SELECTIVE BREEDING OF *SACCHAROMYCES CEREVISIAE* TO INCREASE GLYCEROL LEVELS IN WINE

PRODUCTION D'UNE SOUCHE DE *SACCHAROMYCES CEREVISIAE* CAPABLE D'AUGMENTER LE TAUX DU GLYCÉROL DANS LES VINS

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Abstract : Glycerol in wines is at levels of 1 to 10 g/l and improves their body. To breed wine yeast for increased glycerol production, we crossed yeast isolated from natural fermentations with a commercial strain. The average glycerol concentration of 187 wine yeast strains was 4.2 g/l. Strain Ba25 had the highest glycerol production and a spore clone of Ba25 was crossed with one of Premier Cuvée, the best commercial strain, by spore-spore pairing and this was repeated three times. Several of the spore clones produced >15 g/l of glycerol. Glycerol and ethanol levels were inversely related.

Résumé : Exception faite pour l'éthanol, le glycérol est le principal produit de fermentation. Ce composé est présent dans les vins à un taux compris entre 1 et 10 g/l. On estime que des concentrations élevées de glycérol améliorent la qualité du vin en augmentant son corps. Pour cette raison, nous avons décidé de produire une souche de levure œnologique capable d'accroître la production de glycérol ; à ces fins, nous avons croisé une souche isolée au cours de fermentation naturelle du vin (autrement dit sans ensemencement artificiel de levures) avec des souches commerciales uilisées pour la production du vin. Nous avons initialement mesuré la production de glycérol de 187 souches naturelles, en utilisant comme milieu de fermentation un vin synthétique. En moyenne, la concentration de glycérol était de 4.2 g/l, excepté pour 10 souches qui produisaient plus de 5 g/l. Les productions d'éthanol de ces dix souches ont été mesurées et le rapport éthanol:glycérol a été calculé. La souche qui possédait une production de glycérol plus élevée et un rapport éthanol:glycérol plus favorable était Ba25. Un clone dérivé d'une ascospore de cette souche a été croisé avec un clone dérivé d'une ascospore du Premier cuvée, la meilleur souche commerciale en ce qui concerne la production de glycérol. Les ascospores séparés de ces deux souches ont été croisées et la fusion des paires d'ascospores a été vérifiée par l'observation microscopique des zygotes primaires. Le clone provenant du zygote a été sporulé et les asques ont été disséquée. Les clones des ascospores, qui avaient les caractéristiques souhaitées en matière de production de glycérol, ont été à nouveau croisés avec un clone dérivant d'une ascospore du Premier cuvée. Le diploide a été sporulé et disséqué afin d'obtenir des clones provenant des ascospores. Ces clones ont été testés pour la production de glycérol et de nouveau le meilleur a été sélectionné et croisé avec un clone dérivant d'une ascospore du Premier cuvée. Ce croisement a été répété une fois. Après la dernière fusion, la viabilité des ascospores était élevée et les asques contenant les quatre ascospores montraient une ségrégation 2:2, pour ce qui est de la production de glycérol, élevée et moyennement élevée. Un certain nombre de clones provenant des ascospores était capable de produire plus de 15 g/l de glycérol et certaines souches plus de 18 g/l. Le taux de production de glycérol et éthanol étaient inversement reliés et pour chaque mole de glycérol formée, deux moles d'éthanol n'étaient pas formées.
Les souches qui produisent des taux élevés de glycérol ont un intérêt pour l'industrie du vin grâce à leurs capacités à réduire les concentrations d'éthanol dans les vins qui étaient produits par fermentation des moûts à grande quantité de sucre et flaveur élevées en les prendant ainsi plus corsés. Elles peuvent aussi être utilisées pour ajouter du corps à des vins qui autrement seraient trop légers. Vingt de ces souches ont été testées dans des barriques de fermentation en Argentine en mars 1998. Des souches ont été également testées en Californie et dans l'État de Washington en septembre 1998.

Key words : glycerol, ethanol, wine yeast, breeding

Mots clés : glycérol, éthanol, levures, sélection

INTRODUCTION

After water and ethanol, glycerol is the most abundant constituent of wines and this compound can affect the quality of wine in a positive way. As a viscous, oily compound with a slightly sweet taste, glycerol is thought to contribute to the body and smoothness of wines (EUSTACE and THORNTON, 1987 ; RANKINE and BRIDSON, 1971). Grape musts contain less than 1 g/l of glycerol whereas, concentrations in wine are between 1 and 10 g/l as a result of the fermentation process MATTICK and RICE, 1970 ; OUGH *et al.* 1972 ; RANKINE and BRIDSON, 1971). The threshhold for detection of glycerol in water was reported to be between 3.8 and 4.4 g/l and in wine the threshold was 5.2 g/l (NOBLE and BURSICK. 1984). The threshhold in wine may be increased by increasing ethanol concentrations and by the degree of acidity (HINREINER *et al.* 1955). At these concentrations, only the sweetness of glycerol is detected because concentrations greater than 25 g/l are required before there is a marked change in the viscosity of the wine (NOBLE and BURSICK, 1984).

