Central Carbon Metabolism in the Biocontrol Yeast *Pichia anomala*

Influence of Oxygen Limitation

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Abstract

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The biocontrol yeast *Pichia anomala* prevents mould damage of moist cereal grain during malfunctioning airtight storage but it can also spoil food and feed. This thesis focuses on the physiology and metabolism of *P. anomala*, in particular during oxygen limitation, a condition relevant to airtight storage of cereal grain.

P. anomala grew under strictly anaerobic conditions, at temperatures between 3ºC and 37ºC, pH values between 2.0 and 12.4, low water activity (0.85), and on many different nutrients. Accumulation of low-molecular compounds in living cells was analysed by HR MAS-NMR. Glycerol, arabitol, and trehalose accumulation increased with reduced oxygen availability, indicating a role during oxygen-limited growth. Regulation of the central aerobic and hypoxic metabolism of *P. anomala* was investigated under controlled fermentor conditions. Oxygen limitation induced alcoholic fermentation as well as activity of the key fermentative enzymes, ADH and PDC. Metabolic flux analysis revealed that the TCA pathway operated as a cycle during aerobic batch culture and as a two-branched pathway under oxygen limitation. Hypoxic conditions also increased the production of ethyl acetate, an ester involved in the biocontrol activity of *P. anomala*.

Genes encoding the ADH and PDC enzymes were cloned, *PaADH1*, *PaADH2*, and *PaPDC1*, and their expression was analysed with real-time RT-PCR. *PaADH1* and *PaPDC1* were expressed during aerobic growth on glucose and ethanol and were up-regulated in response to oxygen limitation. *PaADH2* expression was low during these growth conditions, *i.e.* <1% of the level of its isogene, *PaADH1*. In cells grown on succinate, the expression of the two *ADH* isogenes was the opposite, high expression of *PaADH2* and low expression of *PaADH1*. The up-regulation of gene expression and enzyme activity did not quantitatively correlate with glycolytic flux. Thus, additional regulatory phenomena at the posttranscriptional and posttranslational level are important in the distribution of carbon through the respiratory and fermentative pathways.

Keywords: Crabtree negative, acetate, alcohol dehydrogenase, pyruvate decarboxylase, aldehyde dehydrogenase, *Penicillium roqueforti*

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Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Fredlund, E., Druvefors, U., Lingsten, K-J., Boysen, M. E., and Schnürer J. 2002. Physiological characteristics of the biocontrol yeast *Pichia anomala* J121. *FEMS Yeast Res* 2:395-402.

II. Fredlund, E., Broberg, A., Boysen, M. E., Kenne, L., and Schnürer, J. 2004. Metabolite profiles of the biocontrol yeast *Pichia anomala* J121 grown under oxygen limitation. *Appl Microb Biotech* 64:403-409.

III. Fredlund, E., Druvefors, U. Ä., Olstorpe Nilsson, M., Passoth, V., and Schnürer, J. 2004. Influence of ethyl acetate production and ploidy on the anti-mould activity of *Pichia anomala*. *FEMS Microbiol Lett* 238:133-137.

IV. Fredlund, E., Blank, L., Schnürer, J., Sauer, U., and Passoth, V. 2004. Oxygen and glucose dependent regulation of central carbon metabolism in *Pichia anomala*. *Appl Environ Microbiol*. 70:5905-5911.

V. Fredlund, E., Beerlage, C., Schnürer, J., and Passoth, V. Oxygen and carbon source-regulated expression of *PDC* and *ADH* genes in the respiratory yeast *Pichia anomala*. (manuscript).

Introduction

Yeast metabolism is widely exploited in many biotechnological processes all around the world. The most important yeast related industries are the production of wine, beer, and bread (Boekhout & Robert, 2003; de Winde, 2003). Other areas include fuel alcohol production, expression of heterologous proteins, and yeast in biocontrol applications (Walker, 1998). Yeast metabolism can also cause spoilage of food and drinks by unwanted production of aroma compounds, gas or alcohol (Fleet, 1992).

Yeasts can be regarded as unicellular fungal growth forms, which have resulted as a response to a commonly encountered set of environmental pressures (Kendrick, 1987). Yeasts belong to the ascomycetous or basidiomycetous fungi (van der Walt, 1987; Kurtzman & Fell, 1998) and reproduce by budding or fission. Most yeasts do not produce true hyphae as their relatives, the filamentous fungi (moulds), but many are able to produce pseudohyphae (Kurtzman & Fell, 1998) in response to environmental stress (Zaragoza & Gancedo, 2000). The unicellular growth form facilitates large-scale cultivation of yeasts in biotechnological processes.

The ascomycetous yeast *Saccharomyces cerevisiae* is the most commonly used species in biotechnological applications and is by far the most dominating species in yeast research. As a consequence, the term ”yeast” has in some contexts been used as a synonym to the species name *S. cerevisiae*. However, the properties of *S. cerevisiae* do not represent those of all yeasts. Among the approximately 800 recognized yeast species (Barnett, Payne & Yarrow, 2000), only few species are used commercially and the physiological and metabolic properties of most yeast have not yet been described.

The yeast *Pichia anomala* occurs naturally in many food and feed environments (Kitamoto *et al.*, 1999; Kurtzman, 1998; Lacey & Magan, 1991; Lanciotti *et al.*, 1998; Tokuoka *et al.*, 1985) and has biocontrol activity against several filamentous fungi (Druvefors *et al.*, 2002; Jijakli & Lepoivre, 1998; Masih, Alie & Paul, 2000; Petersson & Schnürer, 1998). As for most non-*Saccharomyces* yeasts, the metabolism of *P. anomala* is not well investigated.

Aims

The ability of *P. anomala* to prevent mould growth when added to malfunctioning airtight storage systems for moist cereal grain is well documented (Druvefors, 2004; Petersson, 1998). However, little attention has been given to the physiological and metabolic properties of the yeast. The general aim of this thesis was to increase the knowledge of *P. anomala* physiology and metabolism, in particular in relation to the biocontrol environment. The effect of oxygen limitation on the metabolic activity of *P. anomala* was of special interest, because this could influence its antifungal activity in the grain biocontrol system.

The specific objectives of this thesis were to:

- characterize the growth ability of *Pichia anomala* on different nutrients and under various environmental conditions (**I**)
- identify the major low-molecular metabolites produced by *Pichia anomala* (**II, IV**)
- study the effect of oxygen limitation on growth (**I, II, IV**), metabolism (**II, IV**) and gene expression (**V**) of *Pichia anomala*
- investigate the regulation of central carbon metabolism in *Pichia anomala* at the level of metabolic flux, enzymatic activity, and gene expression (**IV, V**)
- study the connection between metabolic and biocontrol activity in *Pichia anomala* (**III, IV**)

Pichia anomala

Pichia (syn *Hansenula*) *anomala* (Hansen) Kurtzman is an ascomycetous, heterothallic yeast that reproduces asexually by budding and sexually by the formation of hat-shaped ascospores (Kurtzman, 1998). The ascospores are formed from the diploid cell by direct transformation of the vegetative cell into one to four spores. *P. anomala* is able to grow with branched pseudohyphae, *i.e.* elongated cells that are not released from their mother-cell (Kurtzman, 1998). It is generally believed that the yeast exists in the diploid form in its natural environment (Kurtzman, 1998; Naumov, Naumova & Schnürer, 2001).

P. anomala is present in many types of environments and have been isolated from fruit and plant material (Kurtzman, 1998), cereal grain (Lacey & Magan, 1991), maize silage (Kitamoto *et al.*, 1999), and from high sugar food products (Lanciotti *et al.*, 1998; Tokuoka *et al.*, 1985). *P. anomala* is frequently isolated from wine and has been reported to be a non-*Saccharomyces* wine yeast (Mingorance-Cazorla *et al.*, 2003; Rojas *et al.*, 2003). In addition, it plays a role in the natural fermentation of various fermented drinks and food products (Masoud *et al.*, 2004; Sujaya *et al.*, 2004). *P. anomala* is also an efficient biocontrol agent against mould infections in malfunctioning airtight stored grain (Druvefors *et al.*, 2002; Petersson & Schnürer, 1998), on apples (Jijakli & Lepoivre, 1998), and on grape vine (Masih, Alie & Paul, 2000). Due to its ability to grow at low water activity (a_w) and pH, *P. anomala* may cause food spoilage in high sugar environments (Lanciotti *et al.*, 1998; Tokuoka *et al.*, 1985) as well as increase the pH in grass silage by consumption of lactic acid (Jonsson & Pahlow, 1984). *P. anomala* can also spoil wine by producing high amounts of the aromatic compound ethyl acetate (Rojas *et al.*, 2001), which can add a vinegary taste to the wine (Rapp & Mandery, 1986).

P. anomala is classified as a biosafety level 1 organism that is considered safe for healthy individuals (de Hoog, 1996). In the literature, there are no reports on hazardous mycotoxin formation or the production of allergenic spores from yeast. *P. anomala* infections have been reported in cancer patients, infants and premature neonates, and in patients at a surgical intensive care unit (Aragão *et al.*, 2001; Chakrabarti *et al.*, 2001; Kalenic *et al.*, 2001; Thuler *et al.*, 1997). None of the infections had lethal outcome; yet, *P. anomala* is considered to be an emerging pathogen in severely immunocompromised hosts (Hazen, 1995).

The level of oxygen in the growth environment has a strong regulatory effect on the metabolic activity of yeasts. During malfunctioning storage of moist cereal grain the oxygen availability is rather limited (0-4%; Druvefors *et al.*, 2002). The influence of oxygen limitation on the regulation of central carbon- and energy metabolism in *P. anomala* affects growth but also the antifungal activity of the yeast in the grain biocontrol system.

Yeast metabolism – an overview

Based on the ability to perform and regulate alcoholic fermentation, yeasts are grouped as *obligate aerobes*, *i.e.*, yeasts that are incapable of alcoholic fermentation, or *facultative aerobes*, *i.e.*, yeasts that are capable of producing ethanol (Fig. 1) (Gancedo & Serrano, 1989). The facultative aerobes can be further grouped into the *respiratory* yeasts, *i.e.*, yeasts that induce alcoholic fermentation in response to oxygen limitation, or the *fermentative* yeasts, which are those that produce ethanol during aerobic batch cultivation on glucose, *i.e.,* the so-called Crabtree effect (Gancedo & Serrano, 1989). Though based on the same criteria, different authors use different nomenclature for the two groups (Fig. 1). Most known yeasts are respiratory and include *P. anomala*, *Pichia stipitis*, *Debaryomyces hansenii*, *Candida albicans, C. utilis,* and species of *Kluyveromyces* (de Deken, 1966; Veiga, Arrabaca & Loureiro-Dias, 2003). The *fermentative* yeasts include *S. cerevisiae, Zygosaccharomyces bailii, Dekkera bruxellensis,* and *Schizosaccharomyces pombe* (de Deken, 1966; Veiga, Arrabaca & Loureiro-Dias, 2003). The fermenting yeasts (Fig. 1) can grow with respirofermentative metabolism, *i.e.* with simultaneous respiration and fermentation (Gancedo & Serrano, 1989).

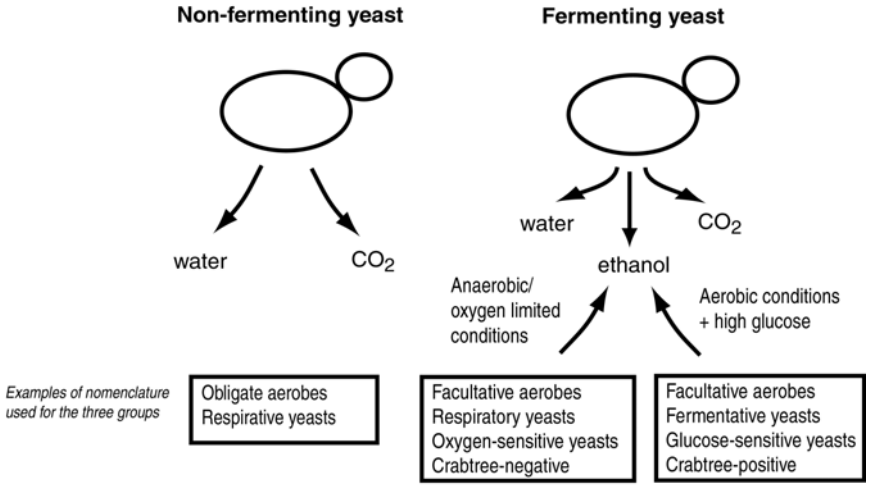

Figure 1. Group assignments of yeasts based on the ability to perform and regulate alcoholic fermentation as proposed by various authors. For a more detailed discussion of the Crabtree effect, see De Deken (1966), of the oxygen and glucose sensitivity, see Fiechter, Fuhrmann, & Käppeli (1981), of the respirative yeasts see Käppeli (1986), and of the obligate and facultative aerobes see Gancedo & Serrano (1989).

