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Dekkera bruxellensis: Studies on carbon and nitrogen sources metabolism and its response to acetic acid stress.

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Moktaduzzaman M., Galafassi S., Capusoni C., Vigentini I., Ling Z., Piškur J., and Compagno C. (2014) Galactose utilization sheds new light on sugar metabolism in the sequenced strain *Dekkera bruxellensis* CBS 2499.

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Moktaduzzaman M., Galafassi S., Vigentini I., Foschino R., Corte L., Cardinali G., Piškur J., and Compagno C. (2014) Response to acetic acid stress in *Dekkera bruxellensis.*

Abstract

The lineages of *Dekkera bruxellensis* and *Saccharomyces cerevisiae* separated approximately 200 million years ago, but they share several industrially relevant traits, such as the ability to produce ethanol under aerobic conditions (Crabtree effect), high tolerance towards ethanol and acid, and ability to grow without oxygen. Beside a huge adaptability, *D. bruxellensis* exhibits a broader spectrum of consumable carbon and nitrogen sources in comparison to *S. cerevisiae*. This yeast is famous as a spoilage yeast in food and beverage industries and contaminates ethanol production process. Despite its economic importance and physiological interest, *D. bruxellensis* has not been well studied yet in detail.

To characterize its carbon metabolism and regulation, we investigated how galactose is used as carbon source by this yeast. Here we show that in *D. bruxellensis* under aerobic conditions and on ammonium-based media galactose is a not-fermentable carbon source, in contrast to *S. cerevisiae* which can ferment also this sugar. The expression of genes involved in different metabolic pathways was also analysed. We report that genes involved in galactose utilization, respiratory metabolism, TCA cycle, glyoxylate cycle and gluconeogenesis are repressed in glucose-based media. These results indicate that in *D. bruxellensis* glucose repression operates similarly to what occurs in *S. cerevisiae*.

In contrast to *Saccharomyces cerevisiae*, *D. bruxellensis* can use nitrate as sole nitrogen source. Our experiments showed that in *D. bruxellensis*, utilization of nitrate determines a different pattern of fermentation products. Acetic acid, instead of ethanol, became in fact the main product of glucose metabolism under aerobic conditions. We have also demonstrated that under anaerobic conditions, nitrate assimilation abolishes the ‘‘Custers effect’’, in this way improving its fermentative metabolism.

Acetic acid, due to its toxic effects, is used in food industry as a preservative against microbial spoilage. We investigated how this yeast responds when exposed to acetic acid. A detailed analysis of acetic acid metabolism was performed on three strains which exhibited a different resistance. Our studies show that *D. bruxellensis* behaves, from a metabolic point of view, more similarly to *S. cerevisiae*, being unable to metabolize acetic acid in presence of glucose. The presence of acetic acid affected the growth, causing a reduction of growth rate, glucose consumption rate, ethanol production rate as well as biomass and ethanol yield. Interestingly, the cells continued to produce acetic acid.

1. State of the Art

1.1 *Dekkera bruxellensis*

Yeasts are ubiquitous unicellular fungi widespread in natural environments colonizing from terrestrial, to aerial to aquatic environments. These are best known for their beneficial contributions to society, and the literature abounds with discussions of their role in the fermentation of alcoholic beverages, bread, and other products.

The genera *Dekkera* has been proposed by van der Walt (1964). The genus *Dekkera/Brettanomyces* comprises five described species: *Dekkera bruxellensis, D. anomala, Brettanomyces custersianus, B. naardenensis* and *B. nanus* (Hulin et al. 2014). *Dekkera bruxellensis*, is a hemiascomycete yeast also known as *Brettanomyces bruxellensis*. The terms *Dekkera* and *Brettanomyces* have been used interchangeably to describe this genus (where *Brettanomyces* is the anamorphic, asexual form of the teleomorph *Dekkera*). *Brettanomyces* was proposed as the generic name for two new anamorphic species, *B. bruxellensis* and *B. lambicus*, isolated from the Belgian beer Lambic in 1921 (Kufferath & Laer, 1921). The first isolation of '*Brettanomyces*' yeast was reported in 1904, when a yeast species was isolated from different kinds of British beers (Claussen 1904). However, *D. bruxellensis* and *B. bruxellensis* can be regarded as synonyms (Smith, 2011). The synonyms of *D. bruxellensis* are shown in Table 1.

Table 1. Synonyms of *D. bruxellensis* (according to Boekhout et al.1994; Smith et al. 1990; Smith 2011)

Synonyms	Synonyms (less frequently used)
D. intermedia, B abstinens, B. custersii, B. lambicus B. intermedius,	*B. bruxellensis var. vini, B. patavinus, B. schanderlii, B. vini, D. abstinens, D. lambica, Mycotorula intermedia.*

The first molecular phylogenic studies of the species in *Dekkera/Brettanomyces* group were done by D. G. Clark-Walker during 80's. Later on, several phylogenic studies have been performed on this group species (Hoeben & Clark-Walker 1986; Hoeben et al. 1993) and finally a phylogenetic tree with the position of *Dekkera/Brettanomyces* group was

published (Kurtzman & Robnett 1998). Another phylogenetical study of this group has been published after the completion of a big part of *D. bruxellensis* genome sequence (Woolfit et al. 2007). The genome sequence database of strain CBS2499 was published in 2012 and revealed that this strain is phylogenetically distant from other food-related yeasts and most related to *Pichia* (*Komagataella*) *pastoris*, which is an aerobic poor ethanol producer (Piškur et al. 2012).

D. bruxellensis cells are ellipsoidal to spherical, often ogival, but also cylindrical to elongated. Pseudomycelium is often formed. The lineages of *D. bruxellensis* and *S. cerevisiae* separated from each other approximately 200 million years ago (Woolfit et al. 2007). However, *D. bruxellensis* and *S. cerevisiae* share several characteristics, such as the production of ethanol under aerobic conditions, the ability to propagate under anaerobic conditions and petite positivity, that are rarely found among other yeasts (Merico et al. 2007; Rozpędowska et al. 2011; Procházka et al. 2010; Hellborg & Piskur 2009). Under aerobic conditions acetic acid is formed in large amounts from glucose (van der Walt, 1964). *D. bruxellensis* is a Crabtree positive yeast, so ferment preferentially in the presence of high glucose under aerobic conditions (Piskur et al. 2006). The main metabolic trait that characterized this species is the "Custrers effect" meaning that the fermentation is inhibited in the absence of oxygen and its stimulation in the presence of oxygen (Custer 1940; Skinner 1947). Custer's effect was initially called the 'negative Pasteur effect', since *Dekkera/Brettanomyces* yeast ferments glucose at a faster rate in aerobic conditions than in anaerobic conditions. This yeast cannot grow in vitamin-free medium and requires biotin and/or thiamine for growth (Barnett et al., 2000). However, It has ability to grow at low pH, high ethanol conditions and can assimilate a wider variety of alternative carbon sources (Conterno et al. 2006). Due to its ability to grow in adverse conditions, *D. bruxellensis* dominate over *S. cerevisiae* in the second phase (maturation phase) of alcoholic fermentation (Renouf et al. 2006).

On account of adaptation capability to survive in physiologically challenging environments, *D. bruxellensis* is sadly famous as a spoilage yeast being a major cause of wine spoilage worldwide. Wines infected with *D. bruxellensis* develop distinctive, unpleasant aromas due to volatile phenols produced by this species. This kind of infected

wines are said to have “Brett” character: they may smell mousy or medicinal or of wet wool, burnt plastic, or horse sweat (J. L. Licker et al. 1998). “Brett” off-flavour is associated in particular with the production of 4-ethylphenol (4-EP) and 4- ethylguaiacol (4-EG) (Pollnitz et al. 2000). *D. bruxellensis* is also regarded as the most common contaminant yeast in industrial ethanol fermentation process (de Souza Liberal et al. 2007).

Despite as a well-known spoilage yeast, *D. bruxellensis* is also used beneficially in the production of Belgianstyle, Lambic and Geuze beers (Daenen et al. 2008; Verachtert & Dawoud 1990). It has also been found in Brazilian alcoholic beverage “Cachaca” (Dato et al. 2005) and certain types of sour dough as well (Meroth et al. 2003).

1.2 Yeast metabolism

Living cells are in a state of ceaseless activity. To maintain its "life," each cell depends on highly coordinated biochemical reactions. Carbohydrates are an important source of the energy that drives these reactions. Metabolism refers to the biochemical assimilation (in anabolic pathways) and dissimilation (in catabolic pathways) of nutrients by a cell.

Yeasts are chemoorganotrophic microorganisms deriving their chemical energy, in the form of ATP, from the breakdown of organic compounds. Carbohydrates are usually the major carbon sources taken in by fungi and these are metabolized to provide energy and precursors for the synthesis of cellular structures. The major source for energy production in the yeast is glucose and glycolysis is the general pathway for conversion of glucose to pyruvate, whereby production of energy in form of ATP is coupled to the generation of intermediates and reducing power in form of NADH for biosynthetic pathways (Fig 1).

1.2.1 Glucose metabolism

1.2.1.1 Uptake of glucose

The passage through cell membranes is a critical step for all carbohydrates. Cell membranes are not freely permeable for a variety of solutes, glucose among them. The first and limiting step of glucose metabolism is its transport across the plasma membrane. Glucose transport across the membrane is carried out by specific hexose transporters encoded by specific genes. Depend on yeast, these hexose transporters transport glucose through carrier-mediated facilitated diffusion systems and also active proton sugar symport system. It is now apparent that multiple pathways regulate the transcription of several members of the *HXT* gene family in response to different levels of extracellular glucose. The transcription of genes encoding these transporters is finely regulated by the glucose concentration on the medium. In *S. cerevisiae* in total 20 putative hexose transporters have been identified which are induced at different extracellular glucose level (Lin & Li 2011; Boles & Hollenberg 1997).

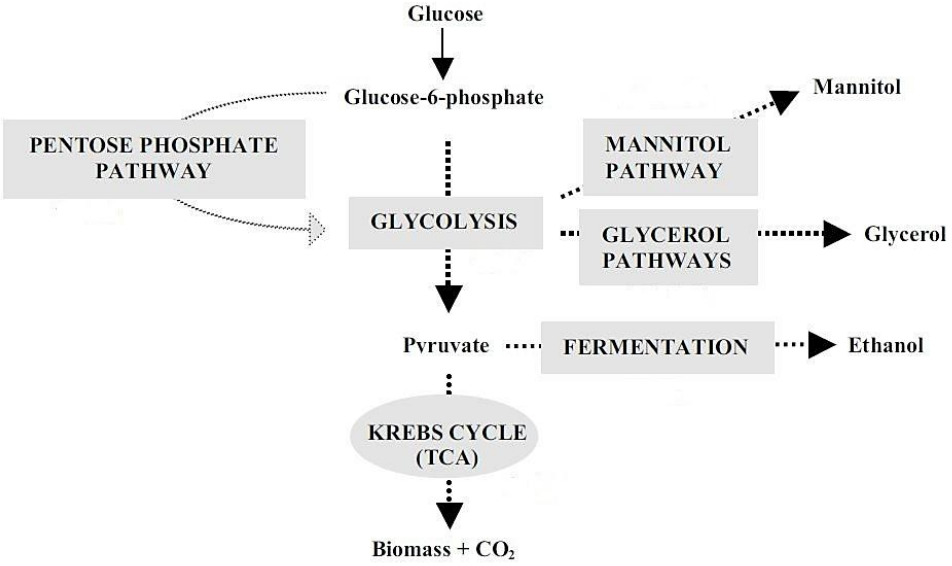

Figure 1 Main pathways approached for glucose metabolism in Yeast (Graca S, 2004).

The putative glucose sensors Snf3 and Rgt2 are 60% identical to each other contain 12 predicted transmembrane-spanning domains. Snf3 and Rgt2 do not appear to act as glucose transporters but instead signal the presence of the sugar. In one pathway, the extracellular glucose concentration is sensed by two proteins, Snf3p and Rgt2p. Snf3p has a high affinity for glucose and acts as a sensor at low glucose concentrations, whilst Rgt2p has low affinity for glucose and functions primarily as sensor at high glucose concentrations (O¨ zcan and Johnston, 1999; Schmidt et al., 1999). The signal triggered by these sensors results in expression of different hexose transporters (encoded by *HXT* genes). Further, it seems clear that the Snf3/Rgt2 regulatory pathway helps to ensure that changes in gene expression are commensurate with the glucose concentration in the environment, a mechanism that is likely to be particularly important in nature.

1.2.1.2 Glycolysis

The most common biochemical pathway used by fungi as a means of energy production from hexoses, namely glucose, is glycolysis, which takes place in cytoplasm (Fig 2). This process makes energy available for cell activity in the form of a high-energy phosphate compound known as adenosine triphosphate (ATP). Once glucose has been transported into the cell, it undergoes phosphorylation to glucose-6-phosphate by a constitutive

hexokinase, a glucokinase can phosphorylated glucose and mannose. This glucose-6-phosphate is a central key for diverse aspects of the cell, since it is the precursor both of the pentose phosphate pathway and for synthesis of oligo and polysaccharides. The enzymatic equipment for hexose phosphorylation varies among different yeasts. *S. cerevisiae* is known to possess two hexokinases, encoded by *HXK1*, *HXK2* (Lobo & Maitra 1977), but *K. lactis* has only one hexokinase encoded by the *RAG5* gene (Petit et al., 1997).

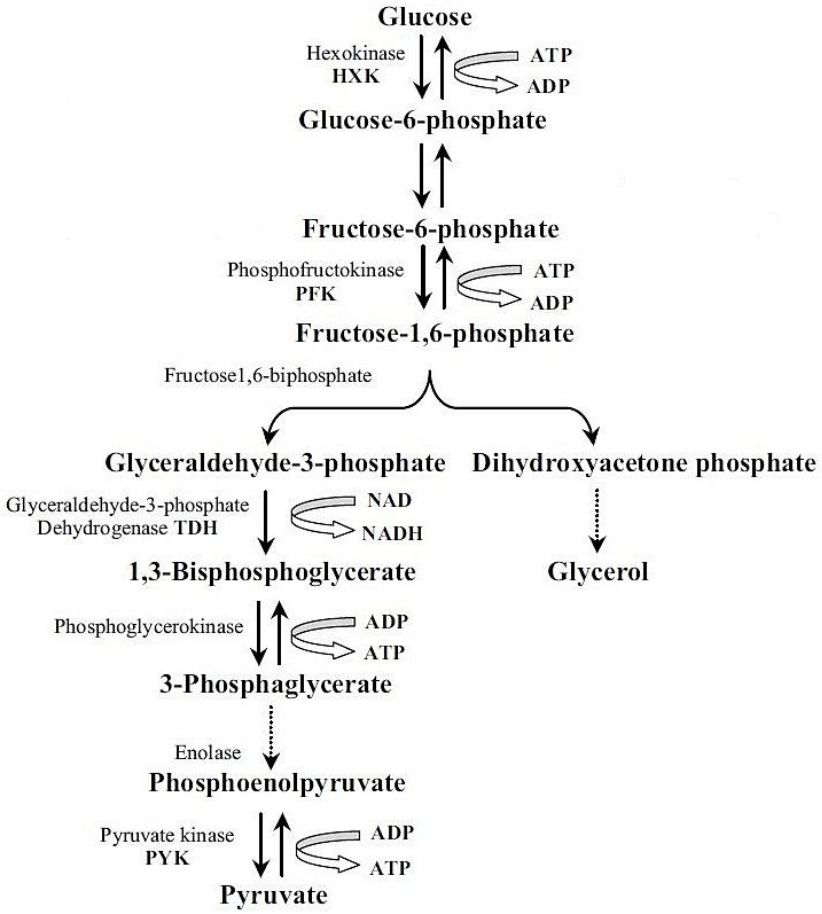

Figure 2 Glycolysis general scheme for glucose in Yeasts. The sequence of enzymatic reactions in the conversion of glucose to pyruvate (Graca S, 2004).

Glucose-6-phosphate is, after being produced, converted to its isomeric form, fructose-6-phosphate, and a second phosphorylation leads to the production of fructose 1, 6 bisphosphate, which is a key intermediate product of glycolysis and is catalysed by an irreversible phosphofructokinase. This phosphofructokinase received much attention in *S. cerevisiae* because it was thought to be the "bottleneck" of glycolysis (Flores et al. 2000). This enzyme and the irreversible pyruvate kinase are considered key regulatory

enzymes in glycolysis, whose activity is in yeasts influenced by numerous effectors, including AMP/ATP ratios, i.e., internal Pi availability, ammonium ions and fructose 2, 6-biphosphate (Gancedo and Serrano, 1989). The interconversion between the two products of fructose diphosphate cleavage, dihydroxyacetone-phoshpate and glyceraldehyde 3-phosphate, is catalysed by triosephosphate isomerase. Another very important step in glycolysis is the conversion of glyceraldehyde-3-phosphate into 1, 3-bisphophoglycerate catalysed by glyceraldehyde-3-phosphate dehydrogenase. In this step, oxidation of the aldehyde generates an energy-rich acyl phosphate bond. Furthermore, NADH is generated and must be reoxidized for glycolysis to proceed and to maintain redox balance. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been considered a classical cytosolic glycolytic protein (Flores et al. 2000). Then, the step catalysed by glyceraldehyde 3-phosphate dehydrogenase is the only oxidation step in the sequence of glycolysis leading to pyruvate.

In glycolysis, two ATP molecules are consumed in the two phosphorylation of glucose, and four ATP molecules are synthesized form each 1, 3 –bisphosphoglyceric acid converted to pyruvate. Thus, the net gain is two molecules of ATP per glucose fermented. Glycolysis leads also to the production of two NADH which will be re-oxidized in the reduction of pyruvate. Pyruvate is subjected to a series of oxidation-reduction leading to fermentation products such as ethanol, lactic acid, CO_2 or be channelled into Krebs cycle for respiration.

1.2.1.3 Pyruvate metabolism

In yeasts, pyruvate is located at major junction of assimilatory and dissimilatory reactions as well as the branch-point between respiratory dissimilation of sugars and alcoholic fermentation. From glycolysis, the end product pyruvates can be either oxidized to CO_2 or its transformation to ethanol (Fig 3). In most yeasts under aerobic conditions, oxidation through the Krebs cycle is predominant, while transformation to ethanol takes place only in anaerobic oxygen limited conditions. In those yeasts that present the so called "Crabtree effect" such as *S. cerevisiae* ethanol is produced even in aerobic condition of high glucose concentration (Pronk et al. 1996; Merico et al. 2007; Hagman et al. 2013).

When pyruvate cannot be completely degraded to CO_2 via TCA cycle, it enters one of two fermentation pathways, lactic or alcoholic. Nevertheless, the most important fermentation pathway occurring in yeasts is the alcoholic fermentation. In this case, pyruvate obtained through this process is decarboxylated to acetaldehyde via pyruvate decarboxylase; the acetaldehyde in then reduced to ethanol via alcohol dehydrogenase.

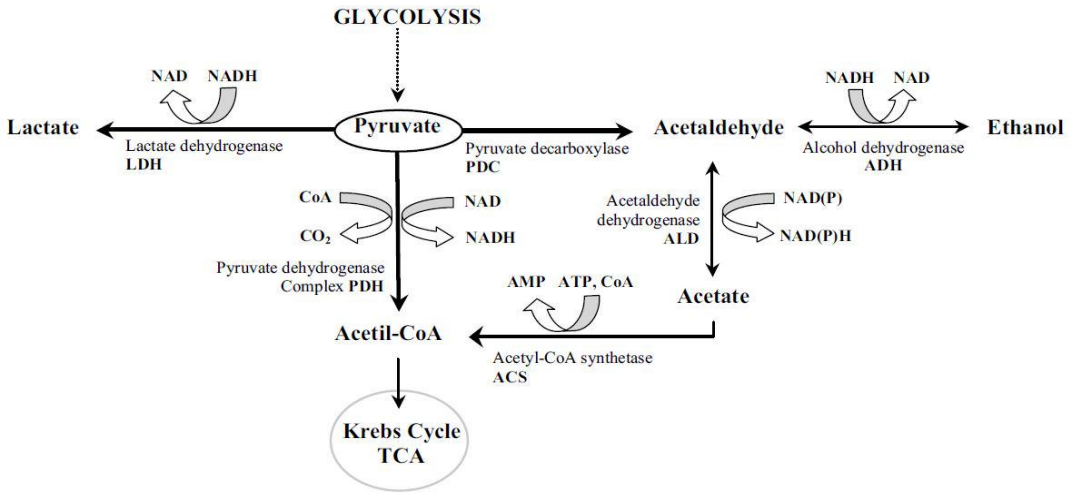

Figure 3 Metabolic fates of pyruvate in yeast (Graca S, 2004).

Pyruvate may be used simultaneously to produce ethanol (fermented) and oxidized through the Krebs cycle (respirated), being this type of metabolism termed respiro-fermentative. Acetic acid is also a by-product of the metabolism of certain yeasts, being synthesized from acetaldehyde in the presence of acetaldehyde dehydrogenase. Simultaneous production of ethanol and acetate is frequent and is regulated by the expression of different genes to transforms with different affinity for the substrate.

Acetyl-CoA, the fuel of the TCA cycle, can be synthesized from pyruvate by a direct oxidative decarboxylation, catalysed by the pyruvate-dehydrogenase complex (Pronk et al. 1996). In contrast to the enzymes of glycolysis, which are all located in the cytosol, the pyruvate dehydrogenase complex is located in the mitochondrial matrix (Holzer & Goedde 1957). Therefore, pyruvate oxidation by the complex requires transport of the substrate across the mitochondrial membranes. In addition to the pyruvate dehydrogenase reaction, conversion of pyruvate to acetyl-CoA can occur in the cytosol through the so-called pyruvate bypass, that involves the synthesis of acetyl-CoA through the concerted

action of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase. These reactions and transport of the so formed acetyl-CoA to the mitochondria could in principle "by-pass" the action of pyruvate dehydrogenase (Pronk et al. 1996).

1.2.1.4 Glucose repression

Glucose is the preferred carbon and energy source in *S. cerevisiae*. It is therefore not surprising that glucose has dramatic effects on the regulation of carbon metabolism and on many other properties of yeast cells. In the presence of glucose, yeast cells repress expression of genes that are required for alternative carbohydrates metabolism, gluconeogenesis and mitochondrial functions, through a widespread phenomenon called glucose repression (Ronne 1995); Gancedo 1992). This repression also involves a regulatory mechanism that makes the difference between fermentative growth and aerobic growth of yeast (Rolland et al. 2002; Santangelo 2006; Ronne 1995).

Glucose repressed genes can be divided into three groups. The first includes genes needed for uptake and metabolism of other carbon sources such as galactose, maltose, ethanol and acetate. Glucose repression of these genes overrides specific induction, such as galactose induction of the *GAL* genes. The second group includes genes needed for the Krebs cycle and oxidative phosphorylation, which are dispensable during fermentative growth. The third group of glucose repressed genes includes those involved in gluconeogenesis, which are needed to be repressed on glucose to prevent futile cycling (Westholm et al. 2008).

Two pathways are mainly involved in glucose repression (Fig 4). Low glucose concentration is sensed by Snf3p, since it has a high affinity for glucose, whilst primary high glucose concentration is sensed by Rgt2p, since it has a low affinity for glucose (Ozcan & Johnston 1999; Schmidt et al. 1999). The signal triggered by these sensors results in expression of different hexose transporters to uptake glucose.

In glucose repression pathway, *MIG1* plays a master role as a main effectors. The DNA-binding domain of Mig1p is composed of two C2H2 zinc fingers which are located at its N-terminal end. This protein can bind to many promoters, repressing their transcription (Nehlin et al. 1991; Ostling et al. 1996; Klein et al. 1998). Repression by *MIG1* can be both direct and indirect, though expression of genes encoding transcriptional activators. One example of this are *GAL* genes which are repressed both directly by *MIG1*, and indirectly through repression of *GAL4* gene (Nehlin et al. 1991). Yeast has two other zinc finger proteins that are closely related to *MIG1*: *MIG2* and *MIG3* (Lutfiyya et al. 1998; Kaniak et al. 2004). *MIG1, MIG2* and *MIG3* have similar DNA-binding zinc fingers, and the residues thought to be important for the DNA specificity are conserved (Lundin et al. 1994). Some glucose repressed genes are synergistically repressed by *MIG1* and *MIG2* while others are repressed only by *MIG1*.

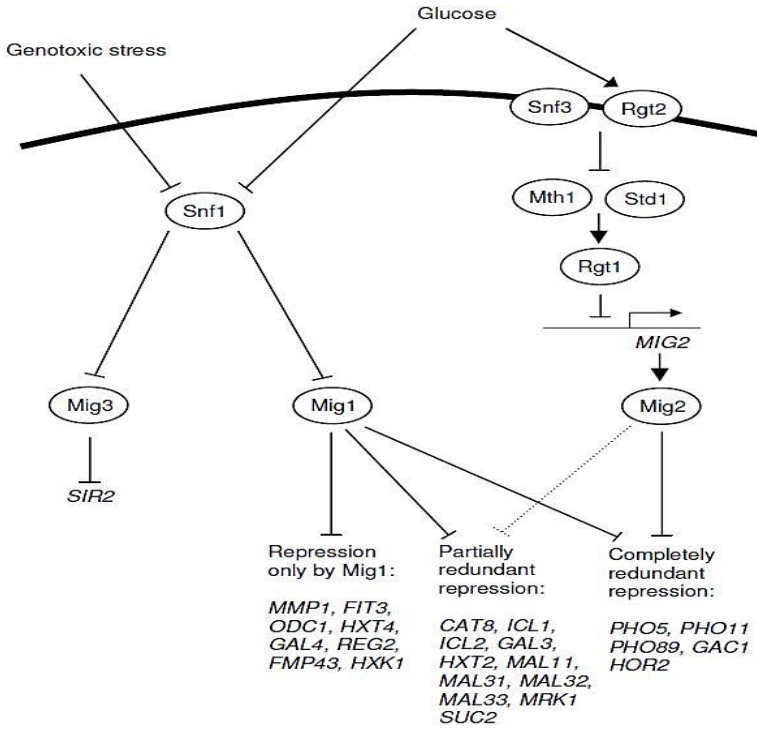

Figure 4 Overview of glucose repression and glucose induction pathways in the yeast *S. cerevisiae*. For each regulatory step, positive control is shown as an arrow, and negative control as a crossbar (Westholm et al. 2008).