A number of factors may affect the glycerol concentrations in wine. These include : 1- the grape variety and sugar concentration (RANKINE and BRIDSON, 1971), 2- the strain of *S. cerevisiae* used (EUSTACE and THORNTON, 1987 ; RANKINE and BRIDSON, 1971), 3- must pH and fermentation temperature (BERG *et al.,* 1955 ; RANKINE and BRIDSON, 1971), 4- sulfur dioxide concentration (RANKINE and BRIDSON, 1971) and 5- nitrogen source (ALBERS *et al.,* 1996 ; RADLER and SCHÜTZ, 1982). The influence of these various factors on glycerol production in wine is complex because they interact with each other (GARDNER *et al.,* 1993 ; SCANES *et al.,* 1998).

It is of considerable interest to the wine industry to have available yeast strains that produce more glycerol during wine fermentations. Two approaches have been tried to achieve this goal : 1- molecular approach using cloning and transformation and 2- breeding for increased glycerol production.

The genes involved in glycerol synthesis and accumulation recently have become better understood (ALBERTYN *et al.,* 1994 ; ERIKSON *et al.*, 1995 ; LUYTEN *et al.*, 1995). This has led to development, using molecular techniques, of strains of *S. cerevisiae* with increased glycerol production (DE BARROS *et al.*, 1996 ; MICHNICK *et al.*, 1997 ; NEVOIGT and STAHL, 1996). Also, selective breeding of *S. cerevisiae* from culture collections has led to the development of strains that produce more glycerol (EUSTACE and THORNTON, 1987 ; OMORI *et al.*, 1995). EUSTACE and THORNTON and OMORI and coworkers were able to obtain significant increases in glycerol production by breeding. However, ZAMBONELLI *et al.* (1994) also made crosses between strains but the segregants nearly all produced glycerol levels intermediate between those of the two parental strains. Diverse strains of wine yeast isolated from natural wine fermentations have, however, not been evaluated for glycerol production and have not been used in breeding programs designed to increase glycerol concentrations. This study demonstrates that such natural strains are a valuable source of genetic material for breeding programs.

MATERIALS AND METHODS

I - YEAST STRAINS

The strains of *S. cerevisiae* used were obtained from the Yeast Genetics Stock Center, University of California, Berkeley, CA USA and from the research collection of one of us (RKM). These included three laboratory strains, six commercial strains of wine yeast, 28 strains obtained from natural fermentations carried out in Italy (MORTIMER *et al.*, 1994) and 159 strains isolated from natural fermentations in California (MORTIMER, 1998). We also used the commercial strains Prise de Mousse and Premier Cuvée, which were obtained from wineries. These two commercial strains appear to be identical (JOHNSTON *et al.*, 1999).

II - MEDIA AND CULTIVATION

The strains were inoculated in 1 ml of YPD broth (1 p. cent glucose, 2 p. cent peptone, 1 p. cent yeast extract) and grown overnight at 300°C with vigorous agitation. The cultures (0.5 ml) were transferred into 5 ml of YEP synthetic must (0.2 p. cent yeast extract, 2 p. cent peptone, 0.1 p. cent KH_2PO_4, 20 p. cent glucose, pH 3.2 (RADLER and SCHÜTZ, 1982) or into must prepared from Zinfandel grapes (Viano Winery, Martinez, CA). The cultures were incubated at 230°C for seven days without agitation. They were then centrifuged and the supernatants were saved for further analyses.

III - GENETIC PROCEDURES

The abilities of the strains to ferment sugars were tested on yeast extract - peptone agar plates containing the relevant sugar plus the pH indicator bromothymol blue and adjusted to pH 7.5 after sterilization. Fermentation-positive cultures form a surrounding yellow zone in a blue background. The other phenotypic properties of the strains were determined as previously

described (MORTIMER and HAWTHORNE, 1969). Spore-spore matings, zygote characterization and single spore isolations were carried out by micromanipulation as described by WINGE and LAUSTEN (1938) and ROMANO *et al.*, (1995). The parental strains in all matings were HO/HO and completely homozygous to ensure that the zygotes will all be the same relative to their nuclear constitution. Homothallism was scored by the ability of single spore clones to form asci. The breeding scheme used is described in figure 1.

IV - CHEMICAL ANALYSES

Glycerol concentrations were determined by using a glycerol test kit (Boehringer Mannnheim Cat. n°148270). Ethanol concentrations were determined either by using a test kit (Boehringer Mannheim Cat. n°176290) or were quantified by gas chromotography using a Hewlett-Packard Model 5710A gas chromatograph. Acetic acid, acetaldehyde, acetoin and 2,3-butanediol were determined by gas chromotography (PRIOR *et al.*, 1980). Statistical analyses were performed using the Sigma Graphical program (Jandel Scientific).

