Letters in Applied Microbiology 1996, **22**, 299–302

Determination of 2,3-butanediol in high and low acetoin producers of *Saccharomyces cerevisiae* wine yeasts by automated multiple development (AMD)

P. Romano, G. Suzzi, V. Brandolini[1], E. Menziani[1] and P. Domizio

Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Università della Basilicata, Potenza, and [1]Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Ferrara, Italy

JES/23: received and accepted 29 September 1995

P. ROMANO, G. SUZZI, V. BRANDOLINI, E. MENZIANI AND P. DOMIZIO. 1996. High performance thin layer chromatography with automated multiple development was used to determine 2,3-butanediol levels in wine produced by high and low acetoin-forming strains of *Saccharomyces cerevisiae*. An inverse correlation between acetoin and 2,3-butanediol content was found suggesting a leaky mutation in acetoin reductase of the low 2,3-butanediol producing strains.

INTRODUCTION

After ethanol, 2,3-butanediol is usually second only to glycerol as the most abundant minor constituent of wines, since it is a by-product of alcoholic fermentation. It originates from the reduction of acetoin, which can derive from the decarboxylation of α-acetolactate, the reduction of diacetyl or the direct condensation of acetaldehyde–TPP complex with free acetaldehyde (for a review see Romano and Suzzi 1996). The amount of 2,3-butanediol found in various wines was summarized by Amerine (1954), ranging from about 250 to 1350 mg l^{-1} for table wines. In Italian wines it can vary from 200 to 1200 mg l^{-1} with an average of 400–900 mg l^{-1} (Pallotta *et al.* 1977). The levels of 2,3-butanediol have been reported to decrease in dessert wines (Amerine 1954) and to increase with malolactic fermentation (Sponholz *et al.* 1993). 2,3-Butanediol cannot be expected to appreciably affect sensory qualities of wine; however, it may contribute somewhat to the body of a wine because of its viscosity and its high content. During fermentation yeasts produce 2,3-butanediol in a mixture of the *laevo* (67%) and the *meso* (33%) forms (Neish 1950) and the amounts produced are at least partly a strain characteristic. Antoniani (1951) reported that actively fermenting yeasts produce only 2,3-butanediol, moderately fermenting yeasts both 2,3-butanediol and acetoin, and weakly fermenting yeasts only acetoin. A study on 100 strains of *S. cerevisiae* wine yeasts (Romano and Suzzi 1993) has shown that the low acetoin production is a predominant pattern and only a few strains exhibit a high production. Crosses between high and low acetoin-producing strains yielded tetrads segregating high *vs* low acetoin production with the ratio 2 : 2 (Romano *et al.* 1995).

Taking into account that acetoin is generally transformed in 2,3-butanediol, the different phenotypes found in acetoin production suggest a consequent difference in 2,3-butanediol production. The purpose of this work is to verify the existence of a correlation between acetoin and 2,3-butanediol production in high and low acetoin-producing single spore cultures, generated from a hybrid of *S. cerevisiae* wine yeast.

Among the different analytical methods to determine 2,3-butanediol such as fluorometric, colorimetric, enzymatic and gas chromatographic methods (Aleixandre Benavent 1987; Garcia Moruno and Di Stefano 1989; Sponholz *et al* 1993; Olguin-Castillo *et al.* 1994), recently the advantages of the planar chromatography in analytical field have been demonstrated (Jork 1993a,b). In this study we used the HPTLC plates, which are easy to clean up in complex real-life samples. It is also possible to analyse a great number of samples in the same plate (Jork *et al.* 1990), in combination with the AMD technique. In this way we have pointed out a reliable analytical method to separate and determine quantitatively 2,3-butanediol in wine.

MATERIALS AND METHODS

Organisms

Thirty-two strains derived from eight tetrads of *Saccharomyces cerevisiae* segregating 2 : 2 for high and low acetoin production were analysed.

Correspondence to: Dr Paola Domizio, Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Università della Basilicata, Via Nazario Sauro 85, 85100 Potenza, Italy.

