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Potential Application of a Glucose-Transport-Deficient Mutant of *Schizosaccharomyces pombe* for Removing Gluconic Acid from Grape Must

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Musts from rotten grapes typically contain high levels of gluconic acid, which can raise severe problems in winemaking processes. In this work, the ability of the glucose-transport-deficient mutant *YGS-5* of *Schizosaccharomyces pombe* to completely or partly remove gluconic acid from a synthetic glucose-containing medium and the potential use of this yeast strain for the same purpose in musts and wines were examined. Surprisingly, the *S. pombe YGS-5* strain successfully removed 93% of the initial gluconic acid (2.5 gL^{-1}) and 80% of the initial malic acid (1.0 gL^{-1}) within 30 h after inoculation. Also, the yeast strain produced no volatile compounds other than those obtained in fermentations conducted with the wine yeast *Saccharomyces cerevisiae*. *S. pombe YGS-5* could thus be used to remove gluconic acid present in musts from rotten grapes. On the basis of these results, various ways of using *S. pombe YGS-5* to treat musts containing gluconic acid in order to solve the problems due to the high gluconic acid concentrations in botrytized grape must are proposed.

KEYWORDS: Gluconic acid; glucose-transport-deficient mutant; *Schizosaccharomyces pombe*; volatile compounds

INTRODUCTION

The presence of gluconic acid in musts and wines poses some problems regarding control of alcoholic fermentation, biological aging, and stability of the end product; specifically, gluconic acid appears to be indirectly responsible for 8% of the total amount of bound SO_2 present in musts from botrytized grapes (*1*, *2*). Also, the metabolism of gluconic acid by lactic acid bacteria in wine increases volatile acidity (*3*−*6*). Metabolic activity in fungi (e.g., *Botrytis cinerea*) and acetic acid bacteria (e.g., *Acetobacter* and *Gluconobacter*) is known to result in the production of gluconic acid in rotten grapes. According to Couto et al. (*7*), low levels of gluconic acid (up to 1−2 g L^{-1}) suggest incipient grape infection (mainly by fungi), whereas higher levels can result from the activity of secondary invaders such as acetic acid bacteria. The problems posed by gluconic acid can be solved only by completely or partly removing it from musts and wines. Thus, our group has shown that the wild flor yeast strain *Saccharomyces cerevisiae* var. *capensis* G1 uses some gluconic acid during biological aging of sherry wines and causes significant changes in the final volatile composition of the resulting wines (*8*). Also, our group was the first to successfully use a wild strain of *Schizosaccharomyces pombe* to remove gluconic acid from wines (*9*, *10*). Previous attempts at using *S. pombe* to lower the content of malic acid in wine had resulted in wines of poor quality. In fact, alcoholic fermentation of musts under this fission yeast provides wines with unpleasant odors that are probably due to the presence of large amounts of sulfur-containing compounds (*11*).

S. pombe cells grow on D-gluconate as the sole carbon and energy source (*12*), and this utilization is rapidly inhibited by the addition of D-glucose, as well as by exogenous cAMP (*13*). D-Gluconate is taken up in symport with protons by a specific symporter (*14*). Milbradt and Höfer (*15*) obtained the glucose-transport-deficient mutant, *YGS-5*, in which gluconate is not repressed by D-glucose. The purpose of this work was to examine the potential of this glucose-transport-deficient mutant of *S. pombe* to remove gluconic acid from glucose-containing media such as grape musts obtained from botrytized or rotten grapes, which typically contain high concentrations of gluconic acid. To this end, a comprehensive analysis of chemical changes in the medium resulting from the metabolism of mutant *YGS-5* was carried out. The strain was found to result in slow fermentation of glucose and fructose, which can provide a sequential method for the removal of gluconate in the wine-making process. On the basis of the results, various ways of using the *S. pombe YGS-5* strain to treat musts containing gluconic acid are proposed.