RESULTS

The average glycerol concentration produced by 187 strains of *S. cerevisiae* which had been allowed to ferment YEP broth for 7 days was 4.2 g/l. The distribution of glycerol values produced by these strains is shown in figure 2 and these values ranged between 1.5 g/l and 5.6 g/l. The mean glycerol concentration produced by the three laboratory strains was 2.0 g/l (range 1.5 to 2.5 g/l). The six commercial strains had a mean of 3.9 g/l (range 3.3 to 4.5 g/l) (table I). These values are similar to those observed in various wines (MATTICK and RICE, 1970 ; OUGH *et al.*, 1972, RANKINE and BRIDSON, 1971).

A selection of strains was examined in greater detail for their abilities to produce glycerol and ethanol using Zinfandel grape must (table I). The laboratory strain W303-1A fermented poorly both the synthetic YEP

TABLE I
Glycerol and ethanol production by a laboratory strain, some commercial strains and several natural wine strains

Tableau I - Taux de glycérol et d'éthanol produits par une souche de levure de laboratoire par quelques souches commerciales et par de nombreuses souches naturelles de levure œnologique

Strain	Glycerol g/l		Ethanol g/l		Ethanol/glycerol	
	YEP	Must	YEP	Must	YEP	Must
Laboratory strain						
W303-1A	4.0 (0.6)a	3.3 (0.8)b	39.6)3.2)	21.2 (1.4)	5.3	6.4
Commercial strains						
French Red	4.0 (1.2)	8.5 (0.8)	58.2 (4.3)	94.0 (5.3)	15.8	11.1
Montrachet	3.3 (1.2)	8.3 (1.1)	50.2 (6.1)	64,1 (8,3)	14.4	7.7
Prise de Mousse	3.9 (1.5)	8.7 (0.6)	56.2 (3.1)	63.5 (6.2)	14.4	7.1
Premier Cuvée	4.5 (0.1)	7.7 (0.3)	60.8 (2.6)	72.0 (11.1)	13.5	9.1
Lallemand K1	3.6 (0.2)	7.9 (0.1)	53.8 (3.0)	92.4 (8.9)	14.9	10.4
Bordeaux	4.2 (0.1)	6.6 (0.2)	60.7 (3.9)	74.2 (7.2)	14.5	11.2
Mean wine strains	3.9	8.0	56.6	75.0	14.5	9.4
Natural wine strains						
Ba25	4.9 (1.6)	10.9 (0.9)	65.2 (6.1)	82.6 (7.7)	13.3	7.6
Ba126	3.6 (0.8)	10.3 (0.6)	27.4 (2.5)	83.2 (12.2)	7.6	8.1
Ba137	4.2 (0.9)	7.1 (0.5)	41.2 (0.6)	57.3 (2.9)	9.8	8.1
Ba220	3.0 (0.8)	7.2 (0.8)	62,4 (6.7)	79.8 (11.5)	20.8	11.1
Bb23(1)	5.1 (0.4)	9.9 (1.3)	42.8 (2.1)	83.5 (7.1)	18.4	8.4
Bb25(8)	4.5 (0.6)	7.5 (0.7)	59.2 (2.6)	75.5 (9.9)	10.8	10.1
Bb30(5)	5.1 (0.3)	9.7 (0.2)	68.4 (4.7)	93.9 (6.9)	13.4	9.6
Bb32(5)	4.6 (0.9)	8.9 (0.9)	63.6 (0.7)	95.2 (8.2)	13.8	9.5
Bb19(4)	4.9 (0.2)	7.7 (1.3)	54.6 (2.2)	60.7 (4.2)	11.1	7.9
Bb22(4)	5.6 (0.7)	8.1 (0.2)	58.1 (1.8)	90.0(6.6)	10.4	11.2
Mean natural yeast	4.7	8.7	54.3	79.3	11.6	9.1

a : mean of three determinations with standard deviations in parentheses ; b : Zinfandel must

must and Zinfandel must. The commercial strains and the strains isolated from natural fermentations produced both higher glycerol and ethanol concentrations in the Zinfandel must than in the synthetic must and the ratio of ethanol/glycerol was lower in the Zinfandel must than in the synthetic must indicating that the medium can affect the balance between these two products of fermentation. Numerous factors, including temperature, pH and nitrogen source, can affect this ratio (PRIOR and HOHMANN, 1997).

