# Beer with Reduced Ethanol Content Produced Using *Saccharomyces cerevisiae* Yeasts Deficient in Various Tricarboxylic Acid Cycle Enzymes

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**ABSTRACT**

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A collection of *Saccharomyces cerevisiae* strains deficient in the tricarboxylic acid cycle enzymes activities has been examined for the production of beer with reduced ethanol content. Strains deficient in fumarase and α-ketoglutarate dehydrogenase encoded by the genes *FUM1* (0.48%), *KGD1* (0.42%) and *KGD2* (0.48%) made non-alcoholic beers with an alcohol content lower than 0.5% (v/v). The rest of the yeast mutants also gave rise to low-alcoholic beers but with a slightly elevated ethanol concentration (mostly in the range of 0.57–0.84% and 1.64% for the *lip5* mutant). Low ethanol content was compensated by the considerable increase of organic acids (citrate succinate, fumarate, and malate). In addition, some of the mutants released high levels of lactic acid (144 (*fum1*), 622 (*kgd1*) and 495 (*kgd2*) mg/L). Lactic acid protects beers against contamination and masks an unacceptable worty off-flavour.

**Key words:** beer, *Saccharomyces cerevisiae*, tricarboxylic acid cycle.

## INTRODUCTION

Two nuclear mutants of *Saccharomyces cerevisiae* deficient in tricarboxylic acid cycle (TCA) genes have been reported as suitable strains for the production of non-alcoholic beer[11]. Basic parameters of beer produced by the mutant yeast, such as real residual extract and concentration of some organic acids, with the exception of ethanol content, were comparable to standard brews. Organic acids of the TCA cycle provide antioxidant or prooxidant activity in the beer[13] as well as giving an increase in the microbial stability of the final product. These substances are also important with respect to the taste of the product. The ratio of succinate, malate or fumarate affects the taste in a significant way. Magarifuchi et al.[9] observed that a strain with a disrupted *FUM1* gene displayed no differences in extracellular malate production, whereas fumarate production increased, and the production of succinate was reduced, when compared with the control strain. Sake, a Japanese rice wine fermented with a strain bearing a disruption in the aconitase gene *ACO1,* contained a two-fold higher concentration of malate and a two-fold lower concentration of succinate than sake produced using a wild-type strain. The *KGDI::URA3* disruptant exhibited lower succinate production in the earlier phase of the fermentation, while the *FUMI::URA3* strain yielded lower succinate production later in the fermentation, due to a switch between the oxidative and reductive part of the bio-process[2]. When the *IDH1* or *IDH2* genes encoding $NAD^+$-dependent isocitrate dehydrogenase were disrupted, an increased production of citrate, malate and acetate occurred. Succinate production was reduced to approximately half of the parent strain[3].

Eight enzymes of the TCA cycle are encoded by at least 15 different nuclear genes in *Saccharomyces cerevisiae*[14]. Mutants in the respective open reading frames (ORF's) exhibit growth defects in non-fermentable carbon sources such as ethanol, acetate, glycerol, lactate or pyruvate[18]. A tendency to acidify the CK medium containing bromcresol purple and to form colonies surrounded by yellow zones, enables simple isolation of these groups of mutants, named "*aci*$^+$"[5]. Genetic characterization of *Saccharomyces cerevisiae aci*$^+$ mutants revealed that these types of mutations are localized in the open reading frames (ORF) operating in the citrate or glyoxalate cycle (*SDH3*), or in the *YHR003c* ORF with an unknown function[5,10].

The aim of this work was to investigate the potential of a TCA cycle mutant in the production of low-alcoholic beer from hopped-wort and to compare the analytical parameters as well as properties of such beers with those fermented by a conventional brewing yeast. To achieve this goal we studied a set of yeast deficient in the 14 TCA cycle genes, yeast deficient in a gene encoding the alcohol dehydrogenase subunit *ADH1* or a yeast mutant in ORF for *LIP5*[16] with silenced tricarboxylic acid cycle functions.