Fermentation

White grape must from the Trebbiano cultivar with 18% (w/v) fermentable sugar, 0·65% (w/v) titratable acidity, pH 2·85, was used to determine the strain capacity to produce acetoin and 2,3-butanediol. Fermentations were carried out in 100 ml samples of sterilized must inoculated with a 5% concentration of 48 h precultures in the same must. The samples were incubated at 25°C until CO_2 evolution ceased, then refrigerated for 2 d at 2°C, racked and stored at −20°C until required for analysis.

Gas chromatographic analysis of acetoin production

Acetoin was analysed by injection of 2 μl of fermented grape must into a 180 cm × 2 mm glass column packed with 80/120 Carbopack B/5% Carbowax 20M (Supelco, USA). A Packard 427 gas chromatograph, equipped with a flame ionization detector and linked to a Shimadzu C-R3A Chromatopac integrator and recorder, was used. The column was run from 60°C to 198°C at a rise rate of 6° min^{-1}. The carrier gas was nitrogen at a flow rate of 20 ml min^{-1}. Each sample was loaded with a standard of *n*-butanol at a concentration of 100 mg l^{-1}. A standard solution containing 100 mg l^{-1} of acetoin was used to calibrate the column and recorder.

Determination of 2,3-butanediol

Sample preparation. The samples of wine were applied as directly or diluted to 1 : 32 with acetonitrile immediately before the analysis. Merck silica gel 60 HPTLC precoated plates, twice prewashed with methanol were used. Acetonitrile and methanol for chromatography (Merck, Germany), acetone for chromatography (Baker, Holland) and double-distilled water (over permanganate), were used. The standard of 2,3-butanediol was of analytical grade and it was obtained from Fluka Chemie AG Buchs (CH). Solutions of standards and samples of wine were applied to the plates with Camag Linomat IV as 8–10 mm bands, 6–8 mm apart (1–8 μl; delivery speed 10 s μl^{-1}).

Development. The development was made in the AMD system using five steps. The preconditioning time was 15 s for each step and the drying time between steps was 10 min. The linear gradient based on acetonitrile : acetone : water is depicted in Fig. 1.

Post-chromatographic derivatization. For the detection of 2,3-butanediol the developed silica gel plates were dipped for 10 s in a mixture of lead acetate (2%) in glacial acetic acid (5 ml) and fluorescein sodium (0·2%) in ethanol (5 ml) made up to 200 ml with toluene immediately before use. The plates were dried for few minutes in a stream of warm air and heated to 100°C for 10 min. It was possible to monitor the thermal reaction by inspecting the plate under u.v. light at 366 nm. All the plates were scanned by fluorescence at 366 nm (cut-off filter 400 nm) in the RLC Scanner II Interfaced with IBM compatible system and CATS evaluation software.

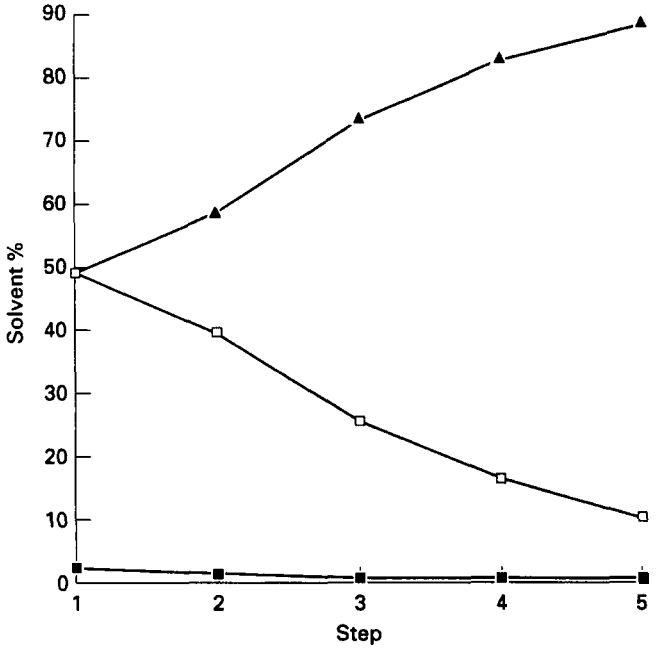

Fig. 1 Solvent (%) gradient employed in the development steps by automated multiple development ▲, Acetonitrile; □, acetone; ■, water