The commercial wine yeast strains Prise de Mousse and Premier Cuvée plus two strains isolated from natural wine fermentations, Ba25 and Bb25 (table I), were selected for the breeding program. These strains were sporulated and at least five asci were dissected from each strain. The spore clones were evaluated for the following abilities : 1) to produce glycerol and ethanol in synthetic must, 2) their segregation patterns for fermentation of sucrose, maltose and galactose, 3) their abilities to grow on minimal medium, 4) their resis-

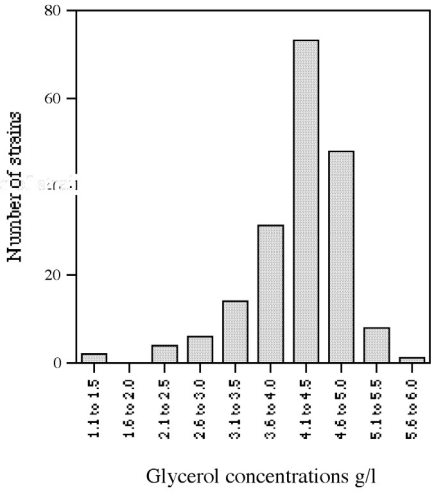

Fig. 2 - Distribution of glycerol concentrations produced by 187 yeast cultures during fermentation of synthetic must.

Fig. 2. - Distribution des concentrations de glycérol produites par 187 cultures de levures pendant la fermentation du moût synthétique.

Figure 1

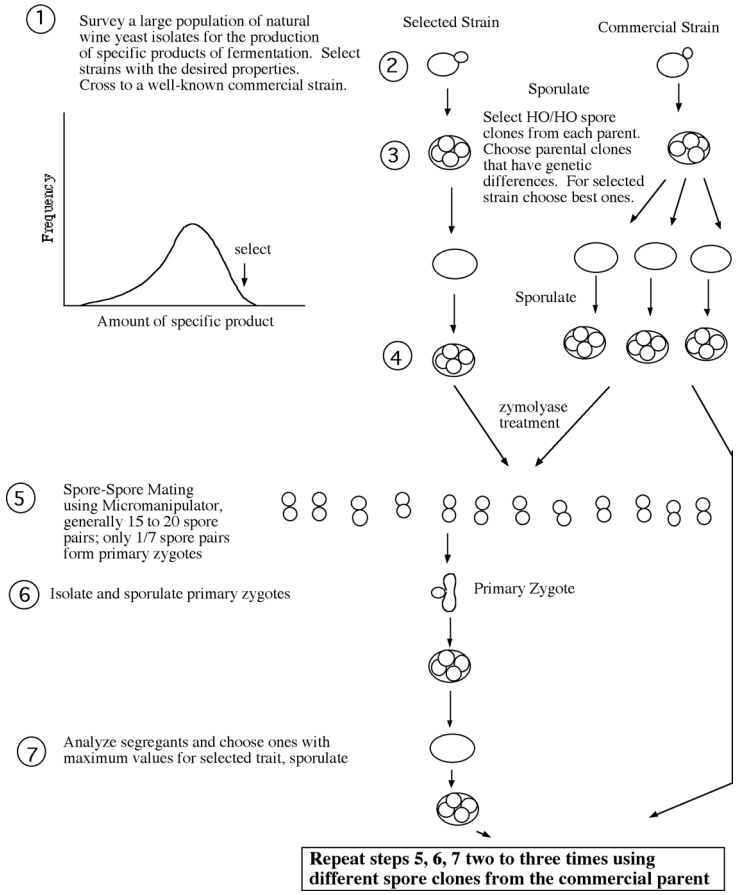

Fig. 1 - Breeding scheme for the development of improved yeast cultures for inoculation of wine fermentations.

Fig. 1 - Schéma des croisements pour le développement de cultures de levures améliorées, qui peuvent être inoculées pendant les fermentations du vin.

tance/sensitivity to copper ion, 5) their abilities to grow on a nonfermentable carbon source, 6) the production of sulfides and 7) their abilities to sporulate. If a spore clone is able to sporulate, that is taken as evidence that this clone carries HO, the homothallism gene (MORTIMER *et al.*, 1994). All spore clones fermented sucrose and maltose and none was petite. The natural wine strains but not the commercial strains fermented galactose.

In our breeding scheme (figure 1), we wanted to use HO/HO spore clones as parental material because they would be completely homozygous and all zygotes from a given cross should be identical, except possibly for some cytoplasmic components. In addition, we wanted at least one phenotypic difference between the two parents so the formation of a zygote could be confirmed in subsequent genetic analyses. We selected spore 25A from Premier Cuvée (PC-25A) and spore 3C from Ba25 (Ba25-3C) as parents for our first cross, XPB1. Both parents sporulated and PC-25A was galactose nonfermenting and Ba25-3C was galactose fermenting. Spores were paired using a micro manipulator and at least 15 such pairs were set up for each cross. Only about 1 in 7 such pairs will form a zygote (WINGE and LAUSTEN, 1938). The spore pairs were observed about once every hour until the pair had either formed a zygote or had started to bud. This is an essential step because zygotes can also be formed by either parental spore by homothallic switching (JENSEN *et al.*, 1983). We selected only « primary zygotes » which were formed by the union of the original two spores.

