidase production ceased after 36 h.



**FIGURE 6.**  $\beta$ -galactosidase specific enzyme activity and ethanol utilization during growth on 20 g ethanol l<sup>-1</sup>. Symbols: Enzyme activity (◆) and ethanol concentration (■)

About a 30% higher maximum specific  $\beta$ -galactosidase activity was observed during growth on 20 g ethanol l<sup>-1</sup> than on 5 g ethanol l<sup>-1</sup>, suggesting that an increase in ethanol concentration enhanced the expression of *ADH2*.

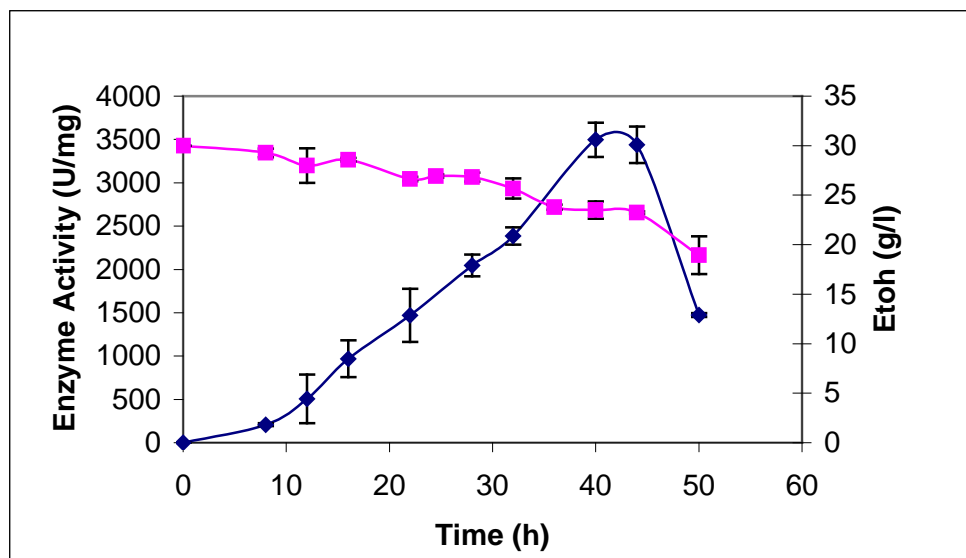
To verify that a high ethanol concentration had a negative effect on the expression of *ADH2*, the yeast was grown on 30 g ethanol l<sup>-1</sup> as sole carbon source. Under these growth conditions the lowest biomass and yield was obtained (Fig. 7 and Table1).



**FIGURE 7.** Cultivation profile obtained during growth on 30 g ethanol l<sup>-1</sup>.  
Symbols: Biomass (●) and ethanol concentration (■)

Most of the ethanol (20 g l<sup>-1</sup>) still remained after a cultivation time of 55 h. A final biomass concentration of 1.4 g l<sup>-1</sup> of biomass was produced (Table 1) from the 10 g ethanol l<sup>-1</sup> utilized by the yeast during this cultivation, which was much less in comparison to the biomass obtained on 20 g ethanol l<sup>-1</sup>. Similar amounts of ethanol were utilized in both cultivations. Even though ethanol was still available at the end of the cultivation on 20 and 30 g ethanol l<sup>-1</sup>, in both cases the growth rate had decreased dramatically. This might have been due to the exhaustion of essential nutrients that may have occurred during growth on ethanol.

