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Glycerol production from sugars with phosphoglycerate mutase-deficient *Saccharomyces cerevisiae* partially resistant to glucose repression

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SUMMARY

Mutants partially resistant to the repressive effect of glucose have been isolated from a *Saccharomyces cerevisiae* strain totally deficient in phosphoglycerate mutase activity (EC 5.4.2.1) by a selection procedure involving the catabolite-repressive effect of 5-thio-D-glucose (5TG). These mutants are able to resist glucose concentrations up to 15 g L^{-1} and exhibit several non-repressed metabolic pathways such as gluconeogenesis, glyoxylic shunt or mitochondrial respiratory chain. Moreover, when these mutants are grown in aerobiosis on ethanol and glucose as sole substrates, glucose is mainly converted into glycerol in order to maintain a normal redox balance. Optimal glucose and oxygen concentrations have been defined for resting cells in order to obtain a glycerol yield from glucose close to 100%. The physiological characteristics of one of these mutants led us to consider an application of this yeast strain in reducing the ethanol content of wines previously lowered in ethanol content by physical processes.

INTRODUCTION

Phosphoglyceromutase (EC 5.4.2.1) converts 3-phosphoglycerate into 2-phosphoglycerate in the lower part of the glycolytic pathway. The corresponding gene *GPM1* in *Saccharomyces cerevisiae* has been cloned [21], sequenced and mapped on chromosome XI [14]. Deletion mutants have been constructed by substitution of the chromosomal copy of *GPM1* by a *URA3* marker gene [21]. These mutants do not grow on glucose, ethanol or glycerol as sole carbon sources, like other glycolytic mutants lacking enzyme activity involved in both glycolysis and gluconeogenesis, and that cannot be by-passed [4,5]. Only a combination of glycerol and ethanol promotes growth: under these conditions, gluconeogenesis is fed by glycerol while energy is provided by respiration of ethanol. At a concentration of 2%, glucose inhibited growth of the *gpm1* deletion mutant on medium containing glycerol and ethanol. This feature has been explained by carbon catabolite repression [12] and/or inactivation exerted by glucose [15] inhibiting the utilization of glycerol and ethanol, glucose not being fermented itself because of the block in the phosphoglycerate mutase reaction. In order to study this phenomenon a deletion mutant lacking most of the *GPM1* structural gene and unable to revert has been used to select mutants resistant to the inhibitory and repressive effects of glucose. These mutants were able to grow on a mixture of glucose and ethanol as sole substrates: they consumed up to 2% glucose and metabolized it mainly into glycerol with a high productivity (from 75 to 100%).

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MATERIALS AND METHODS

Strains. The following strain of *S. cerevisiae* was used: HC38-3D (*Matα gpm1::URA3 ura3-52*) constructed by Rodicio and Heinisch [21].

Media and growth conditions. YEP (2% peptone, 1% yeast extract) supplemented with 2% glucose (YEPD), 3% (v/v) ethanol (YEPE), 3% (w/v) glycerol (YEPG), or 3% (v/v) ethanol plus 3% (w/v) glycerol (YEPEG) were used as rich media. Glucose was used instead of glycerol in the range of 0.2 to 2% in presence of 3% (v/v) ethanol ($YEPD_nE$ medium where n represents the amount of glucose added to the medium in per cent). Media were solidified with 1.5% agar. Liquid cultures were grown on a rotary shaker at 28 °C, or in a 1.5-L aerobic fermentor (SGI, Toulouse, France).

The following medium (RC) was used with resting cells in the 1.5-L aerobic fermentor: potassium phosphate buffer 50 mM (pH 6.0), 3% (v/v) ethanol, glucose between 0.2 and 2%.

Biomass determination. The number of cells was determined with an electronic particle counter (model ZBI; Coulter-Counter Coultronics, Margency, France).

Ethanol, glycerol and glucose determinations. Ethanol and glycerol determinations were carried out using commercial enzymatic kits (Boehringer-Mannheim, Germany) by measuring NADH formation at 340 nm during oxidation of ethanol to acetic acid by alcohol dehydrogenase/acetaldehyde dehydrogenase, and NADH consumption at 340 nm during reduction of glycerol to L-lactate by glycerokinase/pyruvate kinase/L-lactate dehydrogenase respectively. Glucose was determined as reducing sugar by using dinitrosalicylic reagent according to Miller [19].