For Cross XPB1, we examined 18 spore pairs and obtained 2 zygotes. One of these zygote clones was sporulated, 10 asci were dissected and 28 viable spore clones were obtained. The spore clones from these asci were tested for glycerol and ethanol production and for the other traits scored in the parents. Galactose fermentation segregated in a 2:2 fashion indicating that the zygote was formed by a union of the original two spores and was not due to homothallic switching. The range of glycerol concentrations produced by the spores from XPB1 was 5.3 to 14.1 g/l with a mean of 9.0 g/l which is higher than that of the parents (table II).

A spore clone from XPB1, XPB1-8B, that had a low ethanol/glycerol ratio and a high glycerol concen-

TABLE II
Means of glycerol and ethanol concentrations produced by *Saccharomyces cerevisiae* parents and subsequent crosses and their segregants

Table II - Moyennes des concentrations de glycérol et d'éthanol produites par des souches parentales de *Saccharomyces cerevisiae*, par leurs croisements consécutifs et les produits de leurs segregations

Strain	Glycerol concentration (g/l)	Ethanol concentration (g/l)	Ratio : ethanol/glycerol
Parents			
Premier cuvée (PC)	4.5 (0.1)[b]	60.8 (2.6)[b]	13.5
PC spores [14][a]	5.2 (4.1 - 7.0)[c]	60.6 (42.7 - 79.8)[c]	11.7
PC- 25A	5.3 (1.4)[b]	43.9 (3.6)[b]	8.2
Prise de mousse (PDM)	3.9	56.2	14.4
PDM spores [8][a]	2.7 - 6.7	40.4 - 71.2	7.3 - 22.4
Ba25	7.3 (2.0)[b]	51.0 (7.8)[b]	7.0
Ba25 spores [16][a]	6.7 (4.2 - 9.3)[c]	46.7 (26.1 - 63.6)[c]	7.0
Ba25-3C	8.2 (0.7)[b]	56.5 (1.7)[b]	6.9
Bb25	5.0	66.3	13.3
Bb25 spores [9][a]	4.0 - 7.6	56.0 - 72.0	9.5 - 15.7
Crosses			
XPB1: PC-25A x Ba25-3C			
XPB1 spores [28][a]	9.0 (5.3 - 14.1)[c]	81.3 (60.6 - 92.2)[c]	9.0
XPB1-8B	13.3 (0.6)[b]	80.0 (1.6)[b]	6.0
XPB2: PC-25A x XPB1-8B			
XPB2 spores [51][a]	9.2 (5.2 - 16.6)[c]	64.7 (51.5 - 78.6)[c]	6.6
XPB2-2D	15.0 (0.2)[b]	73.6 (1.5)[b]	4.9
XPB3: PC-25A x XPB2-2D			
XPB3 spores [20][a]	11.5 (4.9 - 18.1)[c]	80.2 (71.1 - 92.0)[c]	6.9

a : number of spores tested ; b : standard deviation of at least three determinations ; c : range of concentrations

tration was selected and was backcrossed to PC-25A. This cross was called XPB2. A total of 35 spore pairs were set up and 2 zygotes were obtained. One zygote clone was examined genetically and it segregated 2:2 for galactose fermentation confirming that the zygote was a primary zygote. A total of 51 spore clones were derived from 16 asci dissected from this cross and they produced glycerol concentrations in the range 5.2 to 16.6 g/l with a mean of 9.2 g/l (table II).

A spore clone from XPB2, XPB2-2D, was again selected and backcrossed to PC-25A to form hybrid strain XPB3. Seventy one spore pairs were examined and three zygotes were obtained. However, two of these three zygote clones segregated for only one parental phenotype which indicated that karyogamy had failed in these spore unions. The third zygote clone, which segregated for galactose fermentation, was considered to be a primary zygote. Five tetrads were dissected from this zygote clone (XPB3) and 20 spore clones were obtained. These produced glycerol with a range of 4.9 to 18.1 g/l with a mean of 11.5 g/l. Among the spore clones from XPB3 are several that produce in excess of 15 g/l of glycerol and have ethanol/glycerol ratios of less than 5 (table III).

It is interesting to note that the frequencies of successful matings in XPB2 and, in particular, in XPB3 are much lower than found for XPB1. In previous studies (WINGE and LAUSTEN, 1938 ; ROMANO *et al.*, 1995) approximately one in seven spore pairings formed a primary zygote. We believe this reduction could be due to cryptic mutations in one of the parents that interfere with matings. These mutations could be in the ste (sterile) or kar (karyogamy) genes that were heterozygous in Premier Cuvée and became homozygous in PC-25A. Back crosses to this strain would allow more of these mutations to be homozygous and expressed.