## MATERIALS AND METHODS

### Microorganisms

*Saccharomyces cerevisiae* BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0*

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**Table I.** Basic parameters of the wort and beers produced by the strains bearing deletions of the genes listed below. *S. cerevisiae* BY4734 (laboratory standard, control) and *S. cerevisiae* W96 (brewing standard) after 9 days of fermentation at 9°C.

| Strain | Reducing saccharides (g/L) | Ethanol (% v/v) | pH | Total nitrogen (g/L) | Total polyphenols (g/L) | Colour (EBC U) | Bitterness (BU) |
|---|---|---|---|---|---|---|---|
| Δ*ACO1* | 55.8 | 0.68 | 3.2 | 0.88 | 169 | 18 | 31 |
| Δ*CIT1* | 45.2 | 0.71 | 3.1 | 0.82 | 175 | 16 | 32 |
| Δ*CIT3* | 44.3 | 0.76 | 3.1 | 0.82 | 177 | 16 | 29 |
| Δ*FUM1* | 51.5 | 0.48 | 3.3 | 0.84 | 162 | 17 | 30 |
| Δ*IDH1* | 51.8 | 0.84 | 3.2 | 0.85 | 182 | 17 | 31 |
| Δ*IDH2* | 44.6 | 0.81 | 3.2 | 0.81 | 185 | 16 | 30 |
| Δ*KGD1* | 54.4 | 0.42 | 3.1 | 0.87 | 177 | 17 | 32 |
| Δ*KGD2* | 53.9 | 0.48 | 3.1 | 0.87 | 172 | 17 | 31 |
| Δ*LSC1* | 45.5 | 0.63 | 3.0 | 0.82 | 165 | 16 | 31 |
| Δ*LSC2* | 48.2 | 0.60 | 3.2 | 0.83 | 166 | 16 | 30 |
| Δ*MDH1* | 51.1 | 0.75 | 3.3 | 0.85 | 182 | 17 | 31 |
| Δ*SDH1* | 53.1 | 0.67 | 3.1 | 0.86 | 164 | 17 | 31 |
| Δ*SDH2* | 51.1 | 0.57 | 3.2 | 0.85 | 177 | 17 | 30 |
| Δ*SDH3* | 51.3 | 0.60 | 3.1 | 0.86 | 174 | 17 | 31 |
| Δ*LIP5* | 35.6 | 1.64 | 4.1 | 0.77 | 162 | 16 | 28 |
| Δ*ADH1* | 51.6 | 0.62 | 3.1 | 0.85 | 181 | 17 | 30 |
| BY4734 | 16.2 | 3.56 | 4.5 | 0.70 | 155 | 15 | 25 |
| W96 | 8.9 | 4.58 | 4.3 | 0.64 | 140 | 14 | 23 |
| Wort | 91.0 | … | 5.6 | 10.5 | 260 | 23 | 38 |

*ura3Δ0 /ura3Δ0*) from the Saccharomyces Genome Deletion Project, in which individual ORF's for *ACO1, CIT1, CIT3, FUM1, IDH1, IDH2, KGD1, KGD2, LSC1, LSC2, MDH1, SDH1, SDH2, SDH3, LIP5* or *ADH1* were replaced with *KanMX4* gene[19]. For more information please see http://www-sequence.stanford.edu/group/yeast_deletion_project/. The strain *Saccharomyces cerevisiae* W96, used as a model of standard brewing yeast, was from the collection of the Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, SR collection. The strain BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0 /ura3Δ0*) a model of a laboratory *Saccharomyces cerevisiae* strain was from the Department of Biochemistry, Faculty of Natural Science, Comenius University, Bratislava, SR collection.

Yeast strains were stored on YPD medium containing 1% (w/v) peptone, 1% (w/v) yeast extract and 2% (w/v) glucose.

### Genetic methods

Strains from the Saccharomyces Genome Deletion Project were converted to the haploid form after sporulation and spore dissection[15]. The presence of a deleted gene was determined according to growth ability on YPD medium containing geneticin sulphate—G-418 (2.25 mg/mL) and debilitated growth on GE medium (glycerol, 3% (w/v); ethanol, 2% (w/v); peptone, 1% (w/v); yeast extract, 1% (w/v) and agar, 2% (w/v)). Only colonies from full tetrads with the phenotype segregation in a 2:2 ratio were selected for the fermentation experiments.

### Cultivation and fermentation

Inoculation was performed by adding yeast to the pre-fermentation medium containing 100 g/L glucose; 5 g/L $(NH_4)_2SO_4$; 3 g/L yeast extract; 2 g/L $KH_2PO_4$; 1 g/L $MgSO_4.7H_2O$; 0.1 g/L $CaCl_2$ and 0.1 g/L NaCl, pH was adjusted to 5.8. Cells were cultivated for 72 h at 28°C under semi-aerobic conditions on a rotary shaker. Prior to fermentation, hopped wort from a local brewery with a concentration of reducing saccharides of 91 g/L was re-inoculated with 2% (v/v) of a rich suspension of cells. The batch fermentation was carried out in 500 mL bottles with fermentation seals, filled with 400 mL of hopped-wort at 9°C.