Glucose transport assay. Yeast cells were harvested during early-exponential growth phase by centrifugation. The high-affinity sugar transport activity was measured according to Bisson and Fraenkel [1] by incubating 5% (dry weight) cell suspensions in 0.05 M KH_2PO_4 buffer (pH 6.0) with 1 mM D-[U-^{14}C]glucose (148 kBq μmol^{-1}) (purchased from Amersham Corp., Bucks, UK with a radiochemical purity of 99.5%). Incubation was performed at 28 °C, for 5 s. Samples (50 μl) were removed and washed with ice-cold distilled water on glass fiber filters. In order to estimate substrate binding to cells, the cells were killed by heating for 90 s at 100 °C, according to Postma et al. [20], and sugar uptake experiments were performed as described previously on these killed cells.

Preparation of crude extracts and enzyme assays. Crude extracts were prepared as described by Ciriacy and Breitenbach [5] using glass beads (0.4–0.5 mm diameter) to break the cells. The supernatant resulting from a 2000 *g*/10 min centrifugation was used for all enzyme determinations described below.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.49) was assayed in the direction of oxaloacetate formation (reverse assay) according to Gancedo and Schwerzmann [13]. Phosphoglycerate mutase (EC 5.4.2.1) and isocitrate lyase (EC 4.1.3.1) were assayed according to Maitra and Lobo [17] and Dixon and Kornberg [8] respectively.

All assays were performed at 30 °C. Specific activities were computed as mU (nmoles of substrate transformed per min) based on protein determination according to the BCA protein assay reagent kit (Pierce Chemicals, Rockford, USA), using bovine serum albumin as a standard.

Cytochromes determinations. The absorption spectra of cytochromes reduced by sodium dithionite addition were obtained by the technique described by Claisse et al. [7]. Readings were made directly on cell slurries 0.7 mm thick using an Uvikon 810 spectrophotometer (Kontron, Switzerland). Spectra were recorded in liquid nitrogen at −196 °C. Cytochrome absorbance at maximum absorption was corrected by the influence of the other cytochromes using the calculation procedure described by Claisse and Pajot [6].

Mutagenesis and mutant selection. Yeast cells (10^7 cells per ml) were mutagenized at 28 °C in 0.1 M potassium phosphate buffer (pH 7.0) containing 1% ethyl methane sulfonate (EMS) to obtain a survival frequency between 2 and 10%. After incubation, the cells were centrifugated and resuspended in 5% sodium thiosulfate for 10 min, and washed three times in 0.1 M potassium phosphate buffer (pH 7.0). Mutants resistant to the repressive effects of the glucose analog 5-thio-D-glucose (5TG) were obtained according to Egilsson et al. [9] by exposing mutagenized cells on selective agar medium YEPEG containing 4 mM of 5-thio-D-glucose at cell densities of 10^8 cells per plate and picking growing colonies. These colonies were tested for resistance to repression by high concentrations of glucose on YEPEG medium.

RESULTS AND DISCUSSION

Isolation of derepressed mutants of gpm1 *deletion strain*

Derepressed mutants of the *gpm1* deletion strain HC38-3D were obtained by mutagenesis with EMS, and selection for resistance to the repressive effects of the glucose analog 5-thio-D-glucose (5TG). Resistant colonies appeared after 5 days at a frequency of 10^{-8} to 10^{-9}. About 50 colonies were able to grow despite high glucose concentrations on YEPEG agar medium. Amongst them, two different mutants, designated MT41 and MT42, were selected for further experiments.

These mutants were checked for growth on different YEPEG liquid media containing rising glucose concentrations. Mutants MT41 and MT42 were able to grow on YEPEG liquid medium containing up to 5% glucose. HC38-3D, MT41 and MT42 strains were not different in their growth phenotypes on YEPEG liquid medium. They exhibited very similar exponential growth rates: 0.102, 0.100, and 0.105 h^{-1} respectively. On the contrary, the presence of glucose in the culture medium affected the growth of MT41 and MT42 strains differently: Fig. 1 showed the influence of initial glucose concentration in $YEPD_nE$ liquid medium on their respective growth rate. At a 5% initial glucose