MIG1 is negatively regulated by Snf1, a protein kinase present in all eukaryotes. This kinase has a general function in energy homeostasis (Hardie et al. 1998). It is activated at low energy conditions and restores the energy level by stimulation energy producing processes and inhibiting energy consuming processes. Yeast Snf1 is inhibited in the

presence of glucose, which can be regarded as a high energy condition. Snf1 is activated through a phosphorylation when the cells sense low level of glucose. The active Snf1p then catalyses the phosphorylation of Mig1p causing its to translocation from the nucleus to cytosol, thereby causing depression of glucose repressed genes.

1.2.2 Nitrate assimilation

Yeast nitrogen sources usually play anabolic roles for the biosynthesis of structural proteins and functional enzymes whereas some sources, notably amino acids, may be catabolized immediately on entry into the cell. Yeasts cannot fix atmospheric nitrogen but are capable of transporting and subsequently utilizing a few inorganic and several organic compounds. The favoured sources of nitrogen for maximizing yeast growth are mainly organic source; e.g. ammonium ions, glutamate and glutamine (Walker 1998). However, some yeasts can utilize other inorganic nitrogen compounds; namely, nitrate and nitrite. Utilization of nitrate and nitrite as major sources of inorganic nitrogen is restricted to relatively few yeast species belonging to various genera, including *Hansenula*, *Leucosporidium*, *Pichia*, *Debaryomyces*, *Trichosporon*, *Rodhotorula*, *Sporidiobolus*, *Williopsis*, *Brettanomyces*, *Candida*, etc. (Barnett et al. 2000). However, the classical model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* do not assimilate nitrate (Barnett et al. 2000).

The nitrate-assimilation pathway in this yeast follows that described for plants and filamentous fungi (Guerrero et al. 1981; Hipkin 1989). Nitrate is transported into the cell by the high-affinity nitrate transporter Ynt1 (Pérez et al. 1997). Kinetically two groups of nitrate transporters have been characterised: one with high affinity, K_m in the µM nitrate range, found in yeasts, filamentous fungi, algae and plants (Daniel-Vedele et al. 1998; Crawford & Glass 1998; Forde 2000) and one low affinity group, K_m in the mM nitrate range, found mainly in plants, although there is indirect evidence of its presence in yeast and algae (Machín et al. 2001; Navarro et al. 2000). Once nitrate enters the cells, (Fig 5) it is reduced to ammonium by the combined action of the enzymes NR (nitrate reductase) and NiR (nitrite reductase) (Guerrero et al. 1981).

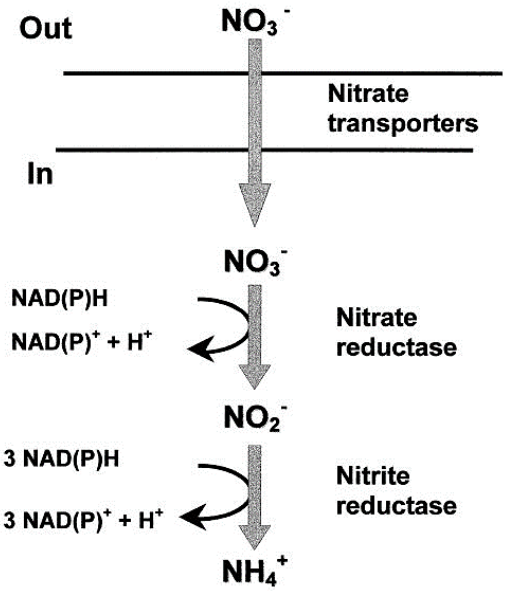

Figure 5. Pathway of nitrate assimilation in yeasts. Nitrate is transported from medium into cell cytosol, where it is reduced to ammonium in two successive reactions catalysed respectively by nitrate and nitrite reductase. Image taken from (Siverio 2002)

Hansenula anomala, along with *Hansenula polymorpha* and *Candida utilis*, are the few yeast species in which the nitrate assimilation pathway has been studied to some extent (Hipkin 1989; Siverio et al. 1993; Avila et al. 1998; Sengupta et al. 1996). In the yeast *Hansenula polymorpha* the genes involved in nitrate assimilation *YNT1*, *YNI1*, *YNA1* and *YNR1* encoding, respectively, a nitrate transporter, nitrite reductase (NiR), a transcriptional factor involved in the induction of the system and nitrate reductase (NR) are clustered (Avila et al. 1998). Two highly similar Zn(II)2Cys6 transcriptional factors encoded by the genes *YNA1* and *YNA2* have been found to be indispensable for nitrate induction in *H. polymorpha*. In general, the *H. polymorpha* genes involved in nitrate assimilation are induced by nitrate and nitrite and repressed by sources of reduced nitrogen, such as ammonium (Crawford & Arst 1993; Marzluf 1997; Avila et al. 1998).

In *Arxula adeninivorans* nitrate assimilation is also mediated by the combined actions of a nitrate transporter, a nitrate reductase and a nitrite reductase. The *AYNT1*, *AYNI1* and *AYNR1* promoters provide attractive control elements of high strength for the *A. adeninivorans* expression platform, and are inducible by a shift from ammonium to nitrate medium (Böer et al. 2009).

1.2.3 Galactose metabolism

In order for galactose to be metabolized by yeast it must first be transported into cell. Gal2p is a high affinity galactose transporter (Tschopp et al. 1986; Huibregtse et al. 1993). *GAL2* gene is expressed in the presence of galactose and repressed in the presence of glucose (Johnston 1987). However, it is not highly specific for galactose and can also transport glucose (Reifenberger et al. 1997; Maier et al. 2002). Furthermore, it is likely that when the organism is growing in galactose, the sugar is transported into the cell by both Gal2p and other less selective hexose transporters of Hxt family (Timson 2007).
During fermentative metabolism, the transcription factor complex of Tup1p, Ssn6p and Mig1p repress the expression of respiratory, gluconeogenic and alternative carbon source utilization genes (Gancedo 1998; Johnston 1999; Rolland et al. 2002). Galactose is metabolised by the enzymes of Leloir pathway (Frey 1996). This pathway, which was named after the Nobel Prize-winning Argentinean biochemist Louis Leloir (Cabib 1970) requires five enzymes to convert galactose to glucose 6-phosphate (Fig 6).

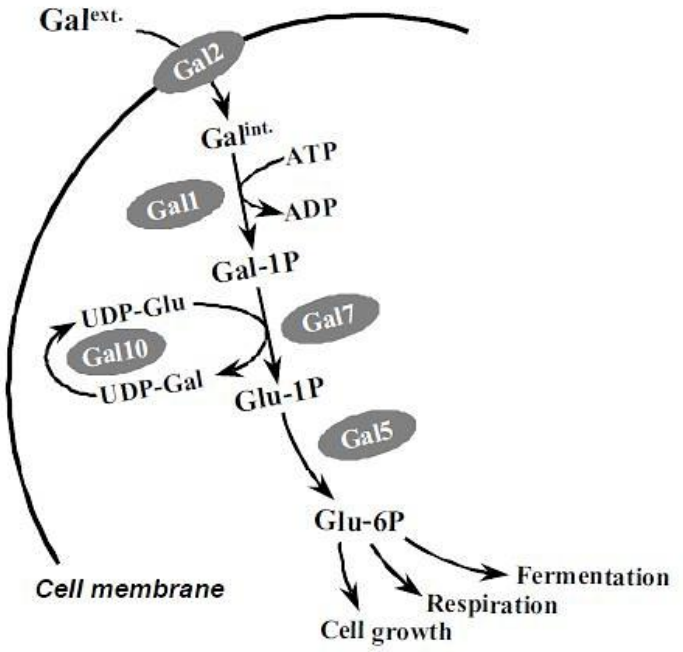

Figure 6. The galactose utilization pathway. Extracellular galactose is transported into the cell and subsequently converted to glucose-6- phosphate by several enzymatic steps. Glucose-6-phosphate may be directed toward respiratory metabolism, fermentative metabolism, or conversion of glucose-6-phosphate may lead to biosynthesis of various precursors required for cell growth. Gal2, galactose permease; Gal1, galactokinase; Gal7, galactose-1-phosphate uridylyltransferase; Gal10, UDP-glucose 4-epimerase; Gal5, phosphoglucomutase. Image taken from (Ostergaard et al. 2000)

These enzymes are encoded by the GAL1, GAL7, GAL10, and GAL5 and, with the exception of GAL5, their synthesis is regulated on the transcription level. Galactose is phosphorylated to galactose-1-phophate by galactokinase (*GAL1*) and then isomerized to glucose-1-phosphate by galactose-1-phosphate uridyltransferase (*GAL7*). UDP-glucose is regenerated from UDP –galactose by the action of UDP-galactose 4-epimerase which is encoded by Gal10p (Fukasawa et al. 1980). The final step in the pathway is the isomerisation of glucose1-phosphate to glucose 6-phosphate catalysed by phosphoglucomutase (*GAL5*). In *S. cerevisiae* there are two phosphoglucomutase isoforms, Pgm1p and Pgm2p. About 80% of the total activity is provided by Pgm2p (Tsoi & Douglas 1964). Phosphoglucomutase is not exclusive to the Leloir pathway; it is also plays a role in glycogen metabolism.

1.2.4 Ethanol metabolism

Ethanol may be used as carbon and energy source by most of the yeast, but is widely known as a by-product of fermentation in fermentative and respire-fermentative yeasts. This is one of the most common gluconeogenic substrate for yeasts in nature. Yeasts can switch from the glycolytic mode in the presence of glucose to a gluconeogenic mode when glucose is exhausted and can use ethanol as carbon source produced by glucose fermentation. This has been called the "make-accumulate-consume" strategy, exhibited mainly by *S. cerevisiae* and other phylogenetically related yeasts (Hagman et al. 2013)

Central to the production or the utilization of ethanol are alcohol dehydrogenases, enzymes that catalyse the reversible reduction of acetaldehyde to ethanol. Utilization of ethanol implicates a first oxidation to acetaldehyde and second oxidation to acetic acid catalysed by an aldehyde dehydrogenase (Fig 7). In *S. cerevisiae*, four genes encode the isoenzymes of alcohol dehydrogenases. It has at least two cytoplasmic alcohol dehydrogenase isoenzymes, encoded by *ADH1* and *ADH2*, and one mitochondrial isoenzymes, encoded by *ADH3* (Reid & Fewson 1994; Leskovac et al. 1997). Aldehyde dehydrogenases are important enzymes not only for the metabolism of acetaldehyde

produced from ethanol, but also for that of toxic aldehydes produced in some stress situations (Flores et al. 2000).

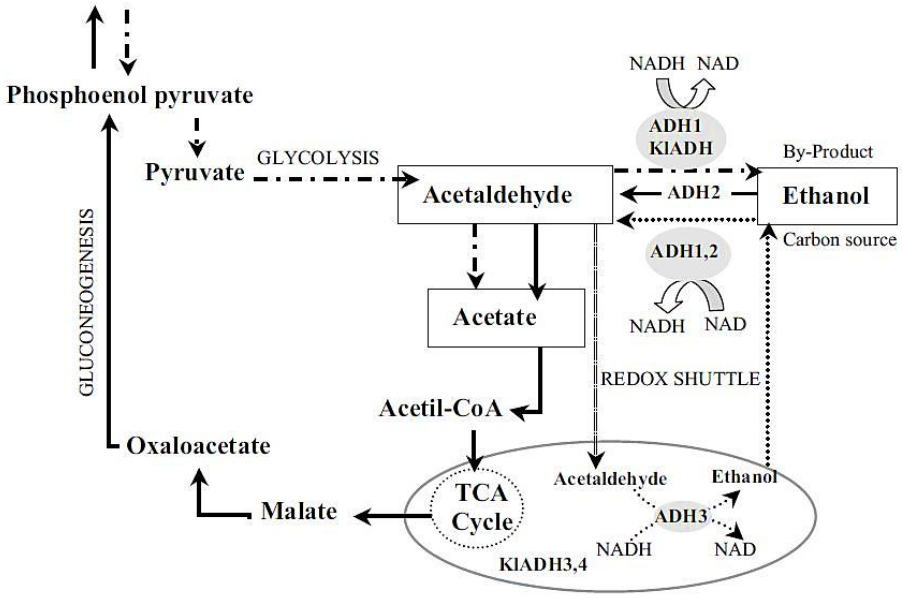

Figure 7. General overview of ethanol metabolism in yeasts (Graca S, 2004).

Ald2p and Ald3p use NAD^+ as cofactor, are stress-induced and repressed by glucose; Ald4p uses NAD^+ or $NADP^+$ and is also repressed by glucose, while Ald5p use $NADP^+$ and is constitutive. Ald4p and Ald6p seem important for growth in ethanol (Tessier et al. 1998). The transcriptional activators Cat8p involve in genes activation required for gluconeogenesis during growth on non-fermentable carbon sources (Rolland et al. 2002; Schüller 2003). On the other hand, respiratory genes in the TCA cycle and respiratory chain are highly induced by the Hap transcription factors, a global activator complex of respiratory genes (Gancedo 1998; Schüller 2003).

1.2.5 Acetic acid metabolism

The transcriptional activators Cat8p involve in genes activation required for gluconeogenesis during growth on non-fermentable carbon sources (Rolland et al. 2002; Schüller 2003). Several yeast species display the ability to use acetic acid and other weak monocarboxylic acids when added to the culture medium as the only carbon and energy sources (Barnett e t al., 1990). In *Saccharomyces cerevisiae*, it is well-established that the

utilization of acetic acid as the only carbon and energy source implies the activity of the anaplerotic glyoxylate cycle and the gluconeogenesis pathways, both of which are regulated by glucose repression (Gancedo & Serrano, 1989). The transport of acetic acid through the plasma membrane is mainly in the anionic form by secondary active transport systems, which behave as proton symports specific for acetate and other monocarboxylates. These transporters are subject to glucose repression (Cássio et al. 1987; Casal et al. 1996), however, in low pH medium the undissociated form of acetic acid can pass through the plasma membrane by simple diffusion (Mollapour et al. 2008).

In cytoplasm, conversion of acetate into acetyl-CoA is catalysed by acetyl-CoA synthetase. *S. cerevisiae* contains two structural genes encoding acetyl-CoA synthetase isoenzymes, *ACS1* and *ACS2*. The *ACS1* gene is subject to glucose repression, whereas *ACS2* is expressed constitutively. This reflecs a specific need by *S. cerevisiae* for the by-pass as the main source of cytoplasmic acetyl-CoA for biosynthetic purpose. The formed acetyl-CoA enter into the mitochondria to use as a fuel of kerbs cycle. Glyoxylate cycle is required for growth in minimal medium in carbon sources of less than three carbon atoms, such as ethanol or acetate. Two characteristic enzymes of the cycle are isocitrate lyase and malate synthase. Isocitrate lyase catalyzes the cleavage of isocitrate to succinate and glyoxylate and malate synthase catalyzes the condensation of glyoxylate with a molecule of acetyl CoA (Flores et al. 2000).

1.3 Acetic acid stress and yeast adaptation

Weak organic acids can act either as inhibitory agents or as carbon sources for microbial growth, depending on their concentrations, their ability to enter the cell, and the capacity of the microorganism to degrade the acid. Weak organic acids such as benzoic, sorbic, propionic and acetic acid have been widely used as inhibitory agents in food industries.

1.3.1 Acetic acid stress

Acetic acid, due to its toxic effects, is used in food industry as a preservative against microbial spoilage. The antimicrobial potential of acetic acid is essentially determined by the acid concentration and the pH of the medium. In aqueous medium, acetic acid exists in dynamic equilibrium between molecular acid and its respective charged anion. Such equilibria are highly pH-dependent and the action of acetic acid increases with acidity, appearing to be proportional to the concentration of undissociated acid. The effects of undissociated acid may reach up to 90% of the total concentration depending on pH and p*Ka*. At low pH (below p*Ka*), acetic acid substantially in the form of the undissociated acid (XCOOH; fig 8) and at neutral pH, it is almost completely dissociated to acid anion ($XCOO^-$; fig 8).

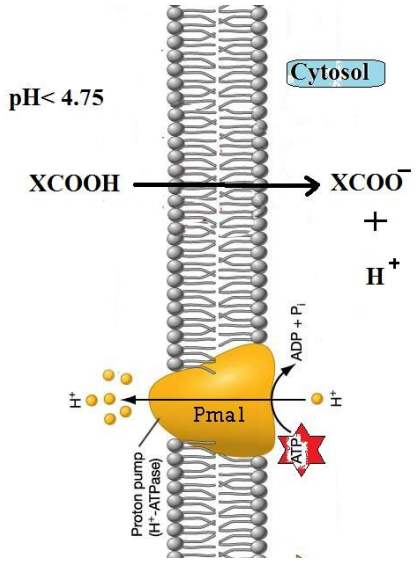

Figure 8. The usual conception of how weak organic acids act on yeast cells. If the undissociated acid (XCOOH) is freely permeable to the membrane, its concentration inside and outside the cell will be governed by the pH on either side of the membrane and the dissociation constant of the acid.

The undissociated form of acid are soluble in lipids, while the charged anions are lipid insoluble. It is generally accepted that the undissociated forms can enter into the cytosol by crossing the plasma membrane much more readily than the charged acid anion. Therefore, the undissociated form of acetic acid permeate the plasma membrane by simple diffusion. However, it has recently been described that acetic acid can also enter the yeast cells by a process of facilitated diffusion, mediated by the aquaglyceronporin Fps1p, (Fig 9) (Mollapour & Piper 2007).

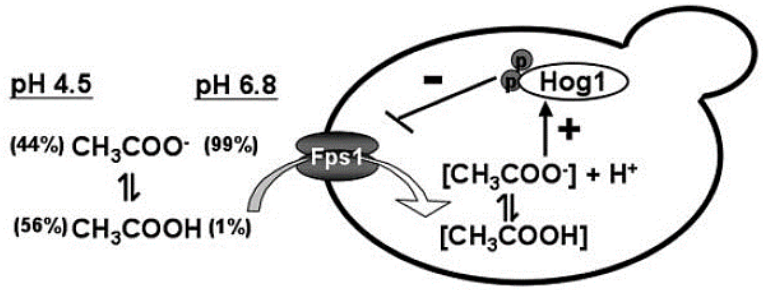

Figure 9. Entry of undissociated acetic acid into the cells in Fps1 facilitated, with the acid that enters the cell in this way then dissociating in the cytosol generating an intracellular pool of the acetate anion. (Mollapour & Piper 2007)

The normal cytoplasmic pH of fungal mycelium has been reported as being close to pH 7.0-7.5 (Legiša & Grdadolnik 2002). Once acetic acid's inside the cell, the undissociated form encounter a more neutral pH in cytoplasm and dissociate into dissociate proton (H^+) and the acid anion. Due to the electric charge, these ions are not able to cross the hydrophobic lipid plasma membrane bilayer and accumulate in the cell interior. The proton release with the intracellular dissociation of these acids potentially acidify the cytosol. This intracellular acidification trigger the stress responses that elevate the acetic acid resistance. The accumulation of protons during acid stress can lead to decreased DNA and RNA synthesis rate, reduced metabolic activity, and disrupted electrochemical proton gradients, while the accumulation of anions primarily results in increased turgor pressure and oxidative stress (Pampulha & Loureiro-Dias 2000; Piper et al. 2001; Carzaniga et al. 2012). Intracellular acidification caused by acetic acid also leads to trafficking defects, hampering vesicle exit from the endosome to the vacuole (Brett et al. 2005) and severe intracellular amino-acid starvation (Almeida et al. 2009).

To counteract this acidification, cells may take several ways, like intracellular pH recovery, detoxification through multidrug resistance, remodelling of the cellular envelope etc.

1.3.2 Intracellular pH recovery

The H^+ -ATPase in fungal plasma membranes functions physiologically to hydrolyse ATP and to pump H^+ out of the cell (Serrano 1988). Several lines of evidence indicate that ATPase activation is due to posttranslational modifications of the Pma1 ATPase (Monteiro et al. 1994; Viegas et al. 1995). Under conditions of cytosolic acidification, Pma1p activity is stimulated, and this stimulation is regarded as central to overall pH homeostasis (Eraso & Gancedo 1987; Yenush et al. 2005). Weak acids induce activation of proton-translocating ATPase Pma1p in yeast plasma membrane, which pumps out the protons generated by weak acid dissociation in the cytosol in an ATP-dependent manner. This activation ensure maintenance of the electro-chemical potential across plasma membrane for nutrient uptake (Carmelo et al. 1997; Guldfeldt & Arneborg 1998; Pampulha & Loureiro-Dias 1989). Together with H^+ -ATPase, vacuolar proton-translocating ATPase (V-ATPase) also plays a central role in organelle acidification in all eukaryotic cells (Fig 10). By sequestering protons into the lumen of the vacuole, V-ATPase activity contributes to the recovery of cytosolic pH and to counteract the acid-induced dissipation of the transmembrane potential across the vacuolar membrane. In yeast, V -ATPase activity plays the role for the efficient cytosolic pH homeostasis and for stable localization of Pma1P at the plasma membrane (Martínez-Muñoz & Kane 2008). Both Pma1p and the V-ATPase use energy from ATP hydrolysis to pump protons out of the cytosol (Serrano 1991). This establishes a pH gradient and membrane potential ($\Delta\psi$) critical for operation of other transporters in their respective membranes but also requires mechanisms, for balancing the generation of membrane potential (Seto-Young & Perlin 1991; Grabe 2001).

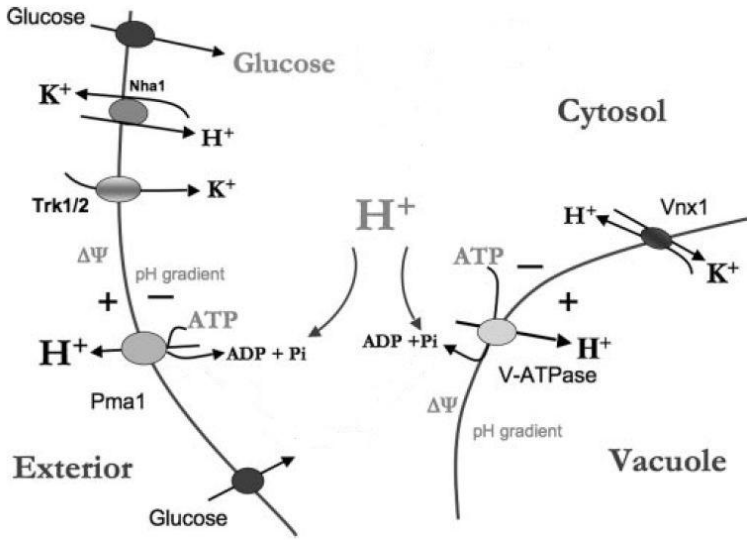

Figure 10. Model for cellular positions of V-ATPase and Pma1p in cytosolic pH homeostasis. Image take and modified from (Martínez-Muñoz & Kane 2008)

1.3.3 Detoxification through multidrug resistance

The main barrier for any transport event is the plasma membrane. Compounds can passively cross this barrier by diffusion. Transport by diffusion is possible only a concentration gradient and is limited to solutes able to partition in hydrophobic membranes. Therefore, transport of most compounds over membranes is mediated by membrane bound proteins with specialized transport functions.

Yeast exposed to acetic acids are therefore subject to a constant acetic stress; the continuous, passive diffusional entry of the acid into the cell increase the pool of acid anions. The reduction of the intracellular pool of the weak acid counterion(s) is therefore essential, and several specific transporters have been implicated in yeast tolerance to different weak acids.

ABC transporters are members of a large superfamily of transporters. Generally, they are located in plasma membranes and intracellular membranes and include both influx and efflux systems. The ABC "core domain" consists of two homologous halves, each containing a membrane spanning domain (MSD) with multiple transmembrane spans and a nucleotide-binding domain (NBD), which couples nucleotide hydrolysis to substrate

transport (Locher 2009; Higgins 1992). The NBDs are required to fuel membrane transport or other functions by hydrolysis of ATP. These are generally referred to as ABC drug efflux pumps. However, ABC proteins not only function as simple membrane transporters, they are also implicated in maintenance of mitochondrial function, maturation of cytosolic Fe/S proteins, pheromone secretion, peroxisome biogenesis, stress response, as well as lipid bilayer homeostasis and lipid uptake .

Members of the ATP-binding cassette (ABC) superfamily catalyse the ATP-dependent transport of chemically diverse compounds across cellular membranes, including the plasma membrane or intracellular organelle membranes (Dean et al. 2001; Higgins 1992). Many ABC transporters are tightly regulated by transcription factors within the so-called PDR network that modulates levels of numerous membrane transporters under physiological as well as adverse conditions. PDR in yeast is similar to multidrug resistance (MDR) phenomena in tumor cells (Dean et al. 2001).

Extrusion of noxious compounds from the cell by efflux pumps is one of the most frequently used strategies for the development of drug resistance in yeast. Yeast cells can quickly counteract toxic environmental challenges through efficient detoxification systems such as the PDR machinery. Pdr12p is a major determinant conferring resistance to sorbate, benzoate and acetate, and it is strongly stress inducible, its induction being essential for the development of weak acid resistance (Piper et al. 1998). War1p is constitutively localized in the nucleus and it is able to form homodimers *in vitro* (Kren et al. 2003). Inside the cell, acid anions activate War1p, a transcription factor that induces the gene for the Pdr12p plasma membrane ATP-binding cassette transporter. This transporter confers resistance by catalysing the active extrusion of the acid anion (against the concentration gradient) from the cell, thereby lowering the intracellular levels of acid (Fig 11) (Mollapour et al. 2008). Pdr12p is essential for growth in the presence of weak organic acids, as cells lacking this efflux pump are hypersensitive to water-soluble monocarboxylic acids with chain lengths from C1 to C7 (18). Recently Pdr12p ABC transporter has determined as the major determinant of weak organic acid tolerance in *S. cerevisiae* (Kren et al. 2003). However, this is a highly ATP energy-demanding process.

