Metabolic Engineering 13 (2011) 263–271

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased *in vivo* flux through the pentose phosphate pathway

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ARTICLE INFO

Article history:
Received 9 August 2010
Received in revised form
22 December 2010
Accepted 26 January 2011
Available online 23 February 2011

Keywords:
Adaptive evolution
Wine yeast
NADPH
Fermentation rate
Aroma
Acetate

ABSTRACT

Amplification of the flux toward the pentose phosphate (PP) pathway might be of interest for various *S. cerevisiae* based industrial applications. We report an evolutionary engineering strategy based on a long-term batch culture on gluconate, a substrate that is poorly assimilated by *S. cerevisiae* cells and is metabolized by the PP pathway. After adaptation for various periods of time, we selected strains that had evolved a greater consumption capacity for gluconate. ^{13}C metabolic flux analysis on glucose revealed a redirection of carbon flux from glycolysis towards the PP pathway and a greater synthesis of lipids. The relative flux into the PP pathway was 17% for the evolved strain (ECA5) *versus* 11% for the parental strain (EC1118). During wine fermentation, the evolved strains displayed major metabolic changes, such as lower levels of acetate production, higher fermentation rates and enhanced production of aroma compounds. These represent a combination of novel traits, which are of great interest in the context of modern winemaking.

1. Introduction

The yeast *Saccharomyces cerevisiae* is one of the most widely used industrial organisms. It has been used in baking and in the production of fermented beverages for thousands of years and has more recently become an attractive cell factory for the production of bioethanol and various chemicals.

The pentose phosphate (PP) pathway plays an important role in yeast metabolism, generating NADPH reducing equivalents, together with various precursors for the biosynthesis of nucleic acids and amino acids. This pathway also protects yeast cells against oxidative stress, as NADPH is an essential cofactor for the glutathione- and thioredoxin-dependent enzymes that defend cells against oxidative damage (Izawa et al., 1998; Shenton and Grant, 2003; Slekar et al., 1996).

The importance of this pathway for various biotechnological applications has been emphasized in recent years. For example, recombinant *S. cerevisiae* strains able to ferment biomass-derived pentose sugars have been developed by expressing heterologous xylose reductase and xylitol dehydrogenase genes that metabolize xylose *via* the PP pathway (Jeffries and Jin, 2004). The amount of xylose metabolized and the production levels of ethanol have been increased by modifying the amounts of PP pathway enzymes (Matsushika et al., 2009).

The PP pathway is also an interesting, yet unexplored, target to develop wine yeasts with new properties. Rerouting of carbon flux through the PP pathway may result in a decreased yield of ethanol because one of the carbon atoms of each glucose molecule entering the PP pathway is excreted as CO_2. The development of low-alcohol yeasts is a current challenge to the wine industry since wines produced by modern winemaking practices have excessively high contents of ethanol (Heux et al., 2008). The other effects of an increased flux through the PP pathway on yeast metabolism are predictable. In particular, these include a reduction of acetate formation, due to the major role of both the PP and acetate pathways in the production of NADPH (Grabowska and Chelstowska, 2003; Saint-Prix et al., 2004). Although wine yeasts have been selected for low acetate production, excessive production — which is detrimental for wine quality — is still observed, with some commercial strains having otherwise good technological features. Moreover, winemaking practices, such as grape must clarification usually favor the production of acetic acid (Ribéreau-Gayon et al., 2005).

During the last 15 years, many efforts have been made to engineer wine yeast strains with new characteristics (Dequin, 2001; Donalies et al., 2008; Husnik et al., 2006; Schuller and Casal, 2005). For example, several attempts have been made to divert sugar metabolism towards products other than ethanol (Cambon et al., 2006; Eglinton et al., 2002; Ehsani et al., 2009;

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doi:10.1016/j.ymben.2011.01.008

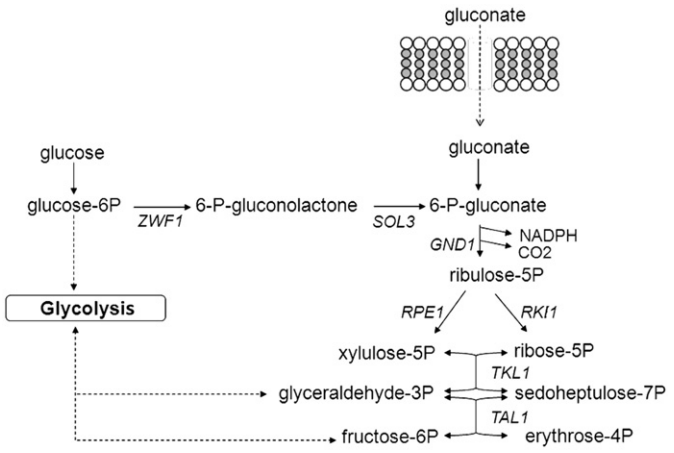

Fig. 1. Gluconate metabolism in *S. cerevisiae.*

Malherbe et al., 2003; Michnick et al., 1997; Nevoigt and Stahl, 1996; Remize et al., 2001; Remize et al., 1999) or to reduce the yield of acetate (Cambon et al., 2006; Remize et al., 2000). However, the use of such approaches is currently limited by poor public acceptance of GMO-based food and beverages. Strategies based on evolutionary engineering are attractive, because they may generate improved strains that in the short-term can be used in industry. Evolution-based approaches have proven valuable — with and without coupling to metabolic engineering — for the generation of new strains with specific phenotypes, such as resistance to various stresses or efficient substrate utilization (Becker and Boles, 2003; Cakar et al., 2009, 2005; Kuyper et al., 2004; Sonderegger and Sauer, 2003; Sonderegger et al., 2004; Wisselink et al., 2007, 2009).