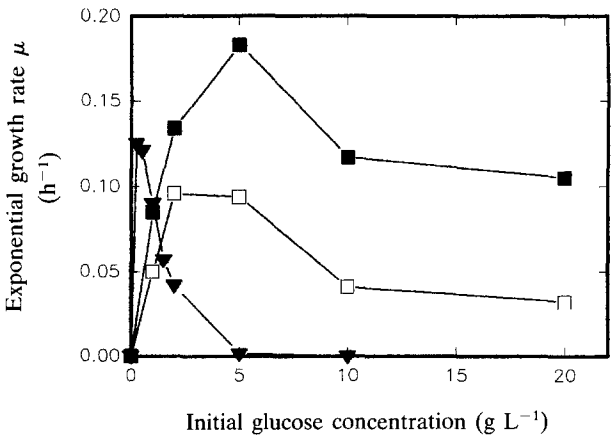

Fig. 1. Influence of initial glucose concentration on exponential growth rates (μ) of *gpm1* deletion strain HC38-3D (▼), MT41 (■), and MT42 (□) mutant strains during growth in $YEPD_nE$ liquid media.

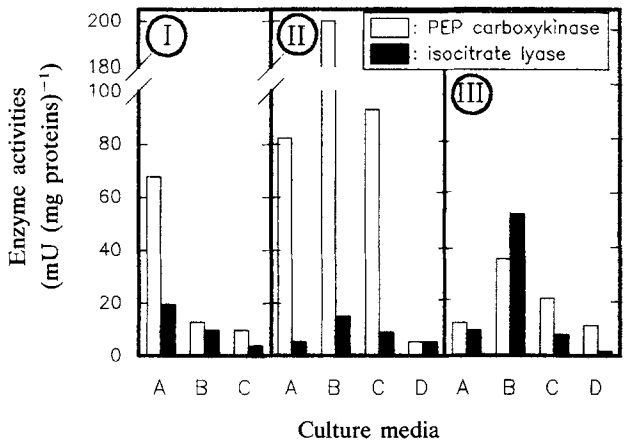

Fig. 2. Phosphoenolpyruvate carboxykinase and isocitrate lyase specific activities of *gpm1* deletion strain HC38-3D (I), MT41 (II), and MT42 (III) mutant strains during growth in YEPEG (A), $YEPD_{0.2}E$ (B), $YEPD_{0.5}E$ (C), and $YEPD_2E$ (D) liquid media.

deletion and bearing mutations involved in MT41 and MT42 strains. Unfortunately, we were unable to sporulate any diploid homozygous for the *gpm1* deletion: this result has been already observed by another laboratory (Gancedo, C., personal communication), and may be due to an impairment of some metabolic pathway involved in sporulation. Nevertheless we crossed MT41 and MT42 strains with a *gpm1* deletion strain of the opposite mating type (constructed from HC38-3D strain by classical genetics) and isolated individual zygotes from the mating mixture by micromanipulation. These putative diploids failed to grow on YEPEG solid medium in the presence of glucose showing that mutation(s) involved in MT41 and MT42 strains were recessive(s). Thus further genetical characterization of the mutants was impossible.

Physiological characterization of mutants of gpm1 *deletion strain resistant to glucose*

First of all the substitution of the chromosomal copy of *GPM1* by the *URA3* marker was checked by Southern blot technique in the mutant strains (data not shown). In addition we were unable to detect any phosphoglycerate mutase