The spore clones from XPB3 segregate in a 2:2 fashion for the level of glycerol production indicating that the differences between these levels is controlled by a single gene. This segregation pattern was also observed for the ethanol/glycerol ratio. A plot of ethanol vs glycerol concentrations in the 20 fermentations by the spore clones from XPB3 (table III) is shown in figure 3. The inverse relationship shows that for each mole of glycerol formed, two moles of ethanol are not formed. This 1:2 ratio is the same as the ratio of the molecular weights of the two compounds, i.i. ethanol – 46, glycerol - 92

We wanted to see if other products may also be inversely related to ethanol production. This led us to examine in more detail the fermentation products of

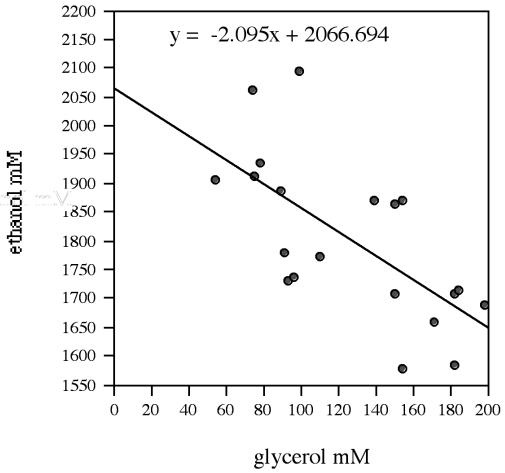

Fig. 3 - Plot of ethanol v.s. glycerol concentrations in fermentations of synthetic must inoculated with 20 strains described in table III.

Figure 3. - Graphique des concentrations dans le éthanol vs glycérol produites par les 20 souches décrites dans le tableau III pendant la fermentation du moût synthétique.

the original parents, Premier Cuvée and Ba25 as well as the four spores from ascus XPB3-5 when allowed to ferment in glucose YEP medium for 210 hours. Acetic acid and acetaldehyde concentrations produced by the two parents and the four spores were similar (0.07 to .0.21 g/l for acetic acid and 0.03 to 0.05 g/l for acetaldehyde). However, for acetoin and 2,3-butanediol production, there were differences. The two spores that produced the most glycerol, XPB3-5B and XPB3-5C, also produced thigher concentrations of these two compounds (table III).

DISCUSSION

These results confirm and extend the data of previous workers who showed that yeast strains could be bred to enhance the levels of glycerol produced (EUSTACE and THORNTON, 1987 ; MICHNICK *et al.*, 1997). Our experimental strategy, however, has led to the development of strains that are able to produce even higher levels of glycerol than those previously reported. This perhaps indicates that selection of strains from natural fermentations may be an important source of genetic diversity for breeding. Also, our breeding strategy of repeated backcrosses to a commercial strain may have played a role in achieving these higher levels.

In contrast to the findings of OMORI *et al.* (1995) who found that their hyper-glycerol producing strains produced by breeding were also more salt tolerant, our high glycerol strains did not appear to be more halo-

tolerant than the parental strains (data not shown). The concentrations of other products found in the fermentations, however, may pose problems for their use in wine making. Acetic acid concentration was near the upper limit (0.3 g/l) above which it is undesirable for the taste and odor of wines. Acetaldehyde levels were high but below the limit of 100 mg/l considered to cause off-odors.

Other workers have had success in producing higher glycerol levels by molecular manipulation of yeast strains using the genes encoding cytosolic glycero ; -3-phosphate dehydrogenase (GPD1 and GPD2). Wine yeast overexpressing GPD1 (under the control of the ADH1 promoter) and GPD2 produced 28.6 g/l (MICHNICK*et al.*, 1997) and more than 18 g/l of glycerol (DE BARROS *et al.*, 1996, respectively. Most yeast produce less than 10 g/l of glycerol during a fermentation (ANSELL *et al.*, 1997). Strains with the ability to produce such high levels could be used to make wines with more interesting sensory properties as well as producing lower ethanol levels. In a strain engineered to overproduce glycerol (MICHNICK *et al.*, 1997) there were significant increases in acetic acid (three-fold), acetaldehyde (at least two-fold), acetoin (at least sixty-fold) and 2,3-butanediol (1.5 fold), respectively. when compared to control yeast (SCANES *et al.*, 1998). An additional problem with the use of bio-engineered yeast is the lack of general acceptability by the industry.

In cross XPB3, there was a 2:2 segregation in each ascus for the levels of glycerol production. Since this cross was the result of a cross of a natural strain to a particular strain, PC-25A, and two back-crosses to the same strain, one would expect that most genes that affect glycerol production to be either homozygous dominant or homozygous recessive. Apparently at least one gene that affected this pathway was still heterozygous and this gave the segregation that was observed. It is very likely that additional crosses could lead to different mixtures of homozygous dominant, homozygous recessive and heterozygosity of the genes affecting this pathway and that could also be interesting. In

TABLE III

XPB3 : Glycerol and ethanol production and galactose fermentation by *Saccharomyces cerevisiae* parental strains and their segregants, measured in glucose (20 percent) - YEP medium.