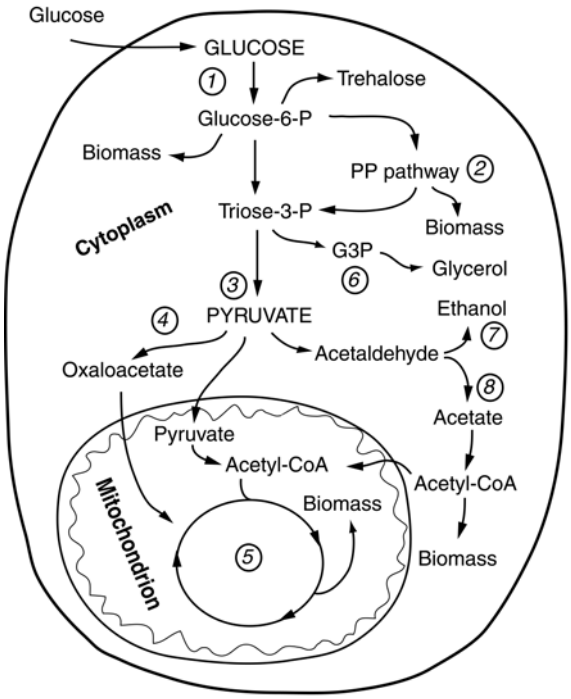

Figure 2. Schematic overview of the main carbon metabolic pathways in yeasts. The numbers represent: (*1*) phosphorylation of glucose, (*2*) the pentose phosphate pathway, (*3*) the pyruvate branchpoint, (*4*) anaplerotic production of oxaloacetate, (*5*) the TCA cycle, (*6*) the reoxidation of NADH by glycerol production, (*7*) the production of ethanol from acetaldehyde, and (*8*) the production of acetate from acetaldehyde. For simplification, production and consumption of ATP, NADH, and CO_2 were not included in the overview. G3P is an abbreviation of glycerol-3-phosphate.

All yeasts described so far can use glucose as the sole carbon source (Barnett, Payne & Yarrow, 2000). In many yeasts, glucose is transported across the membrane by facilitated diffusion (Flores *et al.*, 2000; Gerós, Cássio & Leão, 1999; Maier *et al.*, 2002; Nobre, Lucas & Leão, 1999). Facilitated diffusion is mediated by a membrane protein, a permease, down a concentration gradient without the consumption of energy (Fiechter, Fuhrmann & Käppeli, 1981). Energy dependent H^+-symport systems have also been described (Flores *et al.*, 2000; van Urk *et al.*, 1989). Intracellular glucose is phosphorylated to glucose-6-phosphate (Fig. 2; *1*), which is then distributed between glycolysis, the pentose phosphate (PP) pathway (Fig. 2; *2*), the trehalose synthesis pathway, and biomass. The flux into the PP pathway is regulated by the cellular need for the reducing equivalents NADPH, required in the biosynthetic reactions (Bruinenberg, van Dijken & Scheffers, 1983a; van Dijken & Scheffers, 1986).

Most of the glucose-6-phosphate is directed through glycolysis, which is a series of reactions leading to the formation of pyruvate (Gancedo & Serrano, 1989) (**IV**). At the pyruvate branching point (Fig. 2; *4*), the carbon is distributed between the respiratory and fermentative pathways. Pyruvate can follow three different fates depending on the yeast species and the environmental conditions (Pronk, 1996).

Under respiratory conditions, pyruvate is transported into the mitochondria where it is converted to acetyl CoA by the mitochondrial pyruvate dehydrogenase enzyme complex (Kresze & Ronft, 1981). Acetyl CoA is further oxidized in the TCA cycle. During respiration, intermediates of the TCA cycle are withdrawn as building material for cell biomass. To avoid carbon drainage of the cycle, carbon must be supplied to the cycle via anaplerotic reactions. One such reaction is oxaloacetate production from cytosolic pyruvate (Fig. 2; *3*) (Gancedo & Serrano, 1989). During fermentative conditions, puruvate is decarboxylated to acetaldehyde by the enzyme pyruvate decarboxylase. Acetaldehyde can either be further reduced to ethanol by the enzyme alcohol dehydrogenase (Lutstorf & Megnet, 1968) or oxidized to acetate by aldehyde dehydrogenase (Jacobson & Bernofsky, 1974).

The main purpose of sugar dissimilation is to generate energy in the form of ATP, which is required for cell maintenance and synthesis of essential yeast biomass components. However, during glucose dissimilation, ATP is also consumed in the phosphorylation of glucose and glucose-6-phosphate as well as in the incorporation of CO_2 for the anaplerotic production of oxaloacetate. During fermentation, two ATP molecules per molecule of glucose are formed by substrate level phosphorylation in the glycolytic pathway. During complete respiratory dissimilation of glucose in *S. cerevisiae*, 12 additional ATP molecules per glucose are produced by oxidative phosphorylation in the mitochondrial membrane (Bakker *et al.*, 2001). The efficiency of the oxidative phosphorylation is lower in yeast compared to other organisms and can also vary between yeast species (Verduyn *et al.*, 1991). The reduced ATP production during fermentative glucose dissimilation is also reflected in the reduced biomass yield, which is approximately five-fold lower during fermentation (Verduyn *et al.*, 1990; Weusthuis *et al.*, 1994) (**IV**).

Maintaining the redox balance of the cell is crucial for maintaining a metabolic activity (van Dijken & Scheffers, 1986). During yeast growth, NAD^+ is reduced to NADH in the cytosol as well as in the mitochondria (van Dijken & Scheffers, 1986). To maintain the redox balance, NAD^+ must be regenerated by reoxidation of NADH. Due to the impermeability of the inner mitochondrial membrane to pyridine nucleotide coenzymes (von Jagow & Klingenberg, 1970), reoxidation of NADH must take place in the compartment where it is generated. Cytosolic NADH is reoxidized by different pathways during aerobic and anaerobic (oxygen limited) conditions. In the presence of oxygen, cytosolic NADH is reoxidized by external NADH dehydrogenases in the inner mitochondrial membrane (Luttik *et al.*, 1998; Small & McAlister-Henn, 1998). In contrast, during limited oxygen supply, the co-factor is reoxidized in the ethanol and glycerol production pathways (van Dijken & Scheffers, 1986). NAD^+ cofactors are reduced in the glycolysis and during biomass production. The NADH produced in the glycolytic pathway are reoxidized in the reaction from acetaldehyde to ethanol, *i.e.*, ethanol production is a redox neutral process. NADH produced during biomass formation is reoxidized in the first step of glycerol synthesis, in the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (Gancedo, Gancedo & Sols, 1968) (Fig 2; *6*).

The metabolic pathways of the central carbon metabolism are generally the same between different yeast species, giving the impression that yeasts constitute a homogenous metabolic group. However, the number of isoenzymes and genes, the mechanisms for nutrient uptake and transport, and most importantly, the regulation of fermentation and respiration differ substantially (Flores *et al.*, 2000).

Implication of yeast metabolism in food and feed environments

Production of food and drinks

Yeasts are important fermentation agents in many food products and beverages. Depending on the production conditions and the starting material, *e.g.* dough for bread baking and must for wine making, the yeast contributes differently to the finished product. The two quantitatively most important yeast metabolites are ethanol and CO_2, and their production is exploited in the alcoholic beverages and bread industry. Equally important for high final product quality is the contribution of yeast to aroma and flavour by their production of higher alcohols, esters, and organic acids (Dequin *et al.*, 2003b; Dufour, Verstrepen & Derdelinckx, 2003; Nout, 2003).

During production of wine and beer, the quantitatively most important product of yeast metabolism is ethanol. To facilitate the industrial process, it is important that the yeast strain can efficiently ferment sugar under aerobic conditions. *S. cerevisiae* is the preferred yeast in wine making and is referred to as the "wine yeast" (Dequin *et al.*, 2003b). Two yeast species are commonly used in beer production, the lager brewing (bottom-fermenting) yeast, *S. carlsbergensis* [*S. carlsbergensis* is in fact a hybrid of *S. cerevisiae* and another species of *Saccharomyces* and not a true taxonomical group (Dufour, Verstrepen & Derdelinckx, 2003)], and the ale brewing (top-fermenting) yeast, *S. cerevisiae* (Dufour, Verstrepen & Derdelinckx, 2003). At low glucose concentrations, *S. cerevisiae* stops producing ethanol, favouring biomass production through respiration (Gancedo & Serrano, 1989). In addition, complete exhaustion of the sugar induces ethanol assimilation, reducing the yield of ethanol. Therefore, sugar concentration must be carefully regulated to obtain an efficient fermentation process. Non-*Saccharomyces* wine yeasts, including *P. anomala*, contribute to the flavour and aroma of wine by producing aroma compounds (Mingorance-Cazorla *et al.*, 2003; Rojas*, et al.*, 2001). The spontaneous flora of non-*Saccharomyces* wine yeasts proliferate during the first part of fermentation but subsequently die off as a result of the inhibiting ethanol concentration and the sub-optimal temperature and pH of the fermentation process (Heard & Fleet, 1988).

In bread making, the most valuable product of yeast metabolism is CO_2. CO_2 is formed during both respiratory and fermentative growth but to a higher degree during fermentation, due to the higher glycolytic flux (Lagunas, 1986; Postma *et al.*, 1989; Verduyn *et al.*, 1990). The most important bread-making yeast is *S. cerevisiae* but the stress-resistant yeast *Torulaspora delbrueckii* has been commercialised for frozen dough applications in Japan (Randez-Gil *et al.*, 2003). In the production of baker's yeast, the most wanted end-product is the yeast biomass itself. During biomass production, the sugar level must be carefully

regulated, just as in the production of ethanol, but for the opposite reason. High yield biomass production requires respiratory conditions and to avoid ethanol formation, glucose must be fed to the fermentor continuously (continuous culture) or in doses (fed-batch culture) to keep the glucose concentration low.

All around the world, various yeast species play key roles in the preparation of indigenous fermented alcoholic beverages from cereals and sugary juices (Nout, 2003). In fermented dough and batter, lactic acid bacteria are the most important microbial component of the fermentation process. However, yeasts are often present and contribute to flavour, texture, and to the nutritive value (Nout, 2003). *P. anomala* has been isolated from a variety of alcoholic beverages, including drinks based on cereals, rice, and plants (Sefa-Dedeh *et al.*, 1999; Sujaya *et al.*, 2004; Thapa & Tamang, 2004) and also from fermented coffee beans (Masoud *et al.*, 2004).

Food spoilage

Yeasts can cause food spoilage by their growth and production of metabolic end-products. Due to the low pH and the presence of preservatives in soft drinks and fruit juices, most microorganisms cannot grow. The microorganisms that do grow in this environment include several yeast species (Fleet, 1992), which can thus thrive on the high sugar content, producing as a results ethanol, CO_2, and other fermentation products. Examples of major spoilage yeasts of non-alcoholic beverages are *S. cerevisiae*, *Z. bailii*, and *Dekkera anomala* (Stratford & James, 2003). Alcoholic beverages can also be spoiled by yeast. The indigenous yeast flora present in the first part of fermentation may reduce wine quality through their metabolic activity. *P. anomala* is commonly isolated during the early and middle stages of wine fermentation and may spoil wine through its strong production of ethyl acetate (Rojas*, et al.*, 2001). This ester gives the wine a vinegary taste if present in concentrations exceeding 200 mg per liter (Dequin *et al.*, 2003a; Rapp & Mandery, 1986).

