ANALYSIS OF *SACCHAROMYCES CEREVISIAE* DELETION MUTANTS DISPLAYING A MODIFIED CARBON FLUX UNDER WINE FERMENTATIVE CONDITIONS

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 23/02/2009

SUMMARY

Saccharomyces cerevisiae has been used for millennia for the leavening of dough and in the production of alcoholic beverages such as beer and wine. More recently, it is being used as cell factories for the production of important pharmaceutical products. *S. cerevisiae* has also been extensively used as a model organism for studying many genetic and biochemical processes within the eukaryotic cell. Since the completion of a yeast genome sequence, many functional analysis projects have emerged with the aim of elucidating the functions of the unidentified genes revealed by the genome sequence. One of the most relevant approaches consisted in the construction of a collection of mutants deficient in all single genes, either in a haploid background for non-essential genes, or as heterozygous diploids for essential genes. This collection of strains can be subjected to phenotypic screens that might reveal the function of unknown genes or add to our understanding of already annotated genes. While this approach is promising, it also bears some limitations. For instance, many mutants have no overt phenotypes and some phenotypes do not obviously showcase the function of the encoded protein.

In this study, *S. cerevisiae* strains with single deletions of genes involved in pyruvate metabolism were selected from the Euroscarf deletion library. Pyruvate is a central intermediate of glycolysis, and pyruvate metabolism largely defines the general distribution of carbon flux in the cell. These mutants were screened for modified fermentation kinetics or modified carbon flux under wine fermentative conditions, an environment that had not been previously used for the analysis of these mutants. A strain disrupted in the *PDA1* gene, which encodes the E1α subunit of the pyruvate dehydrogenase showed a significant change in phenotype when grown in wine fermentative conditions. In particular, the mutant displayed a prolonged lag phase, but upon entering exponential growth, fermented significantly faster than the wild type strain and completed alcoholic fermentation in a shorter period of time. This phenotype could be of significant industrial interest.

The mutant phenotype was further investigated through disruption of the gene in the same as well as in different genetic backgrounds, and through complementation of the *PDA1* deletion with a plasmid-born wild type copy. The data show that the *PDA1* gene disruption is not solely responsible for the observed phenotypes under wine fermentative conditions. We therefore propose that secondary mutations have contributed to the mutant phenotype. This study shows that phenotypes attributed to a specific gene in mutants of the Euroscarf library should always be confirmed before performing consequent experiments and drawing significant conclusions.

OPSOMMING

Saccharomyces cerevisiae word reeds vir millennia gebruik in die insuur van deeg en in die produksie van alkoholiese drankies soos bier en wyn. Dit is meer onlangs as selfabrieke vir die produksie van belangrike farmaseutiese produkte gebruik. *S. cerevisiae* is ook op groot skaal gebruik as ‘n modelorganisme in die bestudering van verskeie genetiese en biochemiese prosesse in die eukariotiese sel. Sedert die voltooiing van die gisgenoomvolgorde het baie projekte oor funksionele analise na vore gekom met die doel om die funksies van die ongeïdentifiseerde gene te verklaar wat deur die genoomvolgorde ontdek is. Een van die relevantste benaderings is die konstruksie van ‘n versameling van mutante waarin alle enkelgene ontbreek, óf in ‘n haploïede agtergrond vir nie-noodsaaklike gene óf as heterosigotiese diploïede vir noodsaaklike gene. Hierdie versameling rasse kan aan fenotipiese sifting blootgestel word, wat moontlik die funksie van die onbekende gene kan ontbloot óf ‘n bydrae kan maak tot ons begrip van gene wat reeds geannoteer is. Hoewel hierdie benadering belowend lyk, het dit ook ‘n paar beperkings. Baie van die mutante het byvoorbeeld geen klaarblyklike fenotipes nie and sommige fenotipes vertoon nie duidelik die funksie van die geënkodeerde proteïen nie.

In hierdie studie is *S. cerevisiae*-rasse met enkel delesies van gene wat in piruvaatmetabolisme betrokke is, uit die Euroscarf-delesiebiblioteek geselekteer. Piruvaat is ‘n sentrale tussenproduk van glikolise, en piruvaatmetabolisme bepaal grootliks die algemene verspreiding van koolstofvloei in die sel. Hierdie mutante is gesif vir gemodifiseerde gistingskinetika of gemodifiseerde koolstofvloei onder wyngistingstoestande, ‘n omgewing wat nog nie voorheen vir die analise van hierdie mutante gebruik is nie. ‘n Ras wat in die *PDA1*-geen onderbreek en wat die E1α-subeenheid van die piruvaatdehidrogenase enkodeer, het ‘n beduidende verandering in fenotipe getoon toe dit onder wyngistingstoestande gegroei is. Die mutant het ‘n duidelike verlengde sloerfase getoon, maar toe dit eksponensieel begin groei het, het dit noemenswaardig vinniger as die wilde-tipe ras begin gis en alkoholiese gisting in ‘n baie korter tydperk voltooi. Hierdie fenotipe kan moontlik van groot industriële belang wees.

Die mutantfenotipe is verder ondersoek deur die geen in dieselfde, asook verskillende genetiese agtergronde te onderbreek, en deur komplementering van die *PDA1*-delesie met ‘n plasmiedafkomstige wilde-tipe kopie. Die data toon dat die PDA1-geenonderbreking nie op sy eie vir die waargenome fenotipes onder wyngistingstoestande verantwoordelik is nie. Daar word dus voorgestel dat sekondêre mutasies tot hierdie mutantfenotipe bygedra het. Hierdie studie toon dat fenotipes wat aan ‘n spesifieke geen in mutante van die Euroscarf-biblioteek toegeskryf word, altyd bevestig moet word voordat gevolglike eksperimente uitgevoer en belangrike afleidings gemaak word.

This thesis is dedicated to my mother (Nondzuzo Madlanga) and grandmother (Vuyiswa Ndawule)

BIOGRAPHICAL SKETCH

Ncedile Hamilton Madlanga was born in Matatiele, South Africa on 27 February 1984. He attended Manguzela Junior Secondary School and matriculated in 2001 at Sinako Secondary School.

In 2002, he enrolled at the University of the Western Cape and obtained a BSc degree in Biotechnology. In 2006, he obtained an Honours degree from the Institute for Wine Biotechnology, Stellenbosch University. The following year, 2007, he enrolled for an MSc degree at the same institution.

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PREFACE

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the journal *Yeast*.

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Chapter 2 **Literature review**
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Chapter 3 **Research results**
Screening and analysis of *Saccharomyces cerevisiae* deletion mutants displaying a modified fermentation behaviour and carbon flux under wine fermentative conditions

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Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

A small number of species has been preferentially used for intensive research in order to understand the general cellular and molecular processes that sustain life, and to investigate the evolutionary relationships of organisms. These species are usually referred to as model organisms, and include *Escherichia coli* (bacterium); *Saccharomyces cerevisiae* (budding yeast); *Schizosaccharomyces pombe* (fission yeast); *Caenorhabditis elegans* (nematode); *Drosophila melanogaster* (insect); *Danio rerio* (fish); *Arabidopsis thaliana* (plant) and *Mus musculus* (mammal). Several parameters have led to these species becoming central to molecular investigations of living systems, and include the fact that they are easy to house, propagate and manipulate in the laboratory and ideally have a fairly small or relatively uncomplicated genome (June 2, 2003 V17; *The Scientist*). Among these model organisms, five (baker's yeast, round worm, fruit fly, zebrafish and mouse) are frequently used to discover and validate novel drug targets because of the similarity of their genomic sequences to those of humans (Muda and McKenna, 2004).

S. cerevisiae has been extensively used as a model organism for studying genetic and biochemical processes (Game, 2002) and also in the development of new technologies that are applicable in fundamental and applied research (Muda and McKenna, 2004). The yeast genome contains about ~6000 protein-encoding genes of which over one thousand are still considered of unknown function (www.yeastgenome.org/cache/genomeSnapshot.html) (Game, 2002; Ross-Macdonald, 2000). Assigning biological functions to these genes is one of the major challenges because many of these genes do not result in overt phenotypes upon disruption (Birrell *et al*., 2001; Johnston, 1996; Cutler and McCourt, 2005). This may be due to limitations in the screening platforms or to functional redundancy of these genes due to duplicate genes or alternative metabolic pathways or regulatory networks (Gu *et al*., 2003). The establishment of a yeast deletion library however now allows for systematic screening of phenotypes (Oliver, 1996). This deletion library consists of a collection of yeast mutants deleted for single genes or open reading frames ORFs (Winzeler *et al*., 1999). This collection consists of haploid strains with deletions of non-essential genes, whereas heterozygous diploid strains have been generated for all essential genes. (Game, 2002). Today, the combination of both systematic screening and traditional *ad hoc* approaches promises to reveal the functions of more of the unknown genes (Johnston, 1996).

At present, several methods of phenotypic screening of these mutants are used for functional characterization of genes (Carpenter and Sabatini, 2004). Using these methods, genes that were previously not known to affect certain phenotypes have been identified (Birrell *et al*., 2001). Phenotypic analysis is based on several features such as growth, resistance to toxins such as metal ions (Weiss *et al.*, 2004), metabolite concentrations (Allen *et al*., 2003) and others. The term "metabolomics" describes technologies that allow the identification and quantification of many intracellular and extracellular metabolites, which serve as indicators of metabolic activities. These technologies can also be used for mutant characterization (Allen *et al*., 2003). Indeed, just like proteins, the levels of metabolites change in response to the physiological, developmental, or pathological state of the cell, organ, or organism (Raamsdonk *et al*., 2001).

Although the mutant-based approach is good in allowing the establishment of a link between genotype and phenotype, and therefore in suggesting a function of a gene, it

possesses some limitations. For instance, point mutations in some genes confer phenotypes that are not seen in or different from the deletion mutants (Game, 2002). Phenotypic changes can also occur as a result of secondary mutations, which can include events such as anaeuploidisation or spontaneous diploidization, or that are introduced during transformation (Game, 2002). Such changes can therefore result in false interpretation of the data.

Despite the wealth of data that is available on *S. cerevisiae* at the level of the genome, the transcriptome, the proteome and the metabolome, the amount of information available on wine yeasts or yeasts grown under wine fermentative conditions is limited. This can be attributed to the complexity of the wine making process and to the fact that wine yeasts are mainly diploid, aneuploid or polyploid and show a high level of chromosome length polymorphism (Pérez-Ortín *et al*., 2002), and are therefore more difficult to analyse on a molecular level.

In this project, the impact of gene deletions on fermentation performance and carbon flux in a high sugar environment – synthetic grape must - was assessed. Such conditions are rarely used in laboratories, since they lead to sub-optimal growth. Phenotypic analysis of a selected set of yeast deletion mutants under wine fermentative conditions might therefore reveal unexpected phenotypes. The project forms part of a larger effort to identify genes that show modified carbon flux in such conditions, and focused in particular on the set of genes whose products are responsible for pyruvate metabolism. Pyruvate is a central metabolite in glycolysis, and can be further metabolized through several pathways. Pyruvate production and utilisation indeed depends on many parameters, in particular on the type and concentration of the available carbon source and on the availability of oxygen (Pronk *et al*., 1996).

In general, pyruvate metabolism proceeds via the enzymes of pyruvate carboxylase, pyruvate dehydrogenase and pyruvate decarboxylase. These enzymes are coded for by multiple gene isoforms (Pronk *et al*., 1996) some of which are transcribed at high levels (Pronk *et al*., 1996; Velculescu *et al*., 1997). During growth on fermentable carbon sources, pyruvate production occurs as a result of an irreversible metabolic route that is catalyzed by pyruvate kinase (Pykp). This enzyme is coded for by two gene isoforms, *PYK1* (Sprague Jnr, 1977) and *PYK2* (Boles *et al*., 1997). However, when non-fermentable carbon sources are used pyruvate is formed via pyruvate carboxylase. Under aerobic conditions pyruvate is oxidatively decarboxylated to acetyl-CoA and carbon dioxide by combined activities of the enzymes of the pyruvate dehydrogenase (PDH) complex. This reaction occurs when the sugar concentration does not exceed the capacity of the enzymes of the complex (Pronk *et al*., 1996). However, under fermentative conditions pyruvate is rapidly metabolized into ethanol and carbon dioxide in the process of alcoholic fermentation. During this process pyruvate decarboxylase catalyzes the first irreversible step of pyruvate metabolism. This reaction is highly reinforced by high sugar concentrations. (Pronk *et al*., 1996; Hohmann and Meacock, 1998). More specifically, under both anaerobic and high sugar concentrations the activity of pyruvate decarboxylase is induced (Zimmermann and Entian, 1997). A proportion of pyruvate is metabolized via pyruvate carboxylase (Pyc) enzyme. This metabolic route is apparently not influenced by sugar levels or oxygen, and is important as an *anaplerotic* reaction for the replenishment of the tricarboxylic acid cycle intermediates during growth on fermentable carbon sources (Blazquez *et al*., 1995).

Since pyruvate metabolism plays such a crucial role in the distribution of carbon flux, this study focused on evaluating the impact of deletions in the genes responsible for these metabolic activities on fermentative growth and carbon flux in a high sugar environment. Such environmental conditions have not been assessed previously. Velagapudi *et al*. (2006) showed that metabolic screening of single knockout strains is able to reveal unexpected phenotypes when assessed in unusual environments. In this regard, wine making conditions lead to a combination of several stresses (e.g. high sugar stress initially, ethanol toxicity and nutrient

depletion), which are known to cause enormous changes in the levels of proteins and metabolites. As a consequence, unexpected mutant phenotypes may be revealed.

1.2 PROJECT AIMS

This study forms part of a broader research effort that attempts to gain better understanding of carbon flux in *S. cerevisiae* during high sugar fermentation conditions using single knockout strains from the Euroscarf Yeast Deletion Library.

The specific aims and approaches of this study were as follows:

(i) to select and screen mutant strains from Euroscarf deletion library that are deficient in single genes involved in pyruvate metabolism for modified fermentation kinetics and carbon flux or partitioning under simulated wine fermentative conditions; and

(ii) to do in-depth analysis of the mutant strains selected from Aim (i) with the aim of elucidating the role of the knocked out gene in the modification of carbon flux or fermentation characteristics.

The results obtained in the pursuit of these two aims are described separately in the Result section of this thesis as Chapter 3 and Chapter 4, respectively.

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Chapter 2

LITERATURE REVIEW

2. CARBON METABOLISM IN *SACCHAROMYCES CEREVISIAE*

2.1 INTRODUCTION

The yeast *Saccharomyces cerevisiae* is one of the important micro-organisms and widely used model eukaryotic systems. Since the completion of its genome sequence, many research projects have been dedicated to unravel the different levels of cellular complexity (genome, transcriptome, proteome and metabolome) (Pizarro *et al*., 2007). Furthermore, its eukaryotic nature and GRAS (Generally Regarded As Safe) status have made *S. cerevisiae* gain popularity in many other industrial sectors (Pretorius, 2000). *S. cerevisiae* has a diverse range of industrial applications such as metabolite-directed as well as biomass-directed applications. In metabolite-directed applications, *S. cerevisiae* is used for the production of low-molecular weight metabolites (e.g. ethanol, glycerol, carbon dioxide, flavour compounds) (Barnett, 2003a). For the production of numerous metabolites (e.g. ethanol, glycerol) alcoholic fermentation is desired (Flikweert *et al*., 1997).

The use of *S. cerevisiae* for the production of alcoholic beverages, in particular wine, dates back as early as 3150 BC (Cavalieri *et al*., 2003). Its use in the beverage industry has been improved and it is now used for beer and sake production. *S. cerevisiae* has been of utmost importance in the weaponry industry due to its ability to produce glycerol which was practically used for manufacturing of powerful explosive nitroglycerine during World War I, and it is now used in soap industry (Barnett, 2003a). Its use further extends to the bakery industry where *S. cerevisiae* is used for the leavening of bread (Carnevali *et al*., 2007). In biomass-directed applications, this yeast is being used as a cell factory for the production of industrially important products, such as insulin and heterologous proteins and for baker's yeast production (Romanos *et al*., 1992; Pizarro *et al*., 2007). Maintaining of strict conditions such as sufficient aeration and lower glucose concentrations is necessary during these production processes to avoid occurrence of alcoholic fermentation, a process that becomes a drawback because of low ATP yields (Flikweert *et al*., 1997). Low ATP yields are known to be associated with low biomass yields (Tai *et al*., 2005).

S. cerevisiae is one of the very few species of yeast capable of near-anaerobic growth. Under such conditions sugars are rapidly converted into ethanol and carbon dioxide in a process called alcoholic fermentation. During alcoholic fermentation sugars mainly glucose and fructose are first taken up by the yeast through hexose transporters into the cytoplasm. Within the yeast cell these sugars are consecutively metabolized for the supply of both energy and biosynthetic precursors (Ye *et al*., 1999). The metabolized sugars form two molecules of pyruvate via the glycolytic pathway and this results in the production of two ATP (energy) molecules and two NADH + H^+ from the phosphorylation of two ADP and reduction of two NAD^+ respectively. Both ATP and $NADH^+$ produced within the cell are converted back to the original compounds throughout metabolic reactions. The overall redox balance of the cell is maintained through the continuous regeneration of these compounds (Barnett, 2003a). During anaerobic growth alcoholic fermentation appears to be the only mode of energy production (Postma *et al*., 1989).

In *S. cerevisiae* glucose catabolism mainly occurs through the processes of glycolysis, the pentose phosphate pathway (PPP) and the TCA cycle (respiration) which altogether contribute to the central carbon metabolism (Figure 1). There are a number of factors that determine which pathway dominates (Postma *et al*., 1989). In the presence of oxygen, yeast

physiological changes occur resulting in the oxidation of sugar to form carbon dioxide and thus generate energy via the respiratory pathway of the TCA cycle (Barnett, 2003b). This process involves a series of biochemical reactions. For instance, electrons are being transferred from NADH oxidation to the respiratory chain resulting in the reduction of atomic oxygen to water. Translocation of protons across the mitochondrial inner membrane follows, thereby generating an electro-chemical gradient that is used for the formation of ATP from ADP and inorganic phosphate (de Vries *et al*., 1988). Aerobic conditions appear to greatly reinforce respiration provided that glucose concentration is not in excess. Even under aerobic conditions, high glucose concentrations lead to repression of transcription of genes encoding enzymes involved in the TCA cycle and trigger alcoholic fermentation. This phenomenon is called the Crabtree effect (Petrik *et al*., 1983). Aeration seems to have no profound influence on the PPP if exclusively the carbon source is in excess. However, in carbon-starved cells aeration intensely influences the accumulation of storage carbohydrates via the PPP (Thomsson *et al*., 2003) and ethanol production (Verduyn *et al*., 1990).