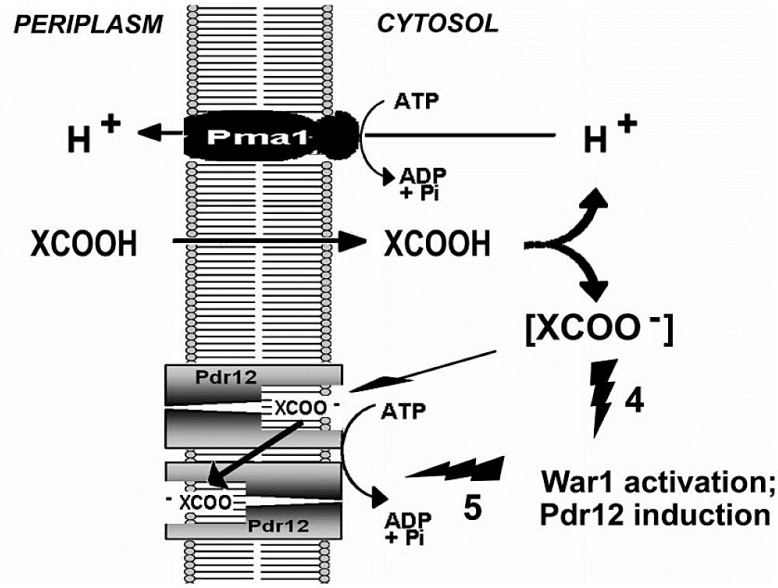

Figure 11. Acetic acid entry to the cell is mainly passive diffusion across the plasma membrane, generating an intracellular acid anion pool ($XCOO^-$) that act as the inducer of War1p, then activated Pdr12p efflux pump (Mollapour et al. 2008).

The normal physiological function of Pdr12 is perhaps to protect against the potential toxicity of weak organic acids secreted by competitor organisms, acids that will accumulate to inhibitory levels in cells at low pH (Piper et al. 1998).

1.3.4 Weak acid-induced remodelling of the cellular envelope

The active expulsion of weak acid anions from the cells interior would be energetically expensive and futile if the undissociated acid could re-enter the cells at similar rate. Therefore, the cell could remodelling its envelope to restrict the re-entry of undissociated acid by passive diffusion. Membrane have proven to be an important target for stress adaptation (Russell et al. 1995). The activation of Hog1p by acetic acid causes the removal of protein-channel Fsp1p from the plasma membrane (Mollapour & Piper 2007). The transcription factor Haa1p also associated with the resistance to acetic acid in glucose medium (Fernandes et al. 2005). This effect was mainly attributed to the downregulation of genes coding for the plasma membrane multidrug transporters, *TOP2* and *TOP3*, and for the cell wall glycoprotein, *YGP1*. A proteomic analysis of *S. cerevisiae* cells treated with acetic acid revealed that proteins from amino-acid biosynthesis, stress response,

protein turnover and cell cycle are affected (Almeida et al. 2009). Upon exposure to acetic acid, *Z. bailii* has the ability to undergo major lipidome rearrangements. This ability in particular concern the enrichment of saturated acyl chains arising from glycerophospholipids and complex sphingolipids (Lindberg et al. 2013).

2. Aim of the Project

Yeasts are best known for their beneficial contributions to the society in the production of alcohol, wine, beer or bread. However, yeasts also cause spoilage leading to a major problem in the food and beverage industries. Among all yeast species, *S. cerevisiae* has dominated the scientific stage and become a synonym of yeast. *D. bruxellensis* and *S. cerevisiae* are considered two phylogenetically distant relatives, but they share several industrial relevant traits. However, *D. bruxellensis* exhibited a more efficient energy metabolism (Blomqvist et al. 2010), and can better grow in high ethanol and acetic conditions compared to *S. cerevisiae*. In parallel, the ability of *D. bruxellensis* to assimilate nitrate may provide a competitive advantage over *S. cerevisiae*. Nevertheless, *D. bruxellensis* is well-known as a spoilage yeast causing enormous economic losses in wine industry due to production of phenolic off-flavor compounds. In lignocellulosic bioethanol production, acetic acid negatively impact on yeast performance leading to inhibit alcoholic fermentation, limiting the productivity of the process. On the other hand, antimicrobial effect of acetic acid is exploited in food industry, where it is used as a preservative. Some non-*Saccharomyces* species including *D. bruxellensis* are highly resistant to acetic acid. Despite its economic importance and physiological interest, *D. bruxellensis* has been poorly studied. The understanding of the molecular basis of this yeast may guide to develop more effective strategies to control spoilage yeasts, and to increase process performance in industrial alcoholic fermentations.

The aims of the study were

-to investigate how nitrate utilization affects carbon metabolism and the fermentation products yields in *D. bruxellensis.*

-to investigate how *D. bruxellensis* use galactose and the role of glucose plays on carbon source utilization.

-to investigate the adaptive stress response and tolerance to acetic acid in *D. bruxellensis.*

3. Main results

3.1 Nitrate utilization under aerobic conditions

In order to obtain a detailed quantitative and qualitative analysis about the effects of utilization of nitrate on the metabolism of glucose and fermentation products in *D. bruxellensis*, batch cultures were performed in a bioreactor under strictly controlled aerobic conditions, controlled pH, and on synthetic media. In the first series of batch cultures, *D. bruxellensis* was cultivated on media containing sodium nitrate as the sole nitrogen source. Under these conditions, the growth rate was similar to the ammonium-based one. Interestingly, the main final product of glucose fermentation was acetic acid instead of ethanol, which reached a 3.5X higher yield and was produced at a 2.5X higher specific production rate than on ammonium-based ones (Table 2). On the other hand, the specific glucose consumption rate as well as the ethanol production rate were both lower on nitrate-based media than on ammonium-based ones (Table 2). The utilization of nitrate determined a slight increase of the biomass yield (Table 2). The use of nitrate as nitrogen source determines in *D. bruxellensis* deep changes in the distribution of the final fermentation products.

Table 2. Growth parameters during aerobic fermentations on glucose mineral medium with ammonium sulphate (5 g L^{-1}), sodium nitrate (6.43 g L^{-1}), or a mixture of ammonium sulphate (5 g L^{-1}) and sodium nitrate (1 g L^{-1}) as nitrogen sources.

	Ammonium[a]	Nitrate	Mixture
Growth rate (h^{-1})	0.11–0.12	0.092 ± 0.006	0.077 ± 0.004
q (mM $g_{dry\ weight}\ h^{-1}$)			
Glucose	3.6–3.7	2.94 ± 0.5	2.42 ± 0.28
Ethanol	3.9–4.4	1.65 ± 0.007	1.30 ± 0.09
Acetate	0.62–0.70	1.83 ± 0.009	1.59 ± 0.10
Y (g $g_{glucose}^{-1}$)			
Biomass	0.17–0.18	0.19 ± 0.004	0.23 ± 0.008
Ethanol	0.320–0.335	0.133 ± 0.006	0.138 ± 0.006
Acetate	0.058–0.060	0.216 ± 0.006	0.166 ± 0.011

[a] Data from Rozpędowska et al. 2011

3.2 Nitrate utilization under anaerobic conditions

D. bruxellensis was cultivated under strictly controlled anaerobic conditions in bioreactor on synthetic media containing a mixture of ammonium and nitrate, due to the fact that we observed no growth on plates containing nitrate as the sole nitrogen source. Under these conditions, cells grew at a higher rate than the one observed on ammonium-based medium enriched with amino acids (Table 3). The growth rate was in fact more similar to the one observed under aerobic conditions (Tables 2 and 3 for comparison). In contrast to what occurred on ammonium-based media, where no acetic acid production was detected, acetic acid was produced under anaerobic cultivation when nitrate was utilized. Noteworthy, the specific acetic acid production rate corresponded to the specific nitrate consumption rate, indicating that there is a strict correlation between nitrate utilization and acetate production (Table 3).

Table 3. Growth parameters during anaerobic fermentations on glucose mineral medium with ammonium sulphate (5 g L^{-1}) or mixture of ammonium sulphate (5 g L^{-1}) and sodium nitrate (1 g L^{-1}) as nitrogen sources.

	Ammonium with casamino acids[a]	Ammonium and nitrate w/o casamino acids
Growth rate (h^{-1})	0.070–0.075	0.084 ± 0.006
q (mM $g_{dry\ weight}$ h^{-1})		
Glucose	1.47–1.60	4.08 ± 0.26
Ethanol	1.74–1.90	5.98 ± 0.42
Acetate	0.02–0.02	0.43 ± 0.02
Nitrate	–	0.46 ± 0.02
Y (g $g^{-1}_{glucose}$)		
Biomass	0.132–0.150	0.10 ± 0.005
Ethanol	0.34–0.35	0.35 ± 0.021
Acetate	0	0.033 ± 0.001

[a] Data from Rozpędowska et al. 2011

Nevertheless, in this case, ethanol was the main product of glucose metabolism and its specific production rate was the highest obtained (Tables 2 and 3, for comparison). Also, the specific glucose consumption rate was the highest estimated, indicating that the redox

unbalance negatively affected glucose metabolism and its fermentative efficiency on ammonium-based media. All these data indicate that the assimilation of nitrate greatly improves the ability to grow under anaerobic conditions, playing like a ‘‘valve’’ to balance the redox potential.

3.3 Analysis of involved enzymatic activities in nitrate assimilation

The activity of the enzymes involved in nitrate assimilation was assayed in *D. bruxellensis* cells growing on nitrate-based media under aerobic as well as under anaerobic conditions. Nitrate reductase was found to use either NADPH or NADH in vitro as the electron donor (Table 4). The activities were higher in cell extracts from anaerobic growth conditions. The activity of nitrite reductase was undetectable in cell extracts from aerobic cultures, but an extremely low activity was assayed in cells grown under anaerobic conditions.

Table 4 Activity of enzymes involved in NAD(P)/NAD(P)H utilization during growth in aerobic or anaerobic conditions, on glucose mineral media with ammonium sulphate (5 g l-1), sodium nitrate (6.43 g L^{-1}), or mixture of ammonium sulphate (5 g L^{-1}) and sodium nitrate (1 g L^{-1}) as nitrogen sources.

Enzyme activity (U $mg^{-1}_{protein}$)	Cofactor specificity	Aerobiosis		Anaerobiosis	
		Ammonium	Nitrate	Ammonium	Ammonium and nitrate
Acetaldehyde dehydrogenase	$NADP^+$	0.23 ±0.007	0.139 ±0.004	0.065 ±0.005	0.115 ±0.003
	NAD^+	0.50 ±0.03	0.24 ±0.014	0.13	0.025 ±0.006
Glucose 6 phosphate dehydrogenase	NADPH	0.21 ±0.09[a]	0.10 ±0.007	0.16 ±0.006	0.025 ±0.003
Nitrate reductase	NADH	–	0.039 ±0.005	–	0.045 ±0.001
	NADPH	–	0.015 ±0.002	–	0.021 ±0.001

In order to understand if nitrate utilization can affect the enzyme activities leading to the increased acetic acid formation, we assayed acetaldehyde dehydrogenase (ACDH). Under

aerobic conditions, the growth on nitrate-based media resulted in a decreased activity of ACDH (Table 4). Interestingly, we found that nitrate utilization under anaerobic conditions determined an increased specific activity of NADP-dependent ACDH, which was in fact higher in nitrate-grown cell extracts than in ammonium-grown ones (Table 4) and, in parallel, a decrease in the NAD-dependent ACDH activity. On the other hand, the activity of glucose 6-phosphate dehydrogenase (G6PDH), which is one of the main sources of NADPH, was lower in nitrate-grown cells (Table 4).

3.4 Galactose metabolism is affected by nitrogen source

In the aim to study galactose metabolism in detail, aerobic cultures of the sequenced strain *D. bruxellensis* CBS 2499 (Piškur et al. 2012) were performed in batch at controlled concentration of dissolved oxygen. Moreover, due to the fact that *D. bruxellensis* is able to use nitrate as nitrogen source (Galafassi, Capusoni, et al. 2013), we tested if the galactose metabolism can be affected by the nitrogen source. When *D. bruxellensis* was cultivated on media containing ammonium salts, galactose metabolism was respiratory, as indicated by the biomass level and by the failed production of metabolites normally resulting from fermentative metabolisms, such as ethanol or acetic acid (Fig. 12 A, Table 5). The growth rate was lower than on glucose (Table 5), and galactose was utilized at a lower specific rate (Table 5). Here we showed that in *D. bruxellensis* CBS 2499 galactose is a not-fermentable carbon source (Table 5). This is in contrast to what occurs in *S. cerevisiae*, which produces ethanol even from galactose (Sierkstra et al. 1993; Ostergaard et al. 2000; Martínez et al. 2014). The scenario that comes out from these new observations is that when the strain of *D. bruxellensis* under study is cultivated on galactose-based media it behaves like a Crabtree-negative yeast, not producing ethanol, in contrast to *S. cerevisiae*, that behaves like a Crabtree-positive yeast both on glucose and on galactose.

Interestingly, when the medium contained nitrate as sole nitrogen source, the final products of galactose metabolism changed: together with biomass also acetic acid and a very low amount of ethanol were produced (Fig. 12 B, Table 5). The rate at which galactose was consumed on nitrate-based media was higher than on the ammonium-based

ones (Table 5), nevertheless the growth rates were very similar. In conclusion, these results indicated that when cells use ammonium as nitrogen source the galactose metabolism is respiratory. Nevertheless, when nitrate is the nitrogen source a partial redirection of pyruvate occurred: some pyruvate was in fact converted by pyruvate decarboxylase to acetaldehyde, which was in turn converted to acetic acid and, at a low level, to ethanol.

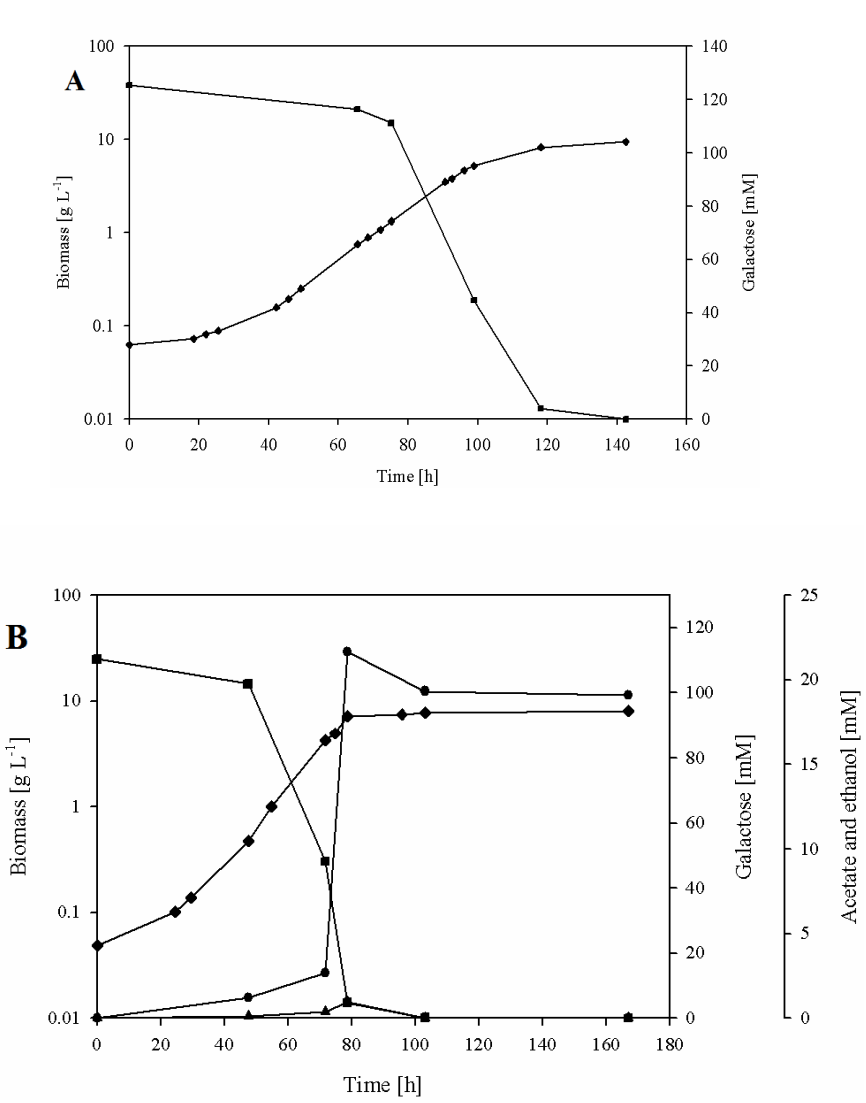

Figure 12: Growth kinetics of cultures on galactose-based media (one example of kinetic is showed) containing ammonium sulphate (A) and sodium nitrate (B) as nitrogen source. Symbols: ■, galactose; ♦, biomass; ●, acetate; ▲, ethanol.

Table 5. Growth parameters of cultivations on galactose-based media containing ammonium sulphate and sodium nitrate as nitrogen source. For comparison, the growth parameters calculated during cultivations on glucose-based media are indicated in brackets.

	q [mmol g DW^{-1} h^{-1}]				Yield [g g^{-1}]			μ [h^{-1}]
	Galactose (Glucose)	Ethanol	Acetate	Nitrate	Biomass	Ethanol	Acetate	
Ammonium	0.82-0.86* (3.6-3.7)[a]	n.d. (3.9 – 4.4)[a]	n.d. (0.62 – 0.070)[a]	- (-)[a]	0.497-0.501* (0.17 – 0.18)[a]	n.d. (0.320 – 0.335)[a]	n.d (0.058 – 0.060)[a]	0.0752-0.0755* (0.11 – 0.12)[a]
Nitrate	1.20-1.3* (2.94 ± 0.006)[b]	0.011-0.011* (1.650 ± 0.007)[b]	0.491-0.496* (1.83 ±0.009)[b]	0.466* (-)[b]	0.374-0.379* (0.19 ± 0.004)[b]	0.002* (0.133 ± 0.006)[b]	0.068-0.072* (0.216 ± 0.006)[b]	0.0714-0.073* (0.92 ± 0.006)[b]

*experiments performed in duplicate (the range of values reported), [a] Data from Rozpędowska *et al.*, 2011, [b] Data from Galafassi *et al.*, 2013, q: specific consumption/production rate, DW: dry weight, n.d.: not detectable.

3.5 Gene expression and enzyme activities for galactose and respiratory metabolism

The availability of the complete genome sequence of the *D. bruxellensis* CBS 2499 strain (Piškur et al., 2012) allowed the identification of genes encoding all the enzymes that in *S. cerevisiae* are required for galactose assimilation. The expression of genes involved in galactose and respiratory metabolism was analyzed, in order to understand the role that the carbon source plays on their transcriptional regulation. In particular, we focused our attention on the expression of genes containing in their promoters putative consensus sequences related to the transcription factor Mig1p, which is known to be one of the key elements in glucose repression in *S. cerevisiae* (Lutfiyya et al. 1998; Westholm et al. 2008). In *S. cerevisiae* these genes are repressed by glucose (Ozcan & Johnston 1999; Schüller 2003; Daran-Lapujade et al. 2004). The same pattern of regulation seems to work in *D. bruxellensis* CBS 2499 as well. Results from genes encoding putative sugar transporters showed that the expression of *HXTA* and *HXTD* was galactose-induced (Fig. 13 A). Also the expression of *DbGAL1*, *DbGAL7* and *DbGAL10* was strongly induced by galactose (Fig. 13 B). The transcriptional analysis of genes for respiratory metabolism, such as those encoding TCA enzymes, cytochrome components, glyoxylate cycle and gluconeogenesis enzymes, showed that also in this yeast glucose can repress these pathways (Fig. 13 C, D, E, F). In the genome of *D. bruxellensis* CBS 2499 we identified a sequence with a high similarity to *C. albicans AOX1*, which encode an oxidase involved in mechanisms of alternative respiration (Huh & Kang 1999). Interestingly, in the strain under study the expression of this gene was lower on galactose than on glucose (Fig. 13 C), despite the respiratory metabolism exhibited on the former sugar.

In order to understand if the respiratory galactose metabolism was due to a lack of enzymes involved in the fermentative pathway, the activity of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ACDH) and alcohol dehydrogenase (ADH) was tested. We found that PDC activity was lower on galactose than on glucose (0.25 U mg^{-1} and 0.48 U mg^{-1}, respectively). The activity of ACDH was higher on galactose than on glucose (1.2 U mg^{-1} and 0.51 U mg^{-1}, respectively), on the contrary ADH exhibited the same activity on both the carbon sources (2.9 U mg^{-1}).

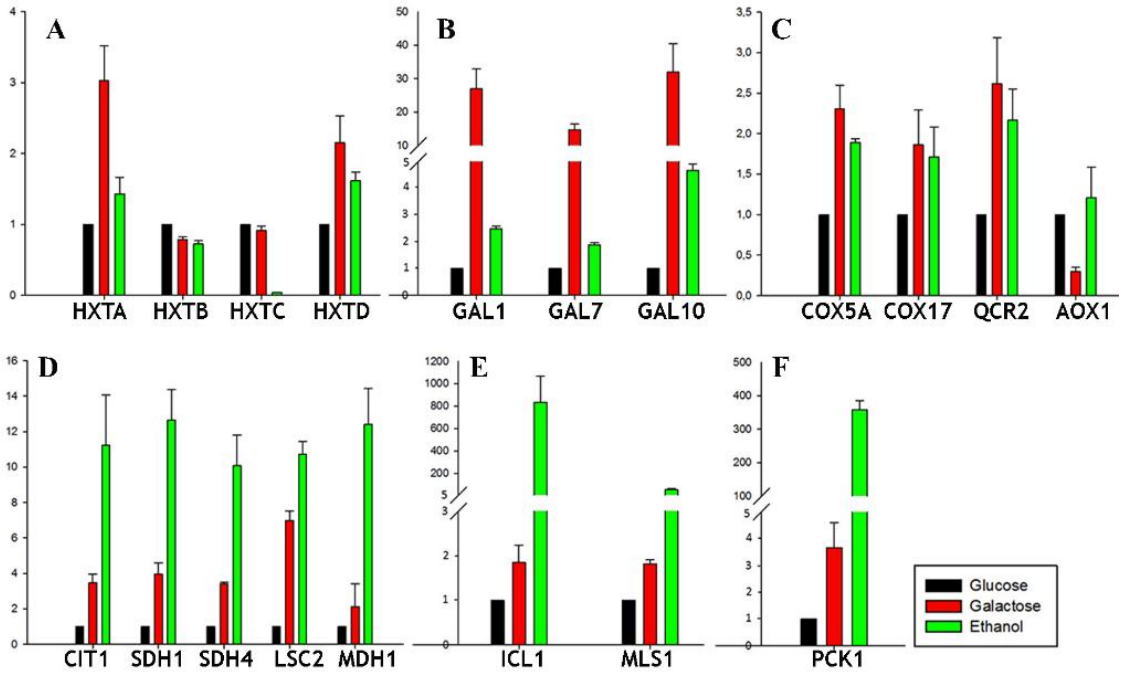

Figure 13. Expression levels of *D. bruxellensis* CBS 2499 genes involved in different metabolic pathways. The transcription level on each carbon source is relative to its expression level on glucose. A: genes involved in hexoses (glucose and galactose) transport; B: genes involved in galactose metabolism; C: genes involved in respiratory pathway; D: TCA genes; E: genes involved in glyoxylate cycle; F: gene involved in gluconeogenesis. Errors bars are the standard deviation of three replicates. The level of statistical significance was set at $p \leq 0.05$.

3.6 Screening for acetic acid resistance in *D. bruxellensis*

The response of *D. bruxellensis* species to the presence of acetic acid was investigated throughout a screening of 29 strains that were cultivated on plates of rich glucose medium (YPD) adjusted to pH 4.5 and containing acetic acid at concentrations ranging from 0 to 120 mM. The results of the screening for acetic acid resistance carried out in this study reveals that all tested *D. bruxellensis* strains were able to face a 120 mM concentration of this compound, though showing differences in adaptation times and growth rates. In particular, strain CBS 4482 showed to be the most resistant and strain CBS 98 the least resistant to acetic acid stress among 29 strains.

3.7 Glucose addition triggers the "short-term Crabtree effect"

The observation that *D. bruxellensis* CBS 2499 metabolizes galactose by a respiratory way prompted us to study in this yeast the presence of the so called "short-term Crabtree effect", the immediate production of ethanol upon the addition of glucose to a culture growing through a respiratory metabolism. This phenomenon has been reported to occur in *S. cerevisiae*, but in some other Crabtree-positive yeasts the production of ethanol is delayed (Pronk et al. 1996; Hagman et al. 2013). To analyze this effect, cells growing in exponential phase on galactose were "pulsed" with glucose, and samples were collected in order to detect if the addition of glucose triggered aerobic fermentation. Ethanol production was detected just after 15 minutes from the glucose addition, and it continued to be produced all along the glucose consumption phase (Fig. 14). Interestingly, also galactose continued to be slowly consumed for at least four hours, then stopped (Fig. 14). Acetic acid production started but delayed in respect to ethanol (Fig. 14). Glucose consumption resulted also in biomass production (Fig. 14).

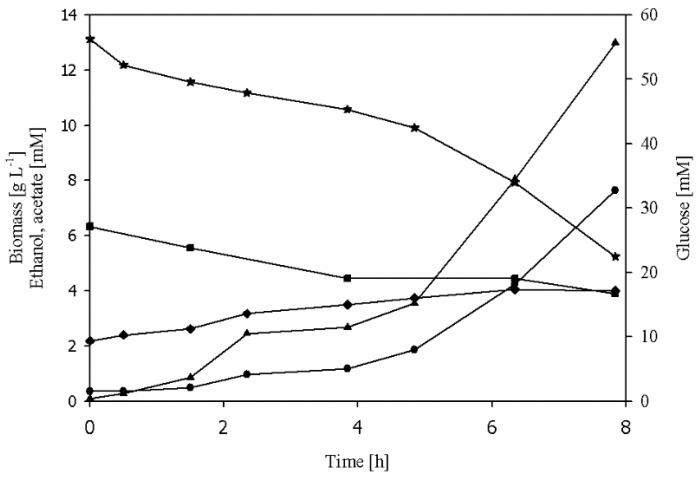

Figure 14: Kinetic of a glucose "pulse" to a galactose-based culture (one experiment is showed). Time starts after glucose addition. Symbols: *, glucose; ■, galactose; ♦, biomass; ●, acetate and ▲, ethanol.