RESULTS AND DISCUSSION

High performance thin layer chromatography (HPTLC) was employed in connection with automated multiple development (AMD) to determine 2,3-butanediol produced in wine by high and low acetoin producing strains of *S. cerevisiae*.

For the calibration purpose the standard concentrations of 2,3-butanediol, diluted in acetonitrile/water (1 : 1), were applied to 20–600 ng absolute due to the excellent linearity and correlation between standard curves (2,3-butanediol $= 103.3 + 0{\cdot}12X$; $r = 0{\cdot}9993$; $n = 6$). Figure 2 shows the concentration range utilized for the determination of our samples.

An important feature of the automated multiple development (AMD) process is the accommodation of many compounds in a single chromatogram by the band-focusing effect (Burger and Tengler 1986). Another remarkable feature of the method is that the migration distance of the individual components is independent of sample nature (Janchen and Issaq 1988).

To assess the accuracy of AMD-HPTLC system to mea-

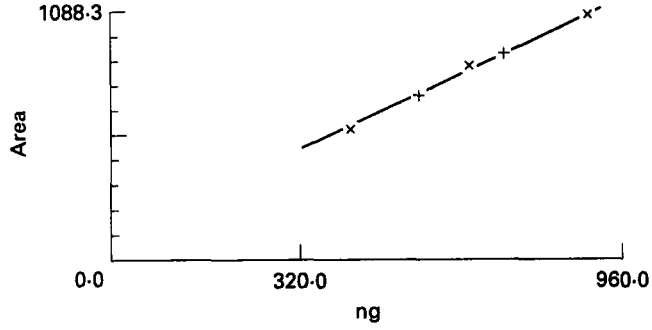

Fig. 2 Calibration curve for the 2,3-butanediol determination in wines

sure 2,3-butanediol in wines, samples were prepared with 50, 100 and 400 mg l^{-1} 2,3-butanediol, prepared and analysed as described. The mg l^{-1} was determined and the per cent recovery calculated. The results indicated that the recovery was quite good (96·6–100·4%) when a sample was spiked with a known quantity of 2,3-butanediol (Table 1).

The 2,3-butanediol produced by the *S. cerevisiae* wine strains are reported in Table 2. The data are given as mean values of four individual measurements. The CV (%) values among the samples indicate a good reproducibility and precision of the method.

The differences among the strains in the 2,3-butanediol production are significant ($P = 0{\cdot}05$) and all the tetrads segregated 2 high : 2 low 2,3-butanediol-producing strains. As expected, an inverse correlation between the acetoin and 2,3-butanediol was found. The two low acetoin producers yield the highest levels of 2,3-butanediol in each tetrad and the two high producers yield the lowest levels of 2,3-butanediol in each tetrad (Fig. 3).

It is well known that in the final stage of fermentation processes, acetoin is rapidly reduced by *S. cerevisiae* to form 2,3-butanediol (Guymon and Crowell 1967; Herraiz 1990), so that generally elevated amounts of the compound are found in wines. The low 2,3-butanediol-producing strains might suggest either a lower fermentative power of these strains (Antoniani 1951) or else a defect in acetoin reductase, the enzyme catalysing the reduction of acetoin to 2,3-butanediol.

Table 1 Automated multiple development-high performance thin layer chromatography recovery of 2,3-butanediol-spiked wine samples

Spiking level ($\mu g\ l^{-1}$)	Recovery*	CV (%)
50	96·6	2·8
100	98·1	1·2
400	100·4	2·7

* Mean of six replicates for each level.