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MATERIALS AND METHODS

Yeast Strain and Inoculum. The yeast strain *YGS-5* used in this study was a glucose-transport-deficient, leucine-auxotrophic mutant *Schizosaccharomyces pombe* strain obtained and supplied by Milbradt and Höfer (*15*), who produced it by genetic purification of *YGS-B22*, which is unable to grow on D-glucose and/or D-fructose as a carbon source and resistant to 2-deoxy-D-glucose (2DG), which was in turn obtained by back-crossing with a leucine-auxotrophic mutant and the wild-type. *YGS-5* had lost 2DG resistance and grown in d-glucose-containing media, albeit very slowly.

The mutant was cultured in YNB synthetic medium with amino acids (Difco), supplemented with a 100 mgL^{-1} concentration of L-leucine (Sigma) and 3% of D-gluconate (potassium salt, Merck). Following incubation at 27 ± 1 °C with shaking for 72 h, fission yeast cells were collected by centrifugation at 3500*g* and used to inoculate the synthetic medium with a population of 10^7 living cells mL^{-1}. The total number of living cells was determined by counting under a light microscope in a Thoma chamber following staining with Methylene Blue (*16*).

Culture Medium. The synthetic medium used contained 200 g L^{-1} D-glucose, 3 g L^{-1} yeast extract, 2.5 g L^{-1} gluconic acid, 3 g L^{-1} tartaric acid, 1 g L^{-1} malic acid, and 100 mg L^{-1} l-leucine. The pH was adjusted to 3.5 with potassium hydroxide and the medium subsequently sterilized by passage through Supra EK filters (Seitz, Germany).

Culture and Experimental Conditions. Three 1 L Erlenmeyer flasks containing 600 mL of synthetic medium prepared as described above were continuously shaken at 27 ± 1 °C until gluconic acid was depleted. Then the flasks were allowed to stand at 21 ± 1 °C until the end of alcoholic fermentation. Samples for analysis of volatile compounds were withdrawn when the gluconic acid was removed and at end of fermentation. The fermentation process was monitored via the amount of CO_2 released as measured from the weight loss (*17*, *18*).

Analytical Methods. Ethanol was quantified using the method of Crowell and Ough (*19*), and titratable acidity, pH, and volatile acidity were determined with the methods of the *Official Report of the European Community* (*20*). Gluconic, acetic, and malic acids were quantified using enzymatic tests from Boehringer Mannheim (Germany).

Major volatile compounds and polyols were quantified on a model 6890 gas chromatograph from Agilent Technologies (Palo Alto, CA), using the method of Peinado et al. (*21*). A CP-WAX 57 CB capillary column (60 m long × 0.25 mm i.d., 0.4 μm film thickness) was used. Aliquots of 0.5 μL from 10 mL samples previously dosed with 1 mL of 1 g L^{-1} 4-methyl-2-pentanol as internal standard were used for injection. Tartaric acid in the wine was removed by precipitation with 0.2 g of calcium carbonate, followed by centrifugation at 300*g*.

Quantification was based on the response factors obtained for standard solutions of each compound. A split ratio of 30:1, an FID, and a temperature program involving an initial temperature of 50 °C (15 min), a 4 °C min^{-1} ramp, and a final temperature of 190 °C (35 min) were used. The injector and detector temperatures were 270 and 300 °C, respectively. The flow rate of carrier gas (helium) was initially set at 0.7 mL min^{-1} (16 min) and followed by a 0.2 mL min^{-1} ramp to the final value (1.1 mL min^{-1}), which was held for 52 min.