The calculated parameters like specific consumption/production rates and yields gave interesting information about the dynamic of glucose utilization during the pulse. Glucose consumption rate was in fact high in the first 30 minutes from the pulse (3.5 mmol g^{-1} h^{-}

^{1}), then decreased, resulting in a value of 2 mmol g^{-1} h^{-1}. This means that glucose was consumed at an higher rate than galactose, as observed also in the batch cultures performed on the single sugar. During the glucose pulse the ethanol production rate and yield increased, from 0.3 mmol g^{-1} h^{-1} in the first 30 minutes to 0.8 mmol g^{-1} h^{-1}after five hours and from 0.013 to 0.12 respectively, indicating that the fermentative pathway could start early (after 15 minutes), but other factors were required to reach a higher fermentative capacity.

3.8. Ability to use acetic acid as carbon source

A more detailed analysis of acetic acid metabolism was performed on three strains: CBS 4482 and CBS 2499, which resulted among the most resistant strains, and CBS 98, one of the least resistant strains. CBS 4482 and CBS 2499 were able to grow in presence of 120 mM acetic acid and consume it. However, CBS 98 grew at acetic acid concentration not over 80 mM. The activity of acetyl CoA synthetase (ACS), the first enzyme in the acetic acid utilization pathway, was found to be similar in cell extracts obtained from CBS 4482 and CBS 2499 strains (Table 6), and even higher in CBS 98 (but in this case it should be noted that the cells were cultivated at a lower acetic acid concentration).

Table 6. Acetyl-CoA synthetase activity of *D. bruxellensis* strains cultivated under different growth conditions.

Strains	Growth conditions	Acetyl-CoA synthetase activity U/mg
CBS 4482	YP + Acetic acid (120 mM, pH 4.5)	0.056±0.004
	YPD, pH 4.5	0.019±0.003
	YPD + Acetic acid (120 mM, pH 4.5)	ND
CBS 2499	YP + Acetic acid (120 mM, pH 4.5)	0.052±0.004
	YPD, pH 4.5	0.021±0.002
	YPD + Acetic acid (120 mM, pH 4.5)	ND
CBS 98	YP + Acetic acid (80 mM, pH 4.5)	0.187±0.02
	YPD, pH 4.5	0.020±0.003
	YPD + Acetic acid (120 mM, pH 4.5)	ND

ND Not Detectable; The average of two replicates with the standard deviation are presented

3.9 The presence of acetic acid affects growth and sugar metabolism

In order to describe the influence of acetic acid on glucose metabolism in *D. bruxellensis*, the selected strains were cultivated in aerobic conditions in media at pH 4.5 containing glucose as carbon source and supplemented with acetic acid. For all the strains the maximum specific growth rates (μ max) were deeply affected, with reductions ranging from 56% to 84% in comparison to the control cultures (Table 7). *D. bruxellensis* strains were unable to use acetic acid in presence of glucose. This could be due to the fact that the activity of ACS, that was lower in cell extracts from glucose cultures in comparison to acetic acid cultures (Table 6), resulted even undetectable in cell extracts from cultures performed in glucose supplemented with acetic acid (Table 6).The presence of acetic acid resulted in an adaptation phase lasting from several days in the case of CBS 98. This prompted us to determine the number of cells that were able to grow in this condition (cultivability). A decrease of 70 % in CFU resulted after 16 hours of growth in presence of 120 mM acetic acid. This result indicated that in the long adaptation phase observed with CBS 98, only a part of population was able to proliferate. When the glucose was exhausted, *D. bruxellensis* started to consume ethanol and converted it to acetic acid; this determined a further increase of the acetic acid concentration, up to 250 mM, and a decrease of pH to 4.0, but the cells maintained a high viability (never below 60%).

Table 7. Growth parameters of *D. bruxellensis* cultured in presence of glucose and acetic acid.

Strains	Growth conditions	Growth rate μ_{max} h^{-1}	Specific consumption rate mmol/g/h	Specific production rate mmol/g/h		Yield g/g			Viability
			Glucose	Ethanol	Acetic acid	Biomass	Ethanol	Acetic acid	
CBS 4482	YPD, pH 4.5 (control)	0.18±0.01	3.8±0.03	3.25±0.25	1.47±0.11	0.27±0.04	0.24±0.01	0.15±0.02	99.9%
	YPD + Acetic acid (120 mM, pH 4.5)	0.072±0.004	1.62±0.09	1.32±0.08	0.94±0.06	0.24±0.01	0.23±0.04	0.20±0.02	93%
CBS 2499	YPD, pH 4.5 (control)	0.197±0.005	4.6±0.11	6.36±0.16	1.15±0.03	0.24±0.01	0.36±0.01	0.08±0.01	99.9%
	YPD + Acetic acid (120 mM, pH 4.5)	0.06±0.003	1.76±0.08	1.40±0.07	0.96±0.05	0.17±0.03	0.22±0.05	0.20±0.06	86%
CBS 98	YPD, pH 4,5 (control)	0.127±0.001	1.9±0.02	2.51±0.03	0.76±0.01	0.24±0.01	0.29±0.07	0.14±0.07	99.9%
	YPD + Acetic acid (120 mM, pH 4.5)	0.019±0.004	0.81±0.14	1.14±0.21	0.27±0.05	0.13±0.01	0.33±0.04	0.15±0.03	73%
	YPD + Acetic acid (80 mM, pH 4.5)	0.044±0.002	1.38±0.06	1.60±0.08	0.61±0.03	0.18±0.02	0.30±0.01	0.15±0.01	84%

The average of two replicates with the standard deviation are presented

3.10 Metabolomic analysis of *D. bruxellensis* cultures grown in presence of acetic acid

The effect of acetic acid on *D, bruxellensis* strains during the cell growth was analyzed by comparing the FTIR profiles of cells cultivated on glucose-based media. The Response Spectra (RS) obtained for the three cultures differed in the amides (W2), carbohydrates (W4) and mixed region (W3) (Fig 15), whereas negligible effects could be observed in the fatty acids (W1) and in the typing (W5) regions. The cellular components mainly involved in this response were carbohydrates and amides. The carbohydrates present in the cell wall could represent one of the acetic acid targets, as supported by recent observations in *S. cerevisiae* and in *Z. bailii* (Mira et al. 2010; Guerreiro et al. 2012). Amides are linked to the AMP/ATP ratio, which is affected by the energy expense required to extrude protons out of the cell.

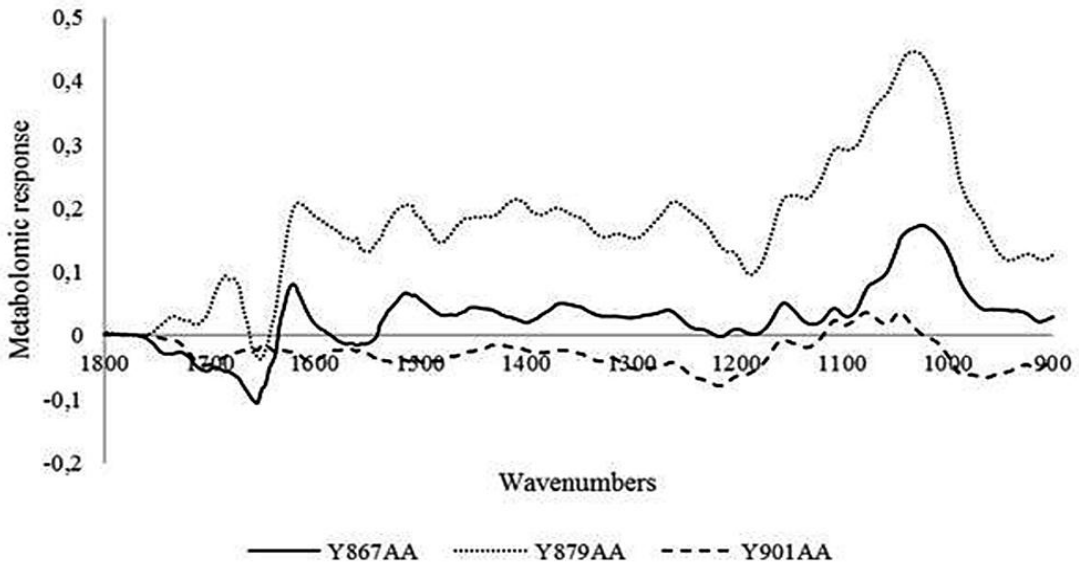

Figure 15. Response spectra (RS) of the three *D. bruxellensis* strains grown in YPD and in presence of acetic acid 120 mM at pH 4.5. Solid line represents the strain CBS 98, dotted line the strain CBS 2499 and dashed line the strain CBS 4482.

3.11 DbHog1p is not phosphorylated upon exposure to acetic acid

To explore whether the presence of acetic acid induced in *D. bruxellensis* the dual phosphorylation of Hog1p, as observed in *S. cerevisiae* (Mollapour & Piper 2006), CBS 2499 cells were collected after exposure to the presence of acetic acid 120 mM. Proteins were examined by Western blot and phosphorylation of Hog1p was tested by using a commercial anti-phospho-p38 antibody, which specifically recognizes the dually phosphorylated form of Hog1p. As reported in (Fig. 16), the incubation of cells in presence of 120 mM acetic acid did not result in any band corresponding to the phosphorylated form of Hog1p, indicating that this regulatory mechanism is not operating as consequence of exposure to acid stress. Therefore, the exposure to acetic acid did not cause in *D. bruxellensis* the activation of the HOG MAP kinase pathway, that on the contrary is involved in the osmotic stress response (Galafassi, Toscano, et al. 2013). Although the HOG pathway appears to be conserved even among distantly related species, the pathway may in each species also be adapted to the specific niche requirements. For example, among pathogenic yeasts the HOG pathway also plays a role in virulence (Rispail et al. 2009).

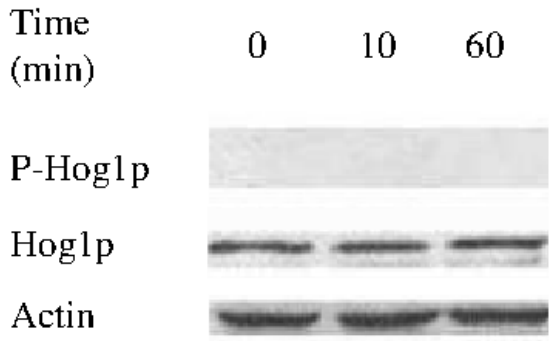

Figure 16 Western blot showing the involvement of HOG1 during the exposure to acetic acid 120 mM in *D. bruxellensis* CBS 2499.

4. Conclusions and future perspectives

The results presented in this thesis show that:

- In *D. bruxellensis*, utilization of nitrate determines a different pattern of fermentation products. Acetic acid, instead of ethanol, became in fact the main product of glucose metabolism under aerobic conditions.
- Under anaerobic conditions, nitrate assimilation abolishes the ‘‘Custers effect’’, in this way improving its growth and fermentative metabolism.
- In *D. bruxellensis*, galactose is a non-fermentable carbon source, in contrast to *S. cerevisiae* that can ferment it.
- The expression of genes involved in galactose utilization and in respiratory metabolism is repressed by glucose, similarly to what occurs in *S. cerevisiae*.
- The galactose metabolism in *D. bruxellensis* is affected by the nature of the nitrogen source. When the cells used nitrate as nitrogen source galactose was metabolized leading to the production of biomass and acetic acid. The galactose consumption rate increased in the presence of nitrate.
- *D. bruxellensis* behaves, from a metabolic point of view, more similarly to *S. cerevisiae*, being unable to metabolize acetic acid in presence of glucose.
- At metabolomics level, the effect of acetic acid mainly related to some cellular components, like carbohydrates and amides.
- The presence of acetic acid affected the growth, causing a reduction of growth rate, glucose consumption rate, ethanol production rate as well as biomass and ethanol yield.

Research interest in the yeast *D. bruxellensis* has been growing due to its role as both a contaminant and a production yeast. This interest is driven by several factors. One of the interesting factor is that the assimilation of nitrate in anaerobic conditions which abolishes the “Custers effect”. This can offer a new strategy besides aeration, to sustain growth and ethanol production for the employment of this yeast in industrial processes. In general, it may also be regarded as a model for a highly competitive yeast, which can help in understanding microbial interactions in industrial ethanol processes. Understanding the molecular determinants underlying such competitiveness would provide opportunities to

improve its performance in ethanol production, but possibly also to prevent contamination by this yeast. Identifying the factors involved in *D. bruxellensis* stress mechanisms in response to acetic acid would help to construct new strain with improved performance. This knowledge would also help to design new strategies aiming the prevention of food and beverage spoilage by resistant yeasts.

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Part II

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Galactose utilization sheds new light on sugar metabolism in the sequenced strain *Dekkera bruxellensis* CBS 2499.

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Abstract

Dekkera bruxellensis and *Saccharomyces cerevisiae* are considered two phylogenetically distant relatives, but they share several industrial relevant traits such as the ability to produce ethanol under aerobic conditions (Crabtree effect), high tolerance towards ethanol and acids, and ability to grow without oxygen. Beside a huge adaptability, *D. bruxellensis* exhibits a broader spectrum in utilization of carbon and nitrogen sources in comparison to *S. cerevisiae*. Whit the aim to better characterize its carbon source metabolism and regulation, the usage of galactose and the role that glucose plays on sugar metabolism was investigated in *D. bruxellensis* CBS 2499. The results indicate that in this yeast galactose is a non-fermentable carbon source, in contrast to *S. cerevisiae* that can ferment it. In particular, its metabolism is affected by the nitrogen source. Interestingly, *D. bruxellensis* CBS 2499 exhibits also the "short-term Crabtree effect", and the expression of genes involved in galactose utilization and in respiratory metabolism is repressed by glucose, similarly to what occurs in *S. cerevisiae*.

Introduction

Sugar metabolism provides an essential source of energy and metabolites for most organisms. To develop industrial strategies and processes based on cell as a factory the understanding of the metabolic pathways and their regulation is mandatory. Although glucose is the preferred sugar by microorganisms and the most abundant component of natural polysaccharides, the use of other sugars is becoming more and more attractive at industrial level to obtain cost efficient bioprocesses and to avoid interfering with the use of food crops for the production of chemicals. Nowadays, research efforts are in fact focused both to obtain monosaccharides from alternative sources than food and to develop microorganisms able to use all of them, hexoses and pentoses as well. In this regard, *Saccharomyces cerevisiae*, which is one of the most frequently used cell factory in industrial biotechnology, prefers glucose as carbon and energy source and is unable to use pentoses. In recent years, yeast strains capable of overcoming this limitation have been developed by metabolic modifications (Van Vleet & Jeffries, 2009). Together with

glucose, galactose is a component of hemicellulose and it is the major sugar in the red seaweed biomass, representing an attractive industrial carbon source (Packer, 2009). It utilization has been extensively studied in *S. cerevisiae* where it occurs through the Leloir pathway. In particular, galactose is phosphorylated to galactose- 1-phosphate by galactokinase (Gal1p) and then isomerized to glucose-1-phosphate by galactose-1-phosphate uridyltransferase (Gal7p). Phosphoglucomutase (Gal5p) converts glucose-1-phosphate into glucose-6-phosphate which can be shunted to glycolysis and pentose phosphate pathway (Bhat & Murthy, 2001). Gal4p and Gal80p are the main transcriptional regulators of GAL genes that are repressed in presence of glucose by the Mig1-Tup1-Ssn6 complex (Nehlin *et al.*, 1991; Traven *et al.*, 2006). *S. cerevisiae* consumes galactose much more slowly compared to glucose and its metabolism is respire-fermentative, ethanol being one of the final products (Sierkstra *et al.*, 1993; Ostergaard *et al.*, 2000).

Another approach to extend the range of carbon sources that can be used at industrial level for biotech processes could be the exploration of the huge yeast biodiversity, in order to find out new species which already possess a wider range of carbon source utilization. The *Brettanomyces/Dekkera* complex is composed of five species, *Dekkera bruxellensis, D. anomala, Brettanomyces naardenensis, B. nanus* and *B. custersianus*. *D. bruxellensis* can be isolated from extreme environments like wine and beer (Fugelsang, 1997; Loureiro, 2003; Vanbeneden *et al.*, 2008), but its presence has been reported also in the continuous fermentation systems for bioethanol production, due to its ability to grow under anaerobic conditions and at high ethanol concentrations (de Souza Liberal *et al.*, 2007; Passoth *et al.*, 2007). Moreover, several *Brettanomyces/Dekkera* strains have been reported to be able to utilize pentose like xylose and arabinose (Toivola *et al.*, 1984; Galafassi *et al.*, 2011), possibly resulting from the high genetic variability observed in the species (Vigentini et al. 2012). Although *D. bruxellensis* and *S. cerevisiae* are considered two phylogenetically distant relatives they share several peculiar traits, such as the ability to produce ethanol under aerobic conditions (Crabtree effect), high tolerance towards ethanol and acids, and ability to grow without oxygen (van Dijken & Scheffers, 1986; Rozpędowska *et al.*, 2011; Blomqvist *et al.*, 2012). Apparently, these traits have evolved in parallel in both groups, but the molecular mechanisms involved could be very different (Rozpędowska *et al.*, 2011). Recently, it has been shown that *D. bruxellensis* can use also

nitrate as nitrogen source, and this characteristic can render *D. bruxellensis* able to overcome *S. cerevisiae* populations, which is unable to use it, in industrial fermentations (de Barros Pita *et al.,* 2011; Galafassi *et al.,* 2013). All these metabolic features have been leading to the idea that *D. bruxellensis* could become a new industrial producing organism (Blomqvist *et al.,* 2010; Galafassi *et al.,* 2011). The present work aimed to investigate how *D. bruxellensis* CBS 2499 can use galactose and the role that glucose plays on carbon source utilization. We show here that under controlled aerobic conditions galactose is a not-fermentable carbon source. Depending on the available nitrogen source (ammonium or nitrate), the pattern of final products obtained from galactose can change. We also show that the addition of glucose to cells growing through a respiratory galactose metabolism triggers the so called "short-term Crabtree effect". The expression of genes involved in different metabolic pathways linked to respiratory metabolism was also investigated.

Materials and Methods.

Yeast strains

The yeast used in this work is the sequenced strain of *D. bruxellensis* CBS 2499. Stocks of the strain were stored at -80 °C in 15% v/v glycerol.

Media and growth conditions

Shake flask cultures in the synthetic medium reported in Merico *et al.* 2007 incubated at 30 °C in a rotary shaker at 200 rpm were used to test the growth with specific carbon sources (glucose 20 g L^{-1} or galactose 20 g L^{-1} or ethanol 2% v/v). Aerobic batch cultivations were performed in a Biostat-Q system bioreactor (B-Braun) with a working volume of 0.8 L. The temperature was set at 30 °C, the stirring speed at 500 rpm, and the pH, measured by a Mettler Toledo pH electrode, was adjusted to 5.0 by automatic addition of 5M KOH. The fermenters were continuously sparged with air, 1 L min^{-1}, and the dissolved oxygen concentration (always higher than 30% of air saturation) was measured by a Mettler Toledo polarographic oxygen probe. For batch cultivations the synthetic medium was that reported above. Carbon sources were, as specified, glucose 20 g L^{-1}, galactose 20 g L^{-1} or ethanol 2% v/v. Nitrogen sources were ammonium sulfate 5 g L^{-1} or

sodium nitrate 6.43 g L^{-1}, as specified. All the experiments were performed in duplicate. The growth was monitored by OD_{600nm} measurement.

Glucose "pulse" experiments

Glucose "pulse" experiments were performed in duplicate using batch cultures of cells growing on synthetic medium reported in Merico *et al.* 2007, and containing ammonium sulfate 5 g L^{-1} as nitrogen source. To obtain a population of cells growing in exponential phase, with a respiratory galactose metabolism and in presence of a lower concentration of residual galactose at the moment of the glucose addition, a concentration of 5 g L^{-1} galactose (instead of 20 g L^{-1}) was used for the pulse trials. When cells reached the exponential growth phase (approx. 6 OD_{600nm}), glucose was added to give a final concentration of 10 g L^{-1}.

Dry weight and metabolites assays

Samples collected at several points during the cultivation were submitted to the dry weight determination after removal of the medium by filtration (0.45 µm glass microfiber GF/A filter; Whatman). The filters were washed with three volumes of de-ionized water and dried overnight at 105 °C. Supernatants were used for glucose, acetic acid, ethanol and nitrate quantification using commercial enzymatic kits (Roche, cat. numb. 1 0716251 035, 1 0148261 035, 1 0176290 035 and 1 0905658 035). All the assays were performed in triplicate.

Enzymatic assays

Cell extracts for enzymatic assays were obtained by extraction with acid-washed glass beads (SIGMA) as described previously (Postma *et al.*, 1989), and total protein concentrations were determined by Bio-Rad kit no. 500-002 (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard. The specific activities of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and alcohol dehydrogenase (ADH) in cells extract were determined at room temperature in a spectrophotometer at 340 nm as previously described (Postma *et al.,* 1989).

RNA extraction and cDNA synthesis.

Pellets for RNA extraction were collected when cultures reached the exponential phase, which ensured that the gene expression analyses were performed from cells in the same physiological state. Harvested cells were centrifuged and pellets were immediately frozen with liquid nitrogen prior to store them at -80 °C until RNA extraction. RNA extraction was performed according to Presto™ Mini RNA Yeast Kit manufacturer's protocol with few changes. Pellets were disrupted with acid washed glass beads (500 µl RB Buffer, 5 µL β-mercaptoethanol and equal volume of glass beads) in a mini-bead beater (Precellys™ 24 homogenizer) instead of using zymolyase enzymatic breakdown. The setting chosen for cell disruption was a run of three repetitions lasting 30 s at high speed. The RNA was quantified by means of a spectrophotometric method, and its integrity was evaluated by running an electrophoresis agarose gel in denaturing conditions. RNA was stored at -80 °C until cDNA synthesis. The cDNA was synthesized with QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. An amount of 1 µg total RNA was used for each reverse transcription reaction. Synthesized cDNA was stored at -20 °C until RT-qPCR assays.

Primers design

The sequences of target genes were obtained from the *D. bruxellensis* CBS 2499 database (DOE Joint Genome institute database, JGI; http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html) after BLASTx analysis using the corresponding potential orthologous sequences in *S. cerevisiae* genome retrieved from the SGD database (http://www.yeastgenome.org/), except for *AOX1* orthologous sequence that was obtained from the *Candida* genome database (http://www.candidagenome.org/). A list of the primers is reported in Table S1.

RT-qPCR analysis

RT-qPCR analyses were performed using SsoFast™ EvaGreen® Supermix (BIO-RAD). 96-well plates were used in a BioRad C1000™ Thermal Cycler machine, and each amplification reaction was composed of 7.5 µl of EvaGreen master mix, 1 µL of each primer (333 nmol L^{-1}, final), 2 µL of cDNA (5 times diluted cDNA synthesized from RNA), and 3.5 µL of MQ water. Cycling parameters were 98 °C for 30 s as hot-start,

followed by 39 cycles of 95 °C for 3 s and 60 °C for 5 s, and melting curve was included at end of each run. Negative PCR control (for unspecific amplification) and negative RT control (for genomic DNA contamination) were run in parallel as internal control. Standard curves were created for each couple of primers by plotting CT (threshold cycle) values of the real-time PCR performed on dilution series of cDNA. From the standard curve, the amplification efficiency (E) was estimated by BioRadCFXManager software (BIO-RAD). The reaction specificity was evaluated by analyzing the melting curve profile. The α-tubulin gene was used as endogenous reference, previously used by Rozpędowska (Rozpędowska *et al.,* 2011), and cDNA samples from cells grown on glucose or ethanol were considered as the reference condition. For each growth condition, a total of 3 independent cDNA samples were prepared from two biological replicates. Each cDNA sample was run in technical triplicates during RT-qPCR assays. Statistically significant differences of each gene expression among three growth conditions were analyzed by ANOVA test. The level of statistical significance was set at $p \leq 0.05$.

Promoter motif presence

The 1000 nt sequence upstream the translation start site of each gene was considered as the promoter sequence of the gene. A home-made python script was used to search the motif sequences presents in the promoters.

Phylogenetic analysis

Protein sequences of homologous genes among different species were aligned by MUSCLE Version 3.8.31. Trimal was used to trim the sequence alignment with the following parameter: -cons 50 –gt 0.5. PhyML was used to build the phylogenetic tree of each group of genes.

Results

Galactose metabolism is affected by nitrogen source

Preliminary cultivation on shake-flasks indicated the ability of several *D. bruxellensis* strains to grow on galactose-based media (data not shown). In the aim to study galactose metabolism in detail, aerobic cultures of the sequenced strain *D. bruxellensis* CBS 2499

(Piškur *et al.*, 2012) were performed in batch at controlled concentration of dissolved oxygen. Moreover, due to the fact that *D. bruxellensis* is able to use nitrate as nitrogen source (Galafassi *et al.*, 2013), we tested if the galactose metabolism can be affected by the nitrogen source. When *D. bruxellensis* was cultivated on media containing ammonium salts, galactose metabolism was respiratory, as indicated by the biomass level and by the failed production of metabolites normally resulting from fermentative metabolisms, such as ethanol or acetic acid (Fig. 1 A, Table 1). The growth kinetic showed a long lag phase when the pre-inoculum was performed on glucose-containing media (not shown), that was not present when the pre-inoculum grew on galactose-containing media (Fig. 1 A). This fact could indicate that in *D. bruxellensis* CBS 2499 the genes for galactose metabolism are repressed by glucose (see below). The growth rate was lower than on glucose (Table 1), and galactose was utilized at a lower specific rate (Table 1). On the other hand the biomass yield was higher, as expected due to the respiratory metabolism exhibited in this condition, in comparison with the respire-fermentative one occurring on glucose-based media (Table 1).