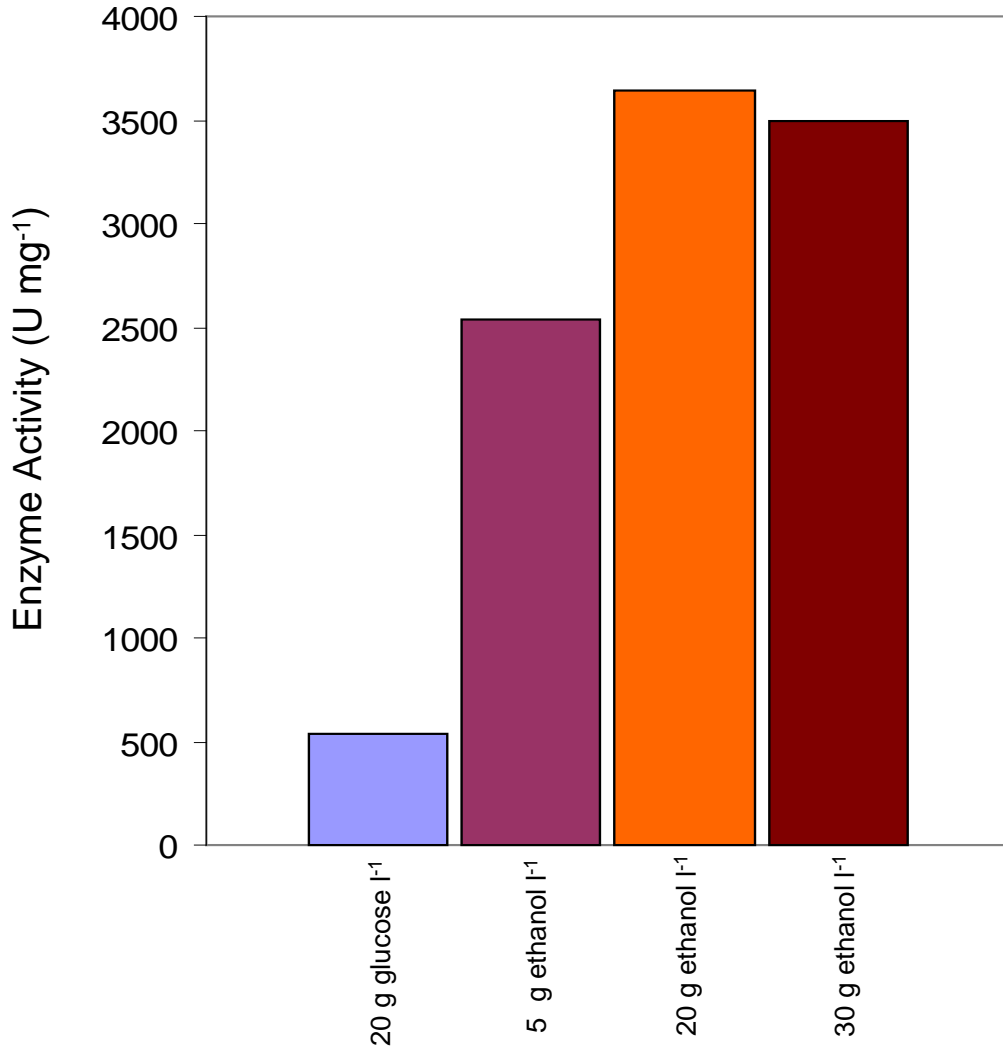
Contrary to the results expected, the specific  $\beta$ -galactosidase activity obtained here was higher than that obtained during growth on 5 g ethanol l<sup>-1</sup> (Fig. 8).



**FIGURE 8.**  $\beta$ -galactosidase specific activity and ethanol utilization during growth on 30 g ethanol l<sup>-1</sup>. Symbols: Enzyme activity (◆) and ethanol concentration (■).

However, this maximum specific  $\beta$ -galactosidase activity was achieved after the level of the ethanol concentration decreased to about 24 g l<sup>-1</sup> (Fig. 8). There was a slow steady increase in the  $\beta$ -galactosidase activity observed during this cultivation (Fig. 8), reaching a maximum of 3 496 U mg<sup>-1</sup> protein when the ethanol concentration reached 24 g l<sup>-1</sup> after 40 h. This slow production of  $\beta$ -galactosidase observed here might have been due to the slow growth of the yeast during this cultivation. Although a maximum specific activity and the maximum volumetric enzyme activity obtained here were almost as high as that obtained during the cultivation on 20 g ethanol l<sup>-1</sup>, the final biomass produced during this cultivation was less by almost a half to that produced on 20 g ethanol l<sup>-1</sup> (Table 1), this therefore indicated that  $\beta$ -galactosidase production was two fold higher. Therefore higher expression occurred when the ethanol concentration of 30 g l<sup>-1</sup> was used than on either 20 or 5 g l<sup>-1</sup>.