Minor volatile compounds were determined by capillary column gas chromatography−mass spectrometry following continuous extraction of 100 mL of medium sample with 100 mL of Freon-11 for 24 h. Samples were adjusted to pH 3.5, and 5 mL of a 30 mg L^{-1} solution of 2-octanol was added as internal standard. The Freon extracts containing the volatile compounds were concentrated to 0.2 mL in a Kuderna−Danish microconcentrator, and 1.5 μL aliquots were injected into an HP-6890 gas chromatograph equipped with an HP MS 5972A mass detector (Agilent Technologies, Palo Alto, CA). An HP-Innowax fused silica capillary column (60 m long × 0.32 mm i.d., 0.25 μm film thickness) was used. The temperature program was as follows: initial temperature, 40 °C, held for 10 min, and 1 °C min^{-1} ramp to 180 °C, held for 35 min. Helium at a constant flow rate of 0.9 mL min^{-1} was used as carrier gas, and a 30:1 split ratio was employed at the injection port. The mass detector was used at a voltage of 1612 V in the scan mode to sweep the mass range from 39 to 300 amu.

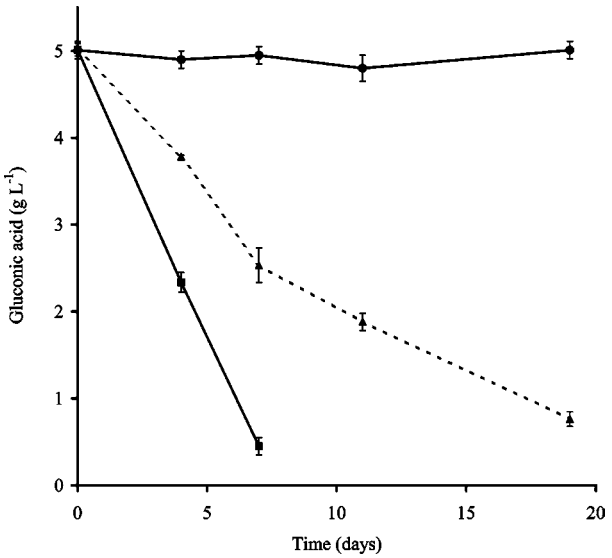

Figure 1. Uptake of gluconic acid by the wild strain (●, static and shaking conditions) and by the mutant strain *YGS-5* of *S. pombe* in the fermentation medium subjected to static (▲) and shaking (■) conditions.

Retention times, spectral libraries supplied by Wiley, and pure chemical compounds obtained from Merck, Sigma-Aldrich, Riedel de Haën, and Fluka were used for identification, confirmation, and preparation of standard solutions of the volatile compounds. Each compound was quantified from its response factor, which was obtained from standard solutions of known concentration subjected to the same treatment as the samples, using the target and qualifier ions for each compound selected by a Hewlett-Packard Chemstation (Palo Alto, CA).

Statistical Treatment. The results given are the averages of three independent experiments. The statistical software package Statgraphics Plus v.2, from STSC, Inc. (Rockville, MD), was used to perform a single analysis of variance (ANOVA) to identify those compounds exhibiting significant differences between the two periods examined: the first period when all gluconic acid had been consumed and the second at the end of alcoholic fermentation.

RESULTS AND DISCUSSION

Prior to examining the effect of shaking, the synthetic fermentable medium containing YNB without amino acids in addition to 200 g L^{-1} D-glucose and 5 g L^{-1} gluconic acid (the highest gluconic acid concentration reported in the winemaking area) was inoculated with pure cultures of *S. pombe* strain *YGS-5*. The continuously shaken fermentable synthetic medium exhibited a decrease of 651 mg L^{-1} day^{-1} in gluconic acid, 90% of the acid being used by the yeast cells within 8 days (**Figure 1**). By contrast, the unshaken fermentable synthetic medium exhibited a decrease by 361 mg L^{-1} day^{-1} over the same period, and use of the acid decreased to 148 mg L^{-1} day^{-1} over the next 12 days (**Figure 1**). Shaking promoted homogeneity in the medium and facilitated a slight oxygenation, which might be the origin of the increased gluconic acid uptake in the shaken cultures. The procedure including shaking was thus adopted for subsequent tests. Also, the wild-type of the mutant was used as a control as it used no gluconic acid in the synthetic glucose-containing medium (**Figure 1**).