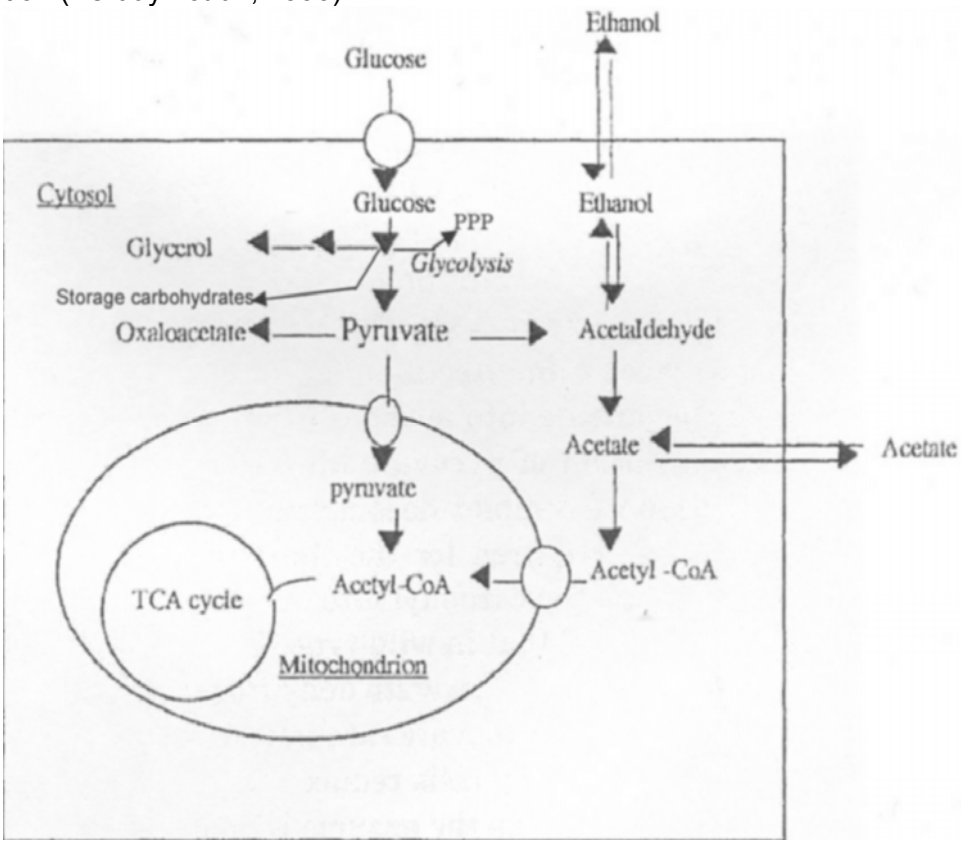

Figure 1. Schematic representation of central carbon metabolism in *S. cerevisiae*: glycolysis as the root of other carbon networks including storage carbohydrates (glycogen & trehalose), the pentose phosphate pathway (PPP), and the TCA cycle (adapted from Boubekeur *et al*., 1999).

2.2 *SACCHAROMYCES CEREVISIAE* AND WINE FERMENTATION

Various yeast species dominate the complex process of wine making. In spontaneous fermentations native microflora derived from grapes and the winery conduct the fermentation process. Non-*Saccharomyces* yeasts usually dominate initially but during the progress of alcoholic fermentation these yeasts are out-competed by the alcohol-tolerant strains of *S. cerevisiae*. *S. cerevisiae* is therefore universally known as the 'wine yeast' and is widely used for the wine making process (Bauer and Pretorius, 2000).

By definition, wine is an alcoholic beverage, a final product of fermentation of grape juice by yeasts (e.g. *S. cerevisiae*) and is extremely popular throughout the world (This *et al*., 2006). Wine making is an ancient art dating back to more than 8000 years and wine production is therefore considered one of the oldest biotechnological processes (Pretorius, 2000; Pizarro *et al*., 2007). Wine is also a highly complex fermentation product because of the large number of flavour and aroma active compounds. All these constituents are derived from grapes, and the metabolism of yeast species (non-*Saccharomyces* and *Saccharomyces*) and acetic and lactic acid bacteria (Pizarro *et al*., 2007).

Wine can be produced by either spontaneous or inoculated fermentations. During spontaneous fermentations there is a progressive growth pattern of indigenous yeasts that sequentially succeed each other as the must conditions change over time. Nevertheless, *S. cerevisiae* invariably dominates the final stages of alcoholic fermentation. Spontaneous fermentation is still employed by many wineries; as a consequence of the scientifically unsupported believe that wines produced from natural microflora possess distinct sensorial quality often described as wines with fuller, rounder palate structure. If true, these wine characters may be attributed to the consequence of higher concentrations of glycerol and other polyols produced by indigenous yeasts. Spontaneous fermentation, however, imposes risk due to the lack of predictability or reproducibility of the final product (Pretorius, 2000).

Nowadays, pure yeast starter cultures are widely used to inoculate grape must. The addition of pure starter cultures allows better control of the fermentation since it ensures the predominance of a known yeast strain and thus minimizes the influence of yeasts other than inoculated strains (Pretorius, 2000; Fleet, 2008; Pizarro *et al*., 2007). More importantly starter cultures should possess a range of desirable properties such as specific flavour production characteristics as well as other metabolic, technological and fermentation properties (Pretorius, 2000). Since it is practically impossible to have a yeast strain possessing all the desired characteristics, many different starter cultures with specific attributes are used for different types and styles of wines. For the production of dry wines yeast starter cultures with improved fermentation performance (e.g. rapid initiation of fermentation, greater efficiency in sugar and nitrogen utilization, increased ethanol tolerance and moderate biomass production) are highly desirable (Pretorius, 2000; Bisson, 2004).

2.3 CENTRAL CARBON METABOLISM: FOCUS ON PYRUVATE METABOLISM

The natural environment where *S. cerevisiae* dwells contains a broad set of carbon sources that the yeast can exploit for its growth. Amongst these carbon sources *S. cerevisiae* preferentially utilize sugars such as hexoses (e.g. glucose, fructose, galactose, and mannose) and disaccharides (e.g. maltose and sucrose) (Zimmermann and Entian, 1997). The metabolism of these sugars occurs via the same pathways of glycolysis, the TCA cycle, and the PPP with the exception of ethanol and acetate, which serve in the anabolic pathway (gluconeogenesis) (Roman, 1957; Rodrigues *et al*., 2006).

2.3.1 THE GLYCOLYTIC PATHWAY

In *S. cerevisiae*, glycolysis plays a central role in sugar metabolism. It is the backbone of several different pathways which lead primarily to the production of biomass, ethanol and carbon dioxide (Rodrigues *et al*., 2006). The first step for glycolysis to begin is the transport of hexoses into the yeast cell. Carrier-mediated hexose transport across the plasma membrane is

an essential step in the metabolism of glucose by *S. cerevisiae* (Diderich *et al*., 1999; Boles and Hollenberg, 1997). This sugar transport system exerts control of the flux and rate of glycolysis and also determines the relative activities of the fermentation and respiratory pathways of glucose metabolism (Boles and Hollenberg, 1997). For example, lower rates of glucose transport reinforce glucose oxidation via the respiratory pathway (Ye *et al.*, 1999).

Sugar transport across the yeast plasma membrane is conducted by a family of hexose transporters (Hxt) and occurs by facilitated diffusion (Carlson, 1998). The *HXT* gene family consists of 20 genes, encoding Hxt1-Hxt17, the Gal2 transporters and *SNF3* and *RGT2* (encoding putative sensors of low and high glucose concentrations, respectively). Amongst this family, hxt1p to hxt4p and hxt6p to hxt7p are considered the major hexose transporters in *S. cerevisiae* (Ozcan and Johnston, 1999). The expression of these *HXT* genes differs according to their affinity for glucose, with hxt6p and hxt7p classified as high affinity transporters and hxt1p and hxt3p as low affinity transporters (Reifenberger *et al*., 1997). At low glucose levels, snf3p is induced and inhibits expression of Rgt1p, a repressor, which consequently results in increased transcription of the major hexose transporters (*HXT1-4* and *HXT6* and *7*). At high glucose concentrations snf3p is however repressed and the high glucose sensor Rgt2p triggers Grr1p-dependant conversion of Rgt1p into a transcriptional activator to allow the expression of *HXT1* gene (Figure 2) (Boles and Hollenberg, 1997).

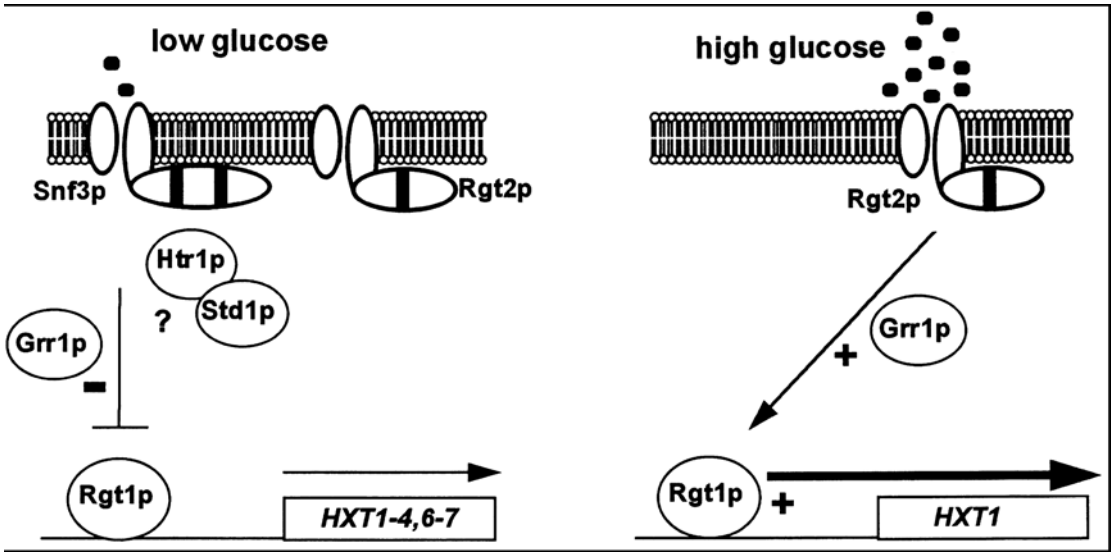

Figure 2. Transport of hexoses across the plasma membrane: the roles of Snf3p and Rgt2p in mediating hexose transport across the membrane and in regulating transcription of *HXT* genes (Adapted from Boles & Hollenberg, 1997).

Gal2p transports glucose with high affinity provided that galactose is present in the medium (Diderich *et al*., 1999; Carlson, 1998; Rintala *et al*., 2008). The *HXT* gene expression is mainly regulated at a transcriptional level by the levels of external glucose. *HXT5* gene is the only exception with its expression being regulated by growth rate rather than the extracellular glucose concentration (Verwaal *et al*., 2002). When glucose is absent *HXT* genes are repressed by the transcription factor Rgt1p. Glucose starvation is not the only factor that imposes inactivation of Hxt proteins, high glucose concentrations have also been shown to inactivate these proteins (Ozcan and Johnston, 1999). Additionally, oxygen levels have been shown to influence the expression of *HXT* genes with the expression of *HXT2, HXT4* and *HXT5* (encoding low affinity transporters) genes being high in aerobic conditions, whereas *HXT6* and *HXT13* (encoding high affinity transporters) expression is high in hypoxic conditions (Rintala *et al*.,

2008). From the natural occurring carbon sources, glucose is utilized preferentially to other carbon sources. The preference for glucose occurs as a result of transcriptional repression of genes that are required for respiratory metabolism and utilization of other carbon sources (Gancedo, 1998). Glucose transport activity also plays an important role in influencing glucose repression of such genes (Ye *et al*., 1999).

Once glucose is inside the cell, a small percentage of sugar is metabolized via the PPP, which plays critical roles of acting as a major cellular source of NADPH and also generating precursors for the synthesis of nucleotides and amino acids (Heux *et al*., 2008). However, a large percentage of glucose enters the ten step enzyme reaction of glycolysis which is considered as the main pathway during the two modes (respiration and alcoholic fermentation) of sugar metabolism. Glycolysis leads to pyruvate formation and a net production of energy (as ATP) and reducing equivalents (Rodrigues *et al*., 2006).

2.3.1.1 PYRUVATE METABOLISM

In *S. cerevisiae*, pyruvate metabolism proceeds exclusively via the thiamine diphosphate (ThDP) enzymes of pyruvate decarboxylase (Pdc) and the pyruvate dehydrogenase (PDH) complex (Hohmann and Meacock, 1998). Pyruvate is located at the important interface between respiratory and fermentative carbon metabolism. At this branch point pyruvate acts as a linkage between glycolysis and the TCA cycle. It is at the pyruvate level where flux is distributed between respiration and fermentation. In *S. cerevisiae*, ATP can be produced by either respiration or alcoholic fermentation and these two processes compete for pyruvate and NADH. Flux distribution at the pyruvate level depends on environmental factors (e.g. oxygen and concentration of sugars) and on the yeast strain used. In addition to the regulation by these factors, the balance between respiration and fermentation in yeasts is determined by several other metabolic phenomena such as the 'Pasteur effect', 'Custers effect', 'Crabtree effect' and 'Kluyver effect'. In general, pyruvate metabolism occurs as a result of three metabolic routes which are the decarboxylation, carboxylation and dehydrogenation of pyruvate (Figure 3). These routes are catalyzed by independent enzymes encoded by different gene families (Table 1) (Pronk *et al*., 1996).

Table1. Enzymes involved in pyruvate petabolism in *S. cerevisiae*, their structural genes, open reading frames, substrates and products.

Enzyme	Structural gene	ORF	Reaction	Product(s)	Reference
Pyruvate carboxylase (EC 6.4.1.1)	*PYC1*	YGL062w	Carboxylation of pyruvate	Oxaloacetate	Pronk *et al*., 1996
	PYC2	YBR218c	Carboxylation of pyruvate	Oxaloacetate	
Pyruvate decarboxylase (EC 4.1.1.1)	*PDC1*	YLR044c	Decarboxylation of pyruvate	Acetaldehyde + CO_2	Pronk *et al*., 1996
	PDC5	YLR134w	Decarboxylation of pyruvate	Acetaldehyde + CO_2	Pronk *et al*., 1996
	PDC6	YGR087c	Decarboxylation of pyruvate	Acetaldehyde + CO_2	Pronk *et al*., 1996
Pyruvate dehydrogenase complex					
E1α subunit (EC 1.2.4.1)	*PDA1*	YER178c	Decarboxylation of pyruvate	Acetyl-CoA	Pronk *et al*., 1996; Wenzel *et al*., 1992
E1β subunit (EC 1.2.4.1)	*PDB1*	YBR221c			Pronk *et al*., 1996; Wenzel *et al*., 1992
E2 subunit (EC 2.3.1.12)	*LAT1*	YNL071w	Transfer of acetyl group to CoA		Pronk *et al*., 1996; Wenzel *et al*., 1992
E3 subunit (EC 1.6.4.3)	*LPD1*	YFL018c	Reoxidation of lipoamide		

Protein X	*PDX1*	YGR193c	Binding of E3 to E2 core		Pronk *et al*., 1996
Pyruvate kinase (EC 2.7.1.40)	*PYK1*	YAL038w	Conversion of PEP to pyruvate	Pyruvate	Portela *et al*., 2006
	PYK2	YOR347c	Conversion of PEP to pyruvate	Pyruvate	Boles *et al*., 1997
Phosphoenolpyruvate carboxykinase (EC 4.1.1.32)	*PCK1*	YKR097w	Conversion of oxaloacetate to PEP	PEP	Valdes-Hevia *et al*., 1989

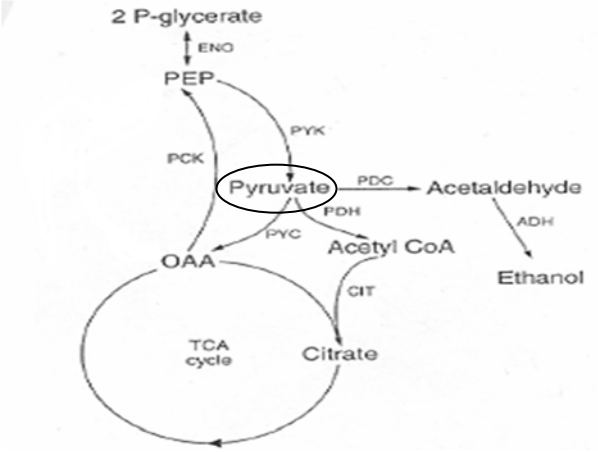

Figure 3. Metabolism of sugars at a pyruvate level occurs as result of 'concerted' action of various gene products encoded by different genes. Abbreviations: PYK, pyruvate kinase; PYC, pyruvate carboxylase; PCK, PEP carboxykinase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase complex; OAA, oxaloacetate; TCA, tricarboxylic acid cycle (This diagram is adopted from Flores and Gancedo, 1997).

The structural genes encoding the enzymes involved in pyruvate metabolism are transcribed at high levels and exist in multiple isoforms (Table1) (Pronk *et al*., 1996; Velculescu *et al*., 1997). For instance, structural genes encoding pyruvate decarboxylase enzymes exist in six isoforms with varying expression levels with *PDC1*, *PDC5* and *PDC6* considered the most influential (Pronk *et al*., 1996). In most cases where genes exist in multiple isoforms, some of these gene-encoded enzymes are not distinctly influential on those metabolic routes and their activity can sometimes only be observed in yeast mutant(s) having one or few gene isoforms mutated or deleted. The increase in the expression levels of the undisrupted gene isoforms and their respective enzyme activities in yeast mutants occur as a way to compensate for the disrupted gene and such phenomenon is called gene compensation (Wagner, 2000).

At the pyruvate branch point multiple gene isoforms exist, whose expression directly contribute to pyruvate synthesis and metabolism. These genes encode the following enzymes: pyruvate kinase, pyruvate carboxylase, pyruvate decarboxylase, phosphoenolpyruvate carboxykinase and enzymes of the pyruvate dehydrogenase complex.

2.3.1.1.1 Pyruvate kinase (*PYK*)

In *S. cerevisiae*, the glycolytic enzymes comprise up to 30 – 50% of total soluble protein and most of these enzymes catalyze reversible reactions that are used during growth on gluconeogenic carbon sources (Zimmermann and Entian, 1997). However, two glycolytic enzymes that are irreversible in reactions are 6-phosphofructo-1-kinase and pyruvate kinase, encoded by *PFK1* and *PYK1* respectively (Pearce *et al*., 2001). For long, *PYK1* gene was considered the only gene responsible for the conversion of phosphoenol pyruvate (PEP) to

pyruvate (Sprague Jnr, 1977). Just after the yeast genome sequence was completed, a second functional pyruvate kinase isoenzyme Pyk2 was identified and characterized (Boles *et al*., 1997).

The structure of Pyk1 enzyme is composed of monomers that are encoded by *PYK1* (Burke *et al*., 1983), a gene that is regarded as highly expressed among glycolytic genes (Nishizawa *et al*., 1989). The Pyk1 enzyme consists of approximately 500 amino acids and has a molecular mass of 54,5 kDa (Boles *et al*., 1997). The catalytic activity of Pyk enzyme is modulated by a number of positive and negative effectors including fructose 1,6 biphosphate (FBP), adenosine 5'-triphosphate (ATP) and citrate (Sprague Jnr, 1977). In the absence of FBP, the Pyk1 enzyme is largely inactive. When large amounts of PEP are present, Pyk activity is re-established (Maitra & Lobo, 1977; Fernandez *et al*., 1967). In other organisms, the activity of Pyk enzymes is regulated by other metabolites with prokaryotic Pyk activity being induced by either FBP or sugar phosphates while in trypanosomes Pyk is activated by fructose-2,6-biphosphate. The regulation of the activity of Pyk isoenzymes seems to differ, with Pyk2p activity displaying insensitivity to FBP (Boles *et al*., 1997). Pyk enzyme catalyzes the terminal and net energy producing reaction of glycolysis (Sprague Jnr, 1977; Fernandez *et al*., 1967; Burke *et al*., 1983). This is an irreversible reaction and results in the addition of a proton and formation of pyruvate and ATP from PEP (Boles *et al*., 1997). Schematically this reaction is presented as follows:

$$\text{ADP} + \text{PEP} \xrightarrow[\text{Pykp}]{P_i,\ H^+} \text{ATP} + \text{Pyruvate}$$

In yeast, the *PYK1* gene is constitutively expressed at a high basal level and such expression is apparently regulated at the level of transcription (Burke *et al*., 1983). Addition of glucose results in significantly enhanced expression of *PYK1* gene (Pearce *et al*., 2001). The expression of both *PFK1* and *PYK1* genes is also subjected to allosteric regulation in yeast. The mammalian Pyk however displays hyperbolic kinetics and lacks allosteric regulation (Boles *et al*., 1997). Furthermore, the regulation of the yeast *PYK1* at both transcriptional and translational levels depends on the *PYK1* promoter and 5' leader sequence (Pearce *et al*., 2001).