The maximum specific enzyme activity values obtained under these three different ethanol concentration and 20 g glucose l<sup>-1</sup> are represented in the Figure 9.



**FIGURE 9.** The maximum specific enzyme activity obtained during growth on 20 g glucose l<sup>-1</sup>, 5, 20 and 30 g ethanol l<sup>-1</sup>.

The maximum specific activity of 519 U mg<sup>-1</sup> was obtained when the ethanol concentration reached 7 g l<sup>-1</sup> during cultivation on 20 g glucose l<sup>-1</sup>. During cultivations under derepressing conditions with the different ethanol concentrations the maximum specific activity was 2 533, 3 496 and 3 643 U mg<sup>-1</sup> during growth on ethanol concentrations of 5, 30, and 20 g l<sup>-1</sup>, respectively. These maximum specific activities were obtained when the ethanol concentration in the medium was 2.2 g l<sup>-1</sup>, 24 g l<sup>-1</sup> and 11 g l<sup>-1</sup> during growth on 5, 30, and 20 g l<sup>-1</sup>, respectively. This suggested that ethanol did not repress the expression of the *ADH2* gene. Cultivation of the yeast on 20 g l<sup>-1</sup> glucose confirmed glucose repression on *ADH2*. The data obtained in this study suggested that ethanol enhanced the expression of *ADH2* when its concentration was increased. However it was shown that above a certain concentration the production rate was slow. According to the above results, to produce heterologous proteins using the *ADH2* promoter effectively, the yeast should be grown on glucose prior to derepression of the promoter through cultivation on ethanol and that a 20 g ethanol l<sup>-1</sup> concentration should be used during derepression as the highest enzyme activity was achieved at this initial ethanol concentration.

## 5. DISCUSSION

The yeast grew better on glucose as opposed to growth on ethanol and this may be attributed to the fact that ethanol at a high concentration is toxic to the yeast, the major targets for ethanol toxicity being the plasma membrane and the cytosolic enzymes: Ethanol alters the membrane organization and permeability and inactivates and unfolds globular cytosolic enzymes (Thomas and Rose, 1979). Ethanol has also been found to induce a delay in the yeast cell cycle (Kubota *et al.*, 2004). The highest growth rate and biomass yield on ethanol was achieved during growth on 5 g l<sup>-1</sup>, the lowest ethanol concentration used in this study.

The above results are consistent with the existing literature in terms of the regulation of *ADH2*. Glucose repression was confirmed by the low  $\beta$ -galactosidase activity obtained during cultivation on 20 g glucose l<sup>-1</sup>. Enzyme production was observed when glucose was almost depleted, relieving the transcription activators from glucose repression (Gancedo, 1998). The increase in  $\beta$ -galactosidase activity in the presence of ethanol supported the theory that the activity of Snf1p, which is at the centre of *ADH2* derepression and is also essential for growth on non-fermentable carbon sources, was inhibited by glucose (Jiang and Carlson, 1996) and was only induced when a non-fermentable carbon source is utilized, resulting in the derepression of *ADH2* by the two transcription activators, Adr1p and Cat8p (Walther and Schüller, 2001).

The results also indicated that the expression of *ADH2* increased in the presence of ethanol. This was indicated by an increase in the maximum specific activity which correlated with an increased ethanol concentration in the medium. However to confirm the possible induction of *ADH2* expression by ethanol, the yeast should be grown on another non-fermentable carbon source such as glycerol to compare the expression levels, since it has been reported that ethanol



does not induce *ADH2* expression (Ciriacy, 1997). According to available literature, the increase in  $\beta$ -galactosidase activity during growth on ethanol was rather due to the absence of glucose repression and not due to ethanol induction, although an increase in ethanol may however lead to a high demand for Adh2p resulting in an increased *ADH2* expression.