### Analytical methods

Ethanol was detected by GC with a PORAPAK Q filled column (3 m × 1.2 mm) with nitrogen as a carrier gas and FID. Temperature was 180°C; injection temperature was 230°C; sample volume was 1 µL. Organic acids were determined by isotachophoresis on a ZKI 01 Analyser with conductivity detector. Electrolyte systems of the following compositions were applied: leading electrolyte – 0.01 M HCl, 0.1% (w/v) methylhydroxyethylcellulose, pH 3 and β-alanine as terminating electrolyte: 0.05 M $CH_3COOH$. The samples were analysed at a driving current of 250 µA[6]. Total nitrogen, total polyphenols, colour and bitterness were all measured according to current European Brewing Convention (EBC) methods[1]. The concentration of reducing saccharides was determined spectrophotometrically after reaction with 3,5-dinitrosalicylic acid at 530 nm[17].

## RESULTS AND DISCUSSION

A collection of *Saccharomyces cerevisiae* TCA cycle mutants has been characterized for the production of beer with a reduced ethanol content. *ACO1, CIT1, CIT3, FUM1, IDH1, IDH2, KGD1, KGD2, LSC1, LSC2, MDH1, SDH1, SDH2, SDH3* (deletions in the genes of TCA cycle), *ADH1* and *LIP5* were examined for alcohol-free beer production. Their potential was compared with the laboratory strain BY4743 (control), as well as with the standard brewing strain W96.

In general, beers prepared by the strains with clean deletions in the mentioned genes had a lower ethanol content, which was accompanied by an increasing concentration of lactate (Table I, Fig. 1A). This trend corre-