Interestingly, when the medium contained nitrate as sole nitrogen source, the final products of galactose metabolism changed: together with biomass also acetic acid and a very low amount of ethanol were produced (Fig. 1 B, Table 1). As a consequence, a lower biomass yield than on ammonium-based media was obtained (Table 1). The rate at which galactose was consumed on nitrate-based media was higher than on the ammonium-based ones (Table 1), nevertheless the growth rates were very similar. Notably, acetic acid and ethanol were produced at lower rates and yields than on glucose. In conclusion, these results indicated that when cells use ammonium as nitrogen source the galactose metabolism is respiratory. Nevertheless, when nitrate is the nitrogen source a partial redirection of pyruvate occurred: some pyruvate was in fact converted by pyruvate decarboxylase to acetaldehyde, which was in turn converted to acetic acid and, at a low level, to ethanol.

Gene expression and enzyme activities for galactose and respiratory metabolism

The availability of the complete genome sequence of the *D. bruxellensis* CBS 2499 strain (Piškur *et al.*, 2012) allowed the identification of genes encoding all the enzymes that in

S. cerevisiae are required for galactose assimilation (Table 2). Interestingly, *DbGAL7*, *DbGAL10* and *DbGAL1* are clustered, as reported in other *Ascomycota* (Martchenko *et al.*, 2007). A phylogenetic tree obtained using sequences identified by similarity with *S. cerevisiae* HXT and *C. albicans* HGT protein (Fig. 2) showed that the majority of *D. bruxellensis* putative sugar transporters map within the *C. albicans* group, but four of them are in the group containing also *S. cerevisiae* HXT. Two sequences, HXTA and HXTD, showed a high similarity to *Sc*Gal2p (53 – 56%). In *S. cerevisiae* galactose is sensed in the cytoplasm via Gal3p (Johnston, 1987), but no *GAL3* orthologue was found in *D. bruxellensis* CBS 2499 genome, as reported also in *C. albicans* (Martchenko *et al.*, 2007). On the other hand, in the analyzed genome, a gene encoding a putative protein which showed a 54% identity with CaHgt4p (Table S2) was found; this gene has been demonstrated to sense both glucose and galactose in *C. albicans* (Brown *et al.*, 2009).

The expression of genes involved in galactose and respiratory metabolism was analyzed, in order to understand the role that the carbon source plays on their transcriptional regulation. In *S. cerevisiae* these genes are repressed by glucose (Johnston, 1999; Schüller, 2003; Daran-Lapujade *et al.*, 2004). The same pattern of regulation seems to work in *D. bruxellensis* CBS 2499 as well. Results from genes encoding putative sugar transporters showed that the expression of *HXTA* and *HXTD* was galactose-induced (Fig. 3 A). Also the expression of *DbGAL1*, *DbGAL7* and *DbGAL10* was strongly induced by galactose (Fig. 3 B). These latter genes were expressed even on ethanol and, by comparison, repressed by glucose (Fig. S1). The transcriptional analysis of genes for respiratory metabolism, such as those encoding TCA enzymes, cytochrome components, glyoxylate cycle and gluconeogenesis enzymes, showed that also in this yeast glucose can repress these pathways (Fig. 3 C, D, E, F). In the genome of *D. bruxellensis* CBS 2499 we identified a sequence with a high similarity to *C. albicans AOX1,* which encode an oxidase involved in mechanisms of alternative respiration (Huh & Kang, 1999). Interestingly, in the strain under study the expression of this gene was lower on galactose than on glucose (Fig. 3 C), despite the respiratory metabolism exhibited on the former sugar.

In order to understand if the respiratory galactose metabolism was due to a lack of enzymes involved in the fermentative pathway, the activity of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ACDH) and alcohol dehydrogenase (ADH) was

tested. We found that PDC activity was lower on galactose than on glucose (0.25 U mg^{-1} and 0.48 U mg^{-1}, respectively). The activity of ACDH was higher on galactose than on glucose (1.2 U mg^{-1} and 0.51 U mg^{-1}, respectively), on the contrary ADH exhibited the same activity on both the carbon sources (2.9 U mg^{-1}).

Glucose addition triggers the "short-term Crabtree effect"

The observation that *D. bruxellensis* CBS 2499 metabolizes galactose by a respiratory way prompted us to study in this yeast the presence of the so called "short-term Crabtree effect", the immediate production of ethanol upon the addition of glucose to a culture growing through a respiratory metabolism. This phenomenon has been reported to occur in *S. cerevisiae,* but in some other Crabtree-positive yeasts the production of ethanol is delayed (Pronk *et al.*, 1996; Møller *et al*., 2002; Hagman *et al.*, 2013). To analyze this effect, cells growing in exponential phase on galactose were "pulsed" with glucose, and samples were collected in order to detect if the addition of glucose triggered aerobic fermentation. Ethanol production was detected just after 15 minutes from the glucose addition, and it continued to be produced all along the glucose consumption phase (Fig. 4). Interestingly, also galactose continued to be slowly consumed for at least four hours, then stopped (Fig. 4). Acetic acid production started but delayed in respect to ethanol (Fig. 4). Glucose consumption resulted also in biomass production (Fig. 4). The calculated parameters like specific consumption/production rates and yields gave interesting information about the dynamic of glucose utilization during the pulse. Glucose consumption rate was in fact high in the first 30 minutes from the pulse (3.5 mmol g^{-1} h^{-1}), then decreased, resulting in a value of 2 mmol g^{-1} h^{-1}. This means that glucose was consumed at an higher rate than galactose, as observed also in the batch cultures performed on the single sugar (see Table 1). During the glucose pulse the ethanol production rate and yield increased, from 0.3 mmol g^{-1} h^{-1} in the first 30 minutes to 0.8 mmol g^{-1} h^{-1} after five hours and from 0.013 to 0.12 respectively, indicating that the fermentative pathway could start early (after 15 minutes), but other factors were required to reach a higher fermentative capacity.

Discussion

Recent studies have highlighted that *D. bruxellensis* shares with *S. cerevisiae* the ability to survive in challenging environments such as the industrial bioethanol production processes. Beside the adaptability to conditions of low pH and high ethanol concentration, *D. bruxellensis* exhibits a broader spectrum of consumable carbon and nitrogen sources in comparison to *S. cerevisiae*, having cellobiose and lactose as well as nitrate-assimilating capacity (Conterno *et al.*, 2006*;* Galafassi *et al.*, 2013), and this can contribute also to its ecological distribution. Here we showed that in *D. bruxellensis* CBS 2499 galactose is a not-fermentable carbon source (Table 1). This is in contrast to what occurs in *S. cerevisiae*, which produces ethanol even from galactose (Sierkstra *et al.*, 1993; Ostergaard *et al.*, 2000; Martinez *et al.*, 2014). Another interesting aspect of the galactose metabolism in *D. bruxellensis* CBS 2499 is that it is affected by the nature of the nitrogen source. When the cells used nitrate as nitrogen source galactose was metabolized leading to the production of biomass and acetic acid. The enzymes involved in nitrate assimilation, nitrate and nitrite reductases, in *D. bruxellensis* have been shown to use, *in vitro*, NADH and NADPH as electron donors (Galafassi *et al.*, 2013), and an increased production of acetic acid has been observed to occur on glucose-based media when nitrate is the sole nitrogen source (Galafassi *et al.*, 2013). This means that acetic acid synthesis can generate the NADPH necessary for nitrate assimilation, on glucose as well as on galactose. The presence of nitrate determined an increase in the galactose consumption rate (Table 1), that could be due to the higher energetic cost for the utilization of this nitrogen source compared to the ammonium sulphate (Siverio, 2002). We suppose that the increased galactose consumption rate can also "push" pyruvate toward acetaldehyde and then to acetic acid and ethanol (this last produced in a very low amount).

The scenario that comes out from these new observations is that when the strain of *D. bruxellensis* under study is cultivated on galactose-based media it behaves like a Crabtree-negative yeast, not producing ethanol, in contrast to *S. cerevisiae*, that behaves like a Crabtree-positive yeast both on glucose and on galactose. This could be related to the galactose consumption rate: in *D. bruxellensis* we observed in fact that galactose was consumed at a slower rate than glucose (Table 1). We think that this factor could

determine an insufficient pyruvate overflow, which is necessary to trigger aerobic ethanol production, as occurs in *S. cerevisiae* (Ostergaard *et al.*, 2000). The ability to produce ethanol aerobically can start when the glycolytic flow increase. The addition of glucose to a galactose-based culture determined a faster sugar consumption (glucose consumption rate 3.5 mmol g^{-1} h^{-1}) and a quick production of ethanol (Fig. 4), causing a shift in the metabolism from respiratory toward aerobic fermentative, the so-called "short-term Crabtree effect". The occurrence of the Crabtree effect has been already demonstrated in *D. bruxellensis* in batch cultures (van Dijken & Scheffers, 1986; Rozpędowska *et al.*, 2011), as well as by glucose pulse to glucose-limited continuous cultures growing at low rate with a respiratory metabolism (Leite *et al.*, 2013). In this study we show that the "short-term Crabtree effect" resulted from a high glycolytic flow occurring when the cells use glucose, which can in turn determine a pyruvate overflow. This is known to be one of the main causes that can activate the "short-term Crabtree effect" in *S. cerevisiae* (Postma *et al.*, 1989; Daran-Lapujade *et al.*, 2004; Huberts *et al.*, 2012). Furthermore, it has been demonstrated that by increasing the galactose consumption rate the ethanol production rate increases (Ostergaard *et al.*, 2000).

Glucose repression is the regulatory mechanism that has been shown to determine the "long-term Crabtree effect" in *S. cerevisiae* (Pronk *et al.*, 1996). The transcription of genes essential for respiratory metabolism and mitochondrial functions, as well as for the utilization of other carbon sources, is repressed when *S. cerevisiae* is cultivated on rapidly fermentable sugars like glucose, fructose and mannose (Verstrepen *et al.*, 2004). In particular, we focused our attention on the expression of genes containing in their promoters putative consensus sequences related to the transcription factor Mig1p (Table S3), which is known to be one of the key elements in glucose repression in *S. cerevisiae* (Lutfiyya *et al.*, 1998; Klein *et al.*, 1998; Westholm *et al.*, 2008). In the genome of *D. bruxellensis* CBS 2499 two sequences that show 39-49% similarity with *S. cerevisiae MIG1* (notice that in *S. cerevisiae* genome the presence of three genes, *MIG1*, *MIG2* and *MIG3*, has been reported, Westholm *et al.*, 2008) were identified. In *C. albicans* CaMig1p has been shown to play a role in the regulation of carbon source utilization and energy production (Murad *et al.*, 2001). Our results indicated that genes involved in galactose utilization, TCA, cytochromes structure, glyoxylate cycle, and gluconeogenesis are repressed by glucose (Fig. 3). On the other hand, a sequence encoding a protein with

a 44% identity to *Ca*Cph1p, which in *C. albicans* acts as a transcriptional factor required for the regulation of galactose metabolism (Martchenko *et al.,* 2007; Brown *et al.,* 2009), was present also in *D. bruxellensis* CBS 2499 genome (Table S2). Moreover, putative regulatory motifs recognized by this transcriptional factor were found in all the promoters of the galactose-related genes in the analyzed strain. (Table S2). Although the structural genes for galactose metabolism in *D. bruxellensis* CBS 2499 seems well conserved by evolution, the regulatory components appear to be also related to other species like *C. albicans* (Ihmels *et al.,* 2005). The molecular mechanisms operating in *D. bruxellensis* are still far to be elucidated, and it will be interesting in next future to demonstrate the specific role of different transcriptional factors.

In conclusion, the present work demonstrates that several metabolic implications are linked to the galactose metabolism in *D. bruxellensis*. Contrarily to *S. cerevisiae*, galactose is respired by cells. However, as far the glucose metabolism, this work suggests that a regulation operated by this sugar occurs. In particular, by proving the "short-term Crabtree effect" in *D. bruxellensis*, here is shown that this species behaves like *S. cerevisiae*, since to obtain aerobic ethanol production from sugars a glycolytic "threshold flow" needs to be reached.

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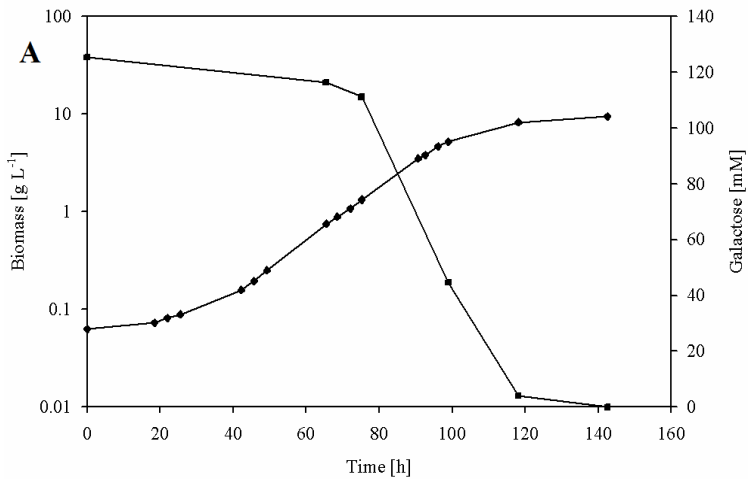
Figure 1: Growth kinetics of cultures on galactose-based media (one example of kinetic is showed) containing ammonium sulphate (A) and sodium nitrate (B) as nitrogen source. Symbols: ■, galactose; ♦, biomass; ●, acetate; ▲, ethanol.



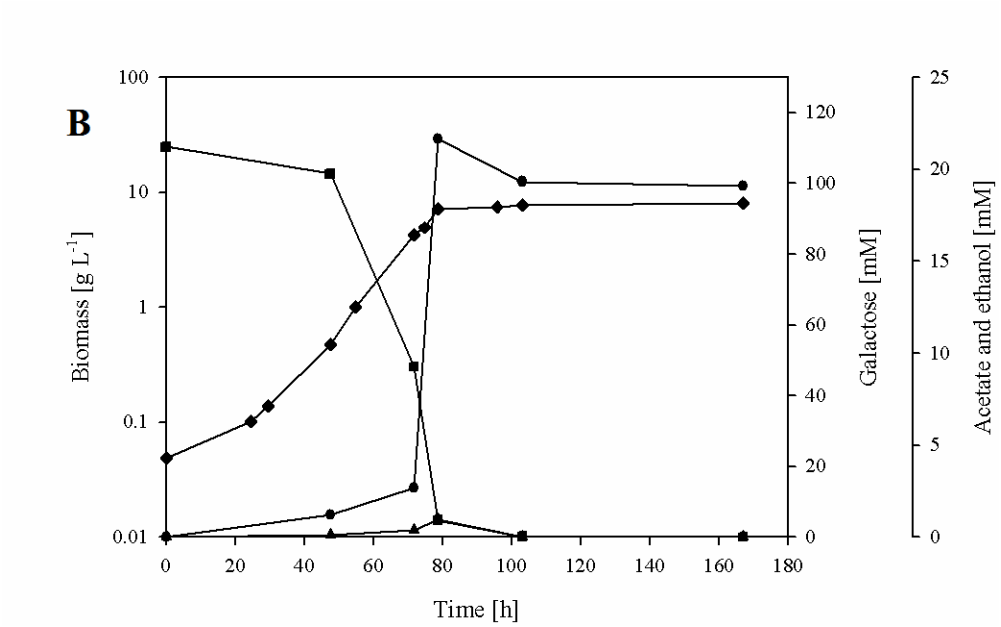


Figure 2: A phylogenetic tree of the *S. cerevisiae* HXTp and the HGTp homologs of *C. albicans* and *D. bruxellensis*. The *HXT* homologs in *D. bruxellensis* are indicated by red box. Note that some *C. albicans* transporters map within the *HXT* group and that a majority of *D. bruxellensis* transporters map within the *C.albicans* HGT group.

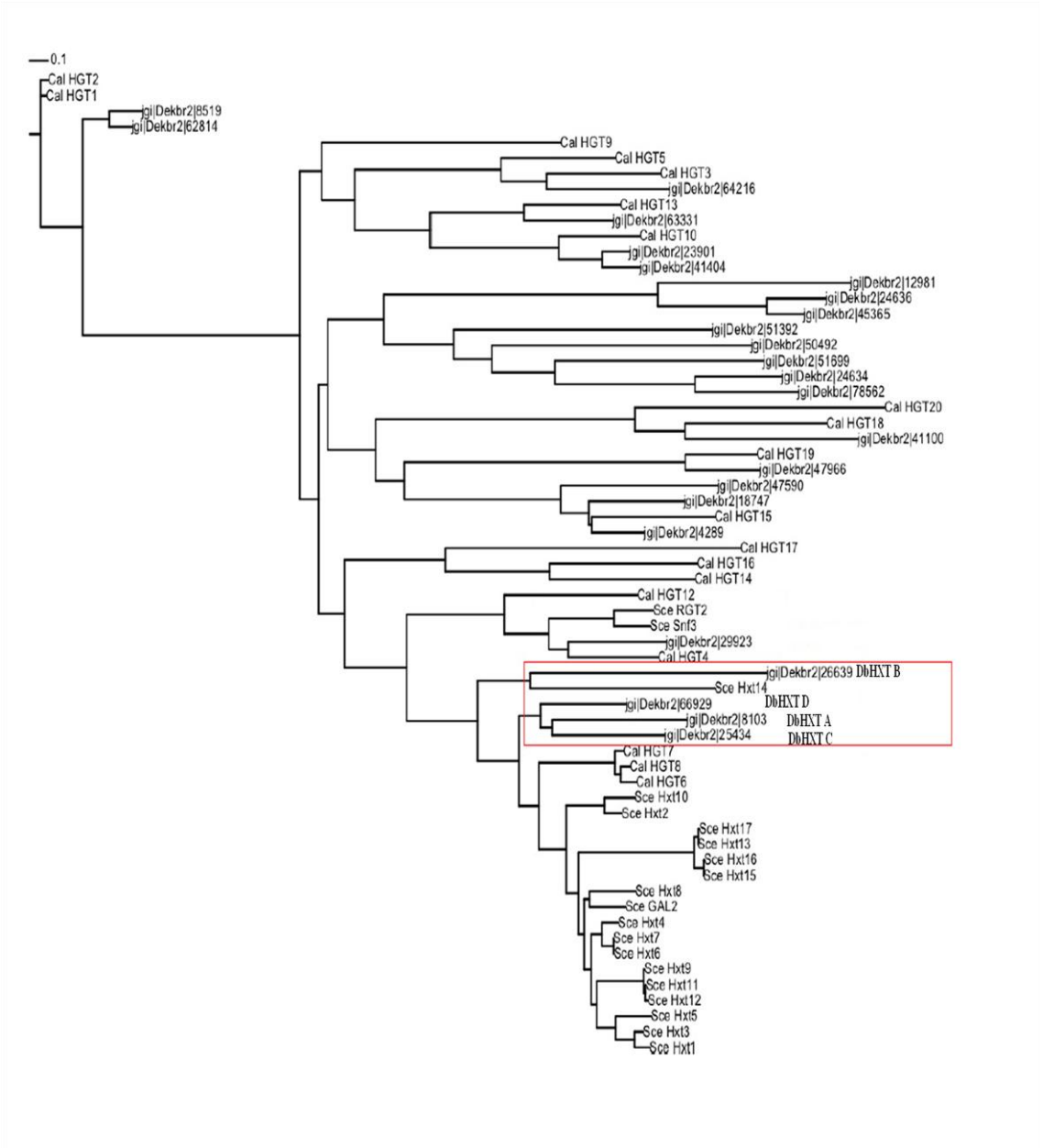


Figure 3: Expression levels of *D. bruxellensis* CBS 2499 genes involved in different metabolic pathways. The transcription level on each carbon source is relative to its expression level on glucose. A: genes involved in hexoses (glucose and galactose) transport; B: genes involved in galactose metabolism; C: genes involved in respiratory pathway; D: TCA genes; E: genes involved in glyoxylate cycle; F: gene involved in gluconeogenesis. Errors bars are the standard deviation of three replicates. The level of statistical significance was set at $p \leq 0.05$.

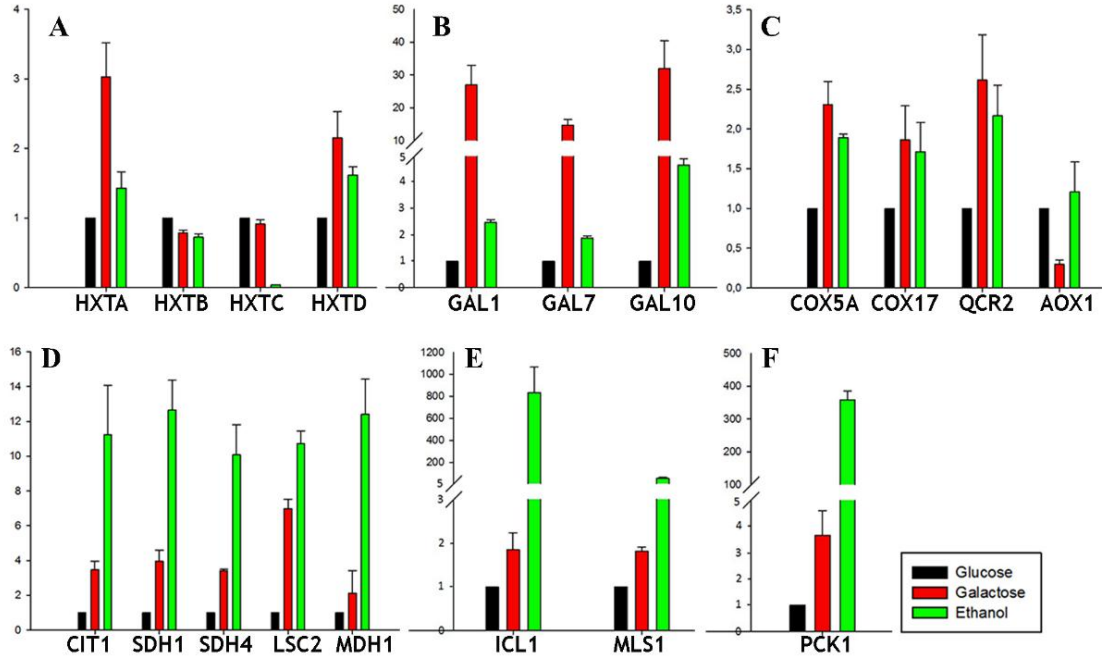


Figure 4: Kinetic of a glucose "pulse" to a galactose-based culture (one experiment is showed). Time starts after glucose addition. Symbols: *, glucose; ■, galactose; ♦, biomass; ●, acetate and ▲, ethanol.

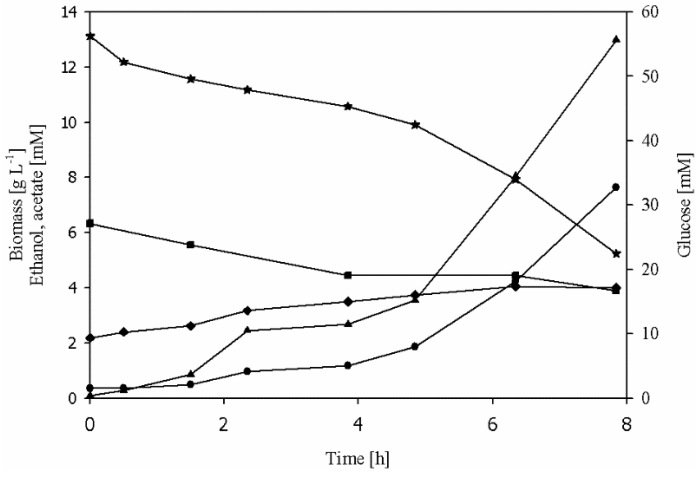


Table 1. Growth parameters of cultivations on galactose-based media containing ammonium sulphate and sodium nitrate as nitrogen source. For comparison, the growth parameters calculated during cultivations on glucose-based media are indicated in brackets.

	q [mmol g DW^{-1} h^{-1}]				Yield [g g^{-1}]			μ [h^{-1}]
	Galactose (Glucose)	Ethanol	Acetate	Nitrate	Biomass	Ethanol	Acetate	
Ammonium	0.82-0.86* (3.6-3.7)[a]	n.d. (3.9 – 4.4)[a]	n.d. (0.62 – 0.070)[a]	- (-)[a]	0.497-0.501* (0.17 – 0.18)[a]	n.d. (0.320 – 0.335)[a]	n.d (0.058 – 0.060)[a]	0.0752-0.0755* (0.11 – 0.12)[a]
Nitrate	1.20-1.3* (2.94 ± 0.006)[b]	0.011-0.011* (1.650 ± 0.007)[b]	0.491-0.496* (1.83 ±0.009)[b]	0.466* (-)[b]	0.374-0.379* (0.19 ± 0.004)[b]	0.002* (0.133 ± 0.006)[b]	0.068-0.072* (0.216 ± 0.006)[b]	0.0714-0.073* (0.92 ± 0.006)[b]

*experiments performed in duplicate (the range of values reported), [a] Data from Rozpędowska *et al.*, 2011, [b] Data from Galafassi *et al.*, 2013, q: specific consumption/production rate, DW: dry weight, n.d.: not detectable.

Table 2: Genes involved in galactose metabolism. For each protein analyzed, the function (Rubio-Texeira, 2005), the ID number (in http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html database), the identity with the related protein in *S. cerevisiae* (http://www.ncbi.nlm.nih.gov/) and the presence or absence of regulative motifs in promoter sequences of *D. bruxellensis* are indicated. Mismatches with the reference motif are underlined.