Table 1 shows the winemaking variables and gluconic acid concentration in the initial medium, after gluconic acid was removed, and at the end of alcoholic fermentation. The gluconic acid concentration used in this test, 2.5 gL^{-1}, is more common in the studied area. The mutant *YGS-5* was found to remove 93% of gluconic acid within 30 h after inoculation, the amount of ethanol produced over such a period being 1.5% (v/v), which

Table 1. Winemaking Variables in a Fermentable Medium Containing Gluconic Acid and Inoculated with Strain *YGS-5*

compound	IM[a]	MAGA[b]	MEF[c]	homogeneous groups[d]
ethanol (% v/v)	0 ± 0	1.5 ± 0.1	6.5 ± 0.2	ABC
pH	3.4 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	ABB
titratable acidity (mequiv L^{-1})	73 ± 1	58 ± 2	59 ± 1	ABB
malic acid (g L^{-1})	1.0 ± 0.1	0.2 ± 0.05	0	ABC
volatile acidity (g L^{-1})	0 ± 0	0.27 ± 0.1	0.30 ± 0.2	ABB
gluconic acid (g L^{-1})	2.5 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	ABB

[a] Initial medium. [b] Medium after gluconic acid was removed. [c] Medium at the end of alcoholic fermentation. [d] Mean analysis comparison. Different letters denote significant differences at the 95% confidence level.

corresponds to a glucose uptake of 25 g L^{-1}. Also, the mutant metabolized 80% of malic acid over the same period of time. After all gluconic acid was removed, tests were continued under static conditions for 35 days, to mimic a potential industrial application and study the contribution of strain *YGS-5* to ethanol production and the formation of volatile compounds. The mutant yeast exhibited a low fermentation capacity; thus, only 6.5% (v/v) ethanol was produced, and a concentration of ~90 g L^{-1} of fermentable sugars remained in the medium. Similarly, Milbradt and Höfer (*15*) had previously observed a slow growth in glucose- and fructose-containing media. The decreased titratable acidity and increased pH may be a result of gluconic acid in the medium being used by the yeast strain. Acetic acid is the major volatile acid in wine; it is always produced by yeasts during fermentation at concentrations that depend on the particular species and strain (*22*). The amounts produced over the period gluconic acid was used and fermentation occurred, 0.27 and 0.30 gL^{-1}, respectively, suggest that this fission yeast strain synthesizes little acetic acid.

Table 2 shows the changes in the volatile composition of the fermentation medium after gluconic acid was removed and at the end of fermentation by *YGS-5*. Only acetaldehyde and propanol exhibited no significant differences after the fermentation process. All other compounds, except acetoin, butanedione (diacetyl), and benzaldehyde, exhibited higher concentrations as a consequence of fermentation, which may be involved in maintaining the redox balance in yeast cells (*23*).

Acetoin is a usual byproduct of alcoholic fermentation; it occurs only in the presence of fermentable carbohydrates or pyruvic acid. This compound is produced from glucose via glycolysis pathway by yeasts. Also, decarboxylation to hydroxyethylthiamin PP_i, which involves thiamin PP_i (TPP), is a key reaction in the transformation of pyruvate into byproducts via the active acetaldehyde or acetaldehyde−TPP complex (*24*). Three different pathways have been proposed for acetoin formation by yeasts. One involves the transformation of active acetaldehyde and pyruvate into α-acetolactate, catalyzed by acetohydroxy acid synthetase. Yeasts can also decarboxylate α-acetolactate to acetoin (*25*). On the other hand, α-acetolactate can be transformed into diacetyl by oxidative decarboxylation and subsequently reduced to acetoin. The second mechanism involves a condensation reaction between active acetaldehyde and acetyl coenzyme, which is then reduced to acetoin by diacetyl reductase (*26*). The third mechanism involves the condensation of active acetaldehyde with free acetaldehyde formed from pyruvate (*27*) without the intervention of α-acetolactate.