Biocontrol activity

P. anomala can inhibit growth of the filamentous fungi *Penicillium roqueforti* in malfunctioning airtight storage silos for cereal grain (Druvefors*, et al.*, 2002; Petersson & Schnürer, 1998). The mode of action behind the inhibitory activity of biocontrol yeasts is generally poorly understood. It has been suggested that competition for nutrients and space as well as the production of inhibitory substances may be involved (Filonow, 2003; Mercier & Jiménez, 2004). Druvefors (2004) tested whether the inhibition of *P. roqueforti* was due to competition for nutrients by adding various concentrations of glucose or nitrogen to grain test tubes. The hypothesis behind the experiment was that nutrient addition should abolish the biocontrol activity if competition was the major mode of action. This has previously been shown for the biocontrol yeasts *Pichia guilliermondi* and *D. hansenii* (Droby *et al.*, 1990; Droby *et al.*, 1989).

In contrast, Druvefors (2004) found that nitrogen addition did not affect the biocontrol activity at all, whereas glucose addition increased, instead of reduced,

the biocontrol effect. The addition of glucose resulted in an increased production of ethanol and ethyl acetate (Druvefors, 2004) and the author proposed that the anti-mould activity of *P. anomala* is, at least partly, due to inhibitory products from glucose metabolism.

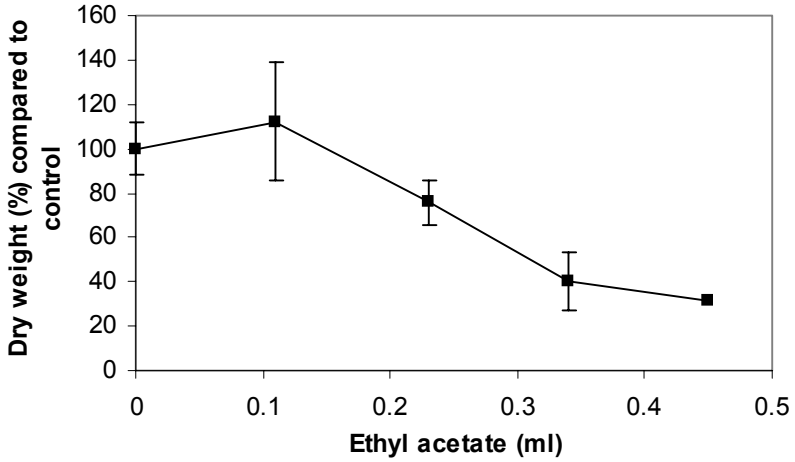

Figure 3. Dose response curve on the inhibition of mould biomass formation by ethyl acetate. *Penicillium roqueforti* was grown on malt extract agar (2%) and incubated in the same atmosphere as different amounts of vaporized ethyl acetate (**III**).

The anti-mould activity of ethyl acetate was investigated *in vitro* with *P. roqueforti* grown in an atmosphere with different amounts of vaporized ethyl acetate (Fig. 3, **III**). In this experimental set-up, ethyl acetate inhibited biomass formation of the mould (Fig. 3, **III**). Volatile compounds from different yeasts have previously been reported to inhibit mould growth (Filonow, 2003; Mercier & Jiménez, 2004). Ethyl acetate can also inhibit growth of *P. anomala* if present in high concentrations (0.20 M) (Tabachnick & Joslyn, 1953b).

Low molecular compounds produced by *Pichia anomala*

Glycerol and arabitol

Glycerol is a sugar alcohol produced from dihydroxyacetone phosphate in a two-step reaction (Fig. 2; *6*). The regeneration of NAD^+ in the first step of the pathway is important during oxygen limitation, when oxidation of NADH in the mitochondrial membrane is inhibited (van Dijken & Scheffers, 1986). In addition to its importance in redox metabolism, glycerol also plays a role in the cellular protection against osmotic stress. During growth under high osmotic pressure, glycerol is produced and retained inside yeast cells where it functions as a compatible solute (Albertyn, Hohmann & Prior, 1994; Nevoigt & Stahl, 1997).

Glycerol accumulates during growth in both high sugar and salt environments (Blomberg & Adler, 1992).

During oxygen-limited growth of *P. anomala*, glycerol accumulated inside the cells as well as in the culture supernatant (Fig. 4, **II**). The accumulation of glycerol was higher in the closed system than in the open system, *i.e.* glycerol accumulation depended on oxygen availability (Fig. 4, **II**). After glucose depletion, glycerol was metabolized by the cells but only in the presence of oxygen (Fig. 4). Glycerol can be utilized as a carbon source by many yeast species (Barnett, Payne & Yarrow, 2000) but its dissimilation requires that NAD^+ is regenerated in the respiratory chain, which is inactive during severe oxygen limitation (van Aelst *et al.*, 1993; Bakker *et al.*, 2001).

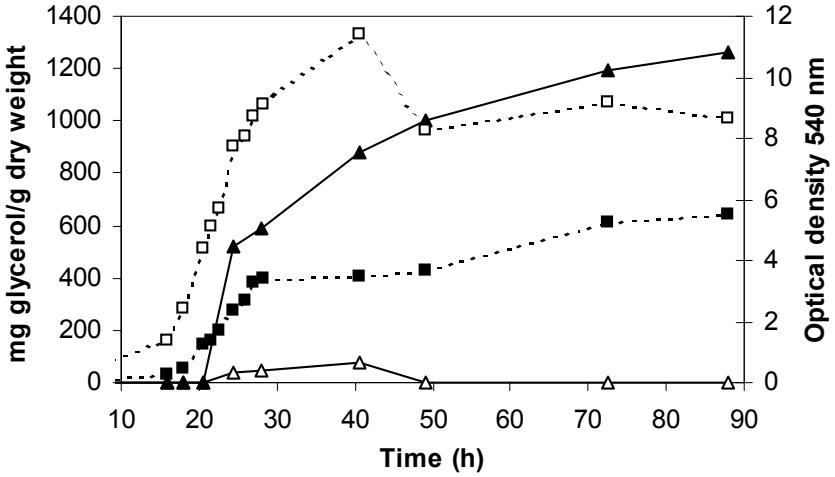

Figure 4. Growth (squares) and extracellular accumulation of glycerol (triangles) in *P. anomala* J121 during growth in closed (filled symbols) and open (white symbols) shake-flasks, representing two conditions of oxygen limitation (modified from **II**).

When *P. anomala* was grown under oxygen limitation, another polyol, arabitol, also accumulated inside the cells (Fig. 5, **II**). Most of the arabitol was produced during stationary phase and was excreted into the culture medium (Fig. 5, **II**). The level of arabitol during stationary phase was higher in the closed system, both inside the cells ($p<0.05$) and in the culture supernatant. This indicates that arabitol is also involved in the specific oxygen-limited growth (Fig. 5). Arabitol was not re-consumed by the cells and the excretion of arabitol during stationary phase implies that the compound is not involved in a general stress response to stationary phase growth. Arabitol has not been reported to be involved in redox metabolism during fermentative growth. Yet, in the last step of arabitol synthesis, one molecule of NAD^+ is regenerated by the enzyme arabitol dehydrogenase (KEGG Database, Kyoto University, Kanehisa Laboratory, Tokyo, Japan; http://www.genome.jp/kegg/). Arabitol is produced by many yeast species, *e.g. D. hansenii*, *Zygosaccharomyces rouxii*, *Z. bisporus*, *Candida sake*, and *Pichia sorbitola* (Abadias *et al.*, 2000; van Eck, Prior & Brandt, 1993; Tokuoka, Ishitani

& Chung, 1992; van Zyl & Prior, 1990) but not by *S. cerevisiae* (Bellinger & Larher, 1988; van Eck, Prior & Brandt, 1993; Tokuoka, Ishitani & Chung, 1992).

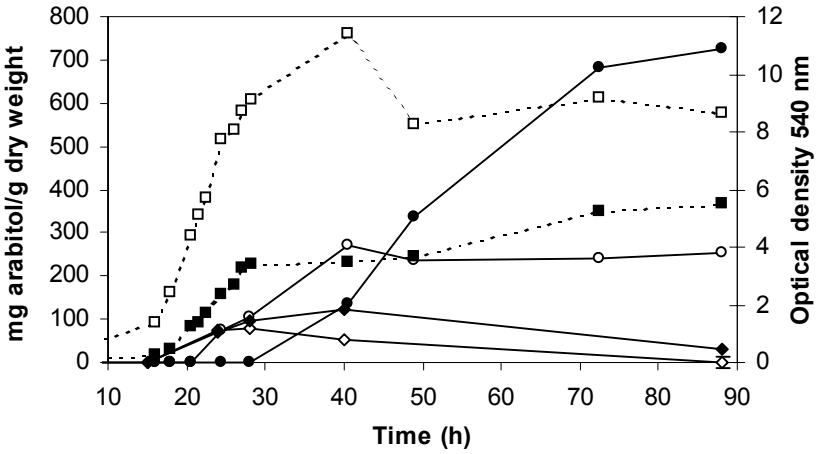

Figure 5. Growth (squares), intracellular (diamonds), and extracellular (circles) accumulation of arabitol in *P. anomala* J121 during growth in closed (filled symbols) and open (white symbols) shake-flasks, representing two conditions of oxygen limitation (modified from **II**).

In *P. anomala* both glycerol and arabitol accumulated during growth in high salt (Bellinger & Larher, 1988) and high sugar substrates (Tokuoka, Ishitani & Chung, 1992), indicating that both polyols function as compatible solutes under these conditions. However, the production patterns of glycerol and arabitol were different, with accumulation of arabitol always occurring at the end of the growth phase and glycerol in the beginning (Tokuoka, Ishitani & Chung, 1992) (**II**). Bellinger *et al.* (1988) also showed that the level of arabitol exceeded that of glycerol during growth in glucose minimal media and that this relation was inverted after the addition of NaCl (2.25 M).

Trehalose

Trehalose is a non-reducing disaccharide that is produced by many organisms, including fungi, plants and bacteria (Argüelles, 2000; Eastmond & Graham, 2003; Thevelein, 1984). Trehalose is accumulated in response to various types of stress, including oxidative stress (Pereira, Panek & Eleutherio, 2003), heat shock (Hottinger, Boller & Wiemken, 1987; de Virgilio *et al.*, 1990) ethanol stress (Mansure *et al.*, 1994; Sharma, 1997), osmotic stress (Hounsa *et al.*, 1998; Sharma, 1997), and dehydration (Gadd, Chalmers & Reed, 1987; Hottinger, Boller & Wiemken, 1987). In several of these studies, trehalose accumulation could be correlated to an increased stress resistance. It has been shown that trehalose can prevent protein denaturation during heat shock *in vitro* (Hottiger *et al.*, 1994; Singer & Lindquist, 1998) as well as increase membrane stability during dehydration (Crowe, Crowe & Chapman, 1984). Trehalose, together with

glycogen, is the major carbon storage compound in *S. cerevisiae* [for review see Francois and Parrou (2001)]. However, both the role of trehalose as a storage compound and as a stress protectant has been questioned by several authors (Alexandre *et al.*, 1998; Lewis *et al.*, 1997; Wiemken, 1990).

In addition to its role in stress protection and as a storage compound, trehalose plays a direct or indirect role in the glucose influx into glycolysis by a mechanism that is not yet completely understood (Thevelein & Hohmann, 1995). In *P. anomala*, trehalose accumulated in cells grown under oxygen limitation and was retained in the cells during stationary phase (Fig. 6, **II**).

In *P. anomala*, trehalose accumulation was higher in the closed system with no oxygen inflow than in the open system with low oxygen inflow. Ethanol formation was induced in both systems (Fig. 7). Under controlled aerobic conditions (respiratory), trehalose accumulation was not detected (Broberg, A. and Håkansson, S., unpublished observations). The fact that trehalose did not accumulate under aerobic conditions and increased with decreased oxygen availability (Fig. 6, **II**) indicates that trehalose plays a role during oxygen-limited growth. During these conditions, trehalose can be involved in the general stress protection against the increasing ethanol concentration in the substrate (Mansure *et al.*, 1994; Sharma, 1997) or in the regeneration of inorganic phosphor (P_i) during high glycolytic flux (Thevelein & Hohmann, 1995). It is also possible that trehalose is involved in a specific stress response to hypoxic conditions.