Deletion of *PYK1* gene renders a mutant strain that has lost the ability to grow on fermentable sugars. Several possible mechanisms for this phenotype have been postulated, such as the repression of cytochromes or other enzymes of the oxidative metabolism, ATP depletion or toxicity of accumulated metabolites such as PEP and phosphoglycerates (Ciriacy and Breitenbach, 1979; Clifton *et al*., 1978; Maitra and Lobo, 1977). However, deletion of *PYK2* gene has no obvious growth phenotypes (Boles *et al*., 1997).

2.3.1.1.2 Pyruvate carboxylase (*PYC*)

During respiratory growth on various carbon sources, the respiratory machinery of the TCA cycle plays a key role in the supply of precursors essential for several reactions including gluconeogenic, amino acid and porphyrin biosynthesis. Without a constant replenishment of the intermediates of the TCA cycle, the TCA cycle will be drained off by these biosynthetic reactions resulting in loss of operational capability of the cycle. To prevent this depletion of intermediates, yeast has two native anaplerotic reactions that replenish the cycle. The first is the pyruvate

carboxylase (Pyc) catalyzed reaction and the second is the glyoxylate cycle (de Jong-Gubbels *et al*., 1998).

In *S. cerevisiae*, the key anaplerotic enzyme is Pyc enzyme, which exists as two isoenzymes. The Pyc isoenzymes are encoded by separate genes, *PYC1* and *PYC2* (Walker *et al*., 1991). The localization of the Pyc enzymes differs from one organism to the other, for instance, in yeast they are located in the cytosol, whereas those in mammalian cells are mitochondrial (Huet *et al*., 2000). Structurally, the Pyc enzyme has three functional domains, namely: an *N*-terminal ATP-binding domain, a central pyruvate-binding domain and a *C*-terminal biotinyl carrier domain (Sueda *et al*., 2004; Val *et al*., 1995). The biotin domain displays some sequence homology to lipoyl domains of the pyruvate dehydrogenase and the protein in the glycine-cleavage system (Val *et al*., 1995). The Pyc isoenzymes exist in two forms: a single polypeptide chain type and subunit type. They are composed of approximately 1200 amino acids and are conserved in eukaryotes and some prokaryotes (Sueda *et al*., 2004). The coding regions of both *PYC* genes exhibit a 90% and 85% sequence similarity at the levels of amino acid and nucleotide, respectively. Furthermore, both Pyc isoenzymes have relatively similar K_m values for ATP and pyruvate (Stucka *et al*., 1991). On the other hand, their open reading frames (ORFs) differ by a few bases, for instance, open reading frame of *PYC2* is reduced by 15 bases due to a single base deletion near the 3' end (Val *et al*., 1995).

The activity of Pyc enzyme is regulated by different effectors that act as activators (e.g. acetyl-CoA and palmitoyl-CoA) and inhibitors (e.g. aspartate) (Brewster *et al*., 1994). On the other hand, the nature of the nitrogen source in glucose mineral medium exerts control over the activity of this enzyme, because substitution of ammonium ions for aspartate results in a three to four fold increase in Pyc enzyme activity (Haarasilta and Oura, 1995). In many organisms including yeast, Pyc enzyme catalyzes a two step carboxylation of pyruvate to oxaloacetate (OAA), a reaction that is important in two levels: firstly, for the supply of OAA essential in gluconeogenesis and secondly for in the replenishment of TCA cycle intermediates (Attwood, 1995). The Pyc catalyzed reaction can be schematically presented as follows:

$$\text{MgATP} + \text{HCO}^-_3 + \text{ENZ-biotin} \xleftrightarrow[\text{Pycp}]{\text{Mg}^{2+}\text{, acetyl CoA}} \text{MgADP} + \text{Pi} + \text{ENZ-biotin-CO}^-_2 \qquad [1]$$

$$\text{ENZ-biotin-CO}^-_2 + \text{pyruvate} \xleftrightarrow[\text{Pycp}]{} \text{ENZ-biotin} + \text{oxaloacetate} \qquad [2]$$

During the first step of this reaction, the Pyc enzyme bound to the biotin moiety catalyzes the Mg^{2+}, ATP dependant carboxylation. In the second step, the enzyme-biotin-carboxyl complex dissociates releasing free biotin dependant Pyc enzyme and a carboxyl group that together with pyruvate forms oxaloacetate (Attwood, 1995).

In *S. cerevisiae*, the expression profiles of *PYC1* and *PYC2* differ with *PYC1* displaying a constant level of expression during growth in minimal glucose medium, whereas the expression *PYC2* is mainly observable in the early phases of growth (Brewster *et al*., 1994). The expression of these genes is regulated in response to carbon and nitrogen source. For instance, the expression of *PYC1* is repressed during growth in minimal glucose medium with ammonium (e.g. aspartate, glutamate) as nitrogen source, whereas arginine, leucine, threonine and methionine results in 1.5 to 3 fold increase in *PYC1* expression compared to ammonium (Huet *et al*., 2000).

However, disruption of *PYC1* leads to an aspartate requiring phenotype during growth on ethanol (Brewster *et al*., 1994), while simultaneous disruption of both *PYC1* and *PYC2* renders a strain that has growth defects on glucose-ammonium medium. Addition of aspartate restores growth of a Δ*pyc1pyc2* double mutant under the same conditions (Stucka *et al*., 1991). The aspartate requiring phenotype has also been observed in other yeasts deficient of Pyc enzyme (Ozimek *et al*., 2003). Aspartate provides OAA via transamination with α-ketoglutarate, thereby replenishing the TCA cycle. In *S. cerevisiae*, only Pyc and enzymes of the glyoxylate cycle are anaplerotic reactions. During growth on glucose enzymes of the glyoxylate cycle are repressed, therefore, Pyc catalyzed reaction becomes the only anaplerotic reaction to fulfil this role. Although Δ*pyc1pyc2* double mutant does not grow on glucose due to the lack of anaplerotic reaction, mutagenesis of Δ*pyc1pyc2* double mutant strain, however, resuscitated growth of this mutant. This mutation suppressed the effect of glucose or carbon catabolite repression on the enzymes of the glyoxylate cycle, thus allowing the replenishment of the TCA cycle to occur (Blazquez *et al*., 1995).

2.3.1.1.3 Pyruvate decarboxylase (*PDC*)

In *S. cerevisiae*, six *PDC* genes have been identified of which three structural genes (*PDC1*, *PDC5* and *PDC6*) encode for active Pdc enzymes independently (Hohmann and Cederberg, 1990). The regulatory genes *PDC2*, *PDC3* and *PDC4* encode proteins that are probably involved in the regulation of *PDC1* and *PDC5* expression. Among the regulatory genes *PDC2* encodes a transcriptional activator that plays a prominent regulatory role in the expression of both *PDC1* and *PDC6*, and a mutation in *PDC2* renders a yeast strain that cannot synthesize Pdc enzyme and therefore cannot grow on glucose (Velmurugan *et al*., 1997). Furthermore, the role of *PDC2* further extends to the regulation of thiamine pyrophosphate (TPP) synthesis (Hohmann and Meacock, 1998).

Structurally, the catalytically active Pdc enzyme is a tetramer composed of two dimers. Each dimer consists of two subunits that are identical and tightly bound. Each subunit has a molecular mass of approximately 60 kDa making up a tetramer of 250 kDa (König *et al*., 1992). The catalytic activity of the Pdc enzyme requires the presence of thiamine diphosphate (ThDP) and metal ion Mg^{2+} as cofactors (Lu *et al*., 2000) with the optimum activity of the tetramer at pH 6.2. Increasing pH towards alkalinity (e.g. pH 8.4) results in the dissociation of the Pdc tetramer into inactive dimers and this dissociation is pH-dependant and reversible (review in König, 1998; König *et al*., 1992). For example, under conditions of glucose excess the concentration of pyruvate increases resulting in significant decrease in pH, thereby favouring formation of the Pdc tetramer. All Pdc enzymes utilize pyruvate as substrate and are all, except the enzyme from the bacterium *Zymomonas mobilis*, subject to substrate activation (Lu *et al*., 2000).

During growth on fermentable carbon sources, Pdc enzymes catalyze an irreversible reaction:

$$\text{Pyruvate} \xrightarrow[\text{Pdcp}]{\text{ThDP} + Mg^{2}} \text{Acetaldehyde} + CO_2$$

During fermentative growth Pdc activity is induced and over 80% of sugar is channelled through Pdc enzyme towards ethanol formation (Zimmermann and Entian, 1997). Acetaldehyde is an intermediate for this reaction and also acts as an electron acceptor to reoxidize the NADH formed in glycolysis in order to maintain redox balance (Pronk *et al*., 1994).

The expression of *PDC1* and *PDC5* genes is enhanced during growth on glucose with *PDC1* mRNA levels being five fold higher than that of the *PDC*5 mRNA levels (Schmitt and Zimmermann, 1982). However, all three structural Pdc isoenzymes contribute directly to ethanol formation. Only *PDC1* and *PDC5* genes are responsible for the Pdc activity in yeast (Ishida *et al*., 2006) and both genes have 88% sequence similarity and are closely related over the entire sequence to *PDC* sequences from other organisms (Eberhardt *et al*., 1999).

Deletion of *PDC1* gene results in a five fold increase in the transcription of *PDC5* mRNA and approximately 60 – 70 % of the wild type Pdc activity is detectable, thus suggesting that the *PDC* genes are subject to autoregulatory mechanism (Hohmann and Cederberg, 1990; Seeboth *et al*., 1990). Alternatively, the autoregulatory mechanism can be explained as gene compensation (Wagner, 2000). Since deletion of either *PDC1* or *PDC5* has no noticeable impact on Pdc activity, disrupting both *PDC1* and *PDC5* renders a mutant strain with no detectable Pdc activity and failure to grow on glucose as a sole carbon source (Seeboth *et al*., 1990; Hohmann, 1991b). Under fermentative conditions this is thought to emanate from insufficient respiratory capacity of the mutant strain to support sugar metabolism because respiratory machinery cannot provide cytosolic Acetyl-CoA required for biosynthetic pathways including lipid biosynthesis (Eberhardt *et al*., 1999). The Pdc-catalyzed reaction appears to be the eminent reaction capable of providing indirectly Acetyl-CoA through the conversion of a proportion of Pdc-produced acetaldehyde (Eberhardt *et al*., 1999). Nevertheless, growth on glucose of ∆*pdc1pdc5pdc6* triple mutant becomes possible following the addition of small quantities of acetate or ethanol in a chemostat, but not batch cultures (Flikweert *et al*., 1999).

From these mutant studies, the lack of Pdc activity in ∆*pdc1pdc5* double mutant somewhat implies that *PDC6* gene cannot be expressed in the absence of other structural *PDC* genes. However, upon spontaneous fusion of *PDC6* gene under the control of *PDC1* promoter in ∆*pdc1pdc5* double mutant yielded a functional Pdc enzyme (Hohmann, 1991a). The role of *PDC6* is still unclear and the optimum conditions for its expression are not known either. Some reports also revealed that a low Pdc activity leads to an increased glycerol production especially under completely anaerobic conditions (Nevoigt and Stahl, 1996). Under sulphur-limited conditions in aerobic chemostat cultures, a 10-50 fold increase in *PDC6* transcription was observed (Boer *et al*., 2003).

2.3.1.1.4 Phosphoenolpyruvate carboxykinase (*PCK*)

During growth on non-fermentable carbon sources (e.g. ethanol, acetate) the gluconeogenic pathway is switched on to enable yeast to utilize these carbon sources (Valdes-Hevia *et al*., 1989). Glycolytic enzymes function by a reversal glycolytic pathway with the exception of fructose 1,6 biphosphatase (Fbp) and phosphoenolpyruvate carboxykinase (Pck) enzymes. Both enzymes catalyze biochemical reactions that bypass the irreversible reactions of 6-phosphofructo-1-kinase and pyruvate kinase. The Fbp and Pck enzymes are encoded by *FBP1* and *PCK1* genes, respectively (Navas *et al*., 1993).

The structure and complexity of Pck enzymes differ from one organism to the other. Pck enzymes from yeast and plants are composed of homotetramers or oligomers of identical subunits, while those from animals and certain bacteria are monomers. The former are ATP dependant and more complex, while the latter are GTP dependant. Although these enzymes display structural differences their active sites are similar (Ravanal *et al*., 2003). It is well known that the higher the complexity of the quaternary structure of the protein is, the more stable it becomes (Frieden *et al*., 1995). In contrast, the complexity of the yeast Pck enzyme does not

confer increased stability when compared to the monomeric Pck enzyme of *E. coli* (Ravanal *et al*., 2003).

In *S. cerevisiae*, Pck enzyme catalyzes the Mn^{2+}- ATP-dependant decarboxylation of OAA to yield PEP, carbon dioxide and ADP. During this reaction there is a transfer of γ-phosphoryl group of ATP to OAA (Ravanal *et al*., 2004). The reaction occurs as follows:

$$ATP + OAA \xrightarrow[Pckp]{Mn^{2+}} PEP + CO^2 + ADP$$

The regulation of Pck enzyme occurs at the level of activity and such activity is repressed by glucose. Addition of glucose to derepressed yeast causes the enzyme to undergo proteolytic degradation, a process of catabolite repression (Navas *et al*., 1993). Site-directed mutagenesis targeted at specific amino acids (e.g. Arg) within the enzyme results in loss of binding affinity for Mn^{2+} and significant decrease in V_{max} (Ravanal *et al*., 2004).

2.3.1.1.5 Pyruvate dehydrogenase (PDH complex)

The yeast *S. cerevisiae* is able to carry out both aerobic and anaerobic fermentation of sugars. Several parameters determining which of the two pathways dominates have been described (Postma *et al*., 1989). Oxygen and low concentrations of glucose are known to have an inductive impact on respiration. During growth on glucose the activity of respiration machinery is influenced by the activity of the enzymes of the pyruvate dehydrogense (PDH) complex (Wenzel *et al*., 1993). The PDH complex is an enzyme complex that occupies a central metabolic position linking the glycolytic carbohydrate metabolic pathway with energy generation via the TCA cycle. Because of its location the PDH complex is considered an important point of regulation in many prokaryotic and eukaryotic organisms (Kresze and Ronft, 1981). The PDH complex consists of four enzymes, which are encoded by separate genes (Table 1). Each individual enzyme catalyzes a specific step in the entire reaction and the combined activities of all four enzymes confer the functional activity of the PDH complex. In the mitochondrial matrix the PDH complex catalyzes the oxidative decarboxylation of pyruvate to yield acetyl-CoA and carbon dioxide. Thus, the overall reaction can be simply presented as:

$$Pyruvate + CoA + NAD^+ \xrightarrow{Pdhp} Acetyl\text{-}CoA + NADH + H^+ + CO_2$$

During this multi-step reaction pyruvate dehydrogenase catalyzes the oxidative decarboxylation of pyruvate. Other components of the complex complete the conversion of pyruvate to acetyl-CoA. The PDH complex plays a crucial role in supplying acetyl-CoA to stimulate the TCA cycle and mitochondrial amino acid biosynthesis. Moreover, the complex is considered to have an alternative function besides sugar metabolism because it remains active even under anaerobic conditions where the complex is not known to have any physiological significance. In fact, the activity of the complex varies depending on the culture conditions. In glucose-grown batch cultures, the activity of the complex is lower when compared to the activity of the chemostat cultures. Such differences are attributed to a higher extent of phosphorylation in batch cultures. Furthermore, under anaerobic growth conditions the enzymes of the TCA cycle are inhibited or present at basal level (Wenzel *et al*., 1993).

Among all the enzymes making up the PDH complex, pyruvate dehydrogenase is regarded as complex-specific. This enzyme consists of two distinct subunits, E1α and E1β,

which are encoded by *PDA1* and *PDB1* genes. During the conversion of pyruvate to acetyl-CoA the first decarboxylation step is catalyzed specifically by the E1α subunit. The function of the E1α further extends to the regulation of the activity of the PDH complex (Pronk *et al*., 1996; Wenzel *et al*., 1993). Phosphorylation of the E1α subunit inactivates the complex, while dephosphorylation in the presence of calcium ions reactivates it (James *et al*., 1995). Similarly, disruption of *PDA1* abolishes the production of the E1α subunit, the activity of the complex and subsequently the mitochondrial efficacy (Steensma *et al*., 1990; Wenzel *et al*., 1992). The above observations explicitly highlight that the regulation of the complex occurs mainly by post-translational modification of the E1α subunit. Because increasing the concentration of the E1α subunit in batch cultures results in 3 – 4 fold increase in the activity of the complex. Cells deficient of the active complex are generally known to grow poorly on glucose (Wenzel *et al*., 1992). Interestingly, these cells can accumulate 20 – 50% more glycogen than their isogenic wild type strain during fermentative growth on glucose. This storage carbohydrate can be readily mobilized when the mutant is subjected in glucose starvation (Enjalbert *et al*., 2000).

Both glucose and oxygen, which are known to influence the activity of the TCA cycle, appear to exert no control over the E1α subunit and the PDH complex because of comparable amounts and stability of *PDA1* mRNA and E1α subunit under various conditions (Wenzel *et al*., 1993). Due to the constitutive expression of *PDA1* gene under various carbon sources, it is now used as a loading standard for quantitative mRNA assays (Wenzel *et al*., 1995).

2.3.2 The TCA cycle and its bypass route

The respiratory metabolism of sugars in *S. cerevisiae* proceeds via the TCA cycle inside the mitochondria (Dickinson and Schweizer, 2004). There are two important parameters influencing this respiratory route of sugar metabolism: (1) the glucose concentration and (2) oxygen availability. Under aerobic conditions pyruvate enters the mitochondrial matrix where it is oxidatively decarboxylated to acetyl-CoA and carbon dioxide. This reaction is catalyzed by the PDH complex, which acts as a link between glycolysis and the TCA cycle. This reaction occurs when the concentration of pyruvate does not exceed the K_m of the enzymes of the PDH complex. Sudden increase in the amounts of glucose causes an immediate shift from oxidative fermentation to alcoholic fermentation via Pdc enzyme. In fact, pyruvate dehydrogenase has a ten fold higher affinity for pyruvate than Pdc enzyme but a much lower capacity (Pronk *et al*., 1996).

The acetyl-CoA formed by the PDH complex serves to stimulate the functional activity of the TCA cycle since the initial step of the TCA requires the presence of OAA and acetyl unit of the acetyl-CoA (Walker, 1998). In yeast, the TCA cycle is important for two reasons: (1) for generating energy and reducing equivalents in the form of ATP and NADH respectively, and (2) for providing acetyl-CoA and other precursors for biosynthesis (Dickinson and Schweizer, 2004). When oxygen is present the respiratory pathway yields approximately 16 ATP molecules per glucose consumed, 0.50g biomass per g glucose (van Maris *et al*., 2001) and five redox equivalents. In the electron transport system, electrons of NADH are transferred to the respiratory chain resulting in the reduction of molecular oxygen to water and regeneration of NAD^+. This process generates a proton gradient across the mitochondrial membrane that can drive ATP-synthase, a mitochondrial membrane-enzyme complex (Snoek and Steensma, 2007). Besides the role in catabolism the TCA cycle also functions in the anabolic pathway when ethanol or acetate is used as a carbon source. Because of this ability to perform both catabolic and anabolic functions, the TCA cycle is commonly referred to as *amphibolic* (Walker, 1998).

As an anabolic pathway, the TCA cycle also provides precursors for amino acid and nucleotide biosynthesis (Menendez and Gancedo, 1998). To achieve this the cycle requires constant replenishment of intermediates (e.g. OAA and α-ketoglutarate) by anaplerotic reactions. The anaplerotic reactions are catalyzed by the Pyc enzymes and enzymes of the glyoxylate cycle (Figure 4) (de Jong-Gubbels *et al*., 1998; Walker, 1998). The replenishment of the TCA cycle by Pyc enzyme occurs when yeasts are grown on fermentable carbon sources (de Jong-Gubbels *et al*., 1998). In contrast, the glyoxylate cycle replenishes the TCA cycle when yeasts are grown on non-fermentable carbon sources because in the presence of glucose the glyoxylate cycle is repressed (Walker, 1998).