The high amount of acetoin produced by *S. pombe* strain *YGS-5* in this study under shaking, while gluconic acid was consumed, is consistent with the results of Crowell and Guymon (*28*) and Cowland and Maule (*29*), who found unusually high concentrations of this compound in aerated fermentation media. In addition, Romano and Suzzi (*30*, *31*) found a clear-cut dependence of acetoin production on the medium composition and the particular *Saccharomyces cerevisiae* yeast strain used. On the other hand, Kuriyama and Kobayashi (*32*) found decreased glycerol production by a *S. cerevisiae* yeast strain in aerated fermentation media as the likely result of NADH coenzymes not being oxidized and pyruvate accumulating in the medium. As noted earlier, pyruvate can become acetoin, which produces reduced coenzymes (NADH), that can be accumulated in cells. At the end of fermentation, however, the concentration of acetoin was lower and that of 2,3-butanediol (*levo* and *meso*) higher. The formation of both butanediol forms may be due to the action of acetoin reductase enzyme, which may have reoxidized the NADH coenzyme, probably accumulating during the preceding shaking period. The latter suggests that reduction reactions in the fermentation period may be favored by the need to reoxidize excess NADH. Similarly, the low concentrations of diacetyl in the shaken medium and zero levels obtained at the end of fermentation may be explained, because this compound is rapidly reduced to acetoin by diacetyl reductase enzyme involving NADH oxidation (*33*).

According to Delfini et al. (*34*) *S. pombe* produces benzaldehyde and benzyl alcohol. Both compounds were detected at low concentrations after gluconic acid was removed. In contrast, only benzyl alcohol was present at a high concentration, at the end of the fermentation period, possibly as a result of the presence of excess NADH.

No ethyl esters were detected in the fermentable medium after gluconic acid was removed; this may have resulted from low ethanol production during the shaking period. By contrast, the

Table 2. Volatile Compounds in a Fermentable Medium Containing Gluconic Acid Inoculated with Strain *YGS-5*

compound	MAGA[a]	MEF[b]	*p* value[c]
acetaldehyde (mg L^{-1})	37 ± 1	29 ± 5	NS
ethyl acetate (mg L^{-1})	ND[d]	2.3 ± 0.2	*
1,1-diethoxyethane (mg L^{-1})	1.2 ± 0.2	6 ± 2	***
1-propanol (mg L^{-1})	4.7 ± 0.5	4.4 ± 0.1	NS
isobutanol (mg L^{-1})	3.5 ± 0.5	14 ± 2	**
isoamyl alcohols (mg L^{-1})	22 ± 2	30 ± 1	**
acetoin (mg L^{-1})	131 ± 7	21 ± 1	***
ethyl lactate (mg L^{-1})	ND	2.1 ± 0.2	***
2,3-butanediol *levo* (mg L^{-1})	88 ± 5	195 ± 20	***
2,3-butanediol *meso* (mg L^{-1})	ND	219 ± 16	***
2-phenylethanol (mg L^{-1})	4 ± 1	9 ± 1	**
glycerol (mg L^{-1})	513 ± 57	2751 ± 61	***
butanedione (μg L^{-1})	277 ± 32	ND	***
benzaldehyde (μg L^{-1})	179 ± 19	ND	***
3-hydroxyethyl butanoate (μg L^{-1})	ND	165 ± 20	***
1-octanol (μg L^{-1})	ND	280 ± 39	***
isobutanoic acid (μg L^{-1})	2521 ± 144	9510 ± 925	***
γ-butirolactone (μg L^{-1})	ND	817 ± 41	***
butanoic acid (μg L^{-1})	680 ± 41	1283 ± 77	***
2- and 3-methylbutanoic acids (μg L^{-1})	1040 ± 110	5354 ± 221	***
diethyl succinate (μg L^{-1})	ND	220 ± 26	***
hexanoic acid (μg L^{-1})	ND	131 ± 17	***
benzyl alcohol (μg L^{-1})	519 ± 84	12207 ± 648	***
pantolactone (μg L^{-1})	ND	753 ± 83	***
octanoic acid (μg L^{-1})	ND	266 ± 24	***
ethyl succinate (μg L^{-1})	ND	24134 ± 1194	***