Tableau III - XPB3 : Production de glycérol et d'éthanol et fermentation sur galactose de souches parentales de *Saccharomyces cerevisiae* et des produits de leur ségrégation, mesurée sur glucose (20 percent) – milieu YEP.

Strain	Glycerol (g/l)	Ethanol (g/l)	Ethanol/ Glycerol	Galactose Fermentation	Acetic acid (g/l)	Acetoin (mg/l)	2,3-butanediol (mg/l)
Premier cuvée-25A	5.3a	43.9	8.2	-	-	-	-
XPB2-2D	15.0	73.6	4.9	+	-	-	-
XPB3-1A	8.8 (0.4)	78.3 (4.4)	8.9	-	-	-	-
XPB3-1B	8.1 (0.4)	85.0 (2.2)	10.5	+	-	-	-
XPB3-1C	13.7 (0.9)	83.9 (1.6)	6.1	-	-	-	-
XPB3-1D	13.7 (1.6)	76.9 (4.3)	5.6	+	-	-	-
XPB3-2A	6.8 (0.4)	92.9(2.0)	13.6	+	-	-	-
XPB3-2B	14.1 (0.7)	94.3 (1.2)	6.0	-	-	-	-
XPB3-2C	16.7 (0.3)	76.9 (1.2)	4.6	+	-	-	-
XPB3-2D	9.1 (1.2)	84.4 (0.6)	9.3	-	-	-	-
XPB3-3A	14.1 (1.8)	71.1 (4.3)	5.0	-	-	-	-
XPB3-3B	6.9 (1.1)	86.1 (1.4)	12.5	+	-	-	-
XPB3-3C	4.9 (0.4)	85.9 (0.8)	17.6	-	-	-	-
XPB3-3D	16.7 (2.4)	71.4 (4.8)	4.3	+	-	-	-
XPB3-4A	7.1 (1.1)	87.1 (0.9)	12.3	+	-	-	-
XPB3-4B	10.1 (1.0)	79.6 (2.7)	7.9	-	-	-	-
XPB3-4C	16.9 (1.6)	77.2 (1.3)	4.6	-	-	-	-
XPB3-4D	15.7 (0.4)	74.7 (3.0)	4.8	+	-	-	-
XPB3-5A	8.3 (0.4)	80.1 (3.6)	9.7	-	0.07	0.025	0.66
XPB3-5B	18.1 (0.6)	76.1 (1.3)	4.2	-	0.21	0.030	1.04
XPB3-5C	12.7 (1.0)	74.2 (1.4)	5.8	+	0.13	0.028	0.85
XPB3-5D	8.5 (0.6)	77.9 (1.1)	9.1	+	0.07	0.020	0.54

a : mean of four independent determinations with standard deviations in parentheses

contrast to the results obtained for glycerol, ethanol concentrations showed a Gaussian unimodal distribution in the spores from XPB3.

CONCLUSION

The use of classical genetic procedures to breed yeast strains to produce higher levels of glycerol appears to be feasible and preferable to the use of molecular techniques to achieve similar results. These strains should be of considerable interest to the wine industry because they lead to the production of wine with greater body and in addition can be used to produce wines with lower ethanol content for a given sugar content of the must.

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REFERENCES

ALBERS E., LARSON C., LIDÉN G., NIKLASSON C. and GUSTAFSSON L., 1996. Influence of nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Appl. Environ. Microbiol.*, **62**, 3187-3195.

ALBERTYN J., HOHMANN S., THEVELEIN J. M. and PRIOR B.A., 1994. GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae* and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol. Cell Biol.*, **14**, 4135-4144.

ANSELL R., GARANATH K., HOHMANN S., THEVELEIN J. M. and ADLER L., 1997. The two isoenzymes for yeast NAD^{+-} dependent glycerol-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. *Embo. J.*, **16**, 2179 - 2187.

BERG H.W., FILIPELLO F., HINREINER F. and WEBB A.D., 1955. Evaluations of thresholds and minimum difference concentrations for various constituents of wine. I. Water solutions of pure substances. *Food Technol.*, **9**, 23 - 26.

DE BARROS M., RECHMAN A., LANGRIDGE P. and HENSCHKE P. A., 1996. Altering glycerol metabolism of yeast for the production of lower alcohol wines. *9th Int. Symp. on Yeast*s, Sydney, Australia, (August 1996).

ERIKSSON P., ANDRÉ L., ANSELL R., BLOMBERG A. and ADLER L., 1995. Cloning and characterization of GPD2, a second gene encoding sn-glycerol-3 phosphate dehydrogenase (NAD^{+}) in *Saccharomyces cerevisiae* and its comparison with GPD1. *Mol. Microbiol.*, **17**, 95-107.

EUSTACE R. and THORNTON R., 1987. Selective hybridization of wine yeasts for higher yields of glycerol. *Can. J. Microbiol.*, **33**, 112-117.