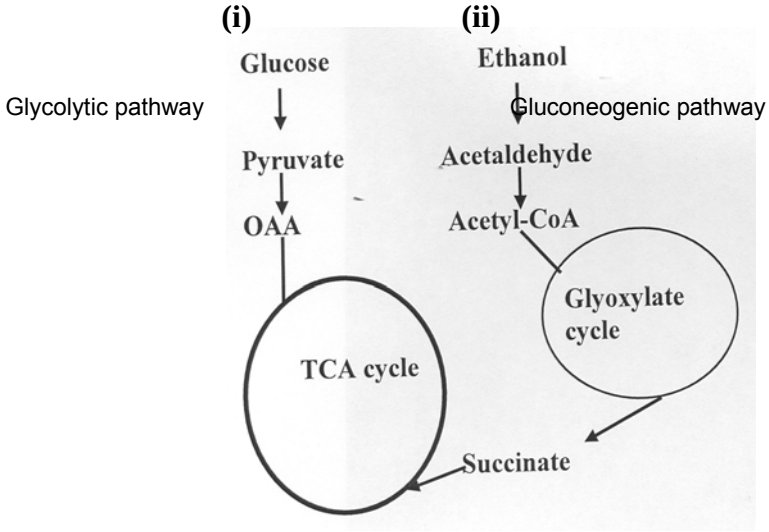

Figure 4. The two anaplerotic reactions serve in the replenishment of the intermediates of the TCA cycle. (i) During growth on fermentable carbon sources Pyc enzyme carboxylates pyruvate to OAA, a compound that replenishes the TCA cycle. (ii) The glyoxylate cycle provides succinate that enters the TCA cycle. This reaction takes place during growth on non-fermentable carbon sources and participates in gluconeogenic pathway.

In yeast mutants lacking the activity of pyruvate dehydrogenase, pyruvate is metabolized in the cytosol through the bypass reaction (Figure 5). This proceeds via the combined activities of Pdc, aldehyde dehydrogenase and acetyl-CoA synthase to form cytosolic acetyl-CoA (Pronk *et al*., 1996). Acetyl-CoA is then used for biosynthetic pathways (e.g. lipids) and a certain proportion of it enters the mitochondria via the carnitine acetyltransferase system. In fact, the bypass reaction serves to compensate for the absence of a functional PDH complex (Pronk *et al*., 1996; Boubekeur *et al*., 1999). The PDH bypass pathway is described as cytosolic because of the cellular compartment where it works (Pronk *et al*., 1996). The mitochondrial PDH bypass reaction has been identified. The mitochondrial and cytosolic PDH bypass reactions differ. In the mitochondrial PDH bypass reaction, acetaldehyde enters the mitochondria where it is converted to acetate by the mitochondrial acetaldehyde dehydrogenase, whereas in the cytosolic one, the conversion of acetaldehyde occurs in the cytosol (Figure 5). These two differently compartmentalized acetaldehyde dehydrogenases play different roles: the cytosolic acetaldehyde dehydrogenase functions in the biosynthetic reactions and the mitochondrial one is required for the bioenergetic pathway for growth on ethanol (Boubekeur *et al*., 1999).

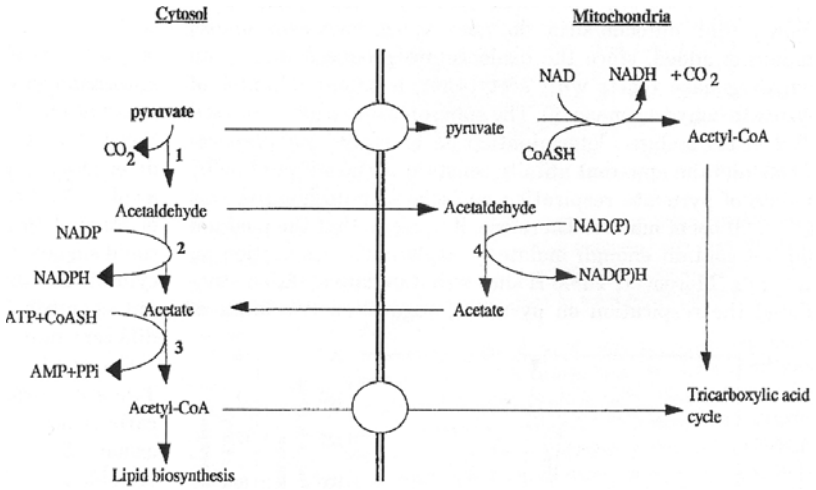

Figure 5. The pyruvate dehydrogenase bypass. Two PDH bypass reactions exist. The first one is catalyzed by the cytosolic acetaldehyde dehydrogenase (2) and the second by mitochondrial acetaldehyde dehydrogenase (4). The numbers represent reactions catalyzed by (1) pyruvate decarboxylase, (2) cytosolic acetaldehyde dehydrogenase, (3) acetyl-CoA synthase and (4) mitochondrial acetaldehyde dehydrogenase (Adapted from Boubekeur *et al*., 1999).

2.4 PYRUVATE METABOLISM AND ANAEROBIC FERMENTATION

In the absence of oxygen the yeast *S. cerevisiae* metabolizes pyruvate by alcoholic fermentation. This type of sugar metabolism is characterized by low ATP yields per sugar consumed and increased accumulation of metabolites. There is a reduction in biomass yield on glucose because of lower ATP yields resulting from alcoholic fermentation. During the production of alcoholic beverages and fuel ethanol, alcoholic fermentation is the preferred mode of sugar metabolism (van Maris *et al*., 2001). During these processes approximately 98% of carbon in the form of pyruvate must be converted to alcohol. To achieve this, a yeast strain with good fermentative efficiency must be used for inoculation. Such a strain must also possess certain other important properties such as ethanol tolerance because high sugar concentrations that are used for wine production leads to increased levels of ethanol (Pretorius, 2000; Bisson, 2004). The accumulation of ethanol becomes toxic to the yeast because it interferes with cell membrane stability and consequently causes cell leakage (Snoek and Steensma, 2007).

Pyruvate is not only essential for alcohol production, but is also required in the production of flavour and aroma compounds during wine production. During the production of wine pyruvate serves as the primary constituent from which different biosynthetic pathways emerge that lead to the formation of higher alcohols, esters, acetaldehyde and fatty acids. The quantitative development of these compounds depends on various factors such as grape cultivar, pH of the must, yeast strain, and the temperature of yeast fermentation (Lilly, 2004).

CARBON FLUX DURING RESPIRATORY METABOLISM OF GLUCOSE

Under aerobic conditions pyruvate produced by glycolysis can be broken down into carbon dioxide and a series of organic acids via the PDH complex and the TCA cycle, which results in the production of hydrogen molecules and five redox equivalents. During the electron transport chain two important reactions are achieved, namely: (1) the phosphorylation of ADP to ATP (the main role of respiration) and the oxidation of the hydrogen molecules to form water (Barnett, 2003). It is worth noting that respiratory metabolism of glucose proceeds for as long as the concentration of pyruvate does not exceed the capacity of the enzymes of the PDH complex (Pronk *et al*., 1996). This metabolic route is exceptionally looked after during the production of

baker's yeast and heterologous proteins since these processes require considerable amounts of cell biomass (Romanos *et al*., 1992). To maintain favourable conditions for these processes is a challenge as such processes can be favourably achieved through the use of chemostats and constant monitoring of various parameters including pH, glucose concentration, and oxygen levels (Walker, 1998).

2.5 CONCLUSION

This review was focused mainly on the pyruvate metabolism which falls under the central carbon metabolism in *S. cerevisiae*. Emphasis was placed on the genes that encode enzymes that are directly involved in pyruvate metabolism, the conditions for their optimum activity and the effects of single and double gene deletions on yeast growth phenotype. In *S.cerevisiae*, metabolism of pyruvate proceeds mainly via the ThDP-dependant enzymes of Pdc and the PDH complex (Hohmann and Meacock, 1998). Both the concentration of sugar and oxygen determine which metabolic routes will prevail (Postma *et al*., 1989). Usually when oxygen is readily available pyruvate is metabolized via the PDH complex for as long as the concentration of pyruvate does not exceed the capacity of the enzymes of the complex. Some reports showed that alcoholic fermentation occurs even under fully respiratory conditions and this is observed in Crabtree positive yeasts such as *S. cerevisiae* (Pronk *et al*., 1996).

A proportion of pyruvate is metabolized via Pyc enzyme and both sugar levels and oxygen appear to have little or no influence on this metabolic route. Because of these observations, Pyc-catalyzed route is not considered to play a significant role in pyruvate metabolism but rather important as an *anaplerotic* reaction for the replenishment of the TCA cycle intermediates during growth on fermentable carbon sources (Blazquez *et al*., 1995).

Gene disruption technologies have long been used to study the effects of gene deletion on carbon flux distribution and redirection. For carbon redirection, a gene catalyzing a competing metabolic route is deleted to allow for a larger proportion of carbon to be directed to the desired product. In many cases disruption of a single gene has little or no effect on flux distribution (Winzeler *et al*., 1999). For example, deletion of *PDC1* gene does not abolish the activity of Pdc and has little effect on the metabolic route (Seeboth *et al*., 1990; Hohmann, 1991b). This is attributed to compensatory role by a gene isoform. Reports showed that mutations in a single gene have little phenotypic effect if there is more than one gene with similar functions unless the disrupted gene catalyzes an irreversible reaction in a pathway (Wagner, 2000).

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Chapter 3

RESEARCH RESULTS

3. RESEARCH RESULTS

Screening and analysis of *Saccharomyces cerevisiae* deletion mutants displaying a modified fermentation behaviour and carbon flux under wine fermentative conditions

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3.1 ABSTRACT

The complete set of *Saccharomyces cerevisiae* deletion strains from the Euroscarf library provides a unique resource allowing for the systematic screening of phenotypes. However, systematic screening generally focused on phenotypes of interest in laboratory environments, and did not include assessment of fermentation performance and carbon flux in high sugar environments such as those encountered in many industrial processes. In this project, which is part of a larger effort to identify genes whose deletion affect carbon partitioning, a subset of seven haploid and two heterozygous diploid deletion mutants that are affected in genes that code for enzymes involved in pyruvate metabolism was screened for modified carbon flux under wine fermentative conditions. The screen identified a mutant deleted in the *PDA1* gene, which encodes the E1α subunit of the pyruvate dehydrogenase. The mutant showed a significant change in phenotype when grown in wine fermentative conditions. In particular, the mutant displayed a prolonged lag phase, but, once entering exponential growth fermented faster than the wild type and completed alcoholic fermentation in a shorter period of time. This phenotype could be of significant industrial interest, and was therefore further investigated.

Keywords: yeast deletion mutant; carbon flux; wine fermentative conditions

3.2 INTRODUCTION

The yeast *Saccharomyces cerevisiae* is the first eukaryotic organism to have its entire genome fully sequenced (Goffeau *et al*., 1996), and most of the protein-encoding genes have by now been at least partially annotated. However, the function of a significant number of genes remains ill-defined or unknown. Traditionally, gene function has primarily been identified through the analysis of mutant phenotypes. This approach received a significant boost when a full set of deletion mutants was generated and made available to researchers (Pues *et al*., 1997; Winzeler *et al*., 1999; Giaever *et al*., 2002; http://sequence-www.stanford.edu/group/yeast/yeast_deletion_project/deletions3.html). The set of deletion strains includes in particular disruptions of every non-essential gene in a haploid genetic background, and of every essential gene in a heterozygous diploid background. This set of deletion strains has been extensively used for many purposes such as identifying genes for ethanol tolerance (Fujita *et al*., 2005), K1 toxin sensitivity (Page *et al*., 2003) and for revealing phenotypes of silent mutations using metabolic footprinting (Raamsdonk *et al*., 2001; Allen *et al*., 2003).

However, most of these efforts have focused on phenotypes that are easily screened in laboratory conditions. As a consequence, few phenotypes of more specific industrial interest

have been investigated. Possible reasons include that the strain in which the deletions were made is a laboratory strain that does not perform well in industrial environments (making screening in such conditions impossible), and that many phenotypes of industrial interest are of a complex nature and differences between strains tend to be small. Yet such small differences may have a significant industrial interest.

For this study, the phenotype of interest is the efficiency and pattern of carbon utilisation in a high sugar environment such as found in wine making. Both of the above reasons for the reduced interest in industrially relevant phenotypes apply to this target, since the laboratory strain is not able to efficiently ferment the amount of sugar found in must, and the differences in phenotypes are expected to be rather small. Nevertheless, such small differences could have significant industrial interest. For example, the wine industry is extremely interested in developing yeast strains that would show only a small reduction in ethanol yield, or slightly increased fermentation performance in extreme conditions. To identify genes that might be of interest as biotechnological targets for such strain improvements, we screened parts of the Euroscarf deletion library for phenotypes related to carbon utilisation patterns. In this specific project, we focused on strains that were deleted for genes that are involved in the metabolism of one of the central metabolites determining overall carbon flux, pyruvate. Indeed, pyruvate is at the intersection between fermentative and respiratory growth, and provides the carbon backbone for the biosynthesis of many other metabolites. Each selected mutant is disrupted in a single gene that is involved in pyruvate metabolism. In *S. cerevisiae*, metabolism of pyruvate occurs via three metabolic routes which are catalyzed by pyruvate carboxylase (Pyc), enzymes of the pyruvate dehydrogenase (PDH) complex and pyruvate decarboxylase (Pdc) (Pronk *et al*., 1996). Each of these enzymes is coded for by multiple gene isoforms. Disrupting one of the gene isoforms may reduce the activity of the enzyme in question (Steensma *et al*., 1990; Wenzel *et al*., 1992) or increase the expression of an undisrupted gene isoform (Hohmann and Cederberg, 1990; Seeboth *et al*., 1990) the latter phenomenon is called gene compensation (Wagner, 2000).

During yeast sugar metabolism environmental parameters determine which metabolic route will dominate. Aerobic, glucose-limited conditions reinforce pyruvate catabolism via the PDH complex (Pronk *et al*., 1996) while anaerobic or glucose excess conditions even in the presence of oxygen favours pyruvate breakdown towards alcoholic fermentation (Postma *et al*., 1989).

In this study we describe the results of the screen of these yeast mutants for modified carbon flux under wine fermentative conditions, and identify one mutant strain which shows a phenotype of significant industrial interest.

3.3 MATERIALS AND METHODS

3.3.1 Yeast strains and culture conditions

The yeast strains used in this study and descriptions of their relevant genotypes are listed in Table 3.1. All yeast strains were rejuvenated on yeast peptone dextrose (YPD) agar plate containing 1% yeast extract, 2% peptone, 2% glucose and 2% agar (Biolab Diagnostics, Gauteng, South Africa) and incubated at 30 ^{o}C for three days.

Table3.1 Yeast strains used in this study

Strain	Genotype	Source or reference
BY4742	*MATα his3 leu2 lys2 ura3*	Euroscarf deletion library
∆*pck1*	*MATα his3 leu2 lys2 ura3* ∆ *ykr097w::KanMX4*	Euroscarf deletion library
∆*pda1*	*MATα his3 leu2 lys2 ura3* ∆*yer178c::KanMX4*	Euroscarf deletion library
∆*pdc1*	*MATα his3 leu2 lys2 ura3* ∆*ylr044c::KanMX4*	Euroscarf deletion library
∆*pdc5*	*MATα his3 leu2 lys2 ura3* ∆*ylr134w::KanMX4*	Euroscarf deletion library
∆*pdc6*	*MATα his3 leu2 lys2 ura3* ∆*ygr087c::KanMX4*	Euroscarf deletion library
∆*pyc1*	*MATα his3 leu2 lys2 ura3* ∆*ygl062w::KanMX4*	Euroscarf deletion library
∆*pyc2*	*MATα his3 leu2 lys2 ura3* ∆*ybr218c::KanMX4*	Euroscarf deletion library
∆*pyk2*	*MATα his3 leu2 lys2 ura3* ∆*yor347c::KanMX4*	Euroscarf deletion library
BY4743	*MATa/α his3Δ1 leu2 lys2/LYS2 MET15/met15Δ0 ura3*	Euroscarf deletion library
∆*pdc2*	*MATa/α his3Δ1 leu2 lys2/LYS2 MET15/met15Δ0 ura3* ∆*ydr081c::KanMX4*	Euroscarf deletion library
∆*pyk1*	*MATa/α his3Δ1 leu2 lys2/LYS2 MET15/met15Δ0 ura3* ∆*yal038w::KanMX4*	Euroscarf deletion library

3.3.2 Confirmation of strains

Individual colonies of each strain from fresh YPD agar plates were inoculated into a test tube containing 5 ml YPD broth and incubated overnight at 30^{o}C and 250 rpm. From these pre-cultures, genomic DNA was isolated according to the standard DNA procedures (Ausubel *et al*., 1994; Sambrook *et al*., 1989). Primers listed in Table 3.2 were used to verify the absence of a gene known to be deleted and the presence of *KanMX4* in place of a deleted gene by means of polymerase chain reaction (PCR) using Takara Ex Taq DNA polymerase (Takara BIO INC., Shiga, Japan). Genomic DNA from all yeast strains in Table 3.1 was used as template. Additionally, the presence of an active *KanMX4* in these mutant strains was also confirmed by plating out each strain on YPD agar containing 0.2 mg/L geneticin (Sigma-Aldrich, Steinheim, Germany).

Table 3.2 Primers used in this study

Primer name	Sequence
PCK1p-spec-FW	5'-GCGCGGCTGTGTTCTACATA-3'
PDA1p-spec-FW	5'-CATCAGCAACAGCAGCCACC-3'
PDC1p-spec-FW	5'-CGTGTGATGAGGCTCGTGGA-3'
PDC2p-spec-FW	5'-CGAACGTCCTCTGTCCCACT-3'
PDC5p-spec-FW	5'-CGCTAACACCTGTATGGTTG-3'
PDC6p-spec-FW	5'-GGTGGGGGCGGTAGTGATAA-3'
PYC1p-spec-FW	5'-GCATCAGATTCGACGAATGG-3'
PYC2p-spec-FW	5'-CGACGCAGCTGCTGATAGCG-3'
PYK1p-spec-FW	5'-GTGTTCCGCACCGTCACAAA-3'
PYK2p-spec-FW	5'-GGACGCCTTGTTGTAACGCT-3'
KanMX4-RV	5'-CTCGCTCAGGCGCAATCACG-3'

3.3.3 Production and analysis of synthetic wine

An aerobically grown overnight culture of all yeast strains in Table 3.1 was inoculated at an initial optical density (OD_{600}) of 0.1 into 250 ml Erlenmeyer flasks containing 100 ml synthetic medium (MS300) (Bely *et al*., 1990) with slight modifications for the purpose of this study. In particular, total sugar levels were adjusted to 100g/L at a ratio of 1:1 glucose: fructose and the pH was adjusted to pH 3.3. The synthetic must otherwise consisted of the following components: citric acid 6 g/L, malic acid 6 g/L, KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.25 g/L, $CaCl_2$. $2H_2O$ 0.155 g/L, NaCl 0.2 g/L and NH_4Cl 120 mg/L N ammonical. Each stock solution was prepared separately prior to adding to the base medium. An amino acid stock contained amino acids to the following final concentrations: tyrosine 18.33 mg/L, tryptophan 179.33 mg/L, isoleucine 32.73 mg/L, aspartic acid 44.51 mg/L, glutamic acid 120.43 mg/L, arginine 374.37 mg/L, leucine 48.43 mg/L, threonine 75.92 mg/L, glycine 18.33 mg/L, glutamine 505.27 mg/L, alanine 145.30 mg/L, valine 44.51 mg/L, methionine 31.42 mg/L, phenylalanine 37.96 mg/L, serine 78.54 mg/L, histidine 32.73 mg/L, lysine 17.02 mg/L, cystein 13.09 mg/L, and proline 612.61 mg/L. All dissolved in 20 g/L $NaHCO_3$ and heated at 65 ^oC for 10 minutes. Uracil stock (2.4 mg/L) was dissolved separately in distilled water and filter-sterilized. An oligoelement stock was prepared and consisted of the following components to the following final concentrations: $MnSO_4.H_2O$ 4 g/L, $ZnSO_4.7H_2O$ 4 g/L, $CuSO_4.5H_2O$ 1 g/L, KI 1 g/L, $CoCl_2.6H_2O$ 0.4 g/L, H_3BO_3 1 g/L and $NH_4.6Mo_7O_{24}$ 1 g/L, all dissolved in distilled water; The vitamins stock consisted of: myo-inisitiol 2000 mg/L, calcium pantothenate 150 mg/L, thiamine hydrochloride 25 mg/L, nicotinic acid 200 mg/L, pyridoxine 25 mg/L, biotin 0.3 mg/L, all dissolved in distilled water and the stock of anaerobic factors contained the following components: ergosterol 15 g/L and oleic acid 5 g/L, both were dissolved in absolute ethanol (99.9%), added to Tween 80 (50 ml + 50 ml, respectively) and dissolved at 65 ^oC. The base medium consisting of sugars and salts was autoclaved at 121 ^oC for 15 minutes, the stocks of amino acids and oligoelements were autoclaved separately. All the stocks of vitamins and anaerobic factors were filtered through a 0.2 µm filters (Acrodisc® Syringe filters). The complete synthetic must was then prepared by adding to the cooled base medium all the stocks to achieve the following final concentrations: amino acid 13.09 ml/L; oligoelements 1 ml/L; vitamins 10 ml/L; anaerobic factors 1 ml/L and uracil 8 ml/L.