The objective of this study was to design an evolution-based strategy to increase flux through the PP pathway and to evaluate the potential of this strategy during wine fermentation. We developed a strategy based on long-term batch cultivation on δ-gluconolactone, which is metabolized *via* the PP pathway after phosphorylation to generate 6-phosphogluconate (Sinha and Maitra, 1992) (Fig. 1). This hexose sugar is a weak carbon source for *S. cerevisiae* and its assimilation requires oxygen (Gancedo and Delgado, 1984; Sinha and Maitra, 1992). Using this evolutionary engineering strategy, we progressively obtained several strains with improved assimilation of gluconate. These strains displayed various novel phenotypes, including modified adhesion properties, improved fermentation performances, lower levels of acetate production and higher levels of aroma production during wine fermentation. Flux distribution analysis revealed that the evolved ECA5 strain displayed a 1.5 times greater flux through the PP pathway and higher levels of lipid production.

2. Materials and methods

2.1. Yeast strain

The wine yeast strain EC1118 was used as the parental strain. the genome of which has recently been sequenced (Novo et al., 2009). *S. cerevisiae* strains were propagated in rich YPD medium (1% Bacto yeast extract, 2% bactopeptone, 2% glucose).

2.2. Growth conditions

2.2.1. Adaptation experiment

The adaptation procedure was based on a long-term serial transfer procedure. The original strain was taken from a −80 °C stock and spread onto a YPD plate. The biomass from this plate was used to inoculate a tube of 50 ml containing 20 ml of YPD broth. After an overnight culture at 28 °C, we used the resulting cell suspension to inoculate two capped tubes (13 ml), each containing 5 ml of SD gluconate medium (0.67% nitrogen base, 2% gluconate) pH 6 (adjusted with a solution of NaOH 32%) to an OD_{600} of 0.1. The tubes were then incubated at 28 °C, with shaking (250 rpm). When the OD_{600} reached 0.8, fresh medium was inoculated to an OD_{600} of 0.1. The population was tested for assimilation of gluconate at regular intervals. Clones isolated from adaptation cultures using a streak-plate isolation procedure and exhibiting the same properties as the total adapted population were selected as evolved strains and stored at −80 °C in 40% glycerol.

2.2.2. Determination of growth parameters

We incubated 50 ml aerobic batch cultures in 250 ml flasks on a rotary shaker (300 rpm), at 28 °C. The medium used was minimal SD medium containing 6.7 g l^{-1} yeast nitrogen base without amino acids (Difco) and 20 g l^{-1} of glucose (pH 5.4) or gluconate (pH 6.5). The pH of the synthetic medium was adjusted to 6.5 when gluconate was used as the carbon source by addition of 32% NaOH solution. The medium was inoculated to an OD_{600} of 0.1, from an overnight culture in 25 ml YPD medium in 50 ml Falcon tubes. The growth rate was determined in an aerobic batch culture, by log-linear regression analyses of OD_{600} against time, with growth rate (μmax) as the regression coefficient. Doubling times (G) were calculated using the following equation: $G = \ln 2/\mu max$.

2.2.3. Oxygenation

Aerobic fermentations were performed in 300 ml fermentors containing 270 ml of SD gluconate, at 28 °C. Fermentors were supplied with 6 and 30 mg l^{-1} of oxygen and agitation was maintained by gentle continuous magnetic stirring at 500 rpm. Dissolved oxygen was measured by an oxygen probe (Clark oxygen probe type YSI5331; Gilson; Middleton, WI, USA). Anaerobic cultures were performed in serum tubing vials of 125 ml containing 100 ml of medium and hermetically closed. Anaerobiosis was obtained by bubbling argon in the medium for 15 min at 95 °C and was monitored *via* the addition of 2 mg l^{-1} resazurin.

2.2.4. Wine fermentation conditions

Batch fermentation experiments were carried out in synthetic medium (MS), which mimics standard grape juice and has been described previously (Bely and Sablayrolles, 1990). The MS medium used in this study contained 240 g l^{-1} glucose, 6 g l^{-1} malic acid, 6 g l^{-1} citric acid, and a nitrogen source composed of 120 mg l^{-1} nitrogen from ammonium chloride and 340 mg l^{-1} nitrogen from amino acids. The following amino acids were used: tyrosine (final concentration 18.5 mg l^{-1}), tryptophane (179 mg l^{-1}), isoleucine (32.7 mg l^{-1}) aspartate (44.5 mg l^{-1}), glutamate (123 mg l^{-1}), arginine (375 mg l^{-1}), leucine (48.5 mg l^{-1}), threonine (76 mg l^{-1}), glycine (18.5 mg l^{-1}), glutamine (505 mg l^{-1}), alanine (145.5 mg l^{-1}), valine (44.5 mg l^{-1}), methionine (31.5 mg l^{-1}), phenylalanine (38 mg l^{-1}), serine (78.5 mg l^{-1}), histidine (32 mg l^{-1}), lysine (17 mg l^{-1}), cysteine (13 mg l^{-1}), and proline (613 mg l^{-1}).

To satisfy the lipid requirements of yeast cells during anaerobic growth, MS medium was supplemented with 7.5 mg l^{-1} ergosterol, 0.21 g l^{-1} Tween 80, and 2.5 mg l^{-1} oleic acid. The pH of the resulting medium was 3.3. We inoculated 250 ml flasks containing 50 ml YPD to an OD_{600} of 0.1 with cells grown overnight in YPD and incubated them for 8 h at 28 or 18 °C. The fermentation culture in MS medium was inoculated with 0.5×10^6 cells per ml and incubated at 28 °C with continuous stirring (350 rpm) in fermentors