GARDNER N., RODRIGUE N. and CHAMPAGNE C.P., 1993. Combined effects of sulfites, temperature, and agitation time on production of glycerol in grape juice by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*, 59, 2022-2028.

HINREINER E., PILIPELLO F., WEBB A.D. and BERG H.W., 1955. Evaluation of thresholds and minimum difference concentrations for various constituents ofwines. III. Ethyl alcohol, glycerol and acicity in aqueous solution. *Food Technol.*, 9, 351-353.

JENSEN R., SPRAGUE C. F. and HERSKOWITZ I., 1983. Regulation of yeast mating type interconversion : Feddback control of HO gene expression by the mating type locus. *Proc. Natl. Acad. Sci. U. S. A.*, **80**, 3035-3039.

JOHNSTON J.R., BACCARI C. and MORTIMER R.K., 1999. Genetic Analysis of Commercial Wine Yeast, (*in preparation*).

LUYTEN K., ALBERTYN J., SKIBBE W.F., PRIOR B.A., RAMOS J., THIEVELEIN J.M. and HOHMANN S., 1995. FPS1, a yeast member of the MIP family of channel proteins is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J.*, **14**, 1360-1371.

MATTICK L.R. and RICE A.C., 1970. Survey of the glycerol content of New-York State wines. *Amer. J. Enol. Vitic.*, 21, 213-215.

MICHNICK S., ROUSTAN J.-L., REMIZE F., BARRE P. and DESQUIN S., 1997. Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for (GPD1) encoding glycerol-3-phosphate dehydrogenase. *Yeast*, **13**, 783-793.

MORTIMER R. K. 1998. Diversity of *yeast*s isolated from natural fermentations. *Proceed. Italian BIodiversity Society*. Alghero, Sicily, September 1998.

MORTIMER R.K. and HAWTHORNE D.C., 1969. *In : The Yeasts, Biology of Yeasts*. A.H. Rose and J.S. Harrison (Editors), pp. 385-460, Academic Press, London.

MORTIMER R.K., ROMANO P., SUZZI G. and POLSINELLI M., 1994. Genome renewal : A new phenomenon revealed from a genetic study of *Saccharomyces cerevisiae* derived from natural fermentation of grape musts. *Yeast*, **10**, 543-552.

NOBLE A.C. and BURSICK G.F., 1984. The contribution of glycerol to perceived viscosity and sweetness in wine. *Am. J. Enol. Vitic*., **35**, 110-112.

NEVOIGT E. and STAHL U., 1996. Reduced pyruvate decarboxylase and increased glycerol-3-phodphate dehydrohrnase [NAD^+] levels enhance glycerol production in *Saccharomyces cerevisiae*. *Yeast*, **12**, 1331-1337.

OMORI T., OGAW K. and SHIMODA M., 1995., Breeding of high glycerol-producing Shochu *yeast* (*Saccharomyces cerevisiae*) with acquired salt tolerance. *J. Ferment Bioengin.*, **79**, 560-565.

OUGH C.S., FONG D. and AMERINE M.A., 1972, Glycerol in wine : determination and some factors affecting. *Am. J. Enol. Vitic.*, **23**, 1-5.

PRIOR B.A., KILLIAN S. and LATEGAN P., 1980, Growth of *Candida utilis* on ethanol and isopropanol. *Arch. Microbiol.*, **125**, 133-136.

PRIOR B.A. and HOHMANN S., 1997. Glycerol production and osmoregulation. *In : Yeast Sugar Metabolism : Biochemistry, genetics, biotechnology and applications*. F.K. Zimmerman and K. Entian (Eds.), pp. 313-337. Technomic. Publ. Co., Lancanster, PA.

RADLER F. and SCHÜTZ H., 1982. Glycerol production of various strains of *Saccharomyces*. *Am. J. Enol. Vitic*, **33**, 36-40.

RANKINE B. C. and BRIDSON H., 1971. Glycerol in Australian wines and factors influencing its formation. *Am. J. Enol. Vitic*., **22**, 6-12.

ROMANO P., SUZZI G., MORTIMER R. and POLSINELLI M., 1995. Production of high levels of acetoin in *Saccharomyces cerevisae* wine *yeast*s is a recessive trait. *J. Appl. Bacteriol.*, **78**, 169-174.

SCANES K.T., HOHMANN S. and PRIOR B.A., 1998. Glycerol production in the yeast *Sacchar omyces cerevisiae* and its relevance to wine. A review. *S. African J. Enol. Vitic*. *(in press)*.

WINGE Ö and LAUSTEN O., 1938, Artificial species hybridization in yeast. *CR Travaux Laboratoire Carlsberg* : *Série physiologique*, **22**, 235 - 244.

ZAMBONELLI C., PASSARELLI P., EAINIERI S. and GIUDICI P., 1994. Genetic study on glycerol prodfuction in *Saccharomyces* yeast breeding. *Ann. Microbiol. Enzimol.*, **44**, 107-118.

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