The synthetic must was fermented anaerobically at room temperature (~25 ^oC) for 21 days and the fermentation progress was monitored by weight loss. All fermentations were done as four biological repeats and two samples per fermentation were collected. For the first sample only the supernatant was collected, filtered and frozen immediately in liquid nitrogen until further analysis. For the second sample the culture was mixed homogeneously and a sample collected for cell growth determination.

For the purpose of determining the concentrations of sugars and organic acids, frozen samples were thawed on ice and analyzed by high performance liquid chromatography fitted with an AMINEX HPX-87H ion exchange column (Biorad) at 55 ^oC. The column was eluted using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 $ml \cdot min^{-1}$. Organic acids were detected by the Agilent 1100 diode-array detector at 210 nm. Sugars were detected by the Agilent 1100 series refractive-index detector (Agilent Technologies, Midrand, South Africa). Culture dry weights were determined via filtration (Postma *et al*., 1989).

3.3.4 Statistical analysis

The statistical differences between results of each mutant and wild type strains were determined using ANOVA and Control test (PractiStat). Differences were considered significant if the $p \leq 0.05$ (Ashcroft, 2002).

3.4 RESULTS

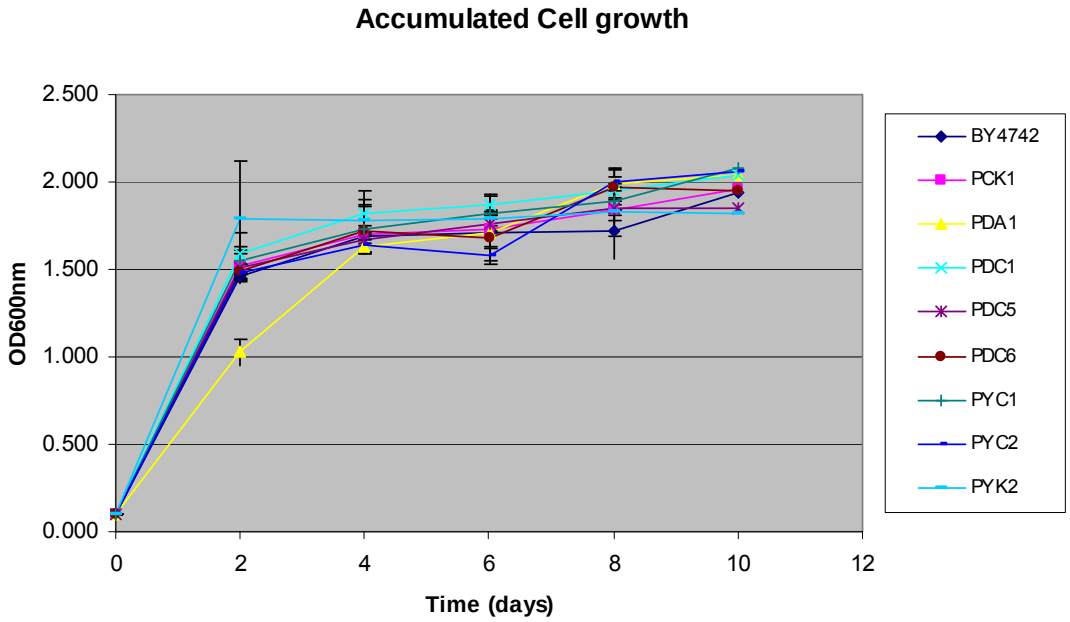
3.4.1 Strain confirmation

All strains were selected from the Euroscarf Yeast deletion library. The presence of *KanMX4* cassette was verified on geneticin-containing medium and all mutants except wild type strains showed resistance to geneticin. To verify that each strain was disrupted in the expected locus, the integration of *KanMX4* cassette in the relevant coding region was confirmed by PCR, using the KanMX4-RV and gene promoter specific primers and the genomic DNA of each mutant as template. The resulting PCR fragments were analysed for the correct sizes.

3.4.2 Screening *S. cerevisiae* mutants for modified carbon flux under wine fermentative conditions

All yeast strains were screened under simulated wine making conditions with glucose and fructose as carbon sources. Growth and fermentation kinetics curves were obtained in MS300 in order to monitor if single gene deletions in these strains have an effect on growth and fermentation rate. As shown in Figure 3.1 A and B, no significant differences in growth characteristics and fermentation kinetics could be observed in all diploid and haploid strains with the exception of the Δ*pda1* mutant that had a prolonged lag phase of approximately 24 hours (Figure 3.1 B). This long lag phase may be attributed to the mitochondrial inefficiency to generate adequate ATP molecules, thereby resulting in low biomass formation. However, after the extended lag phase, the Δ*pda1* mutant fermented faster than the parental strain and exhausted all the sugars before the parental strain making the Δ*pda1* mutant a fermentation efficient strain.

A



B

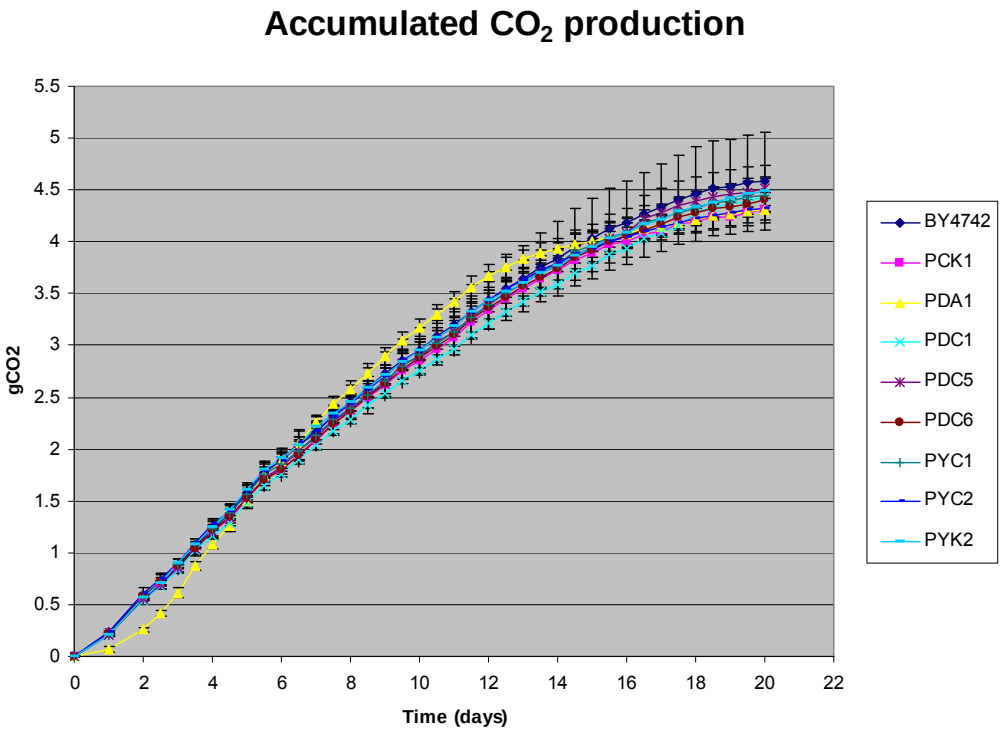


Figure 3.1 Growth and fermentation kinetics curves of yeast mutants bearing single deletions on genes involved in pyruvate metabolism. (**A**) Cell growth, (**B**) Fermentation kinetics. The graph represents the average of four repetitions.

3.4.3 Effect of single gene deletions on the concentrations of sugars and organic acids in synthetic must

The concentrations of the major carbon compounds were determined. When compared to the parental strain, no significant differences could be observed in sugar consumption rates between most strains. However, the synthetic must fermented with the Δ*pda1* mutant showed a behaviour well aligned with the observed release of CO_2. After consuming little carbon in the early stages, the strain consumed more sugars after six days of fermentation, and all sugars had been exhausted before any of the other strains (Figure 3.2 A). However, the ethanol production was slightly higher during the log phase, whereas the amount of glycerol produced was significantly lower in medium fermented with the Δ*pda1* mutant (Figure 3.2 B and C). This suggests that the Δ*pda1* mutant has an improved fermentative efficiency under high sugar concentrations and in simulated wine making conditions.

Analysis of kinetic parameters showed that the Δ*pda1* mutant took approximately 24 hours to acclimatize before the beginning of CO_2 release, while other strains initiated fermentation more rapidly. No significant differences were observed in ethanol yields (determined as g ethanol produced per g sugar consumed) during the course of fermentation (Figure 3.3).

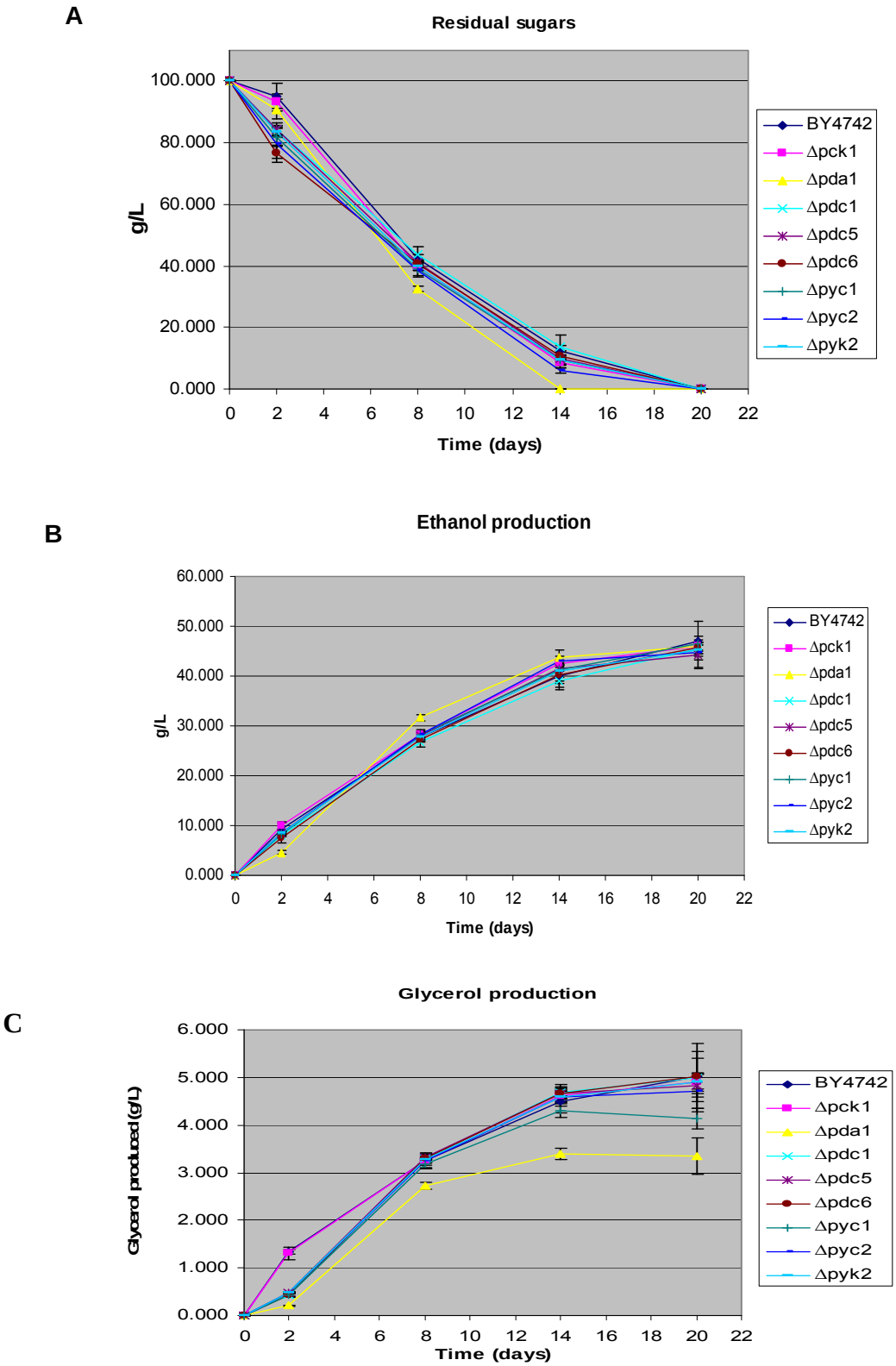


Figure 3.2 The profile of residual sugars and glycerol produced by mutant strains against the parental strain during fermentation. (**A**) Residual sugars (glucose+fructose), (**B**) Ethanol production (**C**) Glycerol production

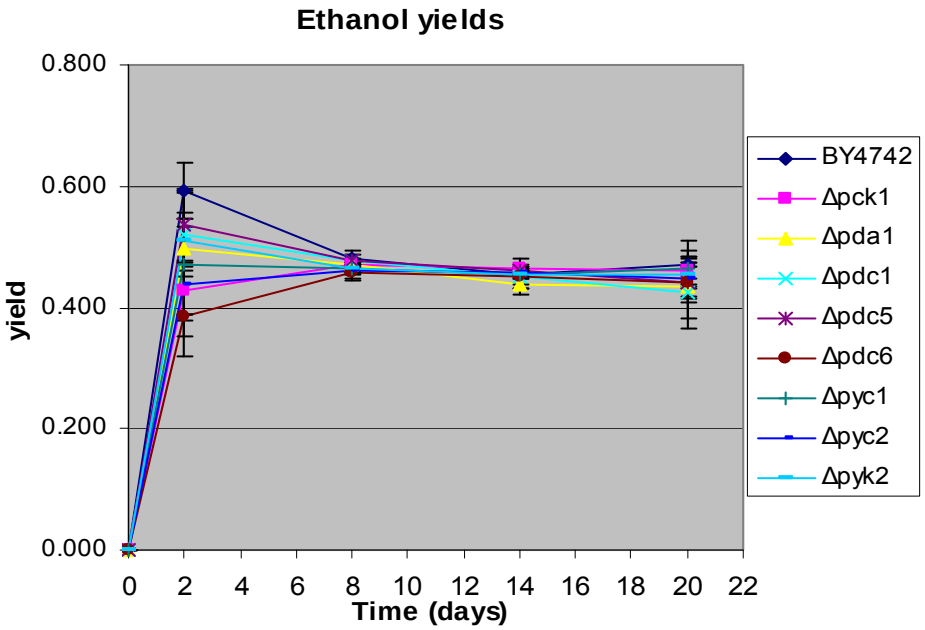


Figure 3.3 Ethanol yields of all the yeast strains during the course of alcoholic fermentation in synthetic must.

3.5 DISCUSSION AND CONCLUSION

In this study, *S. cerevisiae* strains with single deletions of genes involved in pyruvate metabolism were selected from the Euroscarf deletion library. These mutants were then screened for modified carbon flux under wine fermentative conditions. From the screen, all strains grew similarly and fermented at relatively similar rates with the exception of the Δ*pda1* mutant which had a slow fermentation phenotype during the early stages of alcoholic fermentation. The slow growth phenotype of the Δ*pda1* mutant might be due to the lack of fully operational mitochondria in this strain which therefore results in low energy (ATP) generation in the early stage of fermentation when oxygen is still present and consequently low biomass formation (Pronk *et al*., 1994). This slow growth phenotype has been previously described (Wenzel *et al*., 1992).

However, in the high sugar environment, and as fermentation progressed the Δ*pda1* mutant managed to catch up with and fermented better than the wild type strain. The metabolic reasons for this behaviour are unclear, but some hypothesis can be proposed. Firstly, the strain produced a lower amount of glycerol than the wild type strain, suggesting a lower requirement for NAD^+ regeneration. This could indicate that less pyruvate in this strain is diverted away from ethanol and that regeneration of NAD^+ may be more efficient in this strain, allowing faster fermentation. A second explanation may be linked to the role of the PDH bypass reaction in synthesizing acetyl-CoA. The energy demands of this reaction are higher and therefore the amount of glucose that has to be catabolized to provide ATP for biosynthesis is higher than in wild type strain (Pronk *et al*., 1994). Furthermore, previous reports have also shown that a respiratory-deficient strain had an improved rate of fermentation due to reduced accumulation of ATP, a competitor of alcoholic fermentation (de Deken, 1966).

Contrary to this, some studies reported a different observation where mitochondrial mutants and wild type strain had similar growth, alcohol production rates, and total alcohol production under the same culture conditions (Alexander and Detroy, 1983). It is therefore important to note that different researchers obtained different and unique results on the Δ*pda1* mutants. Our data differ from those of Wenzel *et al* (1992) regarding the growth phenotype of

PDA1 deletion strain. In their paper, the strain was described with a slow growth phenotype on glucose. These results may be due to different sugar concentrations and cultivation conditions used in these studies. .

The screening strategy employed in this study worked successfully, since it could discriminate the strains used in terms of rates of fermentation, glycerol production and residual sugars. In this study we observed that the Δ*pda1* mutant is not a slow grower on glucose as has been previously reported but rather requires a certain period of time to acclimatize to the surrounding fermentative conditions prior to efficient fermentation. These observations led us to select the Δ*pda1* mutant for further analysis. The phenotype described here may indeed be of significant biotechnological interest since the modified fermentation kinetics as described here could lead to improved wine yeast strains.

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Chapter 4

RESEARCH RESULTS

4. RESEARCH RESULTS

Elucidating the role of a disrupted *PDA1* gene in modifying carbon flux and fermentation characteristics in *Saccharomyces cerevisiae*

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4.1 ABSTRACT

In a study focusing on the identification of genes whose deletion would significantly impact on fermentation kinetics and carbon flux in a wine environment, the *Saccharomyces cerevisiae PDA1* deletion strain was isolated from the Euroscarf deletion library. After inoculation into synthetic grape must, this strain displayed an extended lag phase, but was able to ferment faster and complete fermentation earlier than the wild type strain. The pyruvate dehydrogenase E1α encoded by the *PDA1* gene is part of an enzyme complex catalysing the transformation of pyruvate to acetyl-CoA, a central reaction during respiratory growth, but which is not expected to play a significant role during fermentation of wine. Such a phenotype could be of significant biotechnological interest since faster fermenting strains continue to be sought-after by wine makers. In this study we further investigated the nature of the observed phenotype. For this purpose, the growth of the mutant and the wild type were compared in media with variable initial sugar concentrations and in aerobic as well as anaerobic conditions. The gene was re-disrupted in three different genetic backgrounds, and the wild type copy of the gene was re-introduced into the initial and the new deletion strains. The data show that the phenotype is only present in the original Euroscarf strain, and is not apparent in the newly disrupted strains. When the growth phenotypes of the Δ*pda1* mutants were compared, our newly generated Δ*pda1* mutants grew similar to the corresponding wild type reference strains under all conditions. These results suggest that secondary mutations in the library strain were responsible for the growth phenotype of the Δ*pda1* mutant.