Table 2 Segregation of the phenotype 2,3-butanediol production in tetrads of *Saccharomyces cerevisiae*

Tetrads	Butanediol (mg l^{-1})	CV (%)
2A	306·0	8·2
2B	148·2	6·4
2C	172·4	5·3
2D	435·8	7·8
3A	550·4	3·2
3B	149·7	2·9
3C	434·5	5·3
3D	208·7	4·4
4A	454·5	6·2
4B	292·1	4·1
4C	278·8	3·8
4D	645·3	7·3
5A	730·0	8·1
5B	564·9	6·5
5C	221·7	6·2
5D	196·0	5·4
6A	1048·8	6·8
6B	482·7	4·6
6C	253·8	5·5
6D	602·8	7·3
7A	966·5	9·0
7B	256·9	6·3
7C	784·7	6·8
7D	435·3	4·1
9A	1053·4	3·7
9B	850·6	4·9
9C	271·4	5·1
9D	164·6	3·2
10A	225·6	8·6
10B	205·2	6·9
10C	516·2	7·2
10D	503·3	5·1

Stivers and Washabaugh (1993) found that in a brewer's yeast (*Saccharomyces carlbergensis*) catalytic reactions of pyruvate decarboxylase isozymes limit acetoin synthesis. A similar mechanism might be involved in the 2,3-butanediol production by the segregants studied.

As differing amounts of secondary products can influence the taste of alcoholic beverages (Rankine 1967; Roset and Margulis 1971; Suomalainen 1971; Nykanen 1986), the inverse differences found in 2,3-butanediol and acetoin production emphasize the importance of strain selection for technological applications.

ACKNOWLEDGEMENTS

This work was supported in part by the National Research Council of Italy, and by a grant from MURST 40%, Italy.

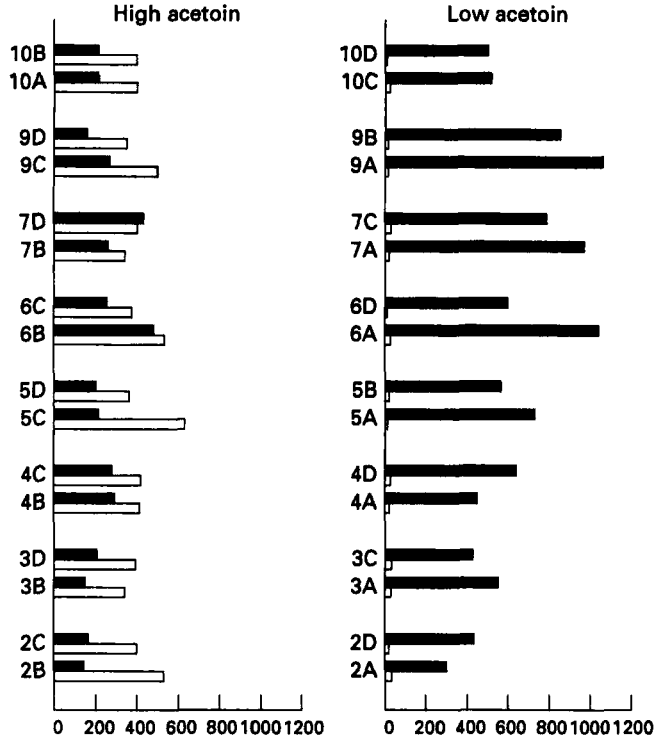

Fig. 3 Acetoin and 2,3-butanediol production in wine by *Saccharomyces cerevisiae*. □, Acetoin; ■, 2,3-butanediol

REFERENCES

Aleixandre Benavent, J.L. (1987) Effect of vinification conditions on glycerol and 2,3-butanediol contents in dry white wine from muscat grapes. *Revista de Agroquimiaca y Technologia de Alimentos* **27(2)**, 225.

Amerine, M.A. (1954) Composition of wines. Organic constituents. *Advances in Food Research* **5**, 353–510.