[a] Medium after gluconic acid was removed. [b] Medium at the end of alcoholic fermentation. [c] *p* values obtained by ANOVA for the different compounds in the two periods: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; NS, no significant differences. [d] Not detected.

fermented medium was found to contain ethyl acetate, ethyl lactate, 3-hydroxyethyl butanoate, ethyl succinate, and diethyl succinate. Except for ethyl succinate, the ester concentrations were low relative to those of dry wines (*8*) as a result of the residual glucose concentration and low ethanol production. On the other hand, 1,1-diethoxyethane, the major acetal in wine, was found at a lower concentration after the gluconic acid was removed than at the end of fermentation.

Propanol, isobutanol, isoamyl alcohols, and 2-phenylethanol are known to be produced at concentrations ranging from 20 to 300 mg L^{-1} from the ketoacids pool by yeasts during alcoholic fermentation. These alcohols were produced in small amounts during both gluconic acid consumption and the fermentation period (**Table 2**). Only isoamyl alcohols were produced preferentially during the former period (shaken medium), possibly as a result of their amino acid precursor, L-leucine, being consumed (*35*).

γ-Butyrolactone and pantolactone (α-hydroxy-β,β-dimethyl-γ-butyrolactone) result from the lactonization of γ-hydroxybutyric acid and pantotenic acid, respectively. No lactones were detected during gluconic acid consumption, but only at the end of fermentation, consistent with the results of Bayonove et al. (*36*). The concentrations of hexanoic and octanoic acids in the fermented medium were low relative to those of young dry wines (*8*). However, those of C_4 acids (butanoic, isobutanoic, and 2- and 3-methylbutanoic) were higher than those found by these authors in young wines.

These findings suggest that reduction reactions are favored in fermentation under the studied conditions. These reactions play a prominent role in yeast metabolism as they enable the reoxidation of NADH to maintain the redox potential of the cells and the removal of toxic compounds such as aldehydes. Thus, the redox potential governs the consumption and production of some compounds such as diacetyl, acetoin, aldehydes, and higher alcohols (*23*).

Our results allow the development of a method for removing gluconic acid from partially rotten grape must with a high gluconic acid concentration to minimize the problems encountered during storage or aging of the resulting wines.

Two findings are especially interesting in relation to the potential use of the glucose-transport-deficient mutant *YGS-5* of *S. pombe*, namely, (i) the mutant efficiently removed gluconic acid from a glucose-containing synthetic medium and (ii) it produced no other compounds than those obtained in a typical fermentation under wine yeast *S. cerevisiae*. The novelty of this work lies in the fact that, for the first time, a *S. pombe* strain was used to remove gluconic acid from glucose-containing media with a view to applying the ensuing method to musts from rotten grapes.

The strain results in slow fermentation of glucose and fructose; this requires the use of a sequential method to remove gluconate under typical winemaking conditions. Some parallel fermentations with *S. cerevisiae* will have to be conducted for reliable comparison and sensory tests conducted to assess the results. There are various choices for the winemaking of grape musts with high levels of gluconic acid. One involves inoculating musts with *S. pombe* strain *YGS-5* to remove gluconic acid, followed by elimination of the yeast cells by centrifugation or filtration and inoculation with *S. cerevisiae* to complete the fermentation. Another method involves sequential inoculation, first with *S. pombe* and, once gluconic acid has been removed, with *S. cerevisiae* (without removing the *S. pombe* strain). A third choice is the simultaneous inoculation of the musts with both yeasts. The effect on the contents in volatile compounds of the resulting wines should be examined and their quality assessed by sensory analysis. The stability of *YGS-5* mutant phenotype under industrial conditions should be checked; thus, reverting mutants for L-leucine phenotype may be desirable, but not essential as grape musts are rich in this amino acid.

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