Keywords: Δ*pda1* mutant; phenotype; secondary mutations

4.2. INTRODUCTION

The yeast *Saccharomyces cerevisiae* is best known for its industrial importance and has been used in the brewing and baking industry for millennia (Walker, 1998). The availability of the genome sequence of this yeast (Goffeau *et al*., 1996) has enabled the generation of a collection of approximately 6000 mutants, each deleted for one individual gene. These mutants provide an excellent tool for the determination of the function of yeast gene products in cellular processes and pathways (Winzeler *et al*., 1999). Indeed, the phenotypic analysis of mutants under defined conditions is a promising strategy to assign gene functions (Carpenter and Sabatini, 2004), because a mutant phenotype is a reflection of a loss of function of the gene (Giaever *et al*., 2000). Such disrupted genes can be investigated further by genetic methods to clearly elucidate their role in cellular processes or pathways.

In a previous chapter, we have described the isolation of a mutant deleted for the *PDA1* gene. The *PDA1* gene is one of the five genes that encode enzymes of the pyruvate dehydrogenase complex. Specifically, the *PDA1* gene encodes the pyruvate dehydrogenase E1α subunit (Pronk *et al*., 1996). The pyruvate dehydrogenase E1α subunit (EC 1.2.4.1) is the first enzyme in a pyruvate dehydrogenase (PDH) complex and is present in both prokaryotic and eukaryotic organisms (Perham, 2000; Smolle and Lindsay, 2006). E1α subunit is a thiamine diphosphate-dependant enzyme that catalyzes the decarboxylation of pyruvate and the reductive acetylation of the lipoyl group bound to the E2β subunit (Wenzel *et al*., 1992; Hohmann and Meacock, 1998; Perham, 2000).

In *S. cerevisiae*, the Pdh-catalyzed metabolic reaction is an important step of regulation in carbohydrate metabolism because it links glycolysis to the tricarboxylic acid (TCA) cycle (Kresze and Ronft, 1981). This reaction is important in the replenishment of the TCA cycle which in turn plays vital roles in energy and biomass generation and biosynthetic reactions (Dickinson and Schweizer, 2004). Moreover, it is highly desired during the production of baker's yeast and heterologous proteins because it is associated with increased cell biomass yields (Kjeldsen, 2000).

In Chapter 3, we examined the behaviour of the *Δpda1* mutant under high sugar wine fermentations and noticed an initial extended lag phase compared to the reference strain. Upon reaching the mid-exponential phase, the *Δpda1* mutant however fermented more vigorously and out-competed the reference strain in CO_2 production rates, which eventually shortened its fermentation time compared to the wild-type reference strain.

So far, little is known about the importance of the E1α-catalyzed reaction under high sugar conditions. High sugar fermentations are sub-optimal growth condition which may present phenotypes that were not previously seen when phenotypes were mainly assessed in standard laboratory conditions (e.g. YPD or SD medium, 2 % glucose). To further investigate this change in fermentative abilities of the Euroscarf Δ*pda1* strain, we compared the Δ*pda1* mutant from the Euroscarf deletion library with self-generated Δ*pda1* mutants in various genetic backgrounds and in various conditions. These data suggest that the phenotype of the Euroscarf strain is specific to this strain, and is not a result of the disruption of *PDA1*. To further confirm this result, a wild type copy of the *PDA1* gene was re-introduced into the original Euroscarf Δ*pda1* strain. These data confirmed the initial findings, suggesting that individual Euroscarf strains harbour additional mutations that may interfere with the phenotypic analysis of mutants.

4.3 MATERIALS AND METHODS

4.3.1 Microbial strains, media and culture conditions

Yeasts BY4742, FY834 and Σ1278b were used as reference strains in this study. All yeast and bacterial strains used in this study and their relevant genotypes are described in Table 4.1. *Escherichia coli* cells were grown in Luria-Bertani broth (1.2% tryptone, 12g NaCl, 0.6% yeast extract) at 37 ^{o}C (Sambrook *et al*., 1989). Yeasts were routinely grown at 30 ^{o}C on complete YPD medium (1% yeast extract, 1% peptone, 2% glucose) as well as on SC medium [containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and 2% (w/v) glucose supplemented with amino acids according to the specific requirements of the respective strains] solidified with 2% agar as necessary. G418 (200μg/ml) (Sigma-Aldrich, Steinheim, Germany) was added to selected strains carrying the Δ*pda1::KanMX4* allele.

For growth measurements, fresh single colonies were taken directly from plates incubated at 30 ^{o}C, inoculated into 5 ml YPD (2%) and incubated at 30 ^{o}C until stationary phase

or until 50 % of the initial sugars had been consumed. A 50 ml YP media with either 2% or 10% glucose was then inoculated to an initial OD_{600} of 0.1 with the 5 ml of the preculture. All these experiments were done in triplicate under both aerobic (flasks with cotton plugs) and anaerobic (flasks with fermentation caps) conditions. Cell growth was monitored at regular intervals until stationary phase. All experiments were carried out in triplicate.

4.3.2 Yeast transformation

Yeast transformations were carried out using a modified version of the yeast transformation procedure described by Gietz *et al* (1992). Reference strains were grown overnight in 200 ml YPD and harvested with OD_{600} of 0.7-1.0. Cells were harvested washed in distilled water twice and resuspended in 1.5 ml freshly prepared sterile 0.01 M Tris-HCl, 1 mM EDTA (TE)/0.1 M LiOAc buffer (pH 7.5). Cells were harvested and resuspended in 200 ul TE/LiOAc buffer. 50 µl of the cell suspension was added to 50 µg of freshly denatured salmon sperm DNA (Sigma-Aldrich, USA), approximately 5 µg of DNA (plasmid or disruption cassette) and 300 µl sterile 40 % PEG 4000 (8:1:1 v/v pH 7.5 of 50 % PEG 4000:0.1 Tris-HCl, 0.01 EDTA: 1 M LiOAc). Cells were incubated at 30 ^{o}C and then 42 ^{o}C for 30 minutes, respectively. Harvested cells were left to grow in YPD for 2-3 hours before plating on YPD supplemented with geneticin G418 or in selective media.

4.3.3 *Δpda1*, pPDA1 and YCplac111 strain construction

Standard procedures for the isolation and manipulation of DNA were used throughout this study (Ausubel *et al*., 1994; Sambrook *et al*., 1989).

To confirm the growth phenotype of Δ*pda1* mutant (BEΔpda1), the *PDA1* gene was deleted in BY4742, FY834 and Σ1278b strains using a *KanMX4* cassette, which confers resistance to G418 to create strains BMΔpda1, FY834Δpda1 and ΣΔpda1 (Table 4.1). The *KanMX4* cassette was amplified from genomic DNA of a Δ*pda1* mutant from a Euroscarf deletion library using primers PDA1-F-EcoR1 and PDA1-R-Xho1 (listed in Table 4.2).

Restriction enzymes (EcoR1 and Xho1) (Roche) and T4 DNA ligase (Promega) were used in the enzymatic manipulation of DNA according to the specifications of the supplier. To confirm if the observed growth phenotype of BEΔpda1 mutant is indeed the sole result of the disrupted *PDA1* gene, the *PDA1* gene with a native *PDA1* promoter and terminator was obtained by PCR with primers PDA1-F-EcoR1 and PDA1-R-Xho1 (Table 4.2) and subsequently cloned back to BEΔpda1 mutant strain. Genomic DNA from parental strain BY4742 (Euroscarf library) was used as a template. The resulting 1.96 kb fragment was ligated into pGEM-TEasy vector and the pGEM-TEasy-*PDA1* was subsequently digested with *Sph*I and *Sal*I. The resulting 2.1kb fragment corresponding to the $PDA1_P$-*PDA1*-$PDA1_T$ cassette, was then cloned into a *Sph*I/ *Sal*I site of a centromeric plasmid YCplac111 and transformed into BY4742, BEΔpda1 and BMΔpda1 strains to create strains BY4742(pPDA1), BEΔpda1(pPDA1) and BMΔpda1(pPDA1). To evaluate the effect of the maintenance and replication of the plasmid on yeast growth, an empty CEN YCplac111 plasmid was also transformed into BY4742, BEΔpda1 and BMΔpda1 strains to create strains BY4742(YCplac111), BEΔpda1(YCplac111) and BMΔpda1(YCplac111). All transformations with YCplac111 were plated out on SC^{-leu} agar plates and incubated at 30 ^{o}C for three days.

The *LEU2* gene was amplified from the YCplac111 plasmid template by PCR, using primers LEU2-pda1-F and LEU2-pda1-R (Table 4.2). The resulting 1.25kb fragment was used to transform BY4742 to create BYLEUΔpda1 mutant.

Table 4.1. Microbial Strains and Plasmids Used in This Study

Strain or plasmid	Genotype or construct	Reference or source
Escherichia coli DH5a	F' *endA1 hsdr17* (r_k^- m_k^+) *supE44 thi-1 recA gyrA* (Nal^r) *relA1* D(*laclzya-argF*)*U169 deoR* [F80d*lac* DE(*lacZ*)M15	GIBCO-BRL/Life Technologies
BY4742	*MATα his3 leu2 lys2 ura3*	Euroscarf
BY4742(YCplac111)	*MATα his3 leu2 lys2 ura3* YCplac111	This study
BY4742(pPDA1)	*MATα his3 leu2 lys2 ura3 PDA1$_p$-PDA1-PDA1$_T$*	This study
BEΔpda1	*MATα his3 leu2 lys2 ura3 Δyer178c::KanMX4*	Euroscarf
BEΔpda1(YCplac111)	*MATα his3 leu2 lys2 ura3 Δyer178c::KanMX4* YCplac111	This study
BEΔpda1(pPDA1)	*MATα his3 leu2 lys2 ura3 Δyer178c::KanMX4 PDA1$_p$-PDA1-PDA1$_T$*	This study
BMΔpda1	*MATα his3 leu2 lys2 ura3 Δpda1::KanMX4*	This study
BMΔpda1(YCplac111)	*MATα his3 leu2 lys2 ura3 Δpda1::KanMX4* YCplac111	This study
BMΔpda1(pPDA1)	*MATα his3 leu2 lys2 ura3 Δpda1::KanMX4 PDA1$_p$-PDA1-PDA1$_T$*	This study
FY834	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63*	This laboratory
FY834Δpda1	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 Δpda1::KanMX4*	This study
YCplac111	*CEN2 LEU2*	Gietz & Sugino 1988
BYLEUΔpda1	*MATα his3 leu2 lys2 ura3 Δpda1::LEU2*	This study
Σ1278b	*α* wild-type *MPR1 MPR2 AZC^r*	This laboratory
ΣΔpda1	*α* wild-type *MPR1 MPR2 AZC^r Δpda1::KanMX4*	This study

All the necessary verifications were carried out with relevant combinations of primers (as listed in Table 4.2). The presence of the *PDA1* gene in strains BY4742(pPDA1), BEΔpda1(pPDA1) and BMΔpda1(pPDA1) was verified by PCR using primers PDA1up-spec-FW and PDA1-R-QRT-PCR. The primer set YCplac111-F and YCplac111-R was used for confirming the presence of an empty CEN plasmid in BY4742(YCplac111), BEΔpda1(YCplac111) and BMΔpda1(YCplac111) strains. The Δ*pda1* mutants (BEΔpda1, BMΔpda1, FY834Δpda1 and ΣΔpda1) were verified by PCR, using primers PDA1up-spec-FW and KanMX-RV, with the exception of BYLEUΔpda1 which was verified using primers PDA1-F-EcoR1 and PDA1-R-Xho1.

Table 4.2. Primers used in this study

Primer name	Sequence
PDA1-F-EcoR1	5'-GTCGGAATTCCCCTCTGGTATAGCGAGA-3'
PDA1-R-Xho1	5'-TGGACTCGAGTTTATCCTTGGGCGGC-3'
PDA1up-spec-FW	5'-CATCAGCAACAGCAGCCACC-3'
KanMX-RV	5'-CGTGATTGCGCCTGAGCGAG-3'
PDA1-F-QRT-PCR	5'-GGAATTTGCCCGTCGTGTT-3'
PDA1-R-QRT-PCR	5'-GCGGCGGTACCCATACC-3'
YCplac111-F	5'-CCGTATTACCGCCTTTGAGTG-3'
YCplac111-R	5'-AGTGAAGGAGCATGTTCGG-3'
LEU2-pda1-F	5'CCCTCTGGTATAGCGAGAAGCAACTTTAGCTTCTTAACGGCAAGAACTTTATGTCTGCCCCTAAGAAGATCGTCGT-3'
LEU2-pda1-R	5'-GCTCGAGTCCATTTATCCTTGGGCGGCTATTTTCCGGTCTGTTAATAACTTAAGCAAGGATTTTCTTAACTTCTTCGGCG-3'

*Underlined sequences indicate introduced restriction sites

4.4 RESULTS

4.4.1 Evaluation of the growth phenotype of Δ*pda1* mutant in different genetic backgrounds

In order to elucidate the role of the disrupted *PDA1* gene in the modification of carbon flux in *S. cerevisiae*, it was important to first confirm the growth phenotype of the Δ*pda1* mutant observed during initial screening (Chapter 3). To achieve this, the *PDA1* gene was disrupted in three yeast strains (BY4742, FY834 and Σ1278b) of different genetic backgrounds and replaced with *KanMX4* cassette amplified from Δ*pda1* mutant from Euroscarf deletion library. This disruption yielded strains BMΔpda1, FY834Δpda1 and ΣΔpda1 from BY4742, FY834 and Σ1278b backgrounds respectively. In addition, a *LEU2* marker gene was also used to disrupt the *PDA1* gene in BY4742 to create BYLEUΔpda1 strain. All of those disruptions were confirmed by PCR (data not shown).

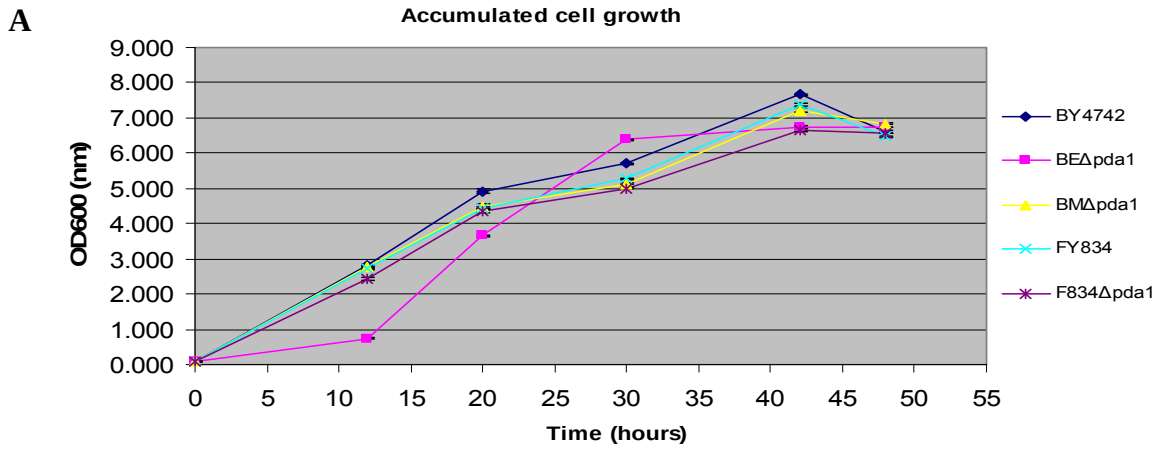
These strains were then inoculated into different growth media to assess their fermentation phenotypes. For this purpose, all strains were inoculated from two different precultures: Precultures were either grown to mid-exponential phase, where sugar concentration are still high and the cells are therefore in a fermentative, non-respiratory physiological state, or to stationary phase when glucose repression has been lifted. In the first case, inoculation occurred when the precultures reached a residual sugar concentration of 50 g/L (i.e. 50% of the initial sugar concentration). These preculture conditions were required to evaluate the impact of the initial physiological state on the prolonged lag-phase that was observed for the *pda1* mutant strain in the previous chapter. Indeed, it is possible that the phenotype is due to difficulties that this strain may experience to switch from respiratory (preculture) to fermentative conditions. In this case, inoculation from a high-sugar preculture should lead to a shortened lag-phase in this strain.

However, no difference in growth patterns due to the difference in pre-culture conditions could be observed for any of the strains (data not shown). Hence, we decided to use precultures grown until stationary phase for further experiments.

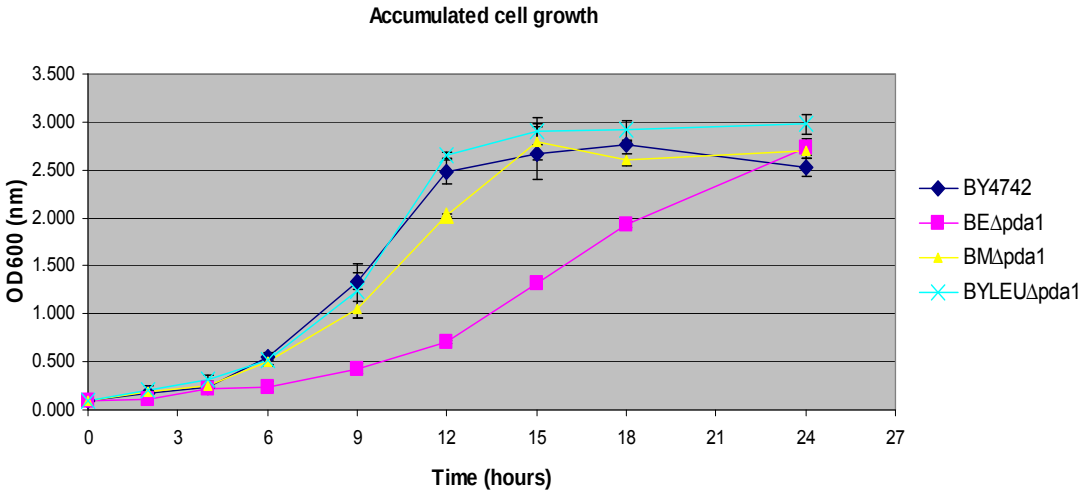
Growth measurements were made in YP medium containing 2% and 10% glucose under anaerobic conditions. As shown in Figure 4.1 A, the data confirmed our previous observation, with a prolonged lag phase of the original BEΔpda1 mutant grown with 10 % glucose. After the

lag phase, the mutant fermented faster than the wild type strain during the mid-exponential growth phase. The accelerated growth in mid-exponential stages was however not observed when the mutant was grown in anaerobiosis with 2 % glucose as carbon source. In this condition, BEΔpda1 grew significantly slower in all stages of the experiment (Figure 4.1 B). This is probably due to the fact that the 2% glucose medium does not provide a sufficient amount of carbon source for this strain to recover from the delayed lag-phase. Once exiting this phase, the sugar concentration may already be low and the strain has to prepare for entry into stationary phase. These data may also explain why the *pda1* mutation has previously been linked to a "slow growth on glucose" phenotype.

In order to link this phenotype to the deletion of the *PDA1* gene, we generated Δ*pda1* mutants in three different backgrounds. A Δ*pda1* mutant was initially generated in the BY4742 background which is identical to the Δ*pda1* mutant from the Euroscarf deletion library. In order to rule out strain specificity of the *PDA1* gene knock-out in the BY background, the knock-out was also generated in FY834 and Σ1278b genetic background strains. No significant differences between the growth of BMΔpda1 mutant and the reference strain BY4742 could be observed. Likewise, the growth characteristics of FY834Δpda1 and ΣΔpda1 mutant were similar to that of the corresponding wild type reference strains (Figure 4.1 B and C). In addition to these tests, disruption of the *PDA1* gene in BY4742 by a *LEU2* marker also resulted in a Δ*pda1* mutant (BYLEUΔpda1) that grew relatively similar to the BY4742 wild type reference strain (Figure 4.1 B). These observations therefore suggest that the interesting growth phenotype of the BEΔpda1 mutant is probably not the result of the disrupted *PDA1* gene, but caused by secondary gene mutations.