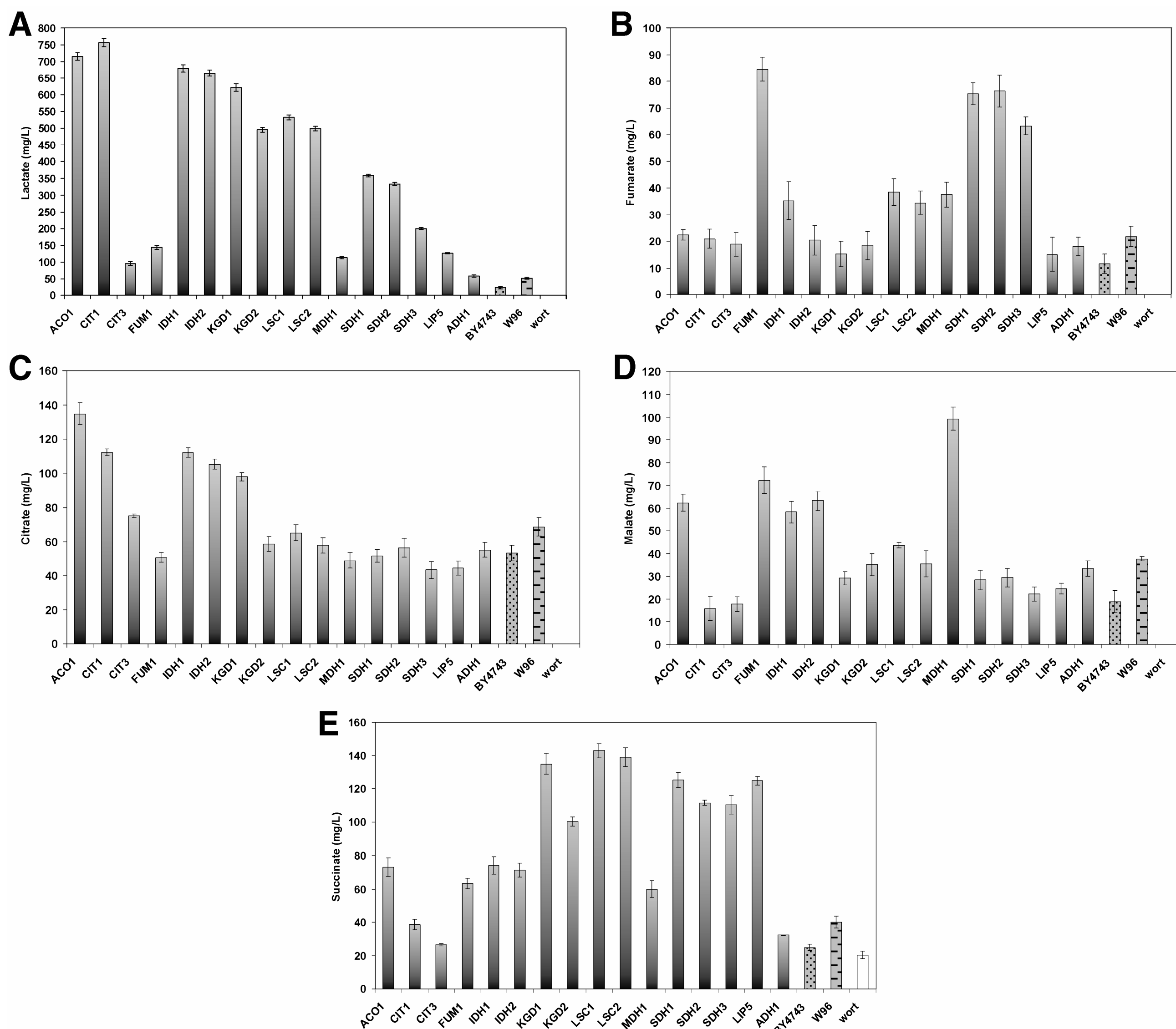


**Fig. 1.** Concentration of acids in the worts and beers produced by the strains with clean deletions in the genes listed below. *S. cerevisiae* BY4734 (laboratory standard, control) and *S. cerevisiae* W96 (brewing standard) after 9 days of fermentation at 9°C. A, lactic acid; B, fumaric acid; C, citric acid; D, malic acid; E, succinic acid.

sponded with slower growth in non-fermentable carbon sources, typical for yeast with defects in the activity of TCA cycle enzymes. The *ADH1* knock out strain also yielded a lower ethanol content, but acid levels were not significantly altered. Each fermented hopped wort with reduced ethanol content exhibited higher residual extract. Colour and bitterness were more intense in comparison to conventional alcoholic beers (W96) or control (BY4743) (Table I).

According to European legislation, non-alcoholic (alcohol-free) beer may not contain more than 0.5% (v/v) ethanol. Beer with an ethanol content in the range 0.5-1.2% (v/v) is termed low-alcoholic. Beers produced by strains deficient in the activity of TCA cycle enzymes and alcohol dehydrogenase were low-alcoholic and contained ethanol from 0.57% (v/v) (*sdh2*) to 0.84% (v/v) (*idh1*). The exceptions were mutants in *FUM1*, *KGD1* and *KGD2* genes, which produced 0.42% and 0.48% (v/v) ethanol, respectively. The strain with the knocked out gene for lipoic acid synthase produced slightly more ethanol, due to TCA cycle weakening. Brewing yeast W96 and control BY4743 gave the expected amounts of ethanol (4.58% (v/v) and 3.56% (v/v) respectively) after 9 days of fermentation. The concentration of reducing saccharides was much higher in the beers made by the strains whose genes for the TCA cycle enzymes were knocked out, when compared with BY4743 or W96. Concentrations between 44.3 and 55.8 g/L indicate that the real attenuation ranged from 51.3% to 38.7% under the given conditions. In the case of the *LIP5* deletion mutant, this parameter was considerably higher (60.9%) and corresponded to the relatively higher ethanol content in the sample.

The pH values of the beers fermented by deletion TCA mutants were one unit lower (3.0–3.3), except for the *LIP5* mutant, compared to the samples fermented using the BY4743 or W96 strain. The typical pH of 4.3 was

achieved only in the W96 sample. Utilization of nitrogen was less intense in samples where the strains with defects in the TCA cycle were used. After 9 days of fermentation, the level of total nitrogen decreased from 10.5 g/L at the beginning to 0.81–0.88 g/L, while in beers fermented using the controls, the final levels of nitrogen were 0.64 g/L (W96) and 0.7 g/L (BY4743). The measured concentrations of the total polyphenols were not easy to interpret, but a general trend can be observed. The samples fermented by the TCA cycle mutants contained more polyphenolic substances due to the lower attenuation. The same trend can be seen in the bitterness of the beers, the samples fermented by the strains with knocked out TCA cycle genes contained slightly higher amounts of hop components, such as iso-α acids, giving the beer its bitterness. The intensive color (16–18 EBC units) relates to a lower utilization of carbonic and nitrogenous sources from the hopped wort. The color of the sample fermented using the W96 strain was 14 EBC units, very close to the color of sample fermented using BY4743 (15 EBC units). The two-fold higher color of the wort was caused by the tendency of the sugars to caramelize during sterilization.

Due to the significant decrease in pH, we analyzed the concentrations of five organic acids (citric, succinic, fumaric, malic and lactic acid) at the beginning (Fig. 1A-E – wort) and at the end of the fermentation (summarized in Fig. 1A-E). Their concentrations in the samples fermented by the standards W96 and BY4743 were negligible. The highest content of citric acid was detected in the samples fermented by the strains with clean deletions in the *CIT1* and *IDH1* genes. The largest amount of succinate was detected in the beers produced by the yeast with knocked out succinate dehydrogenase (encoded by *SDH1, SDH2, SDH3*) and succinyl-CoA ligase (synthase) activity (encoded by *LSC1, LSC2*). Similarly, the highest concentration of fumaric acid was seen in the Δ*FUM1* sample and the major part of the organic acids of the TCA cycle in the Δ*MDH1* sample was represented by malate.