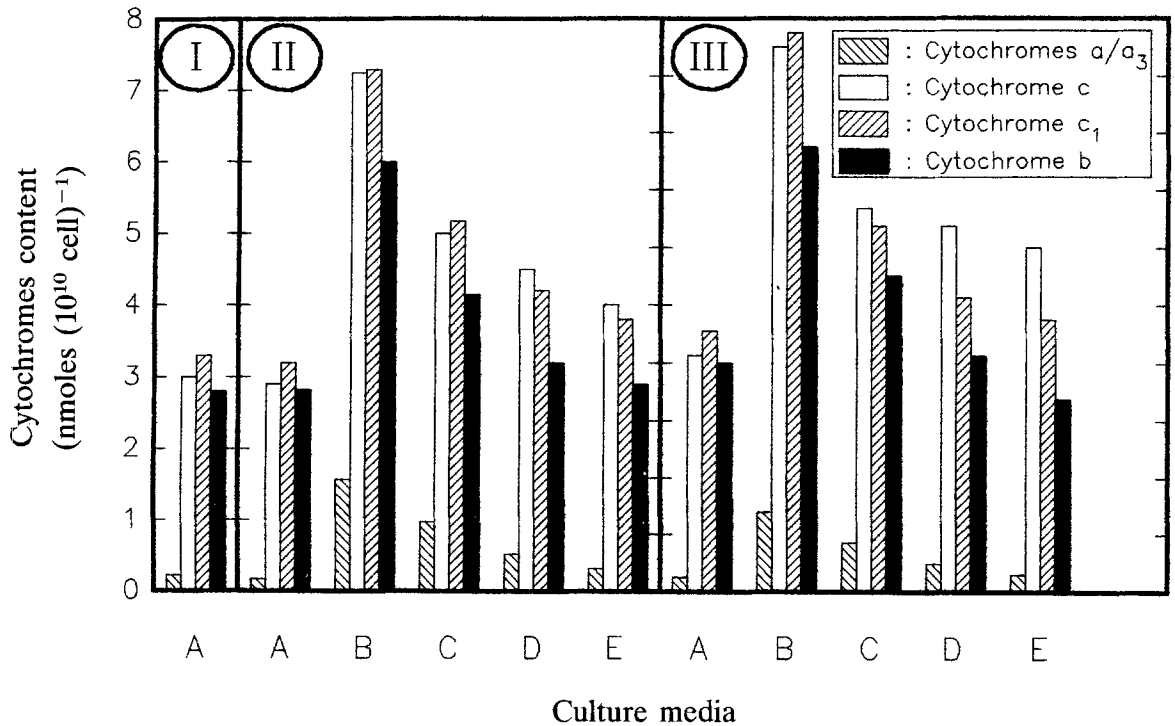

Fig. 3. Cytochromes content of *gpm1* deletion strain HC38-3D (I), MT41 (II), and MT42 (III) mutant strains during growth in YEPEG (A), $YEPD_{0.2}E$ (B), $YEPD_{0.5}E$ (C), $YEPD_1E$ (D) and $YEPD_2E$ (E) liquid media.

concentration these two strains exhibited a better growth than the parental strain. Nevertheless their growth remained still inhibited at higher glucose concentrations, that is 5–10% glucose concentration as compared with 2% for the parental strain. It is noticeable that, in MT41 and MT42 strains, glucose up to a concentration of 5% may serve as a substitute for gluconeogenesis, while energy is provided by respiration of ethanol.

In order to genetically characterize these mutants, we attempted to construct diploids homozygous for the *gpm1* activity in any of the strains tested (data not shown). These results, together with the growth phenotypes of the mutants, prove that the original *gpm1* deletion was preserved in the two mutant strains MT41 and MT42. We assayed several enzyme activities (Fig. 2) and cytochrome contents (Fig. 3) during growth on YEPEG and $YEPD_nE$ liquid medium with increasing glucose concentration in order to assess the extent of glucose resistance in MT41 and MT42 mutants. From the data obtained, MT41 and MT42 mutants seemed to exhibit partially derepressed gluconeogenesis (phosphoenolpyruvate

TABLE 1

High-affinity glucose transport activity (mmol h^{-1} (g dry weight)$^{-1}$) of *gpm1* deletion strain HC38-3D, MT41 and MT42 mutant strains harvested[a] during growth on different liquid media with increasing glucose concentration

	Glucose uptake (mmol h^{-1} (g dry weight)$^{-1}$)			
Culture medium[b]	YEPEG	$YEPD_{0.02}E$	$YEPD_{0.1}E$	$YEPD_{0.25}E$
Strain HC38-3D	5.6[c] ± 0.8[d]	8.9 ± 1.4	5.2 ± 0.7	2.8 ± 0.4
Mutant MT41	2.0 ± 0.4	2.3 ± 0.5	2.0 ± 0.5	1.9 ± 0.3
Mutant MT42	1.6 ± 0.3	1.8 ± 0.7	1.8 ± 0.6	1.6 ± 0.4

[a]In all experiments cells were harvested during early exponential growth phase, so that the substrates would still be present near their original concentrations.
[b]See Materials and Methods for nomenclature.
[c]Mean values and [d]standard deviation of three experiments for each determination.