B



C

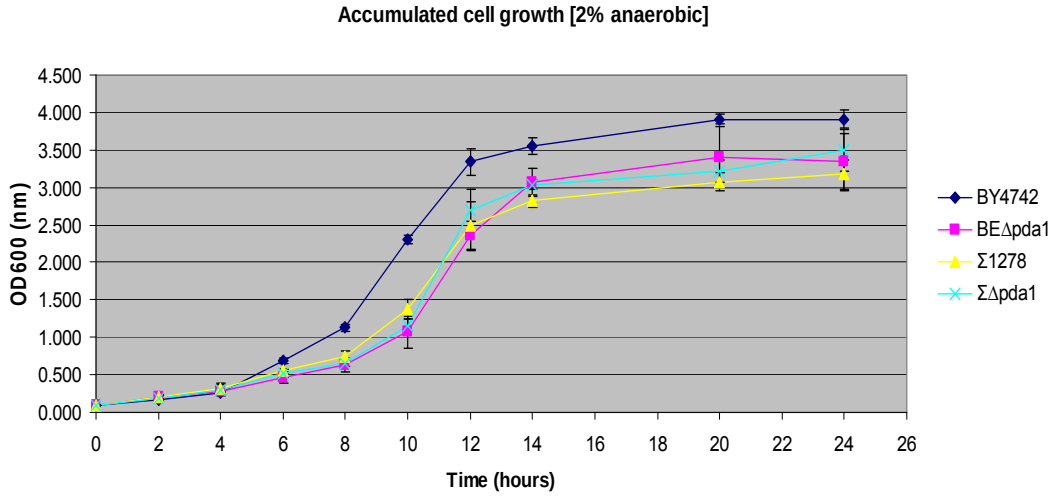


Figure 4.1 Growth curves of Δ*pda1* mutants and their respective wild type strains under anaerobic conditions. (**A**) YP medium with 10% glucose and (**B**) YP media with 2% glucose (**C**) YP media with 2 % glucose. Error margins represent the average of three repetitions.

4.4.2 GROWTH IN AEROBIC AND ANAEROBIC CONDITIONS WITH AND WITHOUT REINTRODUCED *PDA1*

To assess if the deletion of the *PDA1* gene is responsible for the growth phenotype of the Euroscarf strain, BEΔpda1, both BEΔpda1 and BMΔpda1 mutant strains were transformed with a CEN plasmid YCplac111 carrying a $PDA1_P$-*PDA1*-$PDA1_T$ cassette. An empty CEN plasmid was also transformed to both strains to evaluate the effect of plasmid maintenance and replication on yeast growth under both aerobic and anaerobic conditions with either 2% or 10% glucose.

As previously seen, analysis of the growth curves showed that the growth of BEΔpda1 mutant was significantly slower than the growth of BY4742 during all phases of growth in 2% glucose under anaerobic growth conditions (Figure 4.2 A). This slow growth phenotype of the Δ*pda1* mutant has been previously reported (Pronk, *et al.*, 1994; Wenzel, *et al.*, 1992). Upon growing BEΔpda1 in aerobic conditions in 2 % glucose, no significant changes in cell growth between the mutant and the wild type could be detected (Figure 4.2 C). This phenomenon is interesting as it suggest that the reduced growth phenotype is only prevalent during

anaerobiosis, and that molecular oxygen has a distinct role in its fermentative ability. The reason for such a phenotype cannot be elucidated, but may be related to ATP generation. When assessing the BMΔpda1 mutants which were generated in this laboratory, no significant differences in growth could be observed between these strains and BY4742 under both aerobic and anaerobic growth (Figure 4.2 B and D). Those data therefore contradict previous literature regarding the *PDA1* mutant phenotypes (Pronk, *et al*., 1994; Wenzel, *et al*., 1992).

The reintroduction of the *PDA1* gene in BEΔpda1(pPDA1) in 2% glucose medium under anaerobic conditions (Figure 4.2 A) restored growth of the strain back to wild type levels. This suggested that the introduction of the *PDA1* gene could complement the phenotype of the *pda1* gene knock-out. However, as a control for the introduction of pPDA1 in YCplac111, an empty YCplac111 vector was also introduced in BEΔpda1(YCplac111), and this empty vector restored growth to the same degree. This suggested a different type of metabolic regulation possibly linked to the *LEU2* auxotrophic marker.

When the strains were grown in anaerobic YPD medium containing 10% glucose, all strains showed a lag period of approximately six hours before beginning the exponential growth phase except for the BEΔpda1 mutant which had a longer adaptation period of approximately 12 hours (Figure 4.3 A). The growth of BEΔpda1 mutant continued to be significantly slower during the exponential growth phase for both aerobic and anaerobic cultures. In contrast to the BEΔpda1 mutant grown aerobically at 2 % glucose, those grown aerobically at 10 % glucose did show slower growth, but not to the extent of those in 10 % anaerobic conditions. It can be noted that the BEΔpda1 strain only had a growth phenotype similar to the reference strain in 2 % glucose under aerobic conditions. It may seem that the growth phenotype of BEΔpda1 is context dependent, and that the absence of a phenotype at 2 % aerobic growth is linked to conditions where glucose catabolite repression does not supersede oxygen availability. This hierarchical regulation of genes has previously been noted with genes of known targets of the Hap2/3/4/5p complex (Tai *et al*., 2005). The BMΔpda1 strain, as seen with cultures grown in 2 % glucose, did not show significant changes in growth kinetics at 10 % glucose compared to BY4742.

When the *PDA1* gene was re-transformed into the BEΔpda1 mutant using YCplac111 as the vector, the complemented strain BEΔpda1(pPDA1) showed wild type growth. As previously seen in 2 % glucose growth, an empty vector of YCplac111 could also complement this phenotype. This can possibly be linked to presence of the *LEU2* gene on the vector and hence could possibly replenish the auxotrophy for leucine and consequently improve its growth even in a rich medium like YPD (Figure 4.2 A, Figure 4.3 A and C). In contrast to the BEΔpda1 mutant, no growth phenotype was observed for the BMΔpda1 mutant and its derived strains under all conditions tested (Figures 4.2, 4.3 B and D). This result would have been expected as no significant changes in growth phenotype were noticed in the BMΔpda1 mutant itself.

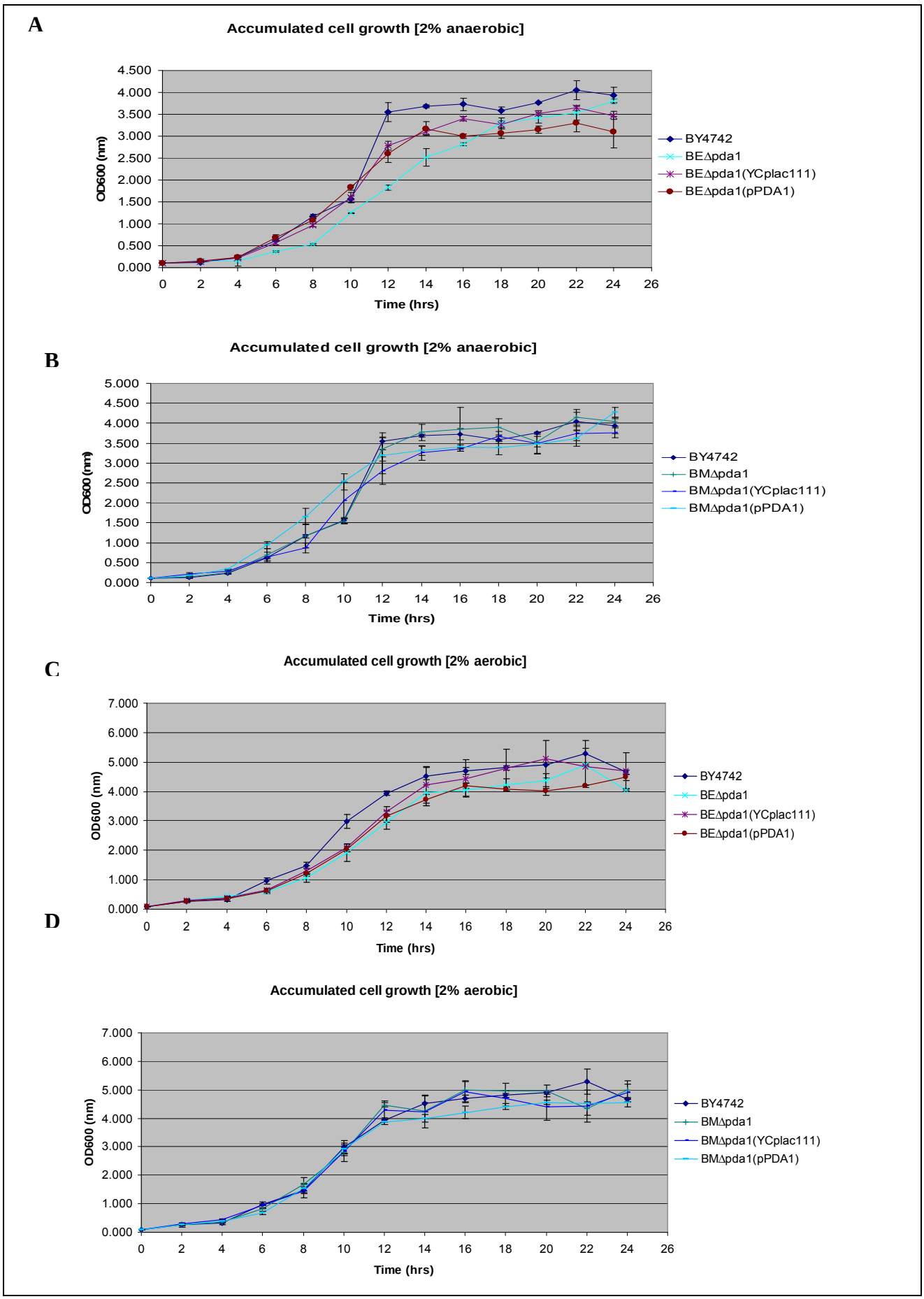


Figure 4.2 Growth analysis of Δ*pda1* mutants, and the *PDA1* restored strains in 2% glucose under both aerobic (C and D) and anaerobic (A and B) conditions.

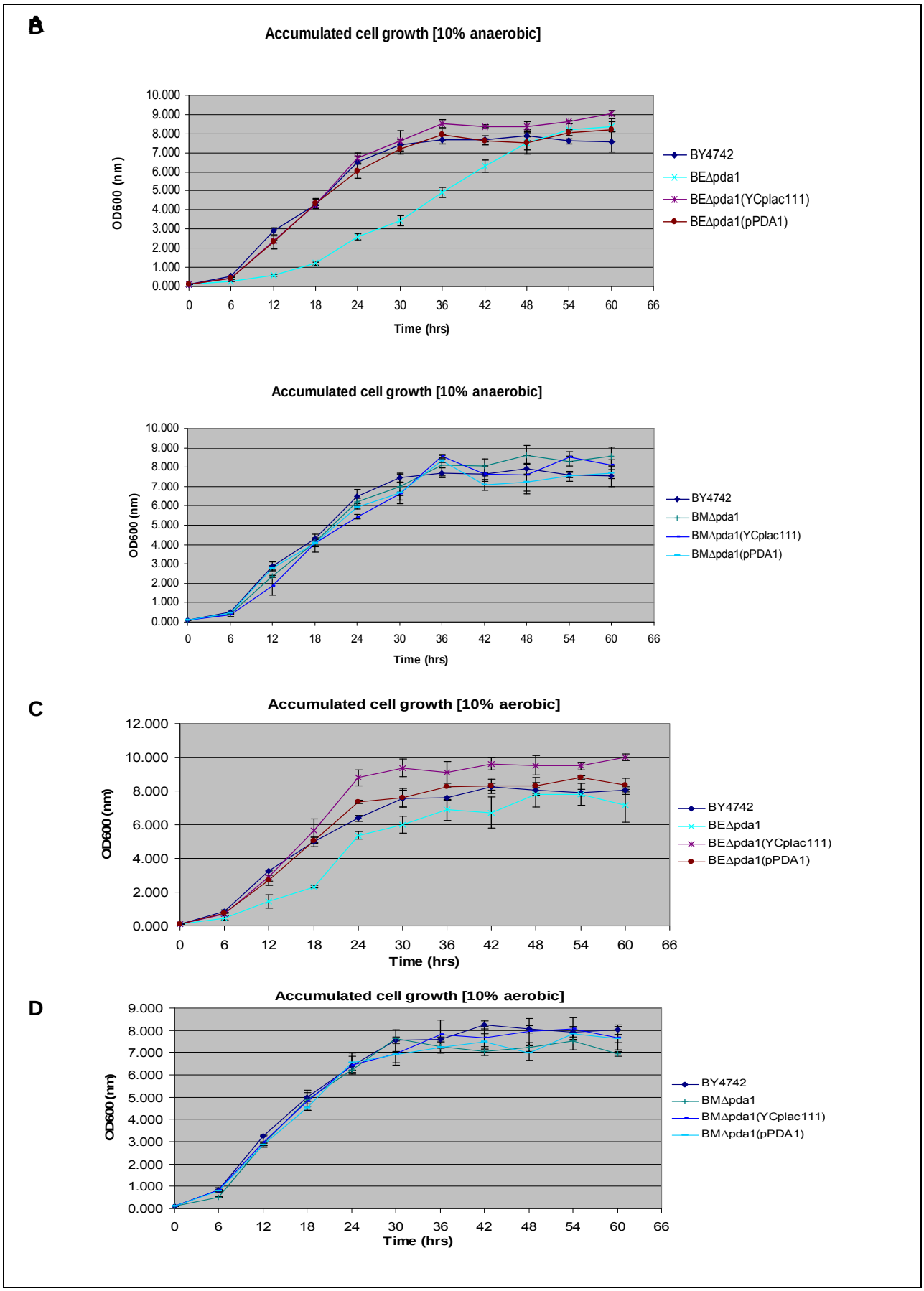


Figure 4.3 Growth analysis of Δ*pda1* mutants, and the corresponding *PDA1* restored strains and the effect of aerobic (C and D) and anaerobic (A and B) growth conditions on these strains in 10% glucose.

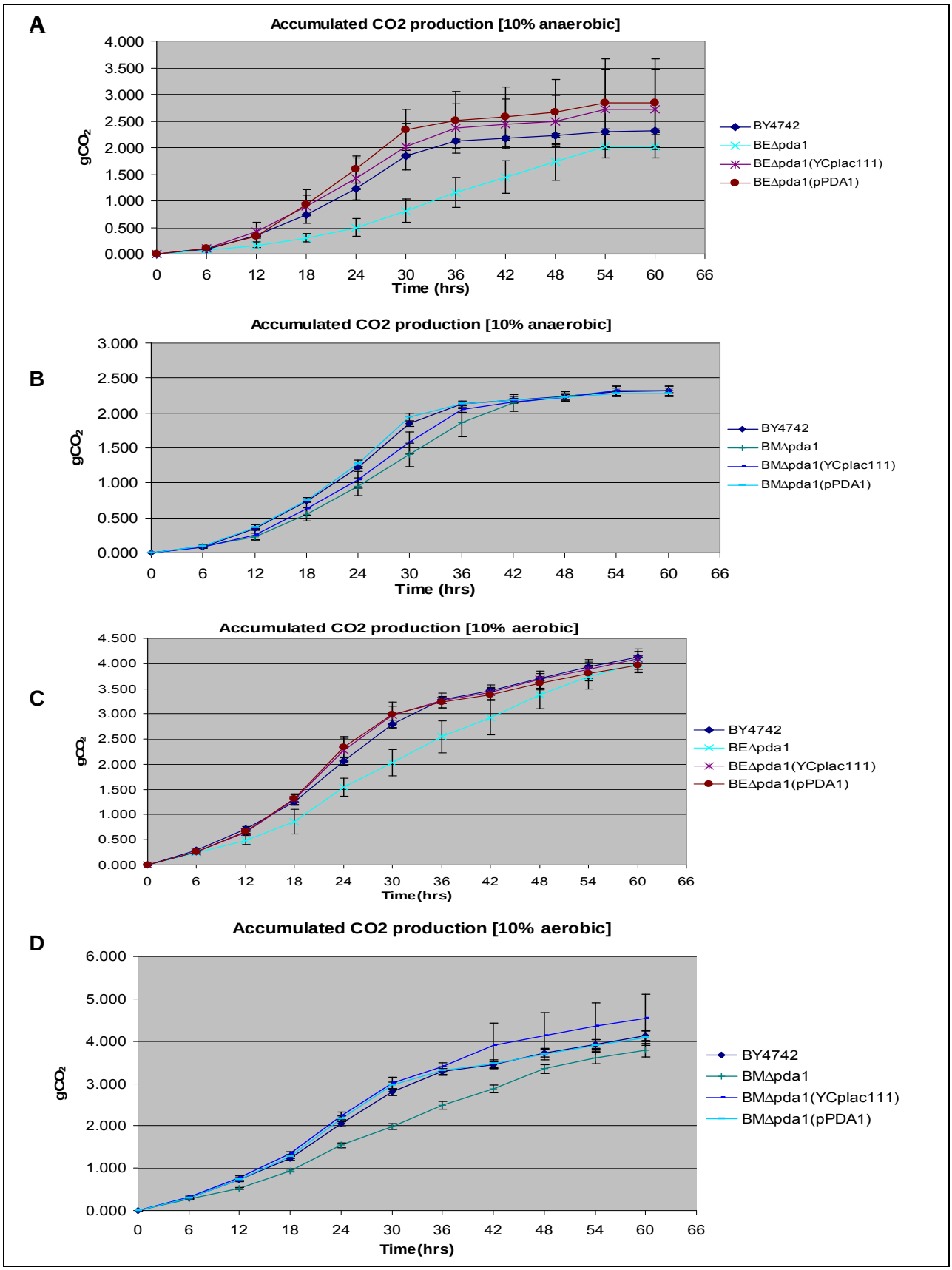


Figure 4.4 CO_2 profiles of Δ*pda1* mutants, and the corresponding *PDA1* restored strains under aerobic (C and D) and anaerobic (A and B) growth conditions in 10% glucose.

In order to monitor the fermentative activity of the cells during fermentation, the production of CO_2 was also measured. CO_2 profiles were only generated for fermentations grown at 10 % glucose and not for 2 % glucose fermentations as those grown at 2 % glucose completed fermentation within 24 hours making weight-loss measurements inaccurate. Analysis of CO_2 profiles showed that the fermentative activity of BE∆pda1 mutant followed those of the growth profile where CO_2 production was significantly lower than that of the wild type. Similarly the effect of this reduced growth/fermentative phenotype was more pronounced during anaerobic growth. On the other hand, both BE∆pda1(YCplac111) and BE∆pda1(pPDA1) strains had similar patterns of CO_2 production profiles as the wild type strain under both aerobic and anaerobic growth conditions (Figure 4.4 A and C).

Upon examining the fermentative behaviour of the BM∆pda1 mutant, this strain displayed similar fermentative abilities as the wild type under anaerobic conditions (Figure 4.4 B). However, the BM∆pda1 mutant showed a similar CO_2 profile as the BE∆pda1 mutant under aerobic conditions (Figure 4.4 D). This result showed discrepancy with cell growth (Figure 4.3 D), where BE∆pda1 mutant showed reduced growth compared to the reference strain, while BM∆pda1 did not. The data suggest that during anaerobiosis with 10 % glucose, BM∆pda1 has lower fermentative efficiency per unit biomass when compared to BE∆pda1.