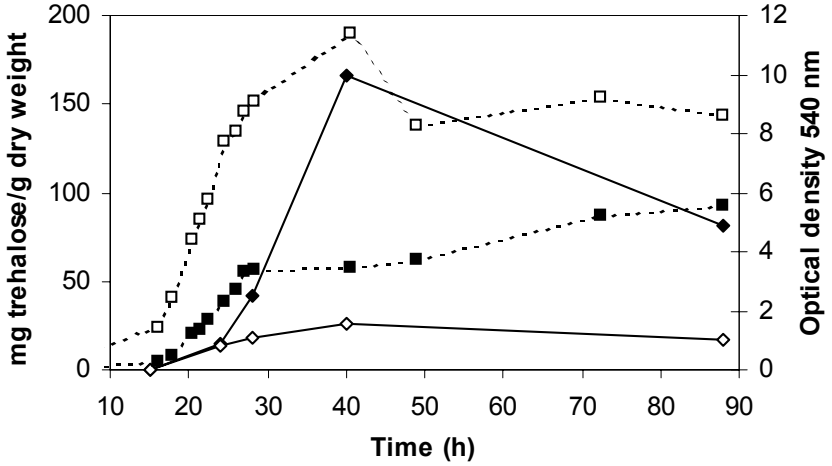

Figure 6. Growth (squares) and intracellular accumulation of trehalose (diamonds) in *P. anomala* J121 during growth in closed (filled symbols) and open (white symbols) shake-flasks, representing two conditions of oxygen limitation (modified from **II**).

S. cerevisiae did not accumulate trehalose during exponential growth on glucose but rather at the entry into stationary phase and during growth on non-fermentable carbon sources (van Dijck *et al.*, 1995; Gadd, Chalmers & Reed, 1987). However, Mansure *et al.* (1997) showed that several wine-strains of *S. cerevisiae* accumulated trehalose during batch fermentation on glucose and that the trehalose level could be correlated to an increased ethanol resistance (Mansure, Souza &

Panek, 1997). The correlation between trehalose accumulation and ethanol tolerance has also been shown in *S. pombe* (Soto *et al.*, 1999).

Trehalose synthesis

Trehalose is synthesised in a two-step reaction pathway. In the first step, glucose-6-phosphate (Fig. 2) and UPD-glucose forms trehalose-6-phosphate, catalysed by trehalose-6-phosphate synthase. In the second step, trehalose-6-phosphate is dephosphorylated by trehalose-6-phosphate phosphatase to generate trehalose and P_i [for a review see Elbein *et al.* (2003)]. Mutants of *S. cerevisiae*, deficient in the trehalose-6-phosphate synthase complex (*tps1*Δ), cannot grow on glucose (Bell *et al.*, 1992; Thevelein & Hohmann, 1995). The connection between trehalose synthesis and the ability to grow on glucose is not completely understood. However, it is generally believed that either trehalose-6-phosphate synthase or trehalose synthesis, or both, controls the glucose influx into the glycolytic pathway (Thevelein & Hohmann, 1995). One model proposes that trehalose plays a role in a metabolic buffer system during high glycolytic flux (Thevelein & Hohmann, 1995). The buffer system would shuttle part of the glucose-6-phosphate into the trehalose synthesis pathway, regenerating inorganic phosphor (P_i) needed in the downstream pathway of glycolysis as a substrate for glyceraldehyde-3-phosphate dehydrogenase. High glycolytic flux is observed in *S. cerevisiae* during growth on high glucose concentration (Lagunas, 1986; Postma *et al.*, 1989) and during oxygen limited, anaerobic growth (Lagunas, 1986; Verduyn *et al.*, 1990). Unrestricted glucose influx results in a drop in intracellular ATP and P_i (van Aelst *et al.*, 1993; Thevelein & Hohmann, 1995).

In *P. anomala*, oxygen limitation induces a higher glycolytic flux (**IV**) as well as trehalose accumulation (Fig. 6, **II**). It is possible that a similar buffer system exists also in this yeast species, triggered by a decreased oxygen availability but not by glucose. Interestingly, the disruption of *TPS1* in *Yarrowia lipolytica* and *Hansenula polymorpha* did not affect aerobic growth on glucose (Gancedo & Flores, 2004; Reinders *et al.*, 1999) consistent with the idea that trehalose accumulation is needed during fermentation but not during respiration.

There are other models that attempt to explain the role of trehalose-6-phosphate synthase during fermentative growth on glucose. These involve trehalose-6-phosphate synthase as a regulator of glucose transport or hexokinase activity, or trehalose-6-phosphate as a direct inhibitor of hexokinase activity (Thevelein & Hohmann, 1995). These models do not propose a direct role of trehalose. Yet, the continuous accumulation of trehalose in *P. anomala* during oxygen limitation (Fig. 6) indicates that the disaccharide in itself is important, not trehalose-6-phosphate synthase or its product, trehalose-6-phosphate.

Ethanol and acetate

Both ethanol and acetate are produced from the metabolic intermediate acetaldehyde (Fig. 2). The enzymatic reaction from acetaldehyde to ethanol is catalysed by alcohol dehydrogenase (ADH), regenerating one molecule of NAD^+.

Acetate is formed by oxidation of acetaldehyde by the enzyme aldehyde dehydrogenase (ALD), reducing one molecule of NAD^+ or $NADP^+$ (Navarro-Aviño *et al.*, 1999).

When ethanol has been synthesised by the cell, it diffuses freely through the membrane and accumulates in the substrate (Gancedo & Serrano, 1989). After the depletion of glucose, ethanol can be used as a carbon source through the complete oxidation to CO_2 and water via the glyoxylate pathway (Gancedo & Serrano, 1989).

P. anomala produced ethanol during growth in both the closed system with no oxygen inflow and in the open system with low oxygen inflow (Fig. 7, **II**). Intracellular ethanol accumulation was low compared to the levels accumulated in the substrate (**II**). In the presence of oxygen, ethanol was re-assimilated by the cells (Fig. 7). In the absence of oxygen, ethanol continued to accumulate in the substrate until the low-molecular carbon compounds in the cell were depleted and the cells died (**II**).

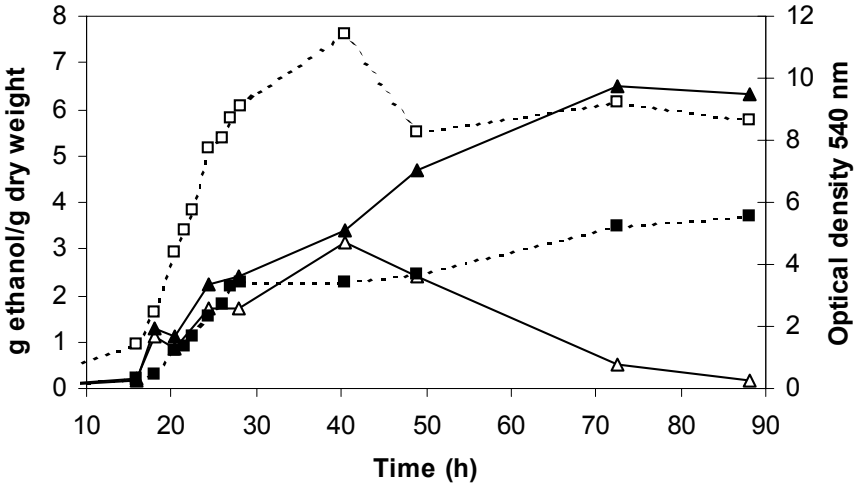

Figure 7. Growth (squares) and extracellular accumulation of ethanol (triangles) in *P. anomala* J121 during growth in closed (filled symbols) and open (white symbols) shake-flasks, representing two conditions of oxygen limitation (modified from **II**).

Acetate accumulation inside the cell can generate a high turgor pressure as well as influence free radical production leading to oxidative stress (Piper *et al.*, 2001). Only undissociated acetic acid, but not the acetate ion, can diffuse freely across the membrane (Conway & Downey, 1949). Most of the intracellular acetate is in the dissociated form (the intracellular pH is higher than 4.75, which is the pK_a value for acetic acid) and the passive diffusion of acetic acid is therefore slow. At least in *S. cerevisiae*, acetate is actively transported out of the cell by ATP-hydrolysing transporter systems, so-called ABC transporters (Piper *et al.*, 2001).

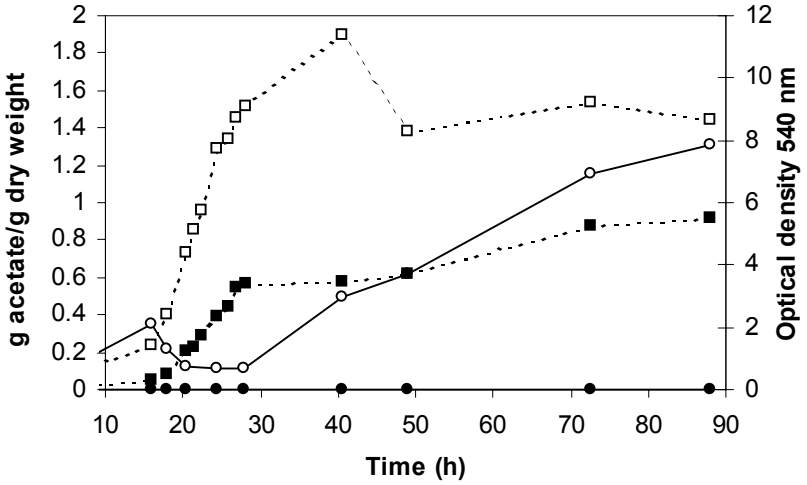

Figure 8. Growth (squares) and extracellular accumulation of acetate (circles) in *P. anomala* J121 during growth in closed (filled symbols) and open (white symbols) shake-flasks, representing two conditions of oxygen limitation (modified from **II**).

P. anomala produced acetate during respiratory growth on glucose (**IV**) and during respiro-fermentative conditions in open shake-flasks (Fig. 8). Acetate was not produced under purely fermentative conditions in the closed shake-flasks (Fig. 8) or during controlled oxygen limitation in the fermentor (**IV**). Acetate production from acetaldehyde is a NAD^+ or $NADP^+$ consuming reaction, which may explain the absence of acetate during fermentative growth. Most of the organic acid rapidly accumulated in the culture substrate, indicative of an active transport system across the membrane (Fig. 8, **II**).

Ethanol and acetate can both inhibit yeast growth if present at high concentrations. Ethanol can alter the membrane structure and permeability (D'Amore & Stewart, 1987; Mansure *et al.*, 1994; van Uden, 1985). *P. anomala* is not particularly tolerant to either ethanol or acetic acid (Kalathenos, Sutherland & Roberts, 1995) and 0.25% acetic acid, supplemented to a glucose-based substrate, reduced the growth of *P. anomala* J121 (**I**).

Ethyl acetate

Yeasts can produce a spectrum of small volatile metabolites, *e.g.* esters, alcohols, and fatty acids (Westall, 1999). The major volatile compound produced from *P. anomala* is ethyl acetate (Fig. 9) (Davies *et al.*, 1951; Gray, 1949; Westall, 1999), but also other compounds such as ethyl propanoate, phenyl ethanol, and 2-phenylethyl acetate (Westall, 1999) are produced.

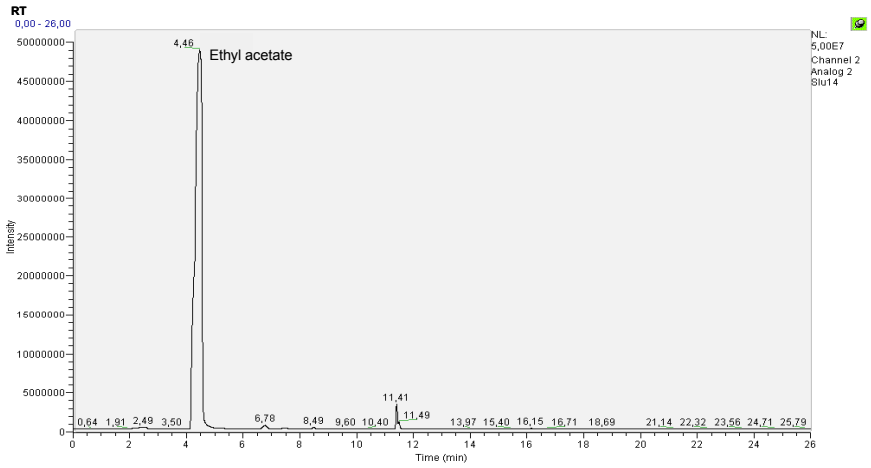

Figure 9. Chromatogram of volatile metabolites produced from *P. anomala* J121 grown in closed vials for five days at 25ºC on malt extract agar (2%, Difco Laboratories, Detroit, USA). Samples of 1 ml were taken out from the vials and automatically injected into the headspace gas chromatograph (ThermoQuest 2000). The carrier gas was H_2 at a flow rate of 35 ml min^{-1}. The column temperature was programmed from 30 to 220°C at a rise rate of 6°C min^{-1} and finally held during 20 min at 220°C. Ethyl acetate was identified by mass spectrophotometry (ThermoQuest HS 2000). Under these growth conditions, ethyl acetate constituted 96±1.5% of the volatile compounds found in the headspace, which corresponds to 3.4±0.4 mg ethyl acetate l^{-1} headspace. The analyses were performed by SIK, Institute of Food and Biotechnology, Gothenburg, Sweden.