It has been found that ethanol limits the growth of *S. cerevisiae* in a concentration dependent manner. An increase in ethanol concentration leads to an increase in the lag phase and a decreased growth rate (Walker-Caprioglio *et al.*, 1985). Alexandre *et al.* (2001) have shown that high ethanol concentrations affect the expression of genes that are involved in protein biosynthesis, cell growth, RNA metabolism and cellular biogenesis and the genes up-regulated by ethanol are mainly involved in energetic metabolism, ionic homeostasis and stress response. One of the genes that was found to be induced by a high ethanol concentration is *ALD4*, a mitochondrial aldehyde dehydrogenase; the expression of this gene was *ADR1* dependent (Young *et al.*, 2003). This indicates that Adr1p is functional under high ethanol concentration conditions, suggesting that *ADH2* is not repressed by high ethanol concentrations. The possibility of the presence of a repressor being involved in *ADH2* regulation was excluded by Irani *et al.* (1987). Therefore, ethanol does not seem to act through any of the known repressors to repress *ADH2*. The low  $\beta$ -galactosidase activity obtained when the ethanol concentration in the medium was above 24 g l<sup>-1</sup> might be a result of the slow growth of the yeast, at high ethanol concentrations (Kubota *et al.*, 2004). Therefore, the apparent slow expression of the reporter gene might be due to the absence/low amounts of the transcription factors Adr1p and Cat8p as a result of the slowed growth during the adaptation of the yeast to the ethanol stress and not repression of the *ADH2* promoter by high ethanol concentration. The results of the  $\beta$ -galactosidase production at 30 g ethanol l<sup>-1</sup> confirmed that the *ADH2* promoter is not repressed by ethanol as similar maximum  $\beta$ -galactosidase specific activities were obtained to the maximum  $\beta$ -

galactosidase specific activity obtained during growth on 20 g ethanol l<sup>-1</sup> even though less biomass was produced on 30 g ethanol l<sup>-1</sup>.

Previous findings by du Preez *et al.* (2001) indicated repression of *ADH2* expression at an ethanol concentration above 17.9 g l<sup>-1</sup>, which is contrary to the results obtained in this study. This suggested that the ethanol effect on the expression of *ADH2* observed by du Preez *et al.* (2001) could be due to the existence of transcriptional misregulation since a multicopy expression system was used in their study or it might have been due to the presence of negative regulatory elements in the expression cassette used in the *ADH2* promoter-*XYN2* construct used in their study.

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## CHAPTER 5

### GENERAL CONCLUSIONS

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The aim of the study was to confirm the negative effect of ethanol on the expression of *ADH2* in the genomic copy of *Saccharomyces cerevisiae*. *ADH2* has been characterized as the major alcohol dehydrogenase isozyme required during ethanol utilization by *S. cerevisiae* (Ciriacy, 1975). Adh2p catalyses the conversion of ethanol to acetaldehyde and this is the initial reaction during ethanol assimilation. Therefore ethanol would not be expected to have inhibitory effect on the expression of this gene. The data obtained during this study is in agreement with this.

To study ethanol repression on *ADH2* expression, *S. cerevisiae* transformed with integrative plasmid cloned with *ADH2::LacZ* expression cassette was grown in different concentrations of ethanol (5, 20 and 30 g l<sup>-1</sup>). The data obtained during this cultivations was however different from that presented in the previous study (du Preez *et al.*, 2001). They showed that *ADH2* expression is repressed during growth at elevated ethanol concentration. They observed that during batch cultivation 17.9 g ethanol l<sup>-1</sup> repressed the expression of *ADH2*. From the chemostat experiment they established that derepression of *ADH2* occurs at ethanol concentration below 2.3 g l<sup>-1</sup>. In this study the lowest maximum  $\beta$ -galactosidase specific activity under derepressing conditions was obtained during growth on 5 g l<sup>-1</sup> with maximum specific activities of 2 533, 3 643 and 3 496 U protein mg<sup>-1</sup> obtained during growth on 5, 20 and 30 g ethanol l<sup>-1</sup> respectively (Chapter 4; Table 1, Fig. 4, 6 and 8). Although high maximum  $\beta$ -galactosidase specific activity was obtained during growth on 30 g l<sup>-1</sup>, the production rate (93 U mg<sup>-1</sup>h<sup>-1</sup>) of  $\beta$ -galactosidase was slow in comparison with the production rate observed during growth on 5 and 20 g ethanol l<sup>-1</sup> concentrations, with the production rates of 128 and 294 U mg<sup>-1</sup> h<sup>-1</sup>, respectively. The maximum specific