4.5 DISCUSSION AND CONCLUSION

4.5.1 Elucidating the role of the *pda1* gene disruption on yeast growth

To confirm the ∆*pda1* mutant phenotype observed in the initial screen, the *PDA1* gene was deleted in three genetically different laboratory *S. cerevisiae* strains (BY4742, FY834 and Σ1278b). In the previous screen the ∆*pda1* mutant displayed a modified carbon flux which was associated with interesting phenotypes: (a) a long lag phase, followed by (b) a faster growth during exponential phase of fermentation, and (c) a shorter total alcoholic fermentation time than the wild type. When the *PDA1* gene was disrupted in these yeast strains and grown in YPD medium containing either 2% or 10% glucose, our newly generated ∆*pda1* mutants grew like their respective wild type strains and differed from the BE∆pda1 mutant from the Euroscarf collection. This is in contrast with the ∆*pda1* cells, which grow poorly on YPD medium (Wenzel *et al*., 1992). Moreover, disrupting the *PDA1* gene with a *LEU2* marker in BY4742 also gave similar growth phenotype. Likewise, growth phenotypes show that BM∆pda1 cells behave like wild type cells in both 2% and 10% glucose under aerobic and anaerobic conditions.

On YPD plates, colonies of BM∆pda1 mutant were similar in size and shape (irregular) to those of BE∆pda1 (data not shown). In addition to the rigorous PCR checks of the *pda1* gene knock-out, this additional test prove the authenticity of our yeast deletions in the strains generated for this study as it seemed to differentiate significantly in fermentative phenotype to that from the Euroscarf deletion library. In fact, our data explicitly show that lower concentrations of glucose in a growth medium (e.g. 2%) is inadequate to reveal the growth behaviour of certain yeast mutants because certain mutants require an extended period of time for adaptation prior to exponential growth. This is clearly seen with the BE∆pda1 mutant during growth at 2 % glucose in aerobic conditions.

The new or altered ∆*pda1* mutant phenotypes were not reproducible in three different genetic backgrounds. When complementation tests were carried out, wild type growth was successfully complemented by BE∆pda1(pPDA1) in 10% glucose and in 2% glucose under both aerobic and anaerobic conditions. However, analysis of the effect of the maintenance and replication of the CEN YCplac111 plasmid on the growth of BE∆pda1 [host] strain showed a restored growth of BE∆pda1 strain. This phenomenon may be explained by the fact that the

CEN plasmid may have complemented the partial leucine auxotrophy of the BE∆pda1 mutant, which has been reported in some ∆*pda1* mutants (Wenzel *et al*., 1992). Even in rich medium, the introduction of an auxotrophic marker may cause a gain-in-function in the organism as in such a case, the organism no longer needs to transport and/or seek this component but is able to synthesize it freely intracellularly.

Identifying new phenotypes poses a serious challenge in molecular studies. The differences in the growth and CO_2 production phenotypes of BE∆pda1 and BM∆pda1 mutants observed proved that the interesting growth phenotype of BE∆pda1 mutant in liquid cultures was not a result of the *PDA1* gene deletion but rather of secondary mutations that are likely to give significance to the growth during high-sugar wine fermentations. A similar situation for *tor1* mutants in the BY background has been reported. The *tor1* mutants displayed a lethality phenotype that was less pronounced and not reproducible even in other genetic backgrounds. This was speculated to be due to mutations that could not be detected (i.e. secondary) or strain differences (Boer *et al*., 2008). In most conditions tested, BM∆pda1 mutant displayed a growth phenotype that was significantly different from that of the BE∆pda1 mutant (Euroscarf collection) even though both mutants were disrupted in the *PDA1* gene in the same BY background.

Phenotypic observations attributed to a specific gene in mutants of the Euroscarf library should always be confirmed before performing consequent experiments and drawing significant conclusions. Similarly, growth conditions play an important aspect as during high-sugar fermentations, new phenotypes such as those seen for these ∆*pda1* mutants were established. These phenotypes may not have been observed during growth in normal laboratory conditions.

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Chapter 5

GENERAL DISCUSSION AND CONCLUSION

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 CONCLUDING REMARKS

For decades *S. cerevisiae* has been used to study many cellular processes and biochemical pathways within the eukaryotic cell because of its ease of propagation, short lifecycle and abundance of online information on its genetic code. The availability of high-throughput technologies have even opened more possibilities to study the molecular mechanisms of many biological processes in great detail and these tools play an important role in modern biology (Ideker *et al*., 2001). One of the developments was the generation of a collection of deletion mutants for each protein-encoding gene (Winzeler *et al*., 1999). Gene deletion is a fundamental tool that allows for the identification of uncharacterized genes as well as their roles in certain cellular processes and pathways. The approach is based on analyzing phenotypes of the mutants under specifically defined conditions, and deriving functional information regarding the roles of genes of previously unknown functions (Carpenter and Sabatini, 2004).

In this study, a phenotypic analysis of ten yeast mutants with single gene disruption involved in pyruvate metabolism were examined for increased fermentative efficiency and/or redirection of carbon fluxes during high-sugar wine fermentations. Chapter 2 of this thesis details the carbon metabolism of *S. cerevisiae* with a particular focus on the pyruvate branch point.

During high-sugar fermentations such as wine fermentations, it is thought that the prolonged fermentation time combined with a number of associated stresses might reveal unexpected phenotypes that would not normally be isolated in standard laboratory growth conditions. In Chapter 3, the initial screen for mutant strains with modified fermentation kinetics and / or carbon flux, revealed one deletion mutant that showed a significantly changed growth and CO_2 production kinetics. The Δ*pda1* mutant showed an interesting phenotype, with a longer lag phase followed by an exponential growth phase where the CO_2 production rate increased tremendously to a point where it out-grew the wild type strain. Because of this phenotype, the BEΔpda1 mutant could complete the alcoholic fermentation before the reference strain.

The *PDA1* gene encodes the E1α subunit that plays a crucial role in the decarboxylation of pyruvate into acetyl-CoA, thereby activating the TCA cycle. The reaction is reinforced by oxygen and limiting sugar concentrations (Pronk *et al*., 1996). On the other hand, in both anaerobic and sugar excess conditions the *PDA1* gene is repressed or expressed at basal levels (Wenzel *et al*., 1993). In Chapter 4, we set to prove that the phenotype obtained in BEΔpda1 as seen in Chapter 3 were indeed the true phenotype caused by the deletion of the *PDA1* gene. Our data showed that the growth profile of BEΔpda1 mutant was characterized by a longer lag phase which was absent in the wild type in both low (2%) and high (10%) sugar concentrations. This effect was more pronounced under anaerobic conditions. Under aerobic and low sugar conditions the growth profile of BEΔpda1 mutant was relatively similar to the wild type strain, thus coinciding with the reports of Pronk *et al* (1996) and Wenzel *et al* (1993). However, in our control strains in both low and high sugar concentrations the growth profile of BMΔpda1, FY834Δpda1 and ΣΔpda1 were similar to that of the wild type. Likewise, the effect of aeration did not affect the growth of the BMΔpda1 mutant significantly.

Previous reports showed that the Δ*pda1* mutant grows poorly on glucose (Pronk *et al*., 1994; Wenzel *et al*., 1992). However, in our study the Δ*pda1* mutant did not display any slow growth phenotype on glucose but rather had a longer adaptation period. It is clear that growth conditions have a significant impact on yeast growth as during high-sugar conditions, the

discrimination of growth phases were made clearer, thus allowing smaller changes in metabolism to be prominent.

The results of this thesis show that the fermentative ability phenotype of the Euroscarf Δ*pda1* mutant observed during high-sugar fermentations is not the sole result of the disrupted *PDA1* gene but rather secondary mutations which could not be screened and identified. This hypothesis is supported by the fact that all other generated Δ*pda1* mutants BMΔpda1, FY834Δpda1, ΣΔpda1 and BYLEUΔpda1 displayed growth profiles that were significantly different from that of the Euroscarf Δ*pda1* mutant and similar to their respective reference strains.

Upon the reintroduction of the *PDA1* gene in the Euroscarf Δ*pda1* strain, BEΔpda1(pPDA1) restored the wild type growth and fermentative ability in 10% glucose but less clearly in 2% glucose under both aerobic and anaerobic conditions. In addition, a reference strain fermentative phenotype was also complemented by the BEΔpda1 mutant transformed with an empty CEN YCplac111 plasmid. This effect may be explained by the fact that since the YCplac111 contain a *LEU2* marker, this marker may have complemented the partial leucine auxotrophy of the BEΔpda1(YCplac111) mutant, which has been reported for some Δ*pda1* mutants (Wenzel *et al*., 1992). Even in rich medium, the introduction of an auxotrophic marker may cause a gain-in-function in the organism as in such a case, the organism no longer needs to transport and seek this component but is able to synthesize it freely intracellularly.

Utilizing large libraries for screening potential targets is an established method in molecular biology especially for the uncovering of novel or previously unidentified gene functions. There have been many successful publications highlighting the use of the yeast deletion library in functional and comparative genomics. However this method of screening also has major pitfalls, such as the large amount of time invested in data analysis and the resources, both manpower and equipment required to carry out such laborious experiments. In addition, because of the size of the library, construction was carried out in different laboratories which may influence the precision and integrity of the library. It is also a difficult task to continuously monitor such a large library to ensure that it remains consistent, unadulterated and intact at all times.

This thesis goes to show that when the library was grown in sub-optimal growth conditions, secondary mutations in the respective strains became apparent. Therefore, phenotypes observed attributed by a specific gene in mutants of the Euroscarf library should always be confirmed before performing consequent experiments and drawing significant conclusions from the mass-screening results. Furthermore, this study opened new avenues for further investigations of screening and identifying possible secondary mutations as this will, of course, provide clues to the regulatory pathways involved.

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Appendix

SUPPLEMENTAL INFORMATION

I TABULAR REPRESENTATIONS OF STATISTICAL ANALYSIS OF DATA

The representations of data in a table form are provided to aid in the understanding of the statistical treatment employed to determine significant differences between the strains used in this study. All time points were analysed separately and only those time points (in hours and days) that are informative are presented in these tables.

Table A. Table showing the Statistical Differences between strains with respect to accumulated cell growth, CO_2 production, glycerol and ethanol production. If $p \leq 0.05$ the difference is significant.

(i)

Statistical Difference

Synthetic wine must (MS300)						
Accumulated Cell growth	Time (days)					
Strains	0	2	4	6	8	10
BY4742 vs Δpck1	NS	NS	NS	NS	NS	NS
BY4742 vs Δpda1	NS	SD **<**	NS	NS	NS	NS
BY4742 vs Δpdc1	NS	NS	NS	NS	NS	NS
BY4742 vs Δpdc5	NS	NS	NS	NS	NS	NS
BY4742 vs Δpdc6	NS	NS	NS	NS	NS	NS
BY4742 vs Δpyc1	NS	NS	NS	NS	NS	NS
BY4742 vs Δpyc2	NS	NS	NS	NS	NS	NS
BY4742 vs Δpyk2	NS	NS	NS	NS	NS	SD **>**

* NS indicates no significant difference, * SD indicates significant difference

(ii)

Statistical Difference

Synthetic wine must (MS300)						
Accumulated CO_2 production	Time (days)					
Strains	1	2	3	4	13	20
BY4742 vs Δpck1	NS	NS	NS	NS	NS	NS
BY4742 vs Δpda1	SD **<**	SD **<**	SD **<**	NS	SD **>**	NS
BY4742 vs Δpdc1	NS	NS	NS	NS	NS	NS
BY4742 vs Δpdc5	NS	NS	NS	NS	NS	NS
BY4742 vs Δpdc6	NS	NS	NS	NS	NS	NS
BY4742 vs Δpyc1	NS	NS	NS	NS	NS	NS
BY4742 vs Δpyc2	NS	NS	NS	NS	NS	NS
BY4742 vs Δpyk2	NS	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(iii)

Statistical Difference

Synthetic wine must (MS300)

Residual sugars	Time (days)				
Strains	0	2	8	14	20
BY4742 vs Δpck1	NS	NS	NS	NS	NS
BY4742 vs Δpda1	NS	NS	SD **>**	SD **>**	NS
BY4742 vs Δpdc1	NS	NS	NS	NS	NS
BY4742 vs Δpdc5	NS	NS	NS	NS	NS
BY4742 vs Δpdc6	NS	NS	NS	NS	NS
BY4742 vs Δpyc1	NS	NS	NS	NS	NS
BY4742 vs Δpyc2	NS	NS	NS	NS	NS
BY4742 vs Δpyk2	NS	NS	NS	NS	NS

(iv)

Statistical Difference

Synthetic wine must (MS300)

Accumulated Glycerol production	Time (days)				
Strains	0	2	8	14	20
BY4742 vs Δpck1	NS	NS	NS	NS	NS
BY4742 vs Δpda1	NS	SD **<**	SD **<**	SD **<**	SD **<**
BY4742 vs Δpdc1	NS	SD **<**	NS	NS	NS
BY4742 vs Δpdc5	NS	SD **<**	NS	NS	NS
BY4742 vs Δpdc6	NS	SD **<**	NS	NS	NS
BY4742 vs Δpyc1	NS	SD **<**	NS	NS	NS
BY4742 vs Δpyc2	NS	SD **<**	NS	NS	NS
BY4742 vs Δpyk2	NS	SD **<**	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(v)

Statistical Difference

Synthetic wine must (MS300)

Accumulated Ethanol production	Time (days)				
Strains	0	2	8	14	20
BY4742 vs Δpck1	NS	NS	NS	NS	NS
BY4742 vs Δpda1	NS	SD **<**	SD **>**	NS	NS
BY4742 vs Δpdc1	NS	NS	NS	NS	NS
BY4742 vs Δpdc5	NS	NS	NS	NS	NS
BY4742 vs Δpdc6	NS	NS	NS	NS	NS
BY4742 vs Δpyc1	NS	NS	NS	NS	NS
BY4742 vs Δpyc2	NS	NS	NS	NS	NS
BY4742 vs Δpyk2	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

Table B. Table showing the Statistical Differences between strains with respect to accumulated cell growth. If $p<0.05$ the difference is significant.

(i)

Statistical Difference

YP medium + 10% glucose

Accumulated cell growth	Time (hours)				
Strains	12	20	30	42	48
BY4742 vs BEΔpda1	SD **<**	SD **<**	SD **>**	SD **<**	NS
BY4742 vs BMΔpda1	NS	NS	NS	NS	NS
FY834 vs FY834Δpda1	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(ii)

Statistical Difference

YP medium + 2% glucose

Accumulated cell growth	Time (hours)				
Strains	2	12	15	18	24
BY4742 vs BEΔpda1	SD **<**	SD **<**	SD **<**	SD **<**	SD **>**
BY4742 vs BMΔpda1	NS	SD **<**	NS	NS	NS
BY4742 vs BYLEUΔpda1	NS	NS	NS	NS	SD **>**

* NS indicates no significant difference, * SD indicates significant difference

(iii)

Statistical Difference

YP medium + 2% glucose

Accumulated cell growth	Time (hours)				
Strains	2	6	12	18	24
BY4742 vs BEΔpda1	NS	SD **<**	SD **<**	SD **<**	SD **>**
∑1278 vs ∑1278Δpda1	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

Table C. Table showing the Statistical Differences between strains with respect to accumulated cell growth. If $p<0.05$ the difference is significant.

(i)

(**Statistical Difference**

YP medium + 2% glucose (anaerobic)

Accumulated cell growth	Time (hours)						
Strains	2	6	12	14	16	20	24
BY4742 vs BEΔpda1	NS	SD **<**	SD **<**	SD **<**	SD **<**	NS	NS
BY4742 vs BEΔpda1(YCplac111)	NS	NS	SD **<**	SD **<**	NS	NS	NS
BY4742 vs BEΔpda1(pPDA1)	NS	NS	SD **<**	SD **<**	SD **<**	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(ii)

Statistical Difference

YP medium + 2% glucose (anaerobic)

Accumulated cell growth	Time (hours)						
Strains	8	10	12	14	16	20	24
BY4742 vs BMΔpda1	NS	NS	NS	NS	NS	NS	NS
BY4742 vs BMΔpda1(YCplac111)	NS	NS	NS	NS	NS	NS	NS
BY4742 vs BMΔpda1(pPDA1)	SD **>**	SD **>**	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(iii)

Statistical Difference

YP medium + 2% glucose (aerobic)

Accumulated cell growth	Time (hours)			
Strains	8	14	20	24
BY4742 vs BEΔpda1	NS	NS	NS	NS
BY4742 vs BEΔpda1(YCplac111)	NS	NS	NS	NS
BY4742 vs BEΔpda1(pPDA1)	SD **>**	SD **>**	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(iv)

Statistical Difference

YP medium + 2% glucose (aerobic)

Accumulated cell growth	Time (hours)			
Strains	8	14	20	24
BY4742 vs BMΔpda1	NS	NS	NS	NS
BY4742 vs BMΔpda1(YCplac111)	NS	NS	NS	NS
BY4742 vs BMΔpda1(pPDA1)	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

Table D. Table showing the Statistical Differences between strains with respect to accumulated cell growth. If $p<0.05$ the difference is significant.

(i)

Statistical Difference				
YP medium + 10% glucose (anaerobic)				
Accumulated cell growth	Time (hours)			
Strains	6	24	48	60
BY4742 vs BEΔpda1	SD **<**	SD **<**	NS	NS
BY4742 vs BEΔpda1(YCplac111)	NS	NS	NS	NS
BY4742 vs BEΔpda1(pPDA1)	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(ii)

Statistical Difference				
YP medium + 10% glucose (anaerobic)				
Accumulated cell growth	Time (hours)			
Strains	6	24	48	60
BY4742 vs BMΔpda1	NS	NS	NS	NS
BY4742 vs BMΔpda1(YCplac111)	NS	NS	NS	NS
BY4742 vs BMΔpda1(pPDA1)	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(iii)

Statistical Difference						
YP medium + 10% glucose (aerobic)						
Accumulated cell growth	Time (hours)					
Strains	6	24	42	48	54	60
BY4742 vs BEΔpda1	SD **<**	NS	SD **<**	NS	NS	NS
BY4742 vs BEΔpda1(YCplac111)	NS	NS	NS	SD **>**	SD **>**	SD **>**
BY4742 vs BEΔpda1(pPDA1)	NS	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(iv)

Statistical Difference						
YP medium + 10% glucose (aerobic)						
Accumulated cell growth	Time (hours)					
Strains	6	24	42	48	54	60
BY4742 vs BMΔpda1	NS	NS	NS	NS	NS	NS
BY4742 vs BMΔpda1(YCplac111)	NS	NS	NS	NS	NS	NS
BY4742 vs BMΔpda1(pPDA1)	NS	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

Table E. Table showing the Statistical Differences between strains with respect to accumulated cell growth. If $p<0.05$ the difference is significant.

(i)

Statistical Difference						
YP medium + 10% glucose (anaerobic)						
CO_2 profiles	Time (hours)					
Strains	6	24	42	48	54	60
BY4742 vs BEΔpda1	SD **<**	SD **<**	SD **<**	NS	NS	NS
BY4742 vs BEΔpda1(YCplac111)	NS	NS	NS	NS	NS	NS
BY4742 vs BEΔpda1(pPDA1)	NS	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(ii)

Statistical Difference						
YP medium + 10% glucose (anaerobic)						
	Time (hours)					
Strains	6	24	42	48	54	60
BY4742 vs BMΔpda1	NS	NS	NS	NS	NS	NS
BY4742 vs BMΔpda1(YCplac111)	NS	NS	NS	NS	NS	NS
BY4742 vs BMΔpda1(pPDA1)	NS	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(iii)

Statistical Difference						
YP medium + 10% glucose (aerobic)						
	Time (hours)					
Strains	6	24	42	48	54	60
BY4742 vs BEΔpda1	SD **<**	SD **<**	SD **<**	NS	NS	NS
BY4742 vs BEΔpda1(YCplac111)	NS	NS	NS	NS	NS	NS
BY4742 vs BEΔpda1(pPDA1)	NS	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference

(iv)

Statistical Difference						
YP medium + 10% glucose (aerobic)						
	Time (hours)					
Strains	6	24	42	48	54	60
BY4742 vs BMΔpda1	SD **<**	SD **<**	SD **<**	SD **<**	NS	NS
BY4742 vs BMΔpda1(YCplac111)	NS	NS	SD **>**	SD **>**	SD **>**	NS
BY4742 vs BMΔpda1(pPDA1)	NS	NS	NS	NS	NS	NS

* NS indicates no significant difference, * SD indicates significant difference