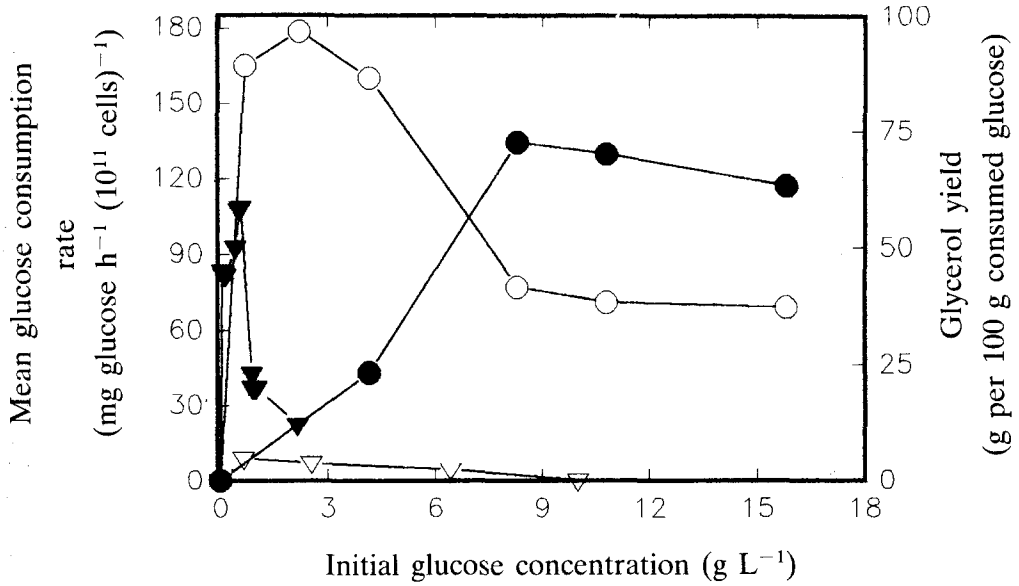

Fig. 4. Effect of initial glucose concentration on the glucose consumption rate (○/▽), and the glycerol yield (●/▼) by resting cells of *gpm1* deletion strain HC38-3D (▽/▼), and MT42 (○/●) mutant strain on RC liquid medium.

carboxykinase), glyoxylic shunt (isocitrate lyase) and mitochondrial respiratory chain (cytochromes). It is noticeable that 2% glucose exerts in the mutant strains the same repressive effect that 0.2% glucose exerts in the *gpm1* deletion strain HC38-3D. This result matched exactly the results obtained during growth rates experiments (Fig. 1).

We tested also the activity of the high-affinity glucose transport system from yeast cells grown on different media (Table 1). Under non-repressing conditions (i.e. in the absence of glucose in the culture medium) MT41 and MT42 strains exhibited a reduced glucose transport activity (about one third of the activity of the *gpm1* deletion strain HC38-3D). In addition catabolite repression by glucose of the high-affinity glucose uptake seemed less efficient in the mutant strains than in HC38-3D strain.

As a conclusion, MT41 and MT42 mutant strains exhibited a pleiotropic phenotype with respect to the expression of glucose-sensitive functions: (1) glucose repression of the synthesis of mitochondrial cytochromes is abolished, (ii) glucose repression of gluconeogenetic enzymes is reduced, (iii) high affinity glucose transport is reduced by two thirds, and seemed to be less sensitive to glucose repression. Considering their low frequency of appearance (10^{-8} to 10^{-9}) and their phenotypes, it is likely that these mutants might be affected in different genes [12].

As stated previously, all the mutant-deficient strains were able to grow on $YEPD_nE$ media. In these conditions the major product of glucose catabolism was glycerol while ethanol was respirated to provide energy. In order to study this peculiar metabolism in the absence of growth, we tested HC38-3D and MT42 resting cells in an aerobic fermentor on RC medium with different initial glucose concentrations (Fig. 4). These cells were able to convert glucose into glycerol with a yield close to 0.75 g glycerol produced per