To investigate the effects of oxygen limitation on ethyl acetate production, ester production was measured from glucose grown cultures of *P. anomala* under aerobic and oxygen-limited conditions (**IV**). We found that the production rate increased approximately 10-fold within one hour after the shift to oxygen limitation (Fig. 10). Thereafter, the production rate decreased (Fig. 10). Previous results on the oxygen dependence of ethyl acetate production are contradictory. Several authors have reported that increased oxygenation reduce ethyl acetate production (Gray, 1949; Tabachnick & Joslyn, 1953a). In contrast, Rojas *et al.* (2001) reported a higher ester production from *P. anomala* and several other non-*Saccharomyces* yeasts during highly aerated conditions compared to that during minimally aerated conditions. This can be explained by the experimental system used by Rojas *et al.* (2001), *i.e.* rotating and non-rotating shake-flasks to obtain highly and minimally aerated conditions, respectively. Our experience is that both these systems rapidly become oxygen limited (**II**). The authors did not relate the ester production to biomass formation. It is likely that the lower level of ethyl acetate in the non-rotating culture is a consequence of reduced biomass formation and not of a reduced specific ethyl acetate production. Though induced by oxygen limitation, ethyl acetate production in *P. anomala* depends on the presence of oxygen and is inhibited by complete anaerobic conditions (Davies *et al.*, 1951; Tabachnick & Joslyn, 1953a).

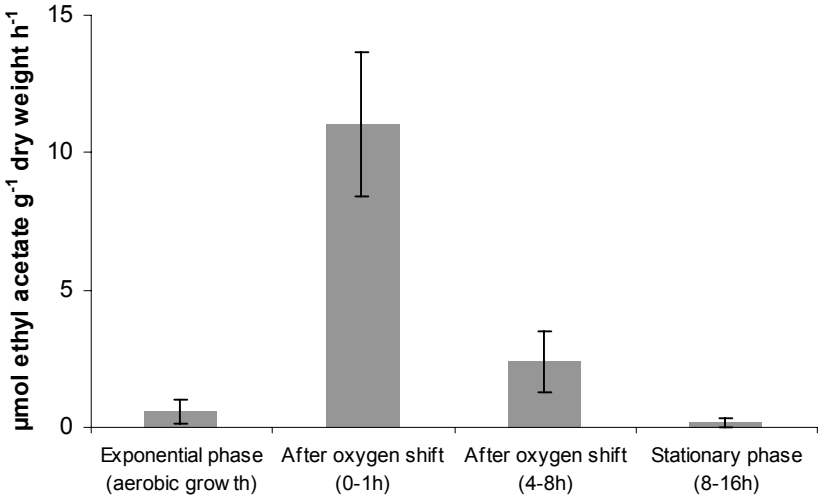

Figure 10. Production of ethyl acetate under controlled aerobic and oxygen limited growth conditions (**IV**).

Ethyl acetate synthesis

Ethyl acetate can be synthesized via two pathways. Both pathways use ethanol as one of the substrates, either together with acetyl co-enzyme A (Nordström, 1961), catalysed by the enzyme alcohol acetyl transferase (Yoshioka & Hashimoto, 1981) or with acetate, by the reversed reaction of enzyme-catalysed hydrolysis (esterase activity) (Schermers, Duffus & MacLeod, 1976; Soumalainen, 1981). The importance of these two pathways in ester synthesis varies between different yeast species. *S. cerevisiae* primarily uses the alcohol acetyltransferase pathway for the production of isoamyl acetate (with isoamyl alcohol instead of ethanol), but both pathways for the production of ethyl acetate (Yoshioka & Hashimoto, 1981). *P. anomala* differs from *S. cerevisiae* by producing most of both esters by the inverse esterase reaction, using acetate and the corresponding alcohol (Yoshioka & Hashimoto, 1981). The quantitative levels of ester synthesis also differ between yeasts. *P. anomala* produces more ethyl acetate, but less isoamyl acetate, than *S. cerevisiae* (Yoshioka & Hashimoto, 1981). Rojas *et al.* (2002), compared the activity of alcohol acetyl transferase (AATase), ester hydrolase (EHase), and ester synthase (ESase) from cell-free extracts of *P. anomala*, *Pichia heedii*, and *S. cerevisiae*. The authors showed that *P. anomala* had the highest ESase activity and the lowest AATase activity of all three strains. This supports earlier findings by Yoshioka & Hashimoto (1981), that ethyl acetate in *P. anomala* is produced from acetate but not from acetyl CoA. In addition, Rojas *et al.* (2002) showed that the ester hydrolase activity in *P. anomala* was almost 30-fold lower than *P. heedii*. This might affect the absolute production level of ethyl acetate, which was much higher in *P. anomala* (Rojas *et al.*, 2002). From these results, it is clear that ester synthesis in *P. anomala* is different from that of *S. cerevisiae* and other yeast species, both in the synthesis pathway and in the amounts produced.

Role of ethyl acetate

The rapid and transient induction of ethyl acetate production in response to oxygen limitation (Fig. 10) indicates that it may play a role in the metabolic shift from aerobic to oxygen-limited growth. After synthesis, ethyl acetate rapidly diffuses through the membrane and into the medium (Soumalainen, 1981). It has been suggested that the ester production prevents toxic accumulation of acetic acid during growth at low pH (Gordon, 1950; Tabachnick & Joslyn, 1953b). Moreover, ethyl acetate accumulated in the culture substrate when *P. anomala* grew on ethanol as the sole carbon source (Tabachnick & Joslyn, 1953a), also preventing the intracellular accumulation of acetic acid. Acetate transport out of the cell is ATP-dependent (Piper *et al.*, 2001) and during oxygen limitation (fermentative growth), intracellular ATP supply is limited (van Aelst *et al.*, 1993). Thus, to avoid depletion of intracellular ATP under these conditions, ethyl acetate production may provide a way to rapidly reduce the intracellular acetate level without consuming energy.

An effect of anti-fungal ester production may be that the producer organism obtains an advantage over other, more sensitive microorganisms. These microorganisms, including bacteria, moulds, and other yeasts, are inhibited from proliferating in the same environment as the ester producer, reducing the competition for nutrients and space.

Influence of ethyl acetate on the antifungal activity of *Pichia anomala*

Growth of *P. anomala* in the grain silo environment relies on the ability to utilize the available nutrients and to tolerate low oxygen and high CO_2 levels. The biocontrol yeast can inhibit mould growth by winning the competition for nutrients, oxygen, and space, or through the production of anti-mould metabolites. Druvefors (2004) showed that competition for nutrients was unlikely to be involved in the anti-mould mechanism because the addition of various nutrients did not reduce the anti-fungal activity. Instead, biocontrol activity increased with the addition of glucose (Druvefors, 2004). *P. anomala* produced ethyl acetate, a compound that has been shown to inhibit growth of *P. roqueforti* (Fig. 3), when growing on grain (**III**) and the production increased when glucose was added to the grain test tubes (Druvefors, 2004). This implies that ethyl acetate may be involved in the mould inhibitory mechanism. Ethyl acetate production also increased in response to oxygen-limited conditions (Fig. 10, **IV**).

The haploid type strain of *P. anomala* (CBS 1984) had similar biocontrol activity as the diploid type strain (CBS 5759) at a water activity (a_w) of 0.98 but did not inhibit mould growth at a_w of 0.95. Growth of the haploid strain was significantly lower ($p<0.05$) at a_w of 0.95 during the first two days of incubation. However, after four days, the yeast CFU level per gram grain was not significantly

different between the two a_w conditions. Instead, the biocontrol activity could be correlated to the production level of ethyl acetate (Fig. 11, **III**). Other volatiles from *P. anomala* have been observed to have anti-mould properties (Wafa Masoud, KVL, Denmark, personal communication). If these volatiles are co-produced with ethyl acetate, it is likely that the ethyl acetate effect (Fig. 11) is in fact the result of a consortium of anti-mould volatiles produced by *P. anomala*.

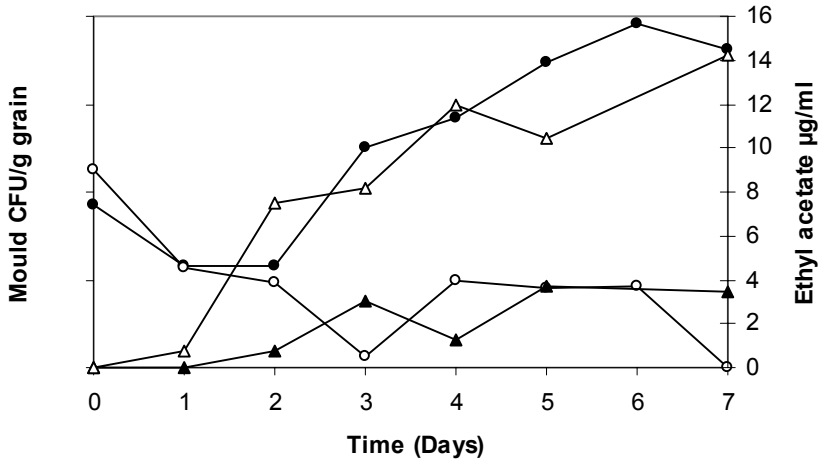

Figure 11. Co-cultivation of *P. roqueforti* and a haploid strain of *P. anomala* (modified from **III**). Mould CFU per g grain (circles) and ethyl acetate production (triangles) at a water activity of 0.95 (closed symbols) and 0.98 (open symbols).

Regulation of respiration and fermentation

Aerobic metabolism

During fully aerated conditions and high glucose concentration (20 g l^{-1}), *P. anomala* dissimilated glucose predominantly by respiration (Fig. 12, **IV**). During these conditions, ethanol was temporarily produced during the exponential phase and re-assimilated by the cells directly after, or simultaneously to, its production. Significant ethanol production is generally absent in Crabtree negative yeasts during aerobic conditions (de Deken, 1966) (**IV**), but a temporary production of ethanol has been seen in both *C. utilis* and *K. lactis* (González-Siso *et al.*, 1996; Verduyn *et al.*, 1984). The signal that induces the temporary ethanol formation in these yeasts is currently not known. Glycerol, acetate, and arabitol were also produced (Fig. 12, **IV**).

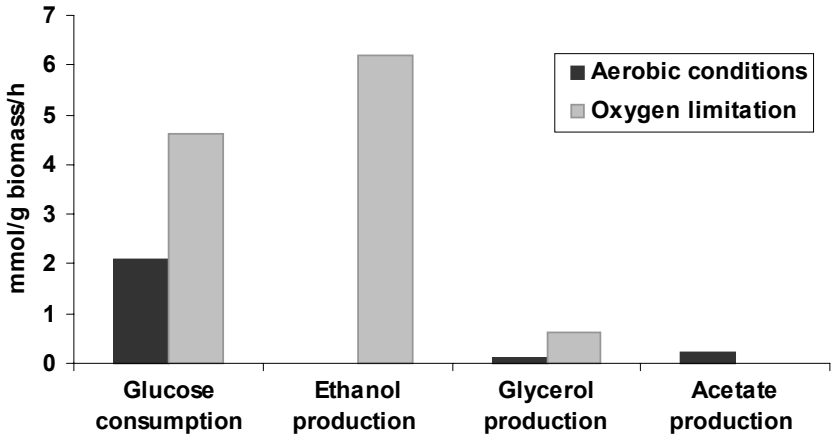

Figure 12. Metabolic changes when *P. anomala* cells are shifted from aerobic to oxygen limited growth. The low specific ethanol production rate found during aerobic conditions (0.14 mmol per g biomass per h) is not discernible in the figure. Acetate production was not detected under oxygen-limited conditions.