Lactate was mostly formed when the genes for aconitase, citrate synthase or $NAD^+$-dependent isocitrate dehydrogenase were knocked out (Fig. 1A). Although 756 mg/L of lactic acid was detected in the Δ*CIT1* sample, only 113 mg/L was present in sample Δ*MDH1*. Navratil *et al.*[11] already reported the presence of lactic acid in non-alcoholic beers made by TCA mutants. They suggested that lactate is formed when pH drops to 2.01–3.25 and alcohol dehydrogenase is deactivated. Under these circumstances, pyruvate is reduced to lactate by lactate dehydrogenase (cytochrome *b*2 enzyme). However, in yeast, D-lactate is synthesized from S-lactoyl-glutathione via glyoxalase I in the methylglyoxal pathway in cytosol where D-lactate dehydrogenase (Dld3) is localized[4,12]. *LD3*D-lactate dehydrogenase is a part of the retrograde regulon, which consists of genes whose expression is stimulated by damage to the mitochondria[8]. The fate of D-lactate is far from being completely elucidated, because two mitochondrial D-lactate dehydrogenases have been reported[4]. In addition, several TCA mutants *CIT1, MDH1 LSC1, LSC2* are capable of utilizing lactate as a carbon source[14]. The *LIP5* mutant exhibited a moderately increased production of succinate (125 mg/L) and lactate (127 mg/L).

There are few reports about the applications of yeast with impaired functions in the TCA cycle enzymes in brewing. The work of Navrátil *et al.*[11] describes the production of non-alcoholic beers by means of batch and continuous fermentation with both, free and immobilized cells. The batch fermentation was carried out at a higher temperature (15°C for continuous process) and on hopped wort of a higher original extract. Hence, they described a more dramatic degree of fermentation. The utilization of nitrogen was even higher than in the beer fermented by the standard strain (W96). The pH values ranged from 2.11 to 3.25, despite a nearly equal concentration of lactic acid in two of their samples. In the remaining three samples, the sum of detected organic acids was lower than in the sample produced using the W96 strain with a pH of 4.11.

## CONCLUSIONS

A collection of *Saccharomyces cerevisiae* strains deficient in 14 genes directly encoding enzymes from the TCA cycle and alcohol dehydrogenase (*ADH1*) has been characterized for the production of beer with a reduced ethanol content. Non-alcoholic beer (with an ethanol content below 0.5% (v/v)) was only made by the yeast with disruptions in the *FUM1*, *KGD1* and *KGD2* genes, corresponding to fumarase and α-ketoglutarate dehydrogenase. The other 11 beers were low-alcoholic (up to 1.2% (v/v) of ethanol). The *LIP5* mutant with a dysfunction in lipoic acid synthase gave rise to beer with an ethanol content of 1.64% (v/v), although it has potential to produce a low-alcoholic beer after some modifications in the engineering parameters of the fermentation. In the sample prepared with knocked-out *ADH1*, the ethanol content reached a value of 0.62% (v/v). However, this value, due to the presence of four other isoforms, may not be stable in other strains of brewing yeast with the same mutation[7]. Considerably higher concentrations of residual saccharides in the beer, or more generally high residual extract values, could pose a problem, but the presence of organic acids, such as lactate and citrate, has a protective effect against bacterial contamination. Lactic acid also helps to mask the unacceptable worty flavour of non-alcoholic beers and along with the other acids serves as an important sensorial factor with a positive effect on the taste and flavour of the final product. Nevertheless, the most important fact is that the described strains enabled us to produce non-alcoholic beers at relatively identical conditions as standard alcoholic beers. Pre-acidification of wort or maturation can be omitted completely, which can bring a significant economic savings and substrates with a lower original extract are favourable for the proliferation of such strains.

It is important to note that all 14 described strains with deletions in TCA cycle genes, as well as mutants mentioned in other works[11], were prepared from laboratory strains, mostly because of their well understood phenotypes. However, a considerable amount of diacetyl and absence of many essential esters, such as ethyl acetate and amyl acetate, can negatively affect the taste of the product. For these reasons, preparation of a hybrid between

brewing yeast and a laboratory strain, carrying all the genetic properties responsible for the positive taste and flavour of the beverage, and deficient in the TCA cycle enzyme genes, would be beneficial.


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