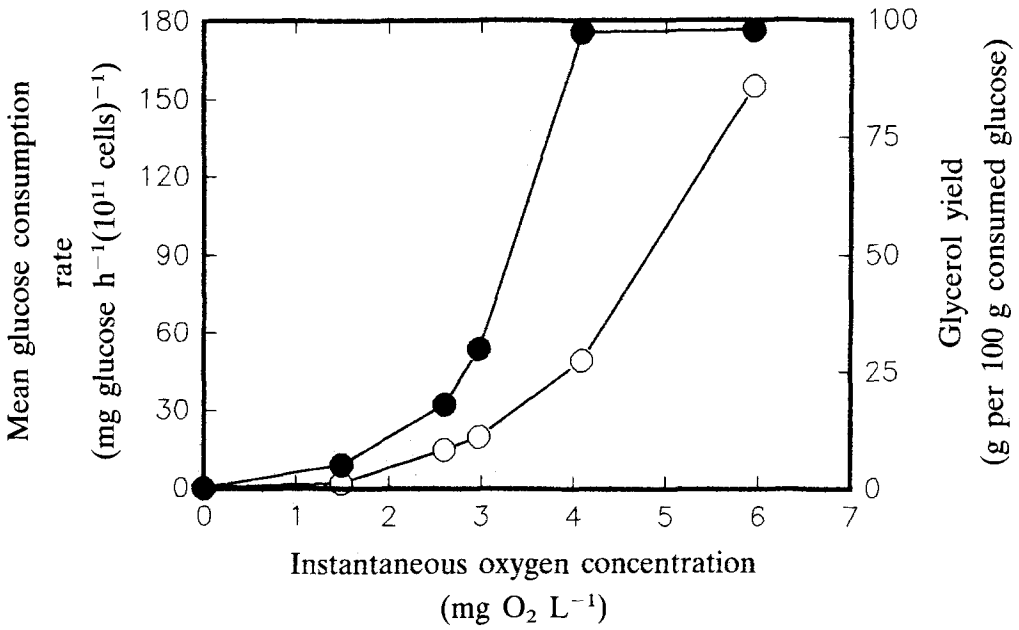

Fig. 5. Effect of the instantaneous oxygen concentration on the glucose consumption rate (○), and the glycerol yield (●) by resting cells of MT42 mutant strain on RC liquid medium containing 2% glucose.

TABLE 2

Metabolic pathways and stoichiometry involved during respiration of MT42 mutant cells on RC medium

Complete ethanol oxidation by citrate cycle
$C_2H_5OH + ADP + P_i + 3\ H_2O + 5\ NAD^+ + UQ^a \rightarrow 2\ CO_2 + AMP + PP_i + 5\ (NADH + H^+) + UQH_2$
Respiratory chain
$NADH + H^+$ (or UQH_2) $+ 0.5\ O_2 + 2\ ADP + 2\ P_i \rightarrow NAD^+$ (or UQ) $+ H_2O + 2\ ATP$
Glycerol fermentation from glucose
$C_6H_{12}O_6 + 2\ ATP + 2\ (NADH + H^+) \rightarrow 2\ C_3H_5(OH)_3 + 2\ ADP + 2\ P_i + 2\ NAD^+$
Total
$C_2H_5OH + C_6H_{12}O_6 + 2\ O_2 + 7\ ADP + 7\ P_i \rightarrow 2\ C_3H_5(OH)_3 + 2\ CO_2 + H_2O + 6\ ATP + AMP + PP_i$

[a]UQ indicates ubiquinone.

g glucose consumed. This glycerol yield may match the theoretical value when air is continuously supplied to the fermentor in order to maintain an instantaneous oxygen concentration close to the oxygen solubility in the RC culture medium containing 2% glucose (7.8 mg L^{-1} (28 °C, 760 mm Hg, air)) (Fig. 5). Ethanol consumption by yeast cells has not been determined because of air stripping which contributes significantly to ethanol disappearance from the medium. All these results may simply be explained by the stoichiometry of the complete respiration of a mixture of ethanol and glucose by a phosphoglycerate mutase-deficient strain (Table 2). If all the respiratory metabolic pathways are derepressed, oxidation of one mole of ethanol is required for conversion of one mole of glucose into two moles of glycerol in order to maintain a redox equilibrium.

Potential technological application of the gpm1 *deletion strain mutant*

Although ethanol and low amounts of glucose or fructose could be respirated by *S. cerevisiae*, this metabolism was never applied to alcoholic beverages and particularly to wines on account of the highly aerobic conditions imposed by this metabolism and of the highly repressive effect of high glucose concentrations on this metabolism.

The potential interest of using a yeast strain like MT42 is that the two main substrates of its respiratory metabolism (glucose/fructose and ethanol) are normally found and easily controlled in wines, and that its main end-product (glycerol) is one of the major by-products of the alcoholic fermentation and is highly desirable in beverages for its potential protective power against myocardial infarction [11]. Moreover less aerobic conditions are sufficient to allow this mutant to use ethanol and sugars to form glycerol, and thus one might expect a lower effect of aeration on the organoleptic properties of the end-product.