Gene	Function	ID number in *D.bruxellensis*	Identity (%)	*MIG1* (C/G)(C/T)GG(G/A)G	*CPH1* TGTAACGTT
ScGAL1	Galactokinase/Bifunctional sensor inducer	jgi|Dekbr2|8686|	61%	GTGGGG GCGGAG GTGGGG	GATACCGTT TTTAACGTGTGTTGCTTT TGCAGCTTT
ScGAL2	Galactose permease	jgi|Dekbr2|66929| jgi|Dekbr2|25434	56-57%	-	GGTAACGTA
ScGAL3	Galactose Sensor	no hit found	-	-	-
ScGAL4	Transcriptional activator	no hit found	-	-	-
ScGAL7	Galactose-1-phosphate uridylyltransferase	jgi|Dekbr2|26690|	66%	CTGGGG CCGGAG CCGGGG	TGTAAAGTT
ScGAL10	Uridine diphosphoglucose 4-epimerase	jgi|Dekbr2|26691|	63%	CTGGAG GTGGGG CCGGGG	GGTAAAGTT
ScGAL80	Gal4p repressor	no hit found	-	-	-

Submitted Manuscript-2

Moktaduzzaman M., Galafassi S., Vigentini I., Foschino R., Corte L., Cardinali G., Piškur J., and Compagno C. (2014) Response to acetic acid stress in *Dekkera bruxellensis*.

Response to acetic acid stress in *Dekkera bruxellensis*.

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Abstract.

Dekkera bruxellensis is associated with wine and beer production and may contribute in a positive or negative way to the flavor development. *D. bruxellensis* has been reported also to compete with *S. cerevisiae* in distilleries producing fuel ethanol due to its ability to grow under anaerobic conditions and to its high ethanol and acid tolerance. In this work we investigated how this yeast responds when exposed to acetic acid. A detailed analysis of acetic acid metabolism was performed on three strains which exhibited a different resistance. Our studies show that *D. bruxellensis* behaves, from a metabolic point of view, more similarly to *S. cerevisiae*, being unable to metabolize acetic acid in presence of glucose. The presence of acetic acid affected the growth, causing a reduction of growth rate, glucose consumption rate, ethanol production rate as well as biomass and ethanol yield. Interestingly, the cells continued to produce acetic acid. The metabolomics fingerprint analysis performed by FTIR technology showed that the response is strain specific.

Key words: *Dekkera bruxellensis*, acetic acid response, acetic acid metabolism, FTIR technology, wine yeast.

1. Introduction

Several yeast species are able to grow at low pH and in environments containing weak acids that have been approved for use as food preservatives, like sorbic acid, benzoic acid, propionic acid and acetic acid. This gives rise to the spoilage of a variety of foods by yeasts, causing alterations of taste, odor, flavor, discoloration and in some cases damaging also the food packaging. In particular acetic acid is a byproduct of sugar fermentation by some yeast genera, like *Brettanomyes/Dekkera*, *Hanseniaspora/Kloeckera,* and *Saccharomyces* group (Dijken and Scheffers, 1986). The effect of acetic acid has been widely investigated in *Saccharomyces cerevisiae* (Piper, 2011), but also in other non-conventional and more tolerant yeasts like *Zygosaccharomyces bailii*, a common food spoilage yeast (Fleet, 1992; Guerreiro et al., 2012). The toxicity of acetic acid is strictly dependent on the pH of the medium: at low pH, in fact, it is mainly present in its undissociated form and it can diffuse into the cell in a passive way. In *S. cerevisiae* an important role for its entrance is played also by the Fps1p aquaglyceroporin channel (Mollapour and Piper, 2007). In the cytosol, due to a higher pH, acetic acid dissociates, producing a decrease of the intracellular pH, which is detrimental to many cellular processes, including the activity of some glycolytic enzymes (Pearce et al., 2001). The generated protons are pumped out by the activity of the ATPase Pma1p and, at the same time, the anion is extruded by the activity of the plasma membrane multidrug resistance transporters (Carmelo et al., 1997; Holyoak et al., 1996; Mira et al. 2010a; Mira et al. 2010b; Stratford et al., 2013a). The function of plasma membrane proteins has been shown to be linked to the lipid composition of the membranes (Wang and Chang, 2002), and a recent work reveals that the acetic acid stress response results in wide lipidome rearrangements (Lindberg et al., 2013). In *S. cerevisiae* the adaptation to acetic acid require also the molecular signalling HOG (High Osmolarity Glycerol) MAP kinase pathway (Hohmann, 2009; Mollapour and Piper, 2006).

In the present study we investigated the acetic acid response in *Dekkera bruxellensis*. This yeast is associated with wine production and lambic beer fermentation, where it may contribute in a negative or positive manner to the flavor development (Fugelsang, 1997; Malfeito-Ferreira, 2011; Zuehlke et al., 2013). Indeed, this species is able to produce

phenolic compounds, such as 4-ethylguaiacol and 4-ethylphenol which could spoil the wine, depending on their concentration (Vigentini et al., 2008). Several strains have been isolated also from other sources, as from apple cider as well as from other sweet drinks (Gamero et al., 2014; Morrissey et al., 2004; Teoh et al., 2004). *D. bruxellensis* has also been reported to contaminate distilleries producing fuel ethanol, especially in continuous fermentation systems where it has been observed that this yeast can outcompete *S. cerevisiae* (de Souza et al., 2007; Passoth et al., 2007). Although *D. bruxellensis* and *S. cerevisiae* are considered as two phylogenetically very distant relatives, they share several peculiar traits, such as the ability to produce ethanol under sugar excess and aerobic conditions, high tolerance towards ethanol and acid, ability to grow without oxygen (Galafassi et al., 2011; Rozpędowska et al., 2011), and cohabitate in several niches. These traits can be important to survive in winemaking conditions. Among the non-*Saccharomyces* species *D. bruxellensis* has been shown to be better adapted than other yeasts to resist in must and during alcoholic fermentation (Renouf et al., 2006). In order to determine the basis of the acetic acid response in this food relevant yeast, we analyzed how the presence of acetic acid affect the growth and sugar metabolism. In recent years Fourier Transform InfraRed Spectroscopy (FTIR) has been applied in microbiological studies to whole cell analysis (Roscini et al., 2010; Szeghalmi et al., 2007; Zhao et al., 2004). FTIR analysis could discriminate among the physiological states of microbial cells throughout their growth and differentiation, irrespectively of the type of cells considered (Adt et al., 2006; Cavagna et al., 2010; Corte et al., 2011). A FTIR-based bioassay was recently developed to determine the presence and the extent of cellular stress, with the rationale that stressing conditions can alter the cell metabolome before and after cell death (Corte et al., 2010). By this tool we obtained information about the effects that the exposure to acetic acid determine in *D. bruxellensis* cells.

2. Materials and Methods

2.1. Yeast strains

Dekkera bruxellensis strains used for the screening were: CBS 72, CBS 73, CBS 74, CBS 75, CBS 78, CBS 96, CBS 97, CBS 98, CBS 1940, CBS 1941, CBS 1942, CBS 1943, CBS 2336, CBS 2499, CBS 2547, CBS 2796, CBS 2797, CBS 3025, CBS 4459, CBS

4602, CBS 6055, CBS 8027, CBS 4480, CBS 4481, CBS 4482 , CBS 4601, CBS 4914, CBS 5206, CBS 5512.

For long term storage, yeast strains were kept in 15% glycerol and 85% YPD (1% yeast extract, 2% peptone and 2% glucose) at -80 °C. Strains from the storage were sub-cultured on YPD or YPD-2% agar plates.

2.2. Screening for acetic acid resistance

Overnight YPD cultures at exponential growth phase were collected by centrifugation and washed twice. Ten µl of 10-fold dilution series, ranging from 100000 to 100 cells were spotted onto YPD plates (in triplicate) supplemented with 0 mM, 40 mM, 80 mM, 100 mM and 120 mM of acetic acid and pH adjusted to 4.5. Growth at 30 ^{o}C was monitored from 7 to 11 days.

2.3. Viability and cultivability

To test the effect of acetic acid on cell viability under different conditions of growth, aliquots from liquid cultures growing exponentially were collected, centrifuged, washed and incubated with a methylene blue solution (0.2 g l^{-1} methylene blue; 27.2 g l^{-1} KH_2PO_4; 0.071 g l^{-1} Na_2HPO_4) for 15 minutes. Blue (dead) and white (live) cells were counted at the microscope. The percentage of viable cells respect to the total counted cells is given.

Cultivability was determined by collecting aliquots from liquid cultures growing on YPD pH 4.5, serially diluting (10-fold dilution series) and plating 100 µl in triplicate, on plates containing YPD pH 4.5 (control) and YPD supplemented with acetic acid 120 mM pH 4.5. The short-term effect on cell cultivability was evaluated by collecting cells in exponential growth phase on YPD pH 4.5, centrifuged, washed twice with distilled sterile water and re-suspended in phosphate buffer (pH 4.5) with or without glucose (10 g/l), in order to have a standardized OD_{600nm} around 5.0. Each sample, except the control, was exposed to 120 mM acetic acid for 1 h at 30°C in a shaking incubator. Then cells were serially diluted and plated on YPD pH 4.5. The long-term effect of acetic acid on cell cultivability was evaluated by collecting the cells at exponential growth phase in YPD medium pH 4.5 without (control) and with 120 mM acetic acid. Then cells were serially diluted and plated on YPD pH 4.5.

2.4. Growth condition and metabolite assessment.

Strains were pre-cultured in YPD medium at 30 ºC. During the exponential phase, cells from pre-cultures were harvested by centrifugation and, after washing, inoculated at OD_{600nm} 0.1 into specific media. YPD (1% yeast extract, 2% peptone and 2% glucose) at pH 4.5 was prepared for control cultures, and supplemented with acetic acid (120 mM) for acetic acid resistance tests. To test the ability to use the acetic acid as the only carbon source, YP (1% yeast extract, 2% peptone) medium was supplemented with acetic acid (80 mM, 120 mM) and pH adjusted to 4.5. The medium was sterilized by filtration (Millipore, 0.22 µm). Cells from pre-inoculum in YPD were inoculated at OD_{600nm} 0.1 and incubated at 30 °C. All the cultures were performed in two replicates.

Cell growth was monitored through the increase in OD measured at 600nm using a spectrophotometer (Jenway, 7315^{TM}), after appropriate dilution. For dry weight measurements, samples from the shake-flask cultures were collected (in triplicate), the cells were filtered through a glass microfiber GF/A filter (Whatman) and washed with three volumes of de-ionized water and dried at 105 ºC for 24 h. Glucose, ethanol and acetic acid concentrations in the supernatants were assayed (in triplicate) by using commercial enzymatic kits (Roche, cat. numb. 1 0716251 035, 1 0176290 035 and 1 0148261 035). To evaluate the ability to use acetic acid as carbon source (on YP supplemented with acetic acid), the growth rates (μ_{max} reported in Table 1) as well as the specific acetic acid consumption rates were calculated during the exponential growth phase, which corresponded to the acetic acid consumption phase (Fig. 1 and Table 1). For the cultures performed on YPD and YPD supplemented with acetic acid, the growth rates, specific glucose consumption rates, and specific ethanol/acetic acid production rates were calculated during the exponential growth phase, corresponding to the glucose consumption phase (Table 3 and Fig. 2). Product yields were calculated as the total amount of product divided by the total amount of substrate utilized (either acetic acid or glucose).

2.5. Determination of ACS

ACS (Acetyl-CoA synthetase) assays were performed on cells collected during the exponential growth phase from cultures growing on YP supplemented with acetic acid, on YPD and on YPD supplemented with acetic acid (see above). Cell extracts were

prepared by extraction with acid-washed glass beads (SIGMA) as described previously (Postma et al., 1989), and total protein concentrations were determined by Bio-Rad kit no. 500-002 (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard. The specific activity of ACS in cells extract was determined at room temperature by using a spectrophotometer (Jenway, 7315^{TM}) at 340 nm as described previously (Postma et al., 1989), with the only exception that the assay reaction started with 100 mM of potassium acetate instead of 10 mM.

2.6. Metabolomic analysis by FTIR assays

Pre-cultures in YPD were inoculated at 0.1 OD_{600nm} in 100 ml flasks of YPD medium (pH 4.5) and then were incubated at 30°C, under shaking at 110 rpm. Two kind of experiments were carried out to assess short and long-term modifications upon acetic acid exposure. As far the former test, 10 ml of each cell suspension, in exponential growth phase, was centrifuged, washed twice with distilled sterile water and re-suspended in phosphate buffer (pH 4.5) with or without glucose (10 g/l), in order to have a standardized OD_{600nm} around 5.0. Each sample, except the control, was exposed to 120 mM acetic acid for 1 h at 30°C in a shaking incubator. From each sample 1.5 ml of cell suspension was taken, centrifuged, washed twice with distilled sterile water and re-suspended in 1.5 ml HPLC grade water in polypropylene tubes. 105 µl was sampled for three independent FTIR readings (35 µl each, according to the technique suggested by Essendoubi et al., 2005).

2.7. FTIR analysis and spectra processing

FTIR analysis was carried out with a TENSOR 27 FTIR spectrometer, equipped with HTS-XT accessory for rapid automation of the analysis (BRUKER Optics GmbH, Ettlingen, Germany). FTIR measurements were performed in transmission mode. All spectra were recorded in the range between 4000 and 400 cm^{-1}. Spectral resolution was set at 4 cm^{-1}, sampling 256 scans per sample. The software OPUS version 6.5 (BRUKER Optics GmbH, Ettlingen, Germany) was used to carry out the quality test, baseline correction, vector normalization and the calculation of the first and second derivatives of spectral values. The script MSA (Metabolomic Spectral Analysis) employed in this study was developed in "R" language to carry out the following operations on the matrices of

spectral data exported as ASCII text from OPUS 6.5. The analytical procedure consisted of calculating the distance between the spectrum of the cells under test and that of the cells without the stressing agent; this procedure was extended to five different spectral regions in order to differentiate the stress response among the different classes of molecules. In more detail, the procedure could be outlined as follows:

1. Each single spectrum was normalized in order to have the range spanning from 0 to 1 in a way already suggested by Huang et al., 2006). Average spectra from the three repetitions were calculated.
2. Response spectra (hereinafter reported as RS) were calculated as difference between each average spectrum and the average spectrum of the same cells maintained in YPD medium without acetic acid for long term effect analysis and in buffer for short term one (defined as control RS (Corte et al., 2010).

Synthetic stress indexes (hereinafter reported as SI) of metabolomic stress response were calculated as Euclidean distances of the RS under stress and the control RS. SI of the whole spectrum and of the five different spectral regions individuated by Kümmerle et al. (Kümmerle et al., 1998) were calculated. The five regions were defined as follows: fatty acids (W1) from 3000 to 2800 cm^{-1}, amides (W2) from 1800 to 1500 cm^{-1}, mixed region (W3) from 1500 to 1200 cm^{-1}, carbohydrates (W4) from 1200 to 900 cm^{-1} and typing region (W5) from 900 to 700 cm^{-1}. Since the five spectral regions differ in length, their SI were scaled to the length of the whole spectrum, in order to make the different SI comparable on the same scale. Global Stress Index (GSI), i.e. the average response throughout the whole spectrum, is a general measure of stressing efficacy, as GSI values below 1.0 typically indicate scarce metabolomics response (Corte et al., 2010). The long-term effect of acetic acid on cells was evaluated by growing cells (inoculated at OD_{600nm} 0.2) in YPD medium pH 4.5 without (control) and with 120 mM acetic acid. Samples were collected at exponential growth phase and submitted to FTIR analysis and cultivability test (as described above).

2.8. Western blotting

For Western blotting analysis, cells from exponentially growing cultures were harvested and incubated with 120 mM acetic acid for 10 minutes and for 60 minutes. Protein extraction was performed on 5% w/v SDS and cells were mechanically disrupted with

glass beads (425-600 µm) using the Precellys 24 tissue omogenizer (Advanced Biotech Italia Srl, Italy). Proteins extracted were separated by SDS-PAGE on 8% w/v polyacrylamide gel and immunoblotting was performed as previously described (Gatti et al., 1994). Hog1p and phospho-Hog1p were analyzed as reported (Galafassi et al., 2013a; Hernandez-Lopez et al., 2006), with polyclonal anti-Hog1 (y-215) (sc-9079, Santa Cruz Biotechnology) and phospho-p38 MAPK (Thr180/Tyr182) respectively, diluted 1:1000 in TBS-BSA 0.5% w/v and Tween 20 0.3% v/v. Actin was used to check the amount of protein loaded and it was detected with monoclonal anti-actin antibody (cat. no. MAB1501, Chemicon International Inc., Massachusetts, USA) 1000-fold diluted in TBS-BSA 0.5% w/v and Tween 20 0.3% v/v. Anti-rabbit and anti-mouse secondary antibodies were diluted 10000 times. Bounded antibodies were revealed using enhanced chemiluminescent substrate (Lite Ablot Plus, EuroClone, Italy).

3. Results

3.1. Screening for acetic acid resistance in D. bruxellensis

The response of *D. bruxellensis* species to the presence of acetic acid was investigated throughout a screening of 29 strains that were cultivated on plates of rich glucose medium (YPD) adjusted to pH 4.5 and containing acetic acid at concentrations ranging from 0 to 120 mM. After 7 days of incubation all the strains were able to grow on 40 mM acetic acid, while on plates containing higher concentrations several strains failed to grow. Prolonging the incubation to 11 days, all the strains proved to grow at 120 mM acetic acid but at the highest spotted cell concentration (corresponding to 100.000 cells in the spot), whereas only 2 strains, CBS 4482 and CBS 2499, grew even at the lowest cell concentration (corresponding to 100 cells spotted). This prompted us to determine the number of cells that were able to grow in this condition (cultivability). A decrease of 10 % and 55% in CFU due to the presence of acetic acid resulted for CBS 4482 and CBS 2499 respectively. In the case of CBS 98, 250 CFU were obtained from plates that were spread with 10^5 cells.

3.2. Ability to use acetic acid as carbon source

A more detailed analysis of acetic acid metabolism was performed on three strains: CBS 4482 and CBS 2499, which resulted among the most resistant strains, and CBS 98, one of the least resistant strains. At first, the ability to use acetic acid as carbon source was analyzed by liquid cultures in aerobic conditions in rich media (YP) containing acetic acid and adjusted to pH 4.5. CBS 4482 and CBS 2499 were able to grow in presence of 120 mM acetic acid and consume it. Their growth kinetics showed that the growth rates increased when the cells metabolized acetic acid (Fig. 1 and Table 1). CBS 98 grew at acetic acid concentration not over 80 mM (Fig. 1). Also in this case, the growth kinetic showed a first phase characterized by a low growth rate (below 0.01 h^{-1}), then the growth rate increased and acetic acid was completely consumed (Fig. 1 and Table 1). The activity of acetyl CoA synthetase (ACS), the first enzyme in the acetic acid utilization pathway, was found to be similar in cell extracts obtained from CBS 4482 and CBS 2499 strains (Table 2), and even higher in CBS 98 (but in this case it should be noted that the cells were cultivated at a lower acetic acid concentration).

3.3. The presence of acetic acid affects growth and sugar metabolism

In order to describe the influence of acetic acid on glucose metabolism in *D. bruxellensis*, the selected strains were cultivated in aerobic conditions in media at pH 4.5 containing glucose as carbon source and supplemented with acetic acid. In general, the strains showed some differences in the growth parameters (growth rates, specific glucose consumption rates, ethanol and acetic acid production rates) in the control condition (without acetic acid) (Table 3). In particular, the lowest value of specific glucose consumption rate was observed in the CBS 98 (Table 3). Noteworthy, the control culture of CBS 4482 showed the highest biomass yield and the lowest ethanol yield (Table 3), that could reflect a higher contribution of respiratory metabolism in glucose utilization by this strain.

The presence of acetic acid resulted in an adaptation phase lasting from several days in the case of CBS 98 (Fig. 2). This prompted us to determine the number of cells that were able to grow in this condition (cultivability). A decrease of 70 % in CFU resulted after 16

hours of growth in presence of 120 mM acetic acid. This result indicated that in the long adaptation phase observed with CBS 98, only a part of population was able to proliferate. For all the strains the maximum specific growth rates (μmax) were deeply affected, with reductions ranging from 56% to 84% in comparison to the control cultures (Table 3). *D. bruxellensis* strains were unable to use acetic acid in presence of glucose (Fig. 2). This could be due to the fact that the activity of ACS, that was lower in cell extracts from glucose cultures in comparison to acetic acid cultures (Table 2), resulted even undetectable in cell extracts from cultures performed in glucose supplemented with acetic acid (Table 2). The biomass level in term of final amounts and yields was lower in all the cultures exposed to acetic acid (Table 3). This phenomenon could reflect that an higher amount of energy had to be redirected to extrude protons out of the cells, in comparison to the cultures containing only glucose (control cultures). Nevertheless, the reduction of biomass yield was higher in CBS 98 (46%) rather than CBS 4482 and CBS 2499 (10% and 20 % respectively): this could indicate that in the latter strains an higher amount of ATP was available for biomass formation in presence of acetic acid. The stress caused by the presence of acetic acid reduced also the specific glucose consumption rates (Table 3), and the lowest value of glucose consumption rate was observed in the CBS 98. The fermentation products, ethanol and acetic acid, were synthetized also in presence of acetic acid, like in the control cultures (Table 3), but the specific ethanol and acetic acid production rates decreased in all the strains as well as the ethanol yield (Table 3). Surprisingly, the acetic acid yield increased in all the cultures exposed to acetic acid stress. Cells collected from the exponential growth phase showed, as expected, an increased ability to form colony (75 % CFU for all the three strains), and also high viability (Table 3). When the glucose was exhausted, *D. bruxellensis* started to consume ethanol and converted it to acetic acid; this determined a further increase of the acetic acid concentration, up to 250 mM (Fig. 2), and a decrease of pH to 4.0, but the cells maintained a high viability (never below 60%).

3.4. Metabolomic analysis of a short-term exposure to acetic acid stress

In order to test the effect of acetic acid, *D. bruxellensis* cells were exposed for 1 h in phosphate buffer at pH 4.5 containing 120 mM acetic acid, both in absence and in presence of glucose; the latter case had the aim to allow energetic metabolism. This short

exposure time was chosen since cellular stress can induce very fast changes in terms of metabolites, detectable by the FTIR bioassay (Roscini et al., 2010).

As expected, the presence or absence of glucose did not determine a metabolomic response or affected the cell cultivability in absence of acetic acid. When glucose was not present in the buffer, the metabolomic response of CBS 98 and CBS 4482 was very weak, confirmed by the fact that the GSI (Global Stress Index) was around 0.3 for both strains (Table 4), whereas the strain CBS 2499 showed an higher metabolomic response (GSI = 1.2). However, the cultivability was very similar for all the investigated strains, ranging from 92% to 88%. The addition of glucose to the stress tests affected poorly the metabolomic response of CBS 98, but significantly induced the stress response of CBS 4482, determining an increase of the GSI (Table 4). Conversely, in the strain CBS 2499 the presence of glucose strongly reduced the stressing effect exerted by acetic acid (GSI = 0.7). The cultivability was also affected by the presence of glucose, increasing in all the strains (Table 4). Looking at the specific spectral regions the fatty acid region (W1) revealed always an increase of the SI values caused by glucose presence, especially in CBS 4482. The SI index of the amide (W2) and the mixed regions (W3) rose upon sugar addition in CBS 98 and CBS 4482, while decreased in CBS 2499. Furthermore, supplementing the cell suspension with glucose caused a decrease in SI index of carbohydrate region (W4) in CBS 98and in CBS 2499, and an increase in CBS 4482 (Tab 4). In conclusion all these observations indicated that the metabolic response to the acetic acid exposition is strain specific, especially in relation to the metabolomic regions involved. Moreover, the concomitant presence of glucose affected cell mortality in all the strains, but the metabolic response remains strain dependent.

3.5. Metabolomic analysis of D. bruxellensis cultures grown in presence of acetic acid

The effect of acetic acid on *D, bruxellensis* strains during the cell growth was analyzed by comparing the FTIR profiles of cells cultivated on glucose-based media. The Response Spectra (RS) obtained for the three cultures differed in the amides (W2), carbohydrates (W4) and mixed region (W3) (Fig 3), whereas negligible effects could be observed in the fatty acids (W1) and in the typing (W5) regions (data not shown). The strain CBS 2499 showed a positive response from 1800 to 900 cm^{-1}, whereas the other two had a slightly positive (CBS 98) and faintly negative response (CBS 4482). The most intense response

was observed for both CBS 2499 and CBS 98 at ca 1040 cm^{-1} (carbohydrate region) and a weaker at ca 1570 cm^{-1} (amide region), the latter response can be probably ascribed to the GMP and AMP (Harz et al., 2009). An increased level of the AMP/ATP rate has been reported to be essential for the activation of the Snf1p pathway, which is known to occur in response to acetic acid stress (Wilson et al., 1996). Moreover, acetic acid induces a depletion of ATP thus raising the AMP/ATP ratio (Mira et al., 2010; Pampulha and Loureiro-Dias, 2000). The significance and position of these response peaks were confirmed by second derivative analysis of the spectra (data not shown). A relatively intense peak was detected in CBS 98 and CBS 2499 around 1620 cm^{-1}, a region attributed to acetyl C=O (Mäntele et al., 1988), and therefore likely to witness the presence of acetic acid within the cell.

3.6. *DbHog1p is not phosphorylated upon exposure to acetic acid*

To explore whether the presence of acetic acid induced in *D. bruxellensis* the dual phosphorylation of Hog1p, as observed in *S. cerevisiae* (Mollapour and Piper, 2006), CBS 2499 cells were collected after exposure to the presence of acetic acid 120 mM. Proteins were examined by Western blot and phosphorylation of Hog1p was tested by using a commercial anti-phospho-p38 antibody, which specifically recognizes the dually phosphorylated form of Hog1p. As reported in Fig. 4, the incubation of cells in presence of 120 mM acetic acid did not result in any band corresponding to the phosphorylated form of Hog1p, indicating that this regulatory mechanism is not operating as consequence of exposure to acid stress, in contrast to what recently has been reported when the cells were exposed to osmotic stress (Galafassi et al., 2013b).

4. Discussion

The elucidation of the mechanisms underlying the resistance of yeasts to acetic acid may have relevant impacts on the improvement of food related processes as well as the biotechnological ones. Yeast cells are in fact able to circumvent the acid toxicity, survive and spoil the products, causing serious economic losses.