activity during growth on  $30 \text{ g l}^{-1}$  was only obtained when the ethanol concentration in the media was  $24 \text{ g l}^{-1}$ . This can be attributed to the slow growth of the yeast on  $30 \text{ g ethanol l}^{-1}$ . There was also a sudden increase in enzyme production on  $20 \text{ g ethanol l}^{-1}$  as compared to the enzyme production on  $5 \text{ g ethanol l}^{-1}$  and this may have indicated a possible ethanol induction of the *ADH2* gene. This was further supported by the high  $\beta$ -galactosidase production on  $30 \text{ g ethanol l}^{-1}$ . However available literature does not support this. Therefore further studies are required to confirm this, whereby a non fermentable carbon source such as glycerol can be used and the results be compared with those obtained during growth on ethanol.

The data obtained during glucose repression studies were as expected, as the lowest specific maximum  $\beta$ -galactosidase specific activity was achieved here. The  $\beta$ -galactosidase activity profile also showed that a decrease in glucose concentration was essential for the derepression of the promoter (Chapter 4; Fig. 2). The data obtained in this study suggests that the presence of ethanol increased the expression of *ADH2* and does not repress its expression. However since these results were obtained in batch cultivation experiments, a different picture might be observed in chemostat cultivations. Therefore further studies might be necessary to confirm ethanol effect on the expression of *ADH2* in continuous cultivations. Even so, when comparing these results with the results of batch cultivation obtained by du Preez *et al.* (2001), ethanol represses the expression of *ADH2* when the concentration is above  $24 \text{ g l}^{-1}$  as opposed to above  $17.9 \text{ g ethanol l}^{-1}$ .

This might suggest that the use of an integrative plasmid as opposed to a multicopy plasmid was more suitable for the expression study. Therefore these results might represent a true reflection of how ethanol might affect the expression of *ADH2* in the genome of *S. cerevisiae*, or using ethanol as a sole carbon source may affect the regulation of *ADH2* expression differently to when the ethanol is spiked into the medium.



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## CHAPTER 6

### SUMMARY

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**Key terms**

*Saccharomyces cerevisiae*, alcohol dehydrogenase, glucose repression,  $\beta$ -galactosidase, ethanol inhibition

The aim of this study was to investigate the ability of ethanol to repress the expression of *ADH2* in the genome of *Saccharomyces cerevisiae*. To achieve this, an expression cassette (*ADH2::LacZ*) using *LacZ* as a reporter gene was constructed using the YIp356R shuttle vector. A 1000 bp up and downstream region, flanking the open reading frame of *ADH2*, was fused to the 5'-end and 3'-end of the *LacZ* gene in the YIp356R shuttle vector. Numerous attempts were made to transform the expression cassette (*ADH2::LacZ*) into *S. cerevisiae* (strain W303) containing a deleted *ADH2* (*adh2 $\Delta$ ::URA3*), to displace the deletion cassette through homologous recombination, thereby placing *ADH2::LacZ* in the place of the *ADH2* in genome of *S. cerevisiae*. This was unfortunately not successful and it was decided to use an alternative approach. In this case the expression cassette was cloned into the integrative vector YIpIac211 and transformed into *S. cerevisiae*. For initial confirmation, the yeast transformants were grown on selective plates containing X-gal, which allows for the detection of  $\beta$ -galactosidase activity through the production of blue coloured colonies. The detection of the blue colour confirmed that the expression cassette was successfully constructed and integrated into the genome.