Antoniani, C. (1951) Ciò che si sa e ciò che si ignora sul chimismo della fermentazione alcolica. *Rivista di Viticoltura ed Enologia (Conegliano)* **4**, 183–196.

Burger, K.D. and Tengler, H. (1986) Automatic multiple development, a new technique of thin layer chromatography. In *Planar Chromatography* ed. Kaiser, R.E. pp. 193–205. Heidelberg: Huetig Verlag.

Garcia Moruno, E. and Di Stefano, R. (1989) Determinazione del glicerolo, del 2,3-butanediolo e dei glicoli nei vini. *Vini d'Italia* **1**, 41–46.

Guymon, J.F. and Crowell, E.A. (1965) The formation of acetoin and diacetyl during fermentation, and the levels found in wines. *American Journal of Enology and Viticulture* **16**, 85–91.

Herraiz, T. (1990) Production of volatile compounds by *Saccharomyces cerevisiae* during the alcoholic fermentation of grape must in presence or absence of SO_2. *Belgian Journal of Food Chemistry and Biotechnology* **45**, 57–62.

Janchen, D.E. and Issaq, H.J. (1988) Modern thin layer chromatography: advances and perspectives. *Journal of Liquid Chromatography* **11**, 1941–1965.

Jork, H. (1993a) Advances in thin layer chromatography: Part 1. *International Laboratory* **6**, 18–22.

Jork, H. (1993b) Advances in thin layer chromatography: Part 2. *International Laboratory* **7**, 10–14.

Jork, H., Funk, W., Fisher, W. and Winner, H. (1990) *Thin Layer Chromatography, Reagents and Detection Methods. 1.* Weinheim: VHC Verlagsgesllschaft.

Neish, A.C. (1950) Stereoisomers of 2,3-butanediol produced by yeast. *Canadian Journal of Research* **28B**, 660–661.

Nykanen, L. (1986) Formation and occurrence of flavour compounds in wine and distilled alcoholic beverages. *American Journal of Enology and Viticulture* **37**, 84–96.

Olguin-Castillo, C.M., Galindo, E. and Salvador-Figueroa, M. (1994) Factors affecting the consumption of 2,3-butanedione by *Saccharomyces cerevisiae*. *Letters in Applied Microbiology* **18**, 132–134.

Pallotta, U., Amati, A. and Minguzzi, A. (1977) *Lezioni di Enologia.* Ch. VIII: Composizione del vino, pp. 7–8. Bologna: La Clueb.

Rankine, B.C. (1967) formation of higher alcohols by wine yeasts and relationship to taste and thresholds. *Journal of the Science of Food and Agriculture* **18**, 583–589.

Roset, M. and Margulis, H. (1971) On so-called "wild" yeasts and the volatile substances which they produce in grape juice. *Industries Alimentaires et Agricole* **88**, 647–653.

Romano, P. and Suzzi, G. (1993) Acetoin production in *Saccharomyces cerevisiae* wine yeasts. *FEMS Microbiology Letters* **108**, 23–26.

Romano, P. and Suzzi, G. (1996) Origin and production of acetoin during wine yeast fermentation. *Applied and Environmental Microbiology* **62** (in press).

Romano, P., Suzzi, G., Mortimer, R. and Polsinelli, M. (1995) Production of high levels of acetoin in *Saccharomyces cerevisiae* wine yeasts is a recessive train. *Journal of Applied Bacteriology* **78**, 169–174.

Sponholz, W.R., Dittrich, H.H. and Muno, H. (1993) Diols in wine. *Viticulture Enological Science* **49**, 23–26.

Stivers, J.T. and Washabaugh, M.W. (1993) Catalysis of acetoin formation by brewers' yeast pyruvate decarboxylase isozymes. *Biochemistry* **32(49)**, 13472–13482.

Suomalainen, H. (1971) Yeast and its effect on the flavour of alcoholic beverages. *Journal of the Institute of Brewing* **77**, 164–177.