To monitor the global effects of oxygenation on metabolism, the metabolic flux distributions in *P. anomala* were determined (**IV**). For this purpose, extracellular fluxes and the known precursor requirements for the determined biomass formation were balanced in a stochiometric model. The model was based on the isotopomer distribution of amino acids in cells fed with uniformly labeled ^{13}C glucose (**IV**). The flux analysis showed that approximately 50% of glucose-6-phosphate was metabolized via glycolysis and 30% via the PP pathway. 90% of the carbon going through the PP pathway reentered glycolysis at the level of fructose-6-phosphate or triose-3-phosphate (Fig. 13, **IV**). This indicates that the PP pathway is predominantly used for NADPH but not biomass production. It has been suggested that the PP pathway plays a more important role in glucose dissimilation in Crabtree negative yeasts than in Crabtree positive (Bruinenberg, van Dijken & Scheffers, 1983a; Bruinenberg, van Dijken & Scheffers, 1983b; González-Siso *et al.*, 2000).

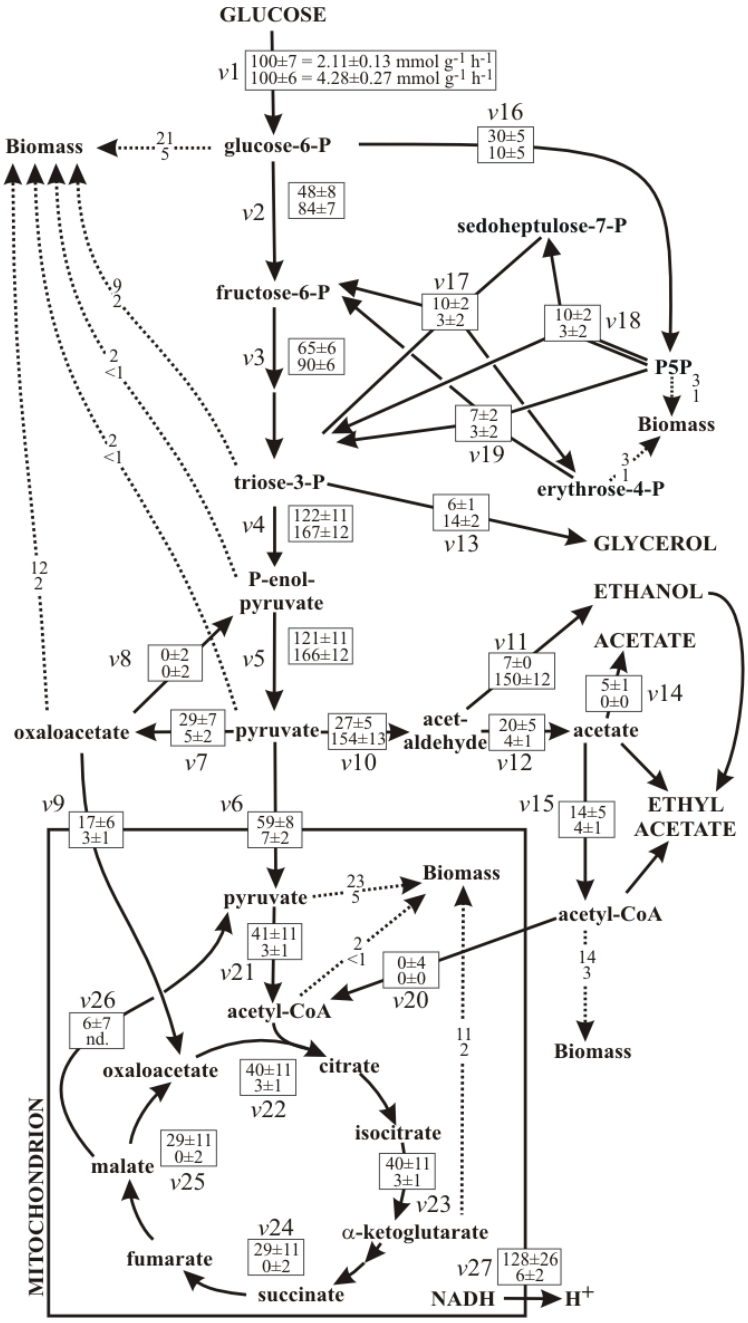

Figure 13. Metabolite flux distributions in *P. anomala* under aerobic and oxygen limited growth conditions on glucose. The vectors (*v*1-*v*27) represent the flux (% carbon passing through each reaction) between the carbon intermediates as calculated from the amino acid distribution. The upper numbers represent flux distribution for an aerobic culture and the lower numbers those of an oxygen-limited culture (**IV**).

The flux analysis showed that 25% of the cytosolic pyruvate is converted to oxaloacetate (Fig. 13), demonstrating the importance of the anaplerotic oxaloacetate pathway (Fig. 2). Approximately 20% of the pyruvate entered the fermentative pathway but most of this carbon was converted to acetate, not ethanol. It was further metabolized into biomass via acetyl CoA in the so-called pyruvate dehydrogenase bypass pathway (Holzer & Goedde, 1957) (Fig. 13). Approximately 50% of the carbon was oxidized completely via the TCA cycle.

Metabolic changes due to oxygen limitation

Induction of alcoholic fermentation

When *P. anomala* was grown under oxygen limitation its metabolism changed from respiration to fermentation (**IV**). The biomass yield was reduced from 0.59 to 0.11 g per g glucose and the growth rate from 0.22 to 0.056 h^{-1} (**IV**). A reduced biomass formation after oxygen limitation is also seen in *S. cerevisiae* and *P. stipitis* (Skoog, Jeppsson & Hahn-Hägerdal, 1992; Verduyn *et al.*, 1990; Weusthuis *et al.*, 1994). The glycolytic flux doubled (Fig. 12), probably to compensate for the reduced ATP supply. The increase in glucose uptake rate during oxygen limitation compared to aerobiosis is described as the Pasteur effect (Lagunas, 1986). Glycerol production increased five-fold (Fig. 12, **IV**), probably to counteract for the inhibition of mitochondrial NAD^+ reoxidation (Bakker *et al.*, 2001). Extracellular acetate accumulation in the substrate stopped after the shift (Fig. 12) and the acetate produced in the cell was converted to acetyl-CoA (Fig. 13).

Other effects

Oxygen limitation reduced the flux through the PP pathway from 30% to 10% (Fig. 13, **IV**). As observed under aerobic conditions, 90% of the carbon that entered the PP pathway returned to glycolysis and 10% was incorporated into biomass (Fig. 13). The reduced flux through the PP pathway may be due to the lower cellular demand for NADPH during reduced biomass formation (Fig. 13, **IV**). Oxygen limitation also induced a shift in the TCA pathway, which operated as a cycle during aerobic growth and as a two-branched pathway under oxygen limitation, sustaining only the synthesis of the biomass precursors oxaloacetate and α-ketoglutarate. The two-branched pathway has also been identified in *S. cerevisiae* during aerobic fermentation on glucose (Gombert *et al.*, 2001).

Effect of glucose on yeast metabolism

Induction of alcoholic fermentation in S. cerevisiae

In *S. cerevisiae* and other Crabtree positive yeasts, alcoholic fermentation is induced by the addition of excess glucose to aerobically grown cells (>0.15 g per liter) (Verduyn *et al.*, 1984). This effect has been subject to many studies and reviews [*e.g.* see van Dijken, Weusthuis & Pronk (1993), Fiechter, Fuhrmann & Käppeli (1981), Gancedo & Serrano (1989), and Käppeli (1986)]. Originally, the Crabtree effect was thought to be due to glucose-induced repression of the

respiratory system (de Deken, 1966; Fiechter, Fuhrmann & Käppeli, 1981). However, several authors have shown that the cytochrome content and oxygen uptake rate did not change after the addition of glucose (Petrik, Käppeli & Fiechter, 1983; Rieger, Käppeli & Fiechter, 1983). Rieger *et al.* (1983) suggested another mechanism in which a limiting step in the respiratory glucose dissimilation causes an "overflow" that directs the carbon into the fermentative pathway during high glycolytic flux. Different parts of the glycolysis have been suggested as the rate limiting step, such as pyruvate accumulation due to a limited capacity of the pyruvate dehydrogenase (van Hoek *et al.*, 1998; Käppeli, 1986; Petrik, Käppeli & Fiechter, 1983), limited capacity of the pyruvate dehydrogenase bypass route (Postma *et al.*, 1989), or limited capacity of the assimilatory pathways (van Urk *et al.*, 1988). Alcoholic fermentation has also been suggested to be due to accumulation of intermediates of the lower part of glycolysis, inducing the enzymes in the fermentation pathway (Boles, Heinisch & Zimmermann, 1993). Most probably, the "overflow" effect is not due to a single rate-limiting enzymatic step but rather due to the simultaneous action of several enzymes (Fell & Thomas, 1995; Jensen *et al.*, 1995).

Glucose effect on P. anomala

P. anomala and other Crabtree negative yeasts do not respond to glucose in the same way as *S. cerevisiae* (de Deken, 1966). *P. anomala* did not induce alcoholic fermentation after a glucose pulse to cells grown on the non-fermentable carbon source succinate (**IV**). After glucose addition, the growth and glucose consumption rates were not significantly different from those during growth in aerobic batch cultures on glucose (**IV**, Table 1).

The fundamental differences between yeasts in the way that they respond to glucose are not understood. Crabtree negative yeasts may possess other systems than the fermentative pathway that can handle the glycolytic intermediates during the high glucose uptake rate that follows glucose addition. Bellaver *et al*. (2004) recently proposed that ethanol formation is prevented by an increased divergence of glucose-6-phosphate into the carbohydrate biosynthesis and the PP pathway. This results in an increased capacity in keeping the glycolytic flux constant. Trehalose production as a glycolytic buffer system has already been discussed. By reducing the influx of glucose during aerobic, high glucose growth of *S. cerevisiae*, Otterstedt *et al.* (2004) showed that the metabolism could be changed from fermentation to respiration. Reduced glycolytic flux in Crabtree negative yeasts during aerobic growth on glucose can be explained by the presence of different glucose uptake systems (van Urk *et al.*, 1989). Facilitated diffusion in Crabtree positive yeasts results in an unrestricted glucose inflow during high glucose concentration, whereas energy-consuming H^+-symport uptake can regulate glucose influx in Crabtree negative yeasts (van Urk *et al.*, 1989). However, glucose uptake systems using facilitated diffusion have also been reported in Crabtree-negative yeasts [for a review see Flores (2000)]. Another alternative to the fermentative pathway is the cyanide insensitive respiration via the alternative oxidase (Veiga, Arrabaca & Loureiro-Dias, 2000).

Cyanide insensitive respiration – an alternative to ethanol formation?

An alternative respiratory pathway has been identified in yeasts, filamentous fungi, and plants (Kirimura, Yoda & Usami, 1999; Minagawa & Yoshimoto, 1987a; Siedow & Berthold, 1986; Veiga, Arrabaca & Loureiro-Dias, 2000; Viola *et al.*, 1986; Yokioka *et al.*, 1998). This pathway bypasses the last two complexes (III and IV) in the respiratory chain by transferring electrons directly from the ubiquinone pool to oxygen, which is reduced to water by the enzyme alternative oxidase (AOX) (Sakajo, Minagawa & Yoshimoto, 1993), [see review by Veiga *et al.* (2003)]. The bypassing of the two cytochrome complexes results in a reduced proton gradient and ATP production compared to that of normal respiration (Veiga, Arrabaca & Loureiro-Dias, 2003). The redox potential is lost as heat instead of energy conservation [reviewed by Moore & Siedow (1991)]. The alternative respiration can be induced by addition of antimycin A or cyanide, which inhibits the main respiratory chain. This implies that the alternative pathway has a role in sustaining growth when the main respiratory chain is inactive (Viola, *et al.*, 1986). The pathway is found in most Crabtree negative yeast and is absent in most Crabtree positive yeasts (Veiga, Arrabaca & Loureiro-Dias, 2000). Lambers *et al.* (1982) proposed that the alternative respiratory pathway serves as an over-flow mechanism to take care of excess electrons when the cytochrome pathway is saturated. During saturated conditions, aerobic alcoholic fermentation in Crabtree positive yeasts and the alternative respiratory pathway in Crabtree negative yeasts may represent two options for maintaining the metabolic flux (Veiga, Arrabaca & Loureiro-Dias, 2000). This implies that a high glucose concentration increases the use of the alternative respiratory pathway in Crabtree negative yeasts. However, at least in *P. anomala*, both the flux through the alternative respiration (Minagawa & Yoshimoto, 1987b) and the expression of the alternative oxidase-encoding gene (Sakajo, Minagawa & Yoshimoto, 1999) are repressed by glucose. If Crabtree negative yeasts use the alternative respiratory pathway and Crabtree positive yeasts use the fermentative pathway, the rate of oxygen consumption should be higher in the former group during these conditions. This has indeed been shown for *K. lactis* (González-Siso *et al.*, 2000) but not for *C. utilis* (van Dijken, Weusthuis & Pronk, 1993).