In other respects recent advances in the production of new products developed in the enological field such as low alcohol content wines implies the use of membrane processes like pervaporation or reverse osmosis technologies [2,3,10,16,23]. These physical processes are always directly applied on normal wines in order to remove ethanol. Nevertheless the poor selectivity of these processes led often to a strong loss of volatile components originally present in wines such as aroma compounds. An alternative process may involve the use of a mutant strain such as MT42 in order to restore numerous typical yeast flavor compounds

TABLE 3

Analytical results of two different wines after different addition of ethanol and subsequent treatment of MT42 mutant strain in a 1.5-L aerobic fermentor, controlled at constant instantaneous oxygen concentrations

	red wine A	red wine B	red wine B
Original wines			
glucose (g L^{-1})	15.7	21.4	21.4
fructose (g L^{-1})	14.9	19.9	19.9
remaining ethanol (g L^{-1})	3.0	1.0	1.0
added ethanol (g L^{-1})	3 × 19.6[a]	40.0[b]	20.0[b]
glycerol (g L^{-1})	7.8	5.1	5.1
lactate (g L^{-1})	4.2	3.0	3.0
acetate (g L^{-1})	0.45	0.5	0.5
fumarate (g L^{-1})	0	0	0
color intensity ($A_{420\ nm} + A_{520\ nm}$, d = 1 mm)	0.88	0.60	0.60
color tint ($A_{420\ nm}/A_{520\ nm}$, d = 1 mm)	0.75	0.70	0.70
Treatment			
initial MT42 cell population (10^8 cells ml^{-1})	0.6	1.3	1.3
treatment duration (h)	350	120	120
instantaneous oxygen concentration (mg O_2 L^{-1})	4.0	3.0	3.0
Final wines after treatment			
MT42 cell population (10^8 cells ml^{-1})	0.9	2.2	2.2
glucose (g L^{-1})	5.1	13.0	12.7
fructose (g L^{-1})	10.5	17.5	17.3
remaining ethanol (g L^{-1})	4.8	27.7	7.0
glycerol (g L^{-1})	11.8	8.4	9.2
lactate (g L^{-1})	0	0	0
acetate (g L^{-1})	0.10	0.15	0.13
fumarate (g L^{-1})	0.10	0.08	0.08
color intensity ($A_{420\ nm} + A_{520\ nm}$, d = 1 mm)	0.78	0.59	0.58
color tint ($A_{420\ nm}/A_{520\ nm}$, d = 1 mm)	0.80	0.79	0.78
Glycerol yield[c]	28.3	33.6	37.6

[a]19.6 g L^{-1} of ethanol was added three times during the process 0, 100, and 227 h after inoculation respectively.
[b]Ethanol was added just before inoculation.
[c]Glycerol yield = g produced glycerol per 100 g of consumed glucose and fructose.

during the partial respiration of ethanol and glucose into glycerol.

We tested such a process on two different low alcohol content red wines obtained at the industrial scale by membrane processes. Ethanol was first added to these wines before inoculation with MT42 yeast cells previously grown on $YEPD_2E$ liquid medium. Incubation was performed in the 1.5-L aerobic fermentor. Air was continuously supplied in order to maintain an instantaneous oxygen concentration much lower than the oxygen solubility in the two different wines (7.8–7.9 mg L^{-1} (28 °C, 760 mm Hg, air)). Analytical results of the final products obtained in these experiments are given in Table 3. In all experiments, glucose and fructose were metabolized into glycerol with a yield close to 33% whatever their initial concentrations in the original wines. Ethanol, lactate and acetate were rapidly oxidized, and little amounts of fumarate were found in the final wines. Other new unidentified organic acids appeared during the process (data not shown). Rough sensory analysis indicated that the final wines had similar aroma intensities to the original wines. Moreover, objective evaluation of color intensity and tint [22] did not reveal dramatic oxidation nor browning of the final products. Nevertheless large amounts of ethanol disappeared simply by air stripping. As a consequence further investigations will consist of studies of experimental devices in order to minimize air stripping during such a process. Complete analysis of organic acid and volatile fractions of the final wines are in progress.

Our pilot scale trial results did not endorse the replacement of physical processes in the production of low alcohol content red wines developed in the enological field, but the possibilities of coupling physical processes and new yeast metabolisms had to be taken into account in such technologies. Moreover further research is needed to determine the actual place of the MT42 yeast strain peculiar metabolism in the area of beverages production.

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