Two randomly selected transformants were cultivated on 20 g glucose l<sup>-1</sup> as sole carbon source, to study glucose repression and on three different ethanol concentrations to study the effect of ethanol on the expression of *ADH2*. Selection was maintained by growing the yeast in a URA<sup>-</sup> chemically defined media (pH 5.5) at 30°C. Samples were taken at appropriate intervals to perform

$\beta$ -galactosidase assay, assess utilization of substrate (ethanol and glucose), ethanol formation and biomass determination.

During growth on 20 g glucose l<sup>-1</sup> the production of  $\beta$ -galactosidase was apparent only when glucose concentrations were very low (2.3 g l<sup>-1</sup>), indicating that glucose levels have to decrease to a critical level before *ADH2* expression can resume. The highest final biomass was produced during growth on 20 g glucose l<sup>-1</sup>. During growth on the three different ethanol concentrations the highest  $\beta$ -galactosidase maximum specific activity was obtained during growth on 20 g ethanol l<sup>-1</sup> (3 643 U mg<sup>-1</sup>) and the lowest during growth on 5 g ethanol l<sup>-1</sup> (2 533 U mg<sup>-1</sup>). Although the maximum specific activity obtained during growth on 30 g ethanol l<sup>-1</sup> were higher than that obtained during growth on 5 g l<sup>-1</sup>, the production rate was the lowest (93 U mg<sup>-1</sup>h<sup>-1</sup>) during growth on 30 g ethanol l<sup>-1</sup>, suggesting that 30 g ethanol l<sup>-1</sup> concentration has negative effect on the expression of *ADH2*. However this slow production might have been due to the slow growth during this cultivation and not due to ethanol repression. The possible repression of *ADH2* is further disputed by the high  $\beta$ -galactosidase production on 30 g ethanol l<sup>-1</sup>.

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# CHAPTER 7

## OPSOMMING

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### Sleutelwoorde

*Saccharomyces cerevisiae*, alkoholdehidrogenase, glukose onderdrukking,  $\beta$ -galaktosidase, etanol-inhibisie

Hierdie studie was onderneem om vas te stel of etanol die uitdrukking van *ADH2* onderdruk. Om dit te bepaal is 'n uitdrukkings-kasset (*ADH2::LacZ*) waar *LacZ* gebruik is as verslaggewer-geen, gemaak deur gebruik te maak van die Yip356R pendelvektor. 'n Eenduisend basispaar gebied, wat weerskante van die oopleesraam van *ADH2* voorkom, is aan die onderskeie 5'- en 3' kant van die *LacZ* geen geheg in die pendelvektor YIp356R. Verskeie pogings is aangewend om die uitdrukkings-kasset (*ADH2::LacZ*) in die gis *Saccharomyces cerevisiae* (stam W303) wat 'n *ADH2* delesie (*adh2 $\Delta$ ::URA3*) bevat, te transformeer om te lei tot die vervanging van die delesie-kasset deur middel van homoloë rekombinasie. Dit was egter nie suksesvol nie en 'n alternatiewe prosedure is gevolg. In die geval is die uitdrukkings-kasset in die integrasievektor YIplac211 geplaas en getransformeer in die gis *S. cerevisiae*. Bevestiging vir die uitdrukking van  $\beta$ -galaktosidase is verkry deur die gemodifiseerde gisselle te groei op selektiewe plate wat X-gal bevat het en sodoende toelaat dat die teenwoordigheid van die ensiem  $\beta$ -galaktosidase bevestig kan word deurdat kolonies blou verkleur. Die verkleuring van kolonies het bevestig dat die uitdrukkings-kasset korrek voltooi is en in die genoom van die gis geïntegreer was.

Twee transformante wat lukraak gekies is, is gegroei in medium wat 20 g glukose l<sup>-1</sup> as enigste koolstofbron bevat het, om die effek van glukose repressie te bestudeer. Om die effek te bestudeer wat etanol uitoefen op die uitdrukking van

*ADH2*, is drie verskillende konsentrasies etanol as enigste koolstof bron gebruik. Gisselle is gegroei in selektiewe URA<sup>-</sup> media (pH 5.5) by 30°C. Monsters is geneem met aanvaarbare tussenposes om sodoende β-galaktosidase aktiwiteit, verbruik van koolstofbron (glukose en etanol), etanol vorming en biomassa te bepaal.