The results of the screening for acetic acid resistance carried out in this study reveals that all tested *D. bruxellensis* strains were able to face a 120 mM concentration of this compound, though showing differences in adaptation times and growth rates. The high resistance of the food spoilage yeast *Z. bailii* to acetic acid has been mainly associated with its ability to metabolize this acid also in presence of glucose (Rodrigues et al., 2012), in contrast to other species like *S. cerevisiae*, due to a specific and not glucose repressed transport system and a higher metabolic flux through acetyl-CoA synthetase (Sousa et al., 1996; Rodrigues et al., 2012). Our study showed that *D. bruxellensis* behaves, from a metabolic point of view, more similarly to *S. cerevisiae*, being unable to co-metabolize glucose and acetic acid simultaneously (Fig. 2 and Table 3). This is due to a glucose repression mechanism on the acetyl-CoA synthetase activity (Table. 2), which is evident in all the strains. Glucose repression of acetyl-CoA synthetase has been reported also in *Dekkera anomala* (Geros et al. 2000). On the other hand, the tested strains were able to use acetic acid as carbon source (Fig. 1). Interestingly, we found that *D. bruxellensis* continued to produce acetic acid as byproduct of its fermentative metabolism, even when this compound was present as a component of the medium, thus resulting in a further increase of its final concentration. This could indicate that a very efficient export system is operating. In *S. cerevisiae* the expression of *TPO2, TPO3,* and *AQR1*, encoding transporters of major facilitator superfamily and supposed to mediate the active expulsion of acetate, is activated in presence of acetic acid (Fernandes et al. 2005; Mira et al., 2010b). The presence of acetic acid causes in yeast cell oxidative stress (Giannattasio et al., 2005; Martani et al., 2013), which increase the demand of NADPH to be counteracted. This fact can play an important role in maintaining acetic acid production, being a way to generate this reduced cofactor. A similar situation has been recently reported for *D. bruxellensis*, due to a specifically increased NADPH demand for nitrate utilization as a nitrogen source (Galafassi et al., 2013a).

Among the analyzed strains, the presence of acetic acid caused the strongest effect for the CBS 98 strain in term of growth rate and biomass yield reduction. We found in fact that the fraction of cells able to proliferate (to form a colony on plate) in presence of glucose and acetic acid is strain specific and is lowest in the CBS 98 strain. This phenomenon has been recently well described in *S. cerevisiae* (Swinnen et al. 2014). It has been demonstrated that only a fraction of cell population resume growth after exposure to

acetic acid, whereas all the other cells persisted in a non-proliferating state or dye after long term exposure, and the size of this fraction is strain-specific and determines the duration of the latency phase. Our results suggest that a similar phenomenon could be present also in *D. bruxellensis*. Studies are now in progress on *D. bruxellensis* to better characterize at single cell level the cellular status all along the different growth phases observed, by analyzing also the influence of intracellular pH, which has been demonstrated to play an important role in *Z. bailii* resistance (Stratford et al., 2013b).

On the other hand we found that a short-term exposure to 120 mM acetic acid did not cause a real cellular damage, being the vitality high and similar for all the three tested strains (Table 4). The metabolomics fingerprint analysis performed by FTIR technology showed that the response to acetic acid is strain specific, both for the short-term exposition as well as in the case of cultivation in presence of acetic acid. The cellular components mainly involved in this response were carbohydrates and amides. The carbohydrates present in the cell wall could represent one of the acetic acid targets, as supported by recent observations in *S. cerevisiae* and in *Z. bailii* (Mira et al., 2010; Guerreiro et al., 2012). Amides are linked to the AMP/ATP ratio, which is affected by the energy expense required to extrude protons out of the cell. The indications that we can deduce from FTIR analysis lead to the idea that in the most resistant strain, CBS 4482, which showed a strong response upon the short-term exposure but a weaker response when cultivated in presence of acetic acid, a more efficient mechanism for the fast export of acetic acid could be operating. This could be linked also to a higher ATP availability, due to a more respiratory metabolism of glucose, as suggested by the lower ethanol yield observed when this strain was cultivated in absence of acetic acid (Table 3). In the more sensitive strain CBS 98, on the contrary, the short-term exposure caused a weak response, but when cultivated in presence of 120 mM acetic acid the response was high (Fig. 3). This could indicate that this strain lack a fast and efficient response mechanism to counteract the acetic acid stress, and that the stress caused by this condition determines the strong negative effect on its growth physiology (Table 3). The exposure to acetic acid did not cause in *D. bruxellensis* the activation of the HOG MAP kinase pathway, that on the contrary is involved in the osmotic stress response (Galafassi et al., 2013b). Although the HOG pathway appears to be conserved even among distantly related species, the pathway may in each species also be adapted to the specific niche requirements. For example, among pathogenic yeasts the

HOG pathway also plays a role in virulence (Rispail et al., 2009). We could speculate that in *D. bruxellensis* it can also have a function under other stress conditions, and this deserves to be studied.

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Table 1. Growth parameters of *D. bruxellensis* strains that use acetic acid as carbon source.

Strain	Growth condition	Growth rate	Specific acetic acid consumption rate	Biomass yield
	acetic acid	μ_{max} (h^{-1})	(mmol/g/h)	(g/g)
CBS 4482	120 mM	0.071±0.003	1.54±0.07	0.81±0.05
CBS 2499	120 mM	0.046±0.001	0.83±0.03	0.86±0.05
CBS 98	80 mM	0.054±0.004	1.27±0.08	0.68±0.03

The average of two replicates with the standard deviation are presented

Table 2. Acetyl-CoA synthetase activity of *D. bruxellensis* strains cultivated under different growth conditions.

Strains	Growth conditions	Acetyl-CoA synthetase activity U/mg
CBS 4482	YP + Acetic acid (120 mM, pH 4.5)	0.056±0.004
	YPD, pH 4.5	0.019±0.003
	YPD + Acetic acid (120 mM, pH 4.5)	ND
CBS 2499	YP + Acetic acid (120 mM, pH 4.5)	0.052±0.004
	YPD, pH 4.5	0.021±0.002
	YPD + Acetic acid (120 mM, pH 4.5)	ND
CBS 98	YP + Acetic acid (80 mM, pH 4.5)	0.187±0.02
	YPD, pH 4.5	0.020±0.003
	YPD + Acetic acid (120 mM, pH 4.5)	ND

ND Not Detectable; The average of two replicates with the standard deviation are presented

Table 3. Growth parameters of *D. bruxellensis* cultured in presence of glucose and acetic acid.

Strains	Growth conditions	Growth rate μ_{max} h^{-1}	Specific consumption rate mmol/g/h	Specific production rate mmol/g/h		Yield g/g			Viability
			Glucose	Ethanol	Acetic acid	Biomass	Ethanol	Acetic acid	
CBS 4482	YPD, pH 4.5 (control)	0.18±0.01	3.8±0.03	3.25±0.25	1.47±0.11	0.27±0.04	0.24±0.01	0.15±0.02	99.9%
	YPD + Acetic acid (120 mM, pH 4.5)	0.072±0.004	1.62±0.09	1.32±0.08	0.94±0.06	0.24±0.01	0.23±0.04	0.20±0.02	93%
CBS 2499	YPD, pH 4.5 (control)	0.197±0.005	4.6±0.11	6.36±0.16	1.15±0.03	0.24±0.01	0.36±0.01	0.08±0.01	99.9%
	YPD + Acetic acid (120 mM, pH 4.5)	0.06±0.003	1.76±0.08	1.40±0.07	0.96±0.05	0.17±0.03	0.22±0.05	0.20±0.06	86%
CBS 98	YPD, pH 4,5 (control)	0.127±0.001	1.9±0.02	2.51±0.03	0.76±0.01	0.24±0.01	0.29±0.07	0.14±0.07	99.9%
	YPD + Acetic acid (120 mM, pH 4.5)	0.019±0.004	0.81±0.14	1.14±0.21	0.27±0.05	0.13±0.01	0.33±0.04	0.15±0.03	73%
	YPD + Acetic acid (80 mM, pH 4.5)	0.044±0.002	1.38±0.06	1.60±0.08	0.61±0.03	0.18±0.02	0.30±0.01	0.15±0.01	84%

The average of two replicates with the standard deviation are presented

Table 4. Stress indexes (SI) derived from FTIR analysis and vitality of different *D. bruxellensis* strains subjected to a short-term exposure to acetic acid (120 mM) in buffer at pH 4.5.

Strains	Glucose (g/L)	W1 region	W2 region	W3 region	W4 region	Global SI	Cultivability %
CBS 4482	0	0.289	0.986	0.452	1.158	0.317	93±2
	10	1.145	2.491	3.575	2.770	1.057	86±3
CBS 2499	0	0.705	2.597	3.575	4.403	1.245	89±1
	10	1.024	2.076	1.554	1.699	0.735	75±1
CBS 98	0	0.400	0.450	0.652	0.849	0.295	90±3
	10	0.736	1.501	1.618	0.692	0.496	84±3

Fig. 1. Growth of *D. bruxellensis* on media containing acetic acid as carbon source at pH 4.5. Strains CBS 4482 (A) and CBS 2499 (B) were cultivated in presence of acetic acid 120 mM; strain CBS 98 (C) was cultivated in presence of acetic acid 80 mM. OD_{600nm} (◊), Acetic acid g/L (○).

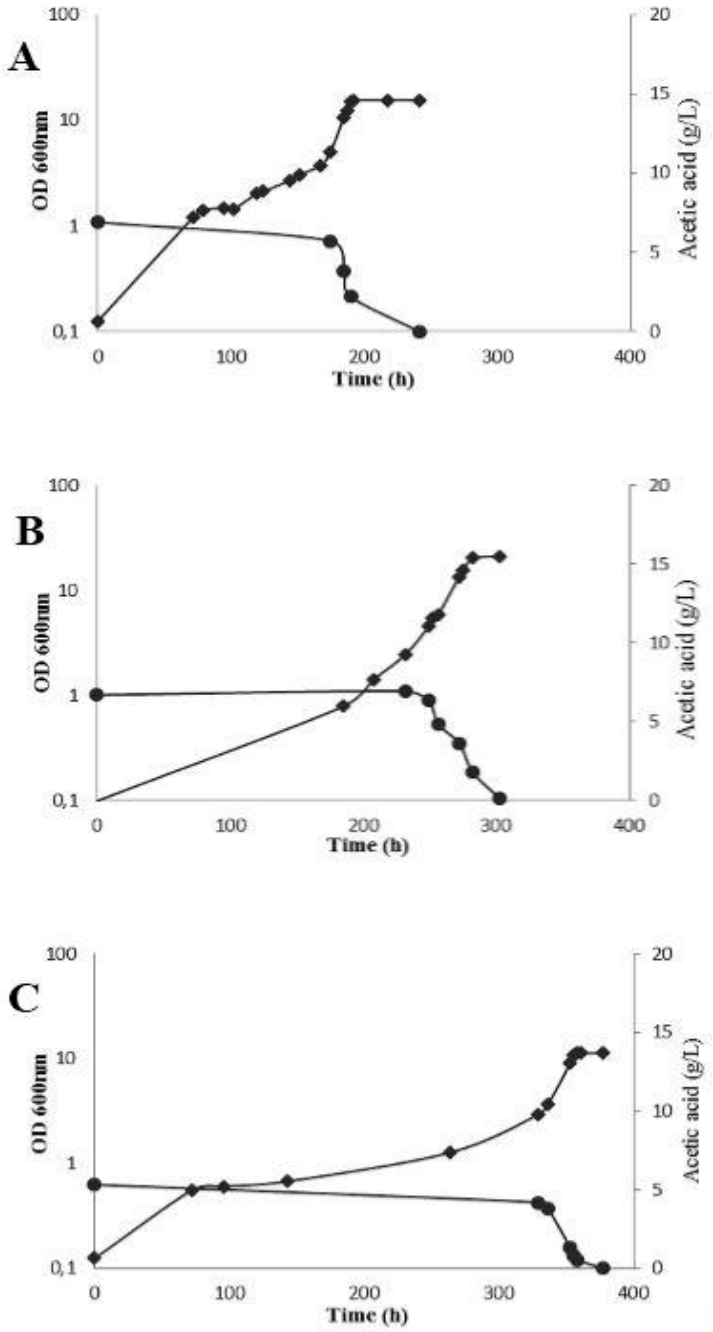


Fig. 2. Growth of *D. bruxellensis* strains in YPD medium in presence of acetic acid 120 mM at pH 4.5. Glucose g/L (□), OD_{600nm} (◊), Acetic acid g/L (○), Ethanol g/L (Δ). Strains: CBS 4482 (A), CBS 2499 (B), CBS 98 (C)

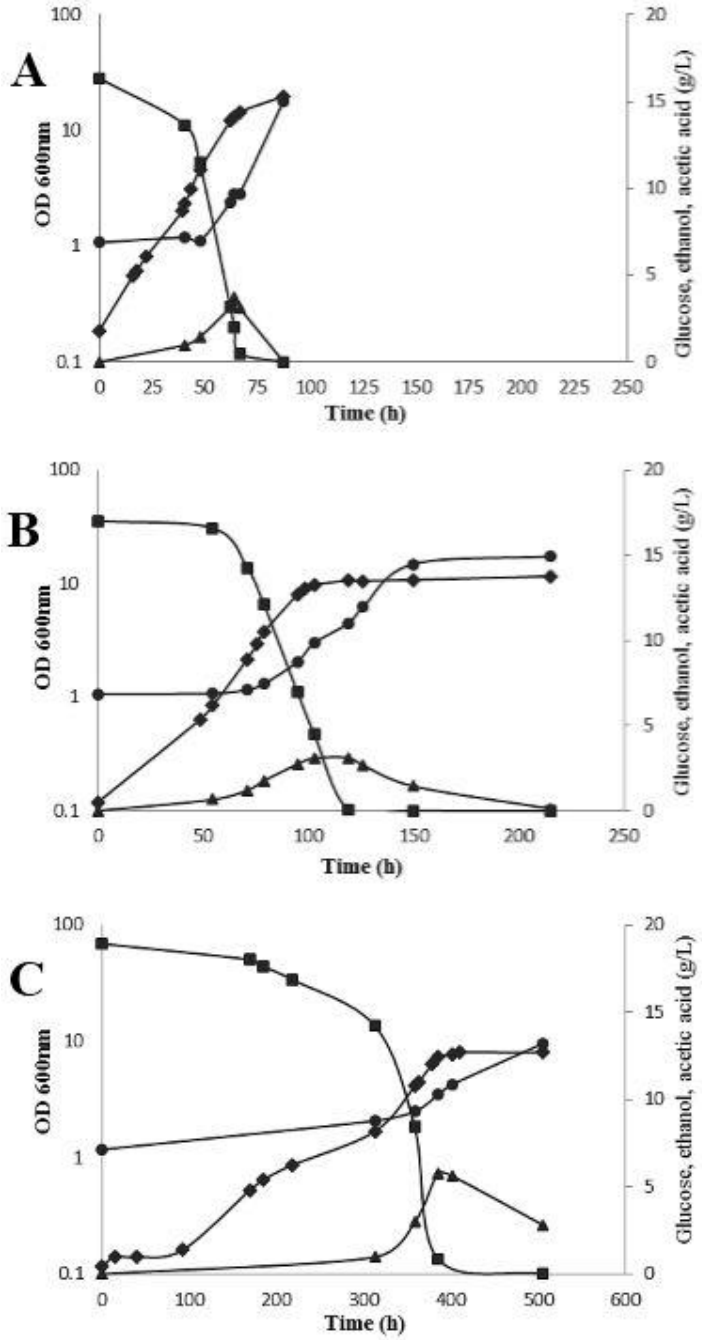


Fig. 3. Response spectra (RS) of the three *D. bruxellensis* strains grown in YPD and in presence of acetic acid 120 mM at pH 4.5. Solid line represents the strain CBS 98, dotted line the strain CBS 2499 and dashed line the strain CBS 4482.

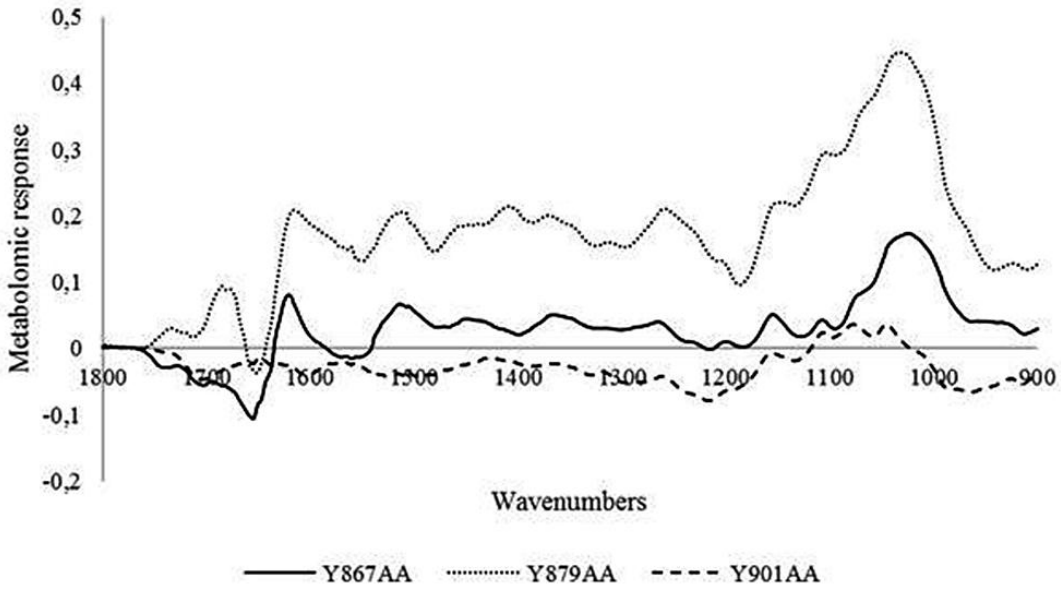
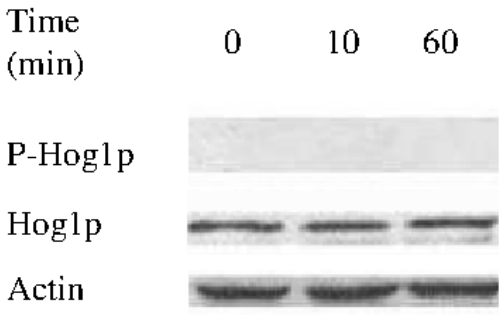


Fig. 4. Western blot showing the involvement of *HOG1* during the exposure to acetic acid 120 mM in *D. bruxellensis* CBS 2499



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BIOENERGY/BIOFUELS/BIOCHEMICALS

Utilization of nitrate abolishes the "Custers effect" in *Dekkera bruxellensis* and determines a different pattern of fermentation products

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Abstract Nitrate is one of the most abundant nitrogen sources in nature. Several yeast species have been shown to be able to assimilate nitrate and nitrite, but the metabolic pathway has been studied in very few of them. *Dekkera bruxellensis* can use nitrate as sole nitrogen source and this metabolic characteristic can render *D. bruxellensis* able to overcome *S. cerevisiae* populations in industrial bioethanol fermentations. In order to better characterize how nitrate utilization affects carbon metabolism and the yields of the fermentation products, we investigated this trait in defined media under well-controlled aerobic and anaerobic conditions. Our experiments showed that in *D. bruxellensis*, utilization of nitrate determines a different pattern of fermentation products. Acetic acid, instead of ethanol, became in fact the main product of glucose metabolism under aerobic conditions. We have also demonstrated that under anaerobic conditions, nitrate assimilation abolishes the "Custers effect", in this way improving its fermentative metabolism. This can offer a new strategy, besides aeration, to sustain growth and ethanol production for the employment of this yeast in industrial processes.

Keywords *Dekkera bruxellensis* · Nitrate metabolism · Custers effect · Ethanol production

Introduction

Nitrate is one of the most abundant nitrogen sources in nature. In the biosphere, nitrate assimilation is the major pathway converting inorganic nitrogen to organic forms. It has been estimated that more than 2×10^4 megatons of organic nitrogen per year are produced by nitrate assimilation in a variety of organisms, including bacteria, fungi, algae, and plants [15]. Several yeast species have been shown to be able to assimilate nitrate and nitrite, but the metabolic pathway has been studied in very few of them so far, especially in the ones which have been receiving biotechnological interest such as *Hansenula polymorpha, Pichia anomala, Pachysolen tannophilus*, and *Arxula adeninivorans* [2, 7, 14, 16]. The nitrate assimilation pathway in yeast is the same as the one described for plants and filamentous fungi [29]. Two kinds of nitrate transporters have been characterized, with high and low affinity [19]. After its uptake, nitrate is converted to ammonium by two successive reductions catalyzed by nitrate reductase and nitrite reductase, respectively (Fig. 1). In *H. polymorpha*, the genes for nitrate transporter, nitrate reductase and nitrite reductase are clustered [3]. These genes are induced by nitrate and nitrite and repressed by ammonium as well as by other factors involved in the utilization of secondary nitrogen sources [27, 29]. This is in agreement with the preference exhibited by yeast to use inorganic compounds as nitrogen sources, like ammonium, as well as various amino acids.

Dekkera bruxellensis is often associated with wine production and lambic beer fermentation and it may contribute in a positive or negative way to the flavor development [8, 12, 21]. *D. bruxellensis* has also been reported to contaminate distilleries producing fuel ethanol, especially in continuous fermentation systems where it has been observed that this yeast can outcompete *S. cerevisiae* [18, 23]. Although *D. bruxellensis* and *S. cerevisiae* are considered as two phylogenetically very distant relatives, they share several peculiar traits, such as

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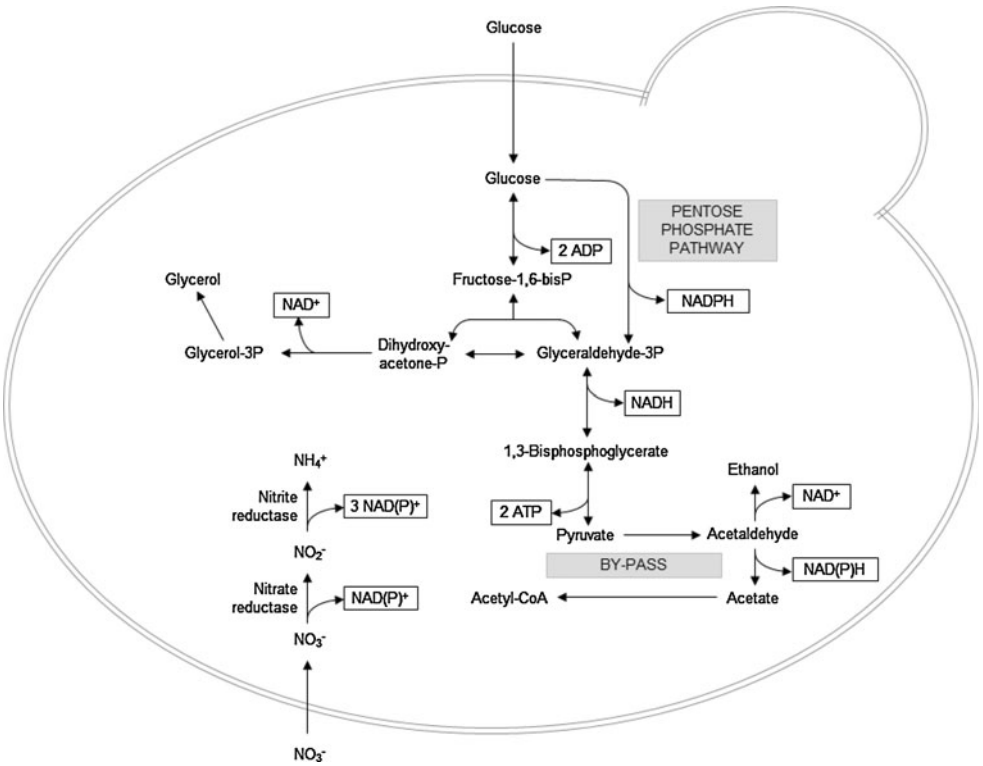

Fig. 1 Schematic representation of pathways involved in glucose metabolism and nitrate utilization with special attention to the steps directly involved in redox balance

the ability to produce ethanol under aerobic conditions, high tolerance towards ethanol and acid, and the ability to grow without oxygen [13, 17, 28]. Apparently, these traits have evolved in parallel in both groups, but the molecular mechanisms involved may be different [28]. *D. bruxellensis* can use nitrate as the sole nitrogen source [10], whereas *S. cerevisiae* cannot. Woolfit et al. [32] reported the presence of five genes encoding for nitrate assimilation pathway in *D. bruxellensis*. Recently, it has been shown that this metabolic characteristic can render *D. bruxellensis* able to overcome *S. cerevisiae* populations in industrial fermentations [11]. The presence of these skills together with a wide range of carbon sources utilization by this yeast species has been leading to the idea that *D. bruxellensis* could become a new industrially relevant ethanol-producing organism [4, 5, 13, 24]. In order to better characterize how nitrate utilization affects carbon metabolism and the yields of fermentation products, we investigated this trait under well-controlled aerobic and anaerobic conditions and in well-defined media. Our experiments showed that utilization of nitrate determines in *D. bruxellensis* a different pattern of fermentation products, in comparison to the one obtained by ammonium utilization. We have also demonstrated that nitrate assimilation abolishes the "Custers effect" under anaerobic conditions, improving its growth and fermentative metabolism.

Materials and methods

Yeast strains

The yeast strain used in this work is *Dekkera bruxellensis* CBS 2499. Stocks of the strain were stored at −80 °C in 15 % v/v glycerol and revitalized prior to each experiment in liquid mineral medium (20 g l^{-1} glucose; 1.7 g l^{-1} YNB w/o amino acid and ammonium sulfate; 5 g l^{-1} ammonium sulfate).