Regulation of metabolic flux

The regulatory networks in the cell determine the flux through a metabolic pathway. Glucose addition and hypoxia are two signals that can induce the same metabolic response, alcoholic fermentation. The signals can affect transcription and translation directly or indirectly at one or several regulatory levels (Fig. 14). Measurement of gene expression (Fig. 14; *1*) is likely to reflect enzyme synthesis and enzyme activity, but only in the absence of other regulatory mechanisms. Regulatory events that affect mRNA translation (Fig. 14; *2*) and protein modification (Fig. 14; *3*) may influence correlations between gene expression and enzyme activity. Moreover, *in vivo* regulation of enzyme activity, such as by allosteric interactions or feedback regulation may change the correlation between *in vitro* enzyme activity and glycolytic flux (Fig. 14; *3*).

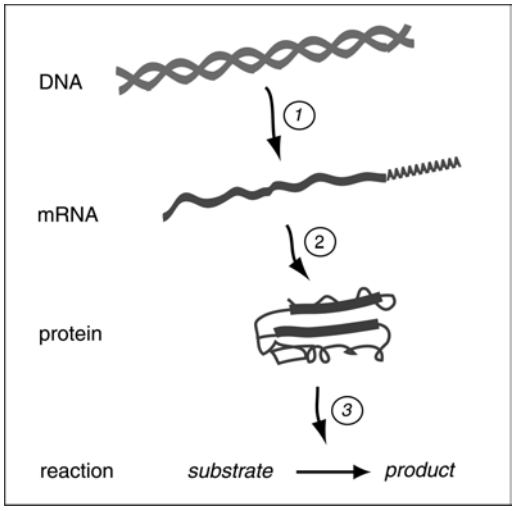

Figure 14. Schematic overview of three different levels of regulation: 1) gene transcription, 2) mRNA translation, and 3) protein activation.

The induction of gene transcription and enzyme activity can occur at different rates. The induction of gene transcription is usually rapid, within one hour, whereas the induction of enzyme activity may require several hours (Gonçalves & Planta, 1997; Møller *et al.*, 2004). Gonçalves & Planta (1998) described two types of transcriptional induction in yeast: the rapid initial response, with a drop immediately after the induction, and the sustained response (Griffioen *et al.*, 1996; de Winde *et al.*, 1996; Winderickx *et al.*, 1996). The sustained response is linked to the physiological relevance of the transcriptional response whereas the initial response is a reflex-like response that may not be linked to a physiological role of that gene product during the changed conditions (Gonçalves & Planta, 1998).

The key enzymes at the two branching points between respiration and fermentation are i) pyruvate dehydrogenase (PDH) and pyruvate decarboxylase (PDC) at the pyruvate branching point and ii) aldehyde dehydrogenase (ALD) and alcohol dehydrogenase (ADH) at the acetaldehyde branching point (Fig. 15). The isogenes of the respective enzyme activity varies in number and mode of regulation between yeast species. We partially cloned two *ADH* genes and one *PDC* gene in *P. anomala* (**V**). However, the total number of *ADH* and *PDC* isoenzymes is presently unknown. Four genes encoding ADH activity have been found in *S. cerevisiae* (Ciriacy, 1975; Lutstorf & Megnet, 1968) and *K. lactis* (Saliola, Shuster & Falcone, 1990; Saliola & Falcone, 1995) and two in *P. stipitis* (Cho & Jeffries, 1998; Passoth *et al.*, 1998). Pyruvate decarboxylase is encoded by one gene in *K. lactis* (Bianchi *et al.*, 1996), two in *P. stipitis* (Lu, Davis & Jeffries, 1998), and three in *S. cerevisiae* (Flikweert *et al.*, 1996).

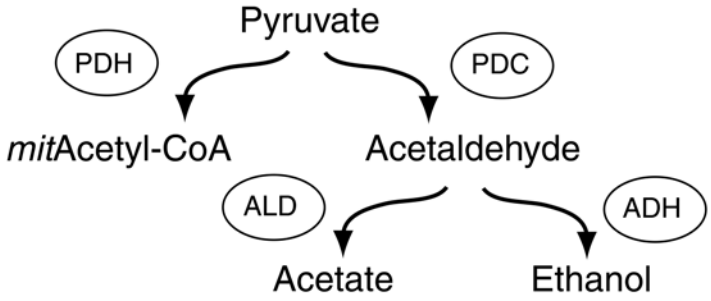

Figure 15. Pyruvate dehydrogenase (PDH) and pyruvate decarboxylase (PDC) at the pyruvate branching point and aldehyde dehydrogenase (ALD) and alcohol dehydrogenase (ADH) at the acetaldehyde branching point.

In *S. cerevisiae*, ADHI is responsible for the production of ethanol whereas ADHII is responsible for the reassimilation of ethanol (Ciriacy, 1975; Lutstorf & Megnet, 1968; Racker, 1955). In *P. stipitis*, *PsADH2* and its encoded enzyme seem to have the dual function of both ethanol formation and assimilation (Cho & Jeffries, 1998; Cho & Jeffries, 1999; Passoth*, et al.*, 1998). *K. lactis* have a complex regulation of ADH activity where the two cytoplasmic genes are expressed during growth on glucose, whereas the two mitochondrial genes are either up- or down-regulated by ethanol (Mazzoni, Saliola & Falcone, 1992; Saliola & Falcone, 1995; Saliola, Shuster & Falcone, 1990). Due to the presence of several isoenzymes, results from alcohol dehydrogenase activity are often difficult to interpret.

Activity of key enzymes in the fermentative pathway

Activities of PDC, ADH, and ALD were measured to investigate whether the induction of fermentation during oxygen-limited growth was correlated with the activation of key fermentative enzymes (Fig. 16, **IV**). Cells grown in aerobic cultures on glucose already expressed high levels of both PDC and ADH, despite the absence of significant ethanol production (Fig. 12 & 16). The metabolic flux to ethanol started immediately after the shift from aerobic to oxygen-limited conditions (Fig. 13) and the activity of both PDC and ADH increased gradually (Fig. 16). Though both enzymatic activity and flux increased, several observations indicated that a substantial portion of regulation occurred beyond the transcriptional and translational level: i) there were high activities of both enzymes without any ethanol formation, ii) ethanol production was instant, whereas enzymatic activity increased gradually, and iii) the ethanol flux increased approximately 20-fold whereas the activity of the enzymes increased only three-fold.

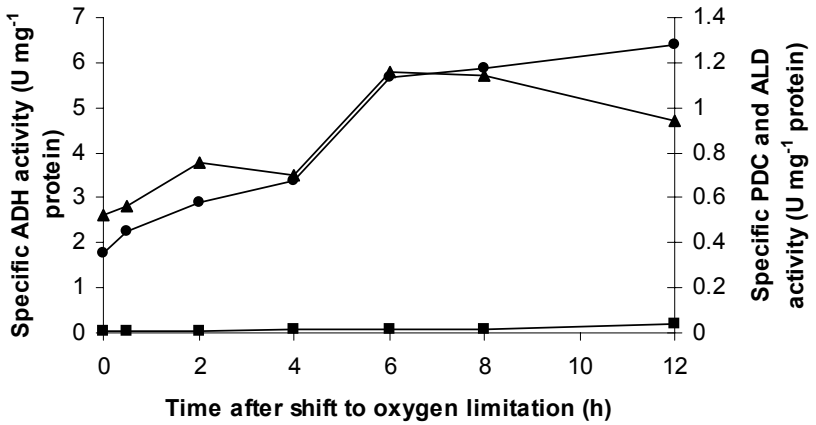

Figure 16. Enzyme activity of PDC (triangles), ADH (circles), and ALD (squares) in *P. anomala* as a response to oxygen limitation (modified from **IV**).

It is possible that the aerobic activities of PDC and ADH are sufficient to account for the ethanol production directly after the exposure to hypoxia. High PDC and ADH activities during respiratory growth have also been reported for *K. lactis* (Kiers *et al.*, 1998; Zeeman *et al.*, 2000) but are generally lower in other Crabtree negative yeasts [*e.g.* (van Urk *et al.*, 1990; Venturin *et al.*, 1995a)]. In *P. stipitis*, no ADH activity is present during aerobic growth on glucose (Passoth, Zimmermann & Klinner, 1996; van Urk *et al.*, 1990). The non-fermentative PDC and ADH activities are higher in *S. cerevisiae* (van Urk *et al.*, 1990) than in *P. anomala* and *K. lactis* (Kiers *et al.*, 1998; Zeeman *et al.*, 2000). Increased PDC activity in response to oxygen limitation has also been seen in other Crabtree negative yeasts, including *C. utilis*, *P. stipitis*, *Hanseniaspora uvarum*, and *K. lactis* (Franzblau & Sinclair, 1983; Kiers *et al.*, 1998; Passoth, Zimmermann & Klinner, 1996; Skoog, Jeppsson & Hahn-Hägerdal, 1992; Venturin *et al.*, 1995b; Weusthuis *et al.*, 1994). A slight induction of PDC in response to anaerobic conditions can also be seen in *S. cerevisiae* (van Hoek, van Dijken & Pronk, 2000). Increased ADH activity in response to oxygen limitation has also been observed in *K. lactis* (Kiers *et al.*, 1998), *H. uvarum* (Venturin *et al.*, 1995a), and *P. stipitis* (Passoth, Zimmermann & Klinner, 1996).

Although glucose does not induce alcoholic fermentation in *P. anomala*, it may induce changes at the levels of enzymatic activity or gene expression (**IV**, **V**). We found that both ADH and ALD activity were higher in crude extract from cells grown on the non-fermentable carbon source succinate than when grown on glucose (Fig. 16 & 17). This was also shown in *C. albicans* (Bertram *et al.*, 1996). Both enzymes were down-regulated after glucose addition (Fig. 17). Down-regulation of ADH and ALD after the addition of glucose has also been observed in *S. cerevisiae* when grown on peptone and yeast extract (Maitra & Lobo, 1971). Down-regulation of ADH was also seen after glucose addition to *Candida parapsilosis* (Hommes, 1965) and *H. uvarum* (Venturin *et al.*, 1995a). However, when *P. anomala* was grown on ethanol, ADH activity was not down-regulated by

glucose (**V**). This indicates that the ADH activity during growth on ethanol and succinate are represented by two different isoenzymes, regulated by different signals.

Figure 17. Enzyme activity of PDC (triangles), ADH (circles), and ALD (squares) in *P. anomala* after the addition of glucose to cells grown on succinate (modified from **IV**).

In *P. anomala*, PDC activity was lower when grown on succinate than when grown on glucose and was not detected at all on ethanol. Glucose addition to both succinate and ethanol grown cells enhanced PDC activity (Fig. 17; **IV**, **V**). Glucose induction of PDC has also been shown in *S. cerevisiae* (Boles & Zimmermann, 1993; Hommes, 1965; Maitra & Lobo, 1971; Schmitt & Zimmermann, 1982), *S. kluyveri* (Møller *et al.*, 2004), and *K. lactis* (Zeeman *et al.*, 2000) but is not a general phenomenon in Crabtree negative yeasts (Passoth, Zimmermann & Klinner, 1996; van Urk *et al.*, 1990; Venturin *et al.*, 1995a).

The enzymatic activities of ADH and PDC during growth on ethanol reflect their physiological roles, *i.e.* high ADH activity for ethanol dissimilation and no PDC activity, due to the lack of flux from glycolysis during these conditions. The high activity of PDC and ALD during growth on succinate (Fig. 17) and the high PDC activity during aerobic growth on glucose (Fig. 16) might reflect their roles in the synthesis of cytoplasmic acetyl CoA. In *S. cerevisiae* aerobic growth on glucose is completely inhibited in *PDC* deletion mutants due to the lack of acetyl CoA synthesis in the cytosol (Flikweert *et al.*, 1996; Hohmann & Cederberg, 1990). In contrast, the high ADH activity in aerobically grown cells on glucose and succinate (Fig. 16 & 17) cannot be explained by the known physiological roles of this enzyme.