Gedurende groei in die teenwoordigheid van 20 g glukose l<sup>-1</sup> is die produksie van β-galaktosidase alleenlik waargeneem indien die vlak van glukose daal na 'n konsentrasie van 2.3 g glukose l<sup>-1</sup>. Dit dui aan dat die vlak van glukose moet verlaag tot 'n kritiese konsentrasie voordat *ADH2* uitdrukking kan plaasvind. Die hoogste finale biomassa is geproduseer gedurende groei in die teenwoordigheid van 20 g glukose l<sup>-1</sup>. Gedurende groei op die drie verskillende konsentrasies etanol was die hoogste spesifieke aktiwiteit van β-galaktosidase waargeneem met groei op 20 g etanol l<sup>-1</sup> (3 643 U mg<sup>-1</sup>) en die laagste vlak tydens groei op 5 g etanol l<sup>-1</sup> (2 533 U mg<sup>-1</sup>). Alhoewel die hoogste spesifieke aktiwiteit van β-galaktosidase, wat waargeneem is gedurende groei op 30 g etanol l<sup>-1</sup>, hoër was as die aktiwiteit wat waargeneem is tydens groei op 5 g etanol l<sup>-1</sup> was die produksie tempo van eersgenoemde (93 U mg<sup>-1</sup>h<sup>-1</sup>) die laagste. Dit mag moontlik aandui dat etanol teen 'n konsentrasie van 30 gl<sup>-1</sup> 'n negatiewe effek het op die uitdrukking van *ADH2*. Dit is egter ook moontlik dat die laer produksie tempo die gevolg was van die verlaagde groei tempo en nie as gevolg van etanol onderdrukking nie. Die verhoogde β-galaktosidase produksie wys egter teen die moontlikheid van etanol onderdrukking op die uitdrukking van *ADH2* in die teenwoordigheid van 30 g etanol l<sup>-1</sup>.

## REGULATION OF *ADH2* IN THE YEAST *SACCHAROMYCES CEREVISIAE*

### ABSTRACT

*ADH2* encodes for the main alcohol dehydrogenase isozyme that catalyses the conversion of ethanol to acetaldehyde during ethanol assimilation. Expression of this gene is known to be under glucose repression and is derepressed by growth on ethanol and other non-fermentable carbon sources. Results obtained by du Preez *et al.* (2001) showed, however, that this gene was repressed by growth on ethanol. These results were obtained using a multicopy expression vector with a promoter-terminator expression cassette derived from the *S. cerevisiae ADH2* gene. The aim of this study is the investigation of ethanol repression of *ADH2* in the genome of *S. cerevisiae*. To achieve this, an expression cassette using LacZ as a reporter gene was constructed using the Ylp356R shuttle vector. A 1000 bp up and downstream region, flanking the open reading frame of *ADH2*, was fused to the 5'-end and 3'-end of the LacZ gene. To study the effect of ethanol on the expression of *ADH2*, *Saccharomyces cerevisiae* transformed with an integrative plasmid containing the *ADH2::LacZ* fusion, was grown on 20 g glucose l<sup>-1</sup> as well as on three different ethanol concentrations (5, 20 and 30 g l<sup>-1</sup>). β-Galactosidase activity was measured as an indicator of *ADH2* expression. The highest β-galactosidase specific activity was obtained during growth on 20 g ethanol l<sup>-1</sup> (3 643 U mg<sup>-1</sup>) and the lowest during growth on 5 g ethanol l<sup>-1</sup> (2 533 U mg<sup>-1</sup>). At ethanol concentrations above 24 g l<sup>-1</sup> low β-galactosidase activity were obtained that might have been due to the low growth rate of the yeast on 30 g ethanol l<sup>-1</sup>. The data suggested that ethanol do not repress the expression of *ADH2*. These results are contrary to previous findings (du Preez et al., 2001) that ethanol at high concentrations repressed *ADH2* expression.