Media and growth conditions

Aerobic batch cultivations were performed in shake-flasks and in a Biostat-Q system bioreactor (B-Braun) with a working volume of 0.8 l. The temperature was set at 30 °C, the stirring speed at 500 rpm, and the pH, measured by Mettler Toledo pH electrode, was adjusted to 5.0 by automatic addition of 2 M KOH. The dissolved oxygen concentration (more than 30 % of air saturation) was measured by Mettler Toledo polarographic oxygen probe. The medium used was a defined synthetic mineral medium as reported by Merico et al. [20] with the only exceptions that nitrogen sources were: ammonium sulfate, 5.0 g l^{-1}; sodium nitrate, 6.43 g l^{-1}; mixtures of ammonium sulfate, 5.0 g l^{-1} and sodium nitrate, 1 g l^{-1}, as specified. The media for anaerobic cultures were supplemented with

uracil, 50 mg l^{-1}; ergosterol, 10 mg l^{-1}; and Tween 80, 420 mg l^{-1}.

The anaerobic batch cultivations were performed in a Biostat-Q system. The bioreactor was flushed with nitrogen (<3 ppm O_2) with a flow of 0.1–0.31 l^{-1} min^{-1}. The stirring was kept constant at 500 rpm. Norprene tubes (Cole-Palmer, General Control, Milan, Italy) were used to minimize the diffusion of oxygen into the bioreactor. All the cultivations were repeated at least two times.

Anaerobic plate test

The plates for anaerobic test were performed on mineral medium (glucose, 20 g l^{-1}; Yeast Nitrogen Base without amino acids and ammonium sulfate, 1.7 g l^{-1}; agarose, 20 g l^{-1}) supplemented with uracil (50 mg l^{-1}), ergosterol (10 mg l^{-1}) and Tween 80 (420 mg l^{-1}) and nitrogen sources (ammonium sulfate, 5.0 g l^{-1}; sodium nitrate 6.43 g l^{-1}; mixtures of ammonium sulfate, 5.0 g l^{-1} and sodium nitrate, 1 g l^{-1}). Cells grown on liquid YPD until the exponential phase were harvested and suspended in distilled water. Approximately 500 cells were spotted on the respective plates and were grown anaerobically for 2 weeks. The anaerobic environment was established using Anaerocult A system (Merck, cat. no. 1138290001 and 116387) and the strength of the anaerobiosis was checked with Anaerotest strips (Merck, cat. no. 115112), ensuring an oxygen content below 1 ppm O_2. The assembly was prepared and the growth was followed according to Merck instructions. Each plate included the positive and negative controls, *S. cerevisiae* and *K. lactis*, respectively.

Biomass and metabolites quantification

Samples were withdrawn from the bioreactor at appropriate intervals and used to monitor the cell growth measuring the optical density at 600 nm with a spectrophotometer, after appropriate dilution. For dry weight determination, washed culture samples were filtered on a 0.45-μm glass microfiber GF/A filter (Whatman) and dried 24 h at 80 °C. The concentration of extracellular metabolites, such as glucose, ethanol, acetate, nitrate, and ammonium in the supernatants were determined by commercial enzymatic kits (Roche, cat. numb. 1 0716251 035, 1 0176290 035, 1 0148261 035, 1 09005658 035, and 1 1112732 035, respectively). All the assays were performed in triplicate and the standard deviations varied between 1 and 5 %.

Specific consumption rates of glucose, nitrate, and ammonium and specific production rates of ethanol and acetic acid were calculated during the exponential phase of growth. The yields of biomass, ethanol, and acetic acid were calculated as the total amount of products divided by the total amount of glucose utilized.

Enzyme activity assays

Cell extracts were prepared by extraction with acid-washed glass beads (SIGMA) according to Postma et al. [26], and the total amount of extracted proteins was quantified using the Bio-Rad kit no. 500-002 (Bio-Rad, Hercules, CA, USA). Acetaldehyde dehydrogenase (ACDH) and glucose 6-phosphate dehydrogenase (G6PDH) were assayed according to Postma et al. [26] with the only exception that the concentration of NADH was increased to 4 mM for the assay of NADH-dependent activity of acetaldehyde dehydrogenase. Nitrate reductase assay was performed in 50 mM potassium phosphate buffer pH 7 and 0.2 mM NADH or NADPH. The reaction was started by the addition of 10 mM sodium nitrate and the formation of NAD^+ or $NADP^+$ was followed at 340 nm. Nitrite reductase assay was performed in 50 mM potassium phosphate buffer pH 7, 10 mM $MgSO_4$, and 0.2 mM NADH or NADPH. The reaction was started by the addition of 1 mM sodium nitrite and the formation of NAD^+ or $NADP^+$ was followed at 340 nm. A unit (U) of enzyme activity is defined as 1 μmol of substrate transformed per minute using an extinction coefficient for NAD(P)H of 6.22 l $mmol^{-1}$ cm^{-1}.

Results

Nitrate utilization under aerobic conditions

In order to obtain a detailed quantitative and qualitative analysis about the effects of utilization of nitrate on the metabolism of glucose and fermentation products in *D. bruxellensis*, batch cultures were performed in a bioreactor under strictly controlled aerobic conditions, controlled pH, and on synthetic media. The *D. bruxellensis* CBS 2499 strain was chosen because its genome has been sequenced [32, 25], and its glucose metabolism has been characterized under aerobic as well as under anaerobic conditions [13, 28]. In the first series of batch cultures, *D. bruxellensis* was cultivated on media containing sodium nitrate as the sole nitrogen source. Under these conditions, the growth rate was similar to the ammonium-based one. Interestingly, the main final product of glucose fermentation was acetic acid instead of ethanol, which reached a 3.5× higher yield and was produced at a 2.5× higher specific production rate than on ammonium-based ones (Table 1). On the other hand, the specific glucose consumption rate as well as the ethanol production rate were both lower on nitrate-based media than on ammonium-based ones (Table 1). The utilization of nitrate determined a slight increase of the biomass yield (Table 1). An analogous redirection of glucose catabolic products was observed also when *D. bruxellensis* was cultivated on

media containing a mixture of ammonium and nitrate as nitrogen sources. In these conditions the growth rate was lower than the one observed on media containing ammonium as the sole nitrogen source (Table 1). Nitrate and ammonium were co-assimilated (Fig. 2). Also in this case the consumption of nitrate resulted in a drastic increase in the production of acetic acid, to about a three times higher yield, and in a parallel decrease of ethanol production, to one-third of the yield calculated on ammonium sulphate-based media, respectively (Table 1).

Table 1 Growth parameters during aerobic fermentations on glucose mineral medium with ammonium sulphate (5 g l^{-1}), sodium nitrate (6.43 g l^{-1}), or a mixture of ammonium sulphate (5 g l^{-1}) and sodium nitrate (1 g l^{-1}) as nitrogen sources

	Ammonium[a]	Nitrate	Mixture
Growth rate (h^{-1})	0.11–0.12	0.092 ± 0.006	0.077 ± 0.004
q (mM $g_{dry\ weight}$ h^{-1})			
Glucose	3.6–3.7	2.94 ± 0.5	2.42 ± 0.28
Ethanol	3.9–4.4	1.65 ± 0.007	1.30 ± 0.09
Acetate	0.62–0.70	1.83 ± 0.009	1.59 ± 0.10
Y (g $g^{-1}_{glucose}$)			
Biomass	0.17–0.18	0.19 ± 0.004	0.23 ± 0.008
Ethanol	0.320–0.335	0.133 ± 0.006	0.138 ± 0.006
Acetate	0.058–0.060	0.216 ± 0.006	0.166 ± 0.011

[a] Data from [28]

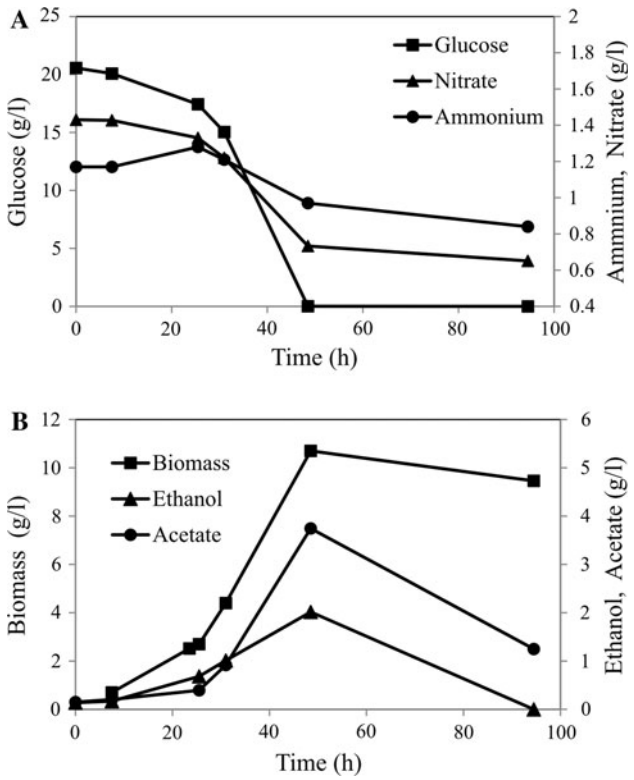

Fig. 2 Batch fermentation on glucose mineral medium with a mixture of ammonium sulphate (5 g l^{-1}) and sodium nitrate (1 g l^{-1}) as nitrogen sources. **a** Substrate consumption. **b** Biomass and metabolites production

Nitrate utilization under anaerobic conditions

Dekkera bruxellensis grows under strict anaerobic condition on synthetic media at a very low rate [28]. This has been ascribed to a redox imbalance due to its scarce ability to produce glycerol, which plays an important role under anaerobic condition for the reoxidation of NADH produced during the amino acids synthesis [30]. The addition of amino acids to the medium has been shown in fact to help its growth, partially alleviating this problem [6]. Due to the dependence by the nitrate-assimilating enzymes for NAD(P)H (Fig. 1), the utilization of nitrate as nitrogen source could work in the cell as a redox sink. Nitrate metabolism has been shown to greatly facilitate growth on xylose under anaerobic conditions in the fungus *Fusarium oxysporum* [22]. To test this hypothesis, *D. bruxellensis* was cultivated under strictly controlled anaerobic conditions in bioreactor on synthetic media containing a mixture of ammonium and nitrate, due to the fact that we observed no growth on plates containing nitrate as the sole nitrogen source (not shown). Under these conditions, cells grew at a higher rate than the one observed on ammonium-based medium enriched with amino acids (Table 2). The growth rate was in fact more similar to the one observed under aerobic conditions (Tables 1 and 2 for comparison). In contrast to what occurred on ammonium-based media, where no acetic acid production was detected, acetic acid was produced under anaerobic cultivation when nitrate was utilized. Noteworthy, the specific acetic acid production

Table 2 Growth parameters during anaerobic fermentations on glucose mineral medium with ammonium sulphate (5 g l^{-1}) or mixture of ammonium sulphate (5 g l^{-1}) and sodium nitrate (1 g l^{-1}) as nitrogen sources

	Ammonium with casamino acids[a]	Ammonium and nitrate w/o casamino acids
Growth rate (h^{-1})	0.070–0.075	0.084 ± 0.006
q (mM $g_{dry\ weight}$ h^{-1})		
Glucose	1.47–1.60	4.08 ± 0.26
Ethanol	1.74–1.90	5.98 ± 0.42
Acetate	0.02–0.02	0.43 ± 0.02
Nitrate	–	0.46 ± 0.02
Y (g $g^{-1}_{glucose}$)		
Biomass	0.132–0.150	0.10 ± 0.005
Ethanol	0.34–0.35	0.35 ± 0.021
Acetate	0	0.033 ± 0.001

[a] Data from [28]

rate corresponded to the specific nitrate consumption rate, indicating that there is a strict correlation between nitrate utilization and acetate production (Table 2). Nevertheless, in this case, ethanol was the main product of glucose metabolism and its specific production rate was the highest obtained (Tables 1 and 2, for comparison). Also, the specific glucose consumption rate was the highest estimated, indicating that the redox unbalance negatively affected glucose metabolism and its fermentative efficiency on ammonium-based media. All these data indicate that the assimilation of nitrate greatly improves the ability to grow under anaerobic conditions, playing like a "valve" to balance the redox potential.

Analysis of involved enzymatic activities

The activity of the enzymes involved in nitrate assimilation was assayed in *D. bruxellensis* cells growing on nitrate-based media under aerobic as well as under anaerobic conditions. Nitrate reductase was found to use either NADPH and NADH in vitro as the electron donor (Table 3). The activities were higher in cell extracts from anaerobic growth conditions. The activity of nitrite reductase was undetectable in cell extracts from aerobic cultures, but an extremely low activity was assayed in cells grown under anaerobic conditions, again using either NADPH and NADH as the electron donor (data not shown). In order to understand if nitrate utilization can affect the enzyme activities leading to the increased acetic acid formation, we assayed acetaldehyde dehydrogenase (ACDH). Under aerobic conditions, the growth on nitrate-based media resulted in a decreased activity of ACDH (Table 3). Moreover, we observed that its affinity for NADP was higher than for NAD (see "Materials and methods"). Interestingly, we found that nitrate utilization under anaerobic conditions determined an increased specific activity of NADP-dependent ACDH, which was in fact higher in nitrate-grown cell extracts than in ammonium-grown ones (Table 3) and, in parallel, a decrease in the NAD-dependent ACDH activity. On the other hand, the activity of glucose 6-phosphate dehydrogenase (G6PDH), which is one of the main sources of NADPH, was lower in nitrate-grown cells (Table 3).

Discussion

The use of nitrate as nitrogen source determines in *D. bruxellensis* deep changes in the distribution of the final fermentation products. This is well evident under aerobic and under strictly anaerobic conditions. Under aerobic conditions, acetic acid resulted in fact as the main product of glucose metabolism, at the expense of the ethanol production (Table 1). In *D. bruxellensis*, nitrate and nitrite reductases can use, in vitro, NADH as well as NADPH as electron donors (Table 3), like most yeast nitrate reductases studied so far [29]. Nitrate assimilatory enzymes and alcohol dehydrogenase (ADH) can then compete for NADH, leading to a reduced ethanol synthesis. As a consequence, acetaldehyde can accumulate and trigger acetic acid formation. Apparently, this is what occurs under aerobic conditions. On the other hand, the stoichiometry of nitrate utilization under anaerobic conditions rather suggested that nitrate reductase could mainly require, in vivo, NADPH as the electron donor (Fig. 1). In fact, the amount of acetic acid produced in this condition corresponded exactly to the amount of NADPH required in the first nitrate-assimilating step, converting nitrate to nitrite through a NADPH-dependent nitrate reductase (10 mmol of nitrate assimilated and 10 mmol of acetic acid produced). It is noteworthy that acetic acid production has never been found under strict anaerobic conditions in *D. bruxellensis* [28], being its production associated to the oxygen concentration [9]. Furthermore, acetic acid-specific production and nitrate-specific consumption showed the

Table 3 Activity of enzymes involved in NAD(P)/NAD(P)H utilization during growth in aerobic or anaerobic conditions, on glucose mineral media with ammonium sulphate (5 g l^{-1}), sodium nitrate (6.43 g l^{-1}), or mixture of ammonium sulphate (5 g l^{-1}) and sodium nitrate (1 g l^{-1}) as nitrogen sources

Enzyme activity (U $mg^{-1}_{protein}$)	Cofactor specificity	Aerobiosis		Anaerobiosis	
		Ammonium	Nitrate	Ammonium	Ammonium and nitrate
Acetaldehyde dehydrogenase	$NADP^+$	0.23 ±0.007	0.139 ±0.004	0.065 ±0.005	0.115 ±0.003
	NAD^+	0.50 ±0.03	0.24 ±0.014	0.13	0.025 ±0.006
Glucose 6 phosphate dehydrogenase	NADPH	0.21 ±0.09[a]	0.10 ±0.007	0.16 ±0.006	0.025 ±0.003
Nitrate reductase	NADH	–	0.039 ±0.005	–	0.045 ±0.001
	NADPH	–	0.015 ±0.002	–	0.021 ±0.001

[a] Data from [13]

same rate, corroborating the strict correlation between nitrate utilization and acetate production. This link can indicate that a specific need of NADPH can be satisfied by a NADP-dependent ACDH activity. In agreement with this hypothesis, an increased level of NADP-dependent ACDH activity was found in nitrate-grown cells in comparison to the ammonium-grown cells (Table 3). Since this cofactor is required for cellular biosynthesis, in the case of nitrate assimilation it could become limiting in cell metabolism, and this can in turn stimulate metabolic pathways able to generate it, as acetic acid formation.

Another important aspect of the redox balance is linked to NAD/NADH ratio, which is an especially critical step under anaerobic conditions. NADH is in fact generated not only by the glycolytic pathway but also by amino acid synthesis. The most important reaction for reoxidation of this surplus NADH under anaerobic conditions is the production of glycerol (Fig. 1) [30]. In *D. bruxellensis*, a very low amount of this compound was produced under those conditions [28] and the inefficiency of this pathway has been indicated as the main cause of the "Custers effect" in this species [31]. Nitrate assimilation could then accomplish the role of balancing the redox status. The higher growth rate and higher specific ethanol production rate obtained in this work under anaerobic conditions (Table 2) indicate that nitrate utilization greatly improves the fermentative metabolism in *D. bruxellensis*. In *S. cerevisiae*, it has been calculated that 13 mmol of glycerol per gram of dry biomass are generated under anaerobic conditions, leading to the reoxidation of 13 mmol of NADH [1]. In *D. bruxellensis*, the assimilation of 10 mmol of nitrate to ammonium through a NADH-dependent nitrite reductase could result in the reoxidation of 30 mmol of NADH (Fig. 1), which fits well with the theoretical formation of 26 mmol of NADH generated from the biosynthesis of 2 g of dry biomass produced during its anaerobic growth.

In conclusion, nitrate assimilation determines in *D. bruxellensis* an improved ability to grow under anaerobic conditions and enhances its fermentative metabolism, working like a redox "valve" and, in this way, abolishing the "Custers effect". This offers a new strategy, besides the controlled aeration, for the employment of this yeast in industrial processes.

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Part III

Supporting information list (Submitted Manuscript-1)

Table S1: Primers used in this study

Table S2: Genes involved in galactose metabolism in *C. albicans*

Table S3: Protein ID numbers in *D. bruxellensis* CBS 2499 database.

Figure S1: Expression levels of *D. bruxellensis* CBS 2499 genes involved in different metabolic pathways. The transcription level on each sugar is relative to its expression level on ethanol.

Table S1: Primers used in this study.

Name	Sequence	Name	Sequence
CIT1 F	5′-CCACTTTGCATCCAATGGCAC-3′	*HXT*-B F	5′-CAAAACTGTTGGAGTGAACAACCC-3′
CIT1 R	5′-AGAGTTGGCAACTTCGCAAGC-3′	*HXT*-B R	5′-GTAAACACACACATTGTAAGAGCC-3′
SDH1 F	5′-ATGCGGTGAAAGTGCATGTGC-3′	*HXT*-C F	5′-GGAGCTGCACCAAACGATGC-3′
SDH1 R	5′-GACTCAAAGCCAATGTCTGCG-3′	*HXT*-C R	5′-ACACATCACACCAGCAAAAGCG-3′
SDH4 F	5′-TCACAGGGTCCAGACATTGG-3′	*HXT*-D F	5′-CAACTTCGCCTCTTCATTTATTGC-3′
SDH4 R	5′-CGATAATGCAACTTTCCCAGCC-3′	*HXT*-D R	5′-CTTGCTGATTAGGTTTGACACCG-3′
LSC2 F	5′-CTCCACCCCAAAGACCATCG-3′	*GAL1* F	5′-CTTTGGTTCATTTCACGACTGAGG-3′
LSC2 R	5′-GATGACAGGAACCTTCAAGCC-3′	*GAL1* R	5′-TTCACAATACAACTTCCAACTGCG-3′
MDH1 F	5′-GTGTTGCTGCTGATCTTTCGC-3′	*GAL7* F	5′-TGGATTGTTCCTGGTTCCACG-3′
MDH1 R	5′-CGTCTCTCGTCATTCCTGGC-3′	*GAL7* R	5′-CCAACTGTTGAAGTTTTGCAGCG-3′
COX5A F	5′-GTTGCTGGTCTTGGTATCTGC-3′	*GAL10* F	5′-AAATTGTTGGCCGTCGGAGTGG-3′
COX5A R	5′-CTGGGAGTAATGGCTGAATGG-3′	*GAL10* R	5′-CCTTGTGGATTCTTTGTGGTCC-3′
COX17 F	5′-TATGAAGAAAGATGACGGAAAGCC-3′	*ICL1* F	5′-TGATTTGCATAAGGGCATCTGC-3′
COX17 R	5′-CTTGAATCCATAACCAGCCATGC-3′	*ICL1* R	5′-GATGTCAGGATTCGTGCTTCC-3′
QCR2 F	5′-TCCCATACGAGGAAGCAGG-3′	*MLS1* F	5′-GATTCATGCTCTCCAACCTGG-3′
QCR2 R	5′-GGAAATTGCCGCCAAATAGCC-3′	*MLS1* R	5′-GCACAATCAGAACTGGAACACG-3′
AOX1 F	5′-CCTTACTTACACAAGATGCTTGC-3′	*PCK1* F	5′-GAAGTCGGAGTCACAGAACC-3′
AOX1 R	5′-CTTCTCTGTGCTTGGCCTCG-3′	*PCK1* R	5′-ATCCAGTATTGAGCAGCCAGG-3′
HXT-A F	5′-ATCACAGCCGCATTCTAGCAG-3′		
HXT-A R	5′-CCAAAGCGTTTAACAAAATCGTCC-3′		

Table S2: Genes involved in galactose metabolism in *C. albicans*. For each protein analyzed, the function (Rubio-Texeira, 2005), the ID number (in *D. bruxelesis* http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html database), the identity with the related protein in *C. albicans* (http://www.ncbi.nlm.nih.gov/) and the presence or absence of regulative motifs in the promoter sequences of *C. albicans* are indicated. Mismatches with the reference motif are underlined.

Gene	**Function**	**ID number in *D.bruxellensis***	**Identity (%)**	***MIG1*** *(C/G)(C/T)GG(G/A)G*	***CPH1*** TGTAACGTT
CaGAL1	Galactokinase	jgi|Dekbr2|8686|	51%	GTGGGG-CTGGAG-CCGGGG	TGTAACGTT
CaGAL2	Hexose transporter	jgi|Dekbr2|66929|	53%	GTGGAG	TGT**T**A**T**GTT -T**T**TA**T**CGTT
CaGAL3	Sensor	No hit found	-	-	-
CaGAL4			DNA binding site	-	T**TT**C**C**GTT TGTAA**GA**T**A** TGTA**C**CG**AC** TGTAA**TTG**T **AAC**AACGTT T**T**T**T**AC**T**TT **AC**TAA**A**GTT - **CAC**AACGTT TATAA**TT**TT C**G**T**C**AC**C**TT
CaGAL7	Galactose-1-phosphate uridylyltransferase	jgi|Dekbr2|26690|	64%	-	TGTAACGTT
CaGAL10	Uridine diphosphoglucose 4-epimerase	jgi|Dekbr2|26691|	56%	CTGGAG	TGTAACGTT- TGTAACGTT
CaCPH1	Transcription factor	jgi|Dekbr2|29960|	44%	-	-

Table S3: Protein ID numbers in *D. bruxellensis* database (http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html) related to genes in *S. cerevisiae* (*AOX1* sequence from *C. albicans*) are listed and the presence or absence of regulative motifs in the promoter sequences is indicated. The percent of identity matrix was created by ClustalW2.

Gene (s)	Functions	Protein ID in *D. bruxellensis* database	Identity (%)	*MIG1* motif(s) (C/G)(C/T)GG(G/A)G
ScCIT1	Citrate synthase activity	7923	66	GCGGAG
ScSDH1	Flavin adenine dinucleotide binding, Succinate dehydrogenase (ubiquinone) activity	25329	70	CCGGGG; CTGGGG; GTGGGG;
ScSDH4	Contributes to succinate dehydrogenase (ubiquinone) activity	26333	57	CCGGAG; GTGGAG
ScLSC2	Succinate-CoA ligase (ADP-forming) activity	8258	65	CCGGGG; CTGGAG; GTGGAG
ScMDH1	L-malate dehydrogenase activity; mRNA binding	8132	65	CCGGAG; GCGGAG
CaAOX1	Alternative oxidase	4905	62	No hit
ScCOX5A	Contributes to cytochrome-c oxidase activity	40584	61	CTGGGG; CTGGAG; GCGGGG
ScCOX17	Copper chaperone activity	123777	67	GCGGGG
ScQCR2	Contributes to ubiquinol-cytochrome-c reductase activity	3933	55	CCGGGG; GCGGGG;
ScGAL1	Galactokinase activity	8686	61	GTGGGG; GCGGAG
ScGAL7	UDP-glucose:hexose-1-phosphate uridylyltransferase activity	26690	66	CCGGGG; CTGGGG; CCGGAG
ScGAL10	Aldose 1-epimerase activity; UDP-glucose 4-epimerase activity	26691	63	CCGGGG; CTGGAG; GTGGGG
ScICL1	Isocitrate lyase activity	29922	671	CCGGGG
ScMLS1	Malate synthase activity	3634	63	GTGGGG; GTGGAG
ScPCK1	Phosphoenolpyruvate carboxykinase (ATP) activity	34770	67	CCGGAG; GTGGGG; GTGGAG
ScGAL2	Galactose and glucose transmembrane transporter activity	DbHXT A 25434	52	GTGGGG
		DbHXT B 26639	68	GCGGGG
		DbHXT C 8103	62	CCGGGG; CTGGAG; GTGGGG
		DbHXT D 66929	57	No hit
ScHXT1	Glucose, galactose, fructose, mannose, pentose transmembrane transport activity	DbHXT A 25434	56	GTGGGG
		DbHXT B 26639	53	GCGGGG
		DbHXT C 8103	80	CCGGGG; CTGGAG; GTGGGG
		DbHXT D 66929	61	No hit

Figure S1: Expression levels of *D. bruxellensis* CBS 2499 genes involved in different metabolic pathways. The transcription level on each sugar is relative to its expression level on ethanol.

A: genes involved in hexoses (glucose and galactose) transport; B: genes involved in galactose metabolism; C: genes involved in respiratory pathway; D: TCA genes; E: genes involved in glyoxylate cycle; F: gene involved in gluconeogenesis. Errors bars are the standard deviation of three replicates. The level of statistical significance was set at $p \leq 0.05$.