Expression of ADH *and* PDC *genes*

Expression of the *PDC* (*PDC1*) and *ADH* (*PaADH1* and *PaADH2*) genes were measured with real-time RT-PCR before and after a shift to oxygen limitation (Fig. 18, **V**). During aerobic and oxygen-limited growth on glucose, *PaPDC1* and *PaADH1* were expressed in the same range as the reference gene (>20% of the level of actin expression) (**V**). A nine-fold induction of *PaADH1* and a four-fold induction of *PaPDC1* were seen 30 min after exposure to oxygen limitation (Fig. 18). The expression level then decreased towards the basal expression levels detected during aerobic growth. The rapid decrease of *PaPDC1* and *PaADH* transcription can either be due to mRNA degradation or decreased mRNA transcription. The expression level of *PaADH2* was below 1% of the expression of *PaADH1* under aerobic conditions and was not up-regulated by oxygen limitation (**V**).

In *K. lactis* (Zeeman *et al.*, 2000) and *P. stipitis* (Passoth *et al.*, 1998), ethanol production is controlled at the transcriptional level, *i.e.* by up-regulation of *PDC* and *ADH* transcription. In *S. cerevisiae*, ADHI activity and ethanol formation is also correlated to changes in gene transcription (van den Berg, de Jong-Gubbels & Steensma, 1998; Denis, Ferguson & Young, 1983; Hauf, Zimmermann & Muller, 2000). Also in *P. anomala, PDC* and *ADH* transcription were up-regulated by oxygen limitation. However, this response was transient and it seems likely that posttranscriptional factors are more important in regulating the metabolic flux to ethanol.

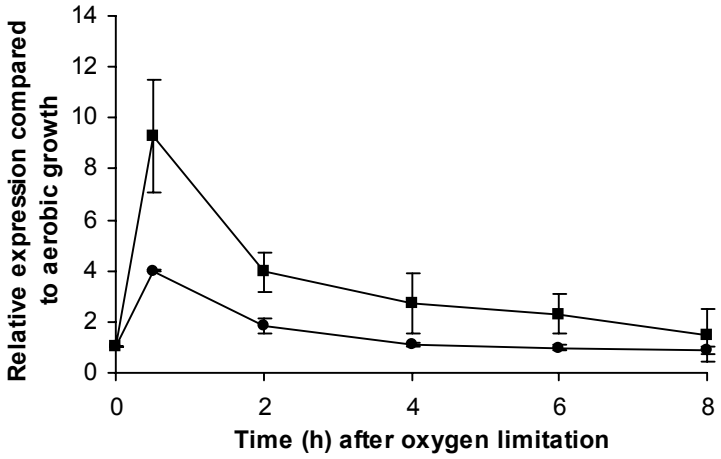

Figure 18. Relative expression of the *PaADH1* (squares) and *PaPDC1* (circles) genes in aerobically grown cells after a shift to oxygen limitation (**V**).

To investigate whether the expression of the three genes is regulated by glucose, mRNA was isolated from cells grown on ethanol and succinate before and after addition of glucose (Fig. 18, **V**). Surprisingly, the expression pattern for the three genes looked completely different in cells grown on ethanol compared to those grown on succinate (**V**). In cells grown on ethanol, the expression level of *PaADH1* and *PaADH2* was similar to that on glucose (**V**). A glucose pulse to the

ethanol grown cells did not affect *ADH* expression (**V**). However, in cells grown on succinate, *PaADH1* expression was only 1% of the actin expression level. Instead, the level of *PaADH2* expression was 90-fold up-regulated, compared to the expression level in cells grown on glucose and ethanol (**V**). Glucose addition drastically reduced the expression of *PaADH2* within 30 min to levels usually found in glucose grown cells (**V**). Simultanously, *PaADH1* expression was three-fold up-regulated and increased 14-fold within four hours (Fig. 19). *PaPDC1* expression was lower in cells grown on ethanol and succinate than on glucose (**V**). After glucose addition to both ethanol- and succinate-grown cells, *PaPDC1* expression increased to the level found during batch cultivation on glucose (**V**).

Figure 19. Expression of the *PaADH1* (squares) and *PaPDC1* (circles) genes in cells grown on succinate after the addition of glucose (**V**).

The high expression of *PaADH1* during both ethanol production and consumption indicates that the ADH enzyme encoded by *PaADH1* performs both reactions. This dual function of ADH has previously been reported in *P. stipitis* (Cho & Jeffries, 1998; Passoth *et al.*, 1998) and *C. albicans* (Bertram *et al.*, 1996). The role of the enzyme encoded by *PaADH2* remains unclear. Gene expression was strongly repressed by glucose and ethanol but was up-regulated in succinate-grown cells (**V**). ADH activity was three-fold higher in cells grown on succinate, than in cells grown aerobically on glucose (Fig. 16 & 17, **IV**). Both *PaADH2* expression and ADH activity was down-regulated after glucose addition (Fig. 17, **IV**). The similar pattern of *PaADH2* expression and ADH activity in succinate grown cells before and after glucose addition suggests that ADH activity during these conditions is encoded by PaADH2p.

Expression of *PaPDC1* was lower in succinate-grown cells than in glucose-grown cells (**V**). This was also reflected in a lower PDC activity during these conditions (**IV**). On ethanol, PDC activity was not detected at all, despite a *PaPDC1* expression corresponding to 10% of the actin expression (**V**). Ethanol has been reported to inhibit PDC activity in *K. lactis* (Bianchi *et al.*, 1996). However, this inhibition was regulated at the transcriptional level, not at the post-transcriptional level, which obviously occurs in *P. anomala*. Glucose addition to

ethanol-grown cells induced *PDC* transcription in both *K. lactis* (Bianchi *et al.*, 1996) and *P. anomala* (**V**).

It is obvious that both short-term transcriptional responses (as induced by oxygen limitation; Fig. 18) and sustained transcriptional responses (as after glucose addition; Fig. 19) exist in *P. anomala*. In *S. cerevisiae*, Gonçalves & Planta (1998) were able to separate the sustained induction of *PDC1* from the short-term induction, showing that both types of inductions may be present simultaneously. They compared the *PDC1* induction after glucose addition in a wild-type strain and a phosphoglucose isomerase deletion mutant (*pgiΔ*). When glucose-6-phosphate could not be converted to fructose-6-phosphate in the *pgiΔ* mutant, the sustained response of the wild type was abolished whereas the short-term response was not affected (Gonçalves & Planta, 1998). The *PDC* induction pattern of the *pgiΔ* mutant was identical to that observed in *P. anomala* after the shift to oxygen limitation (Fig. 18). The rapid induction of both *PDC* and *ADH* in *P. anomala* may be due to a reflex-like response (Gonçalves & Planta, 1998), followed by a phase where the gene expression is adjusted according to the demands and growth abilities of the cell.

Concluding remarks on metabolic regulation

Hypoxia and glucose are the two most important inducers of fermentation in yeast as well as in other organisms. The mechanism by which the two signals affect glycolysis and the fermentative pathway are generally considered to be different. However, a possible overlap between the regulatory responses to the two signals in plants has been discussed (Koch *et al.*, 2000). The regulation of glycolysis is complex due to the many metabolic entry and exit points, making the interpretation of experimental results difficult. In addition, experimental set-ups differ between studies and are therefore difficult to compare. The grouping of yeast species into Crabtree positive and Crabtree negative yeasts is useful at the level of general metabolic flux responses to glucose and oxygen limitation. However, at the level of enzyme activity and gene expression, differences appear to be as great within each group as between the groups. In fact, van Hoek *et al.* (2000) even showed that regulation at the enzyme level differed between two strains of *S. cerevisiae* although the production of biomass and ethanol were similar.

Many studies on the induction of alcoholic fermentation have focused on single carbon intermediates, for example glucose-6-phosphate. Gonçalves & Planta (1998), as well as Boles & Zimmerman (1993), showed that glucose-6-phosphate is involved in the induction of several glycolytic genes and enzymes during aerobic ethanol formation in *S. cerevisiae*. However, oxygen-limited induction of alcoholic fermentation in *P. stipitis* did not correlate with glucose-6-phosphate accumulation (Passoth, Zimmermann & Klinner, 1996). If focus were on studying ratios, instead of absolute levels of intermediates or enzyme activities, it might be possible to find similarities between the glucose and hypoxic induction of fermentation in yeasts. For example, Gonçalves & Planta (1998) suggested that the inductive signal could be the status of the glucose-6-phosphate to ATP ratio. This ratio increases during the first few minutes after glucose addition (Hohmann *et al.*,

1996) due to the increased phosphorylation of glucose to glucose-6-phosphate. It also increases after a shift to anaerobic or oxygen limited conditions. In this case not due to a higher production of glucose-6-phosphate but rather due to the decreased production of ATP.

Our results have shown that enzymatic activity and gene expression levels do not correlate quantitatively with the glycolytic flux. This indicates that other regulatory factors are more important in partitioning the carbon between respiration and fermentation in *P. anomala*. Sierkstra *et al.* (1992) suggested that regulation at the level of transcription and translation is of limited importance for glycolytic flux in *S. cerevisiae*. In a transcriptional analysis of the whole *S. cerevisiae* genome, only small differences were detected between cells grown aerobically or anaerobically at steady state (ter Linde *et al.*, 1999). These authors concluded that the regulation of the flux through either respiration or fermentation was more likely to occur at the posttranscriptional level. The results on the expression of two genes and their corresponding enzymes indicate a similar phenomenon in *P. anomala*.

Conclusions and outlook

In-depth knowledge on yeast growth and metabolism is indispensable for a successful application of yeast in any biotechnological process. Though yeasts constitute a morphologically homogenous group of organisms, their genetic and metabolic diversity makes it difficult to transfer knowledge from one yeast species to another.

P. anomala has potential use in various biocontrol applications (Druvefors*, et al.*, 2002; Jijakli & Lepoivre, 1998; Masih, Alie & Paul, 2000; Petersson & Schnürer, 1998). This thesis has contributed to the increased understanding of metabolite formation, notably the production of trehalose, polyols, and ethanol in *P. anomala*. These compounds may be of importance for storage stability and in optimizing the production of biomass, two prerequisites for an efficient commercialization of the biocontrol agent. Research projects in these areas are ongoing. The mode of action of the biocontrol agent is an important factor in the evaluation of product safety as well as in its regulatory approval. This thesis has established that ethyl acetate, possibly in combination with other anti-mould volatiles, is involved in the biocontrol activity of *P. anomala*. However, the complete picture is difficult to obtain due to the dynamic nature of the storage environment and the multitude of possible interactions between the biocontrol agent and the target organism. Further research on the mode of action of *P. anomala* requires the development of molecular tools, including an efficient transformation system and the construction of a genomic library. Attempts to develop these tools are underway.

The main conclusions of this thesis can be summarized as follows:

- Oxygen limitation is the main inducer of fermentation in *P. anomala.*
- Oxygen availability regulates the levels of gene transcription, enzymatic activity, and metabolic flux.
- Two *ADH* isogenes in *P. anomala* have different physiological roles in the cell and are regulated by different signals.
- Ethyl acetate production is induced as a short-term response to oxygen limitation.
- Ethyl acetate is at least partly responsible for the anti-mould activity of *P. anomala.*

Many studies that aim to understand yeast metabolism (including those presented in this thesis) use simple experimental set-ups that focuses on a few genes or enzymes. However, the connection between physiological function and metabolic fluxes to variations in genetic and enzymatic regulation is complex. To understand the regulation of glycolysis in *P. anomala, S. cerevisiae*, and in other yeasts a broader approach should be used. Metabolic flux analysis is a powerful tool for this purpose, especially when applied on mutants and in combination with multivariate statistics. In addition, comparative analysis between several yeast species will increase the knowledge of single species, and also contribute to our general understanding of fungal metabolism.

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I. Participated in the planning and did most of the data evaluation and writing of the manuscript. Performed minor part of the laboratory work.

II. Most of the planning, data evaluation, and writing of the manuscript. Most of the laboratory work apart from the HR MAS-NMR analysis.

III. Most of the planning, data evaluation, and writing of the manuscript. Performed part of the laboratory work.

IV. Major part of the planning, data evaluation, and writing of the manuscript. Performed all laboratory work, except GC-MS analysis on amino acid isotopomer distributions and the construction of the metabolic flux network.

V. Major part of the planning, data evaluation, and writing of the manuscript. Established and optimised the real-time RT-PCR method and performed the expression analysis under aerobic and oxygen-limited growth.