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Metabolic analysis of *S. cerevisiae* strains engineered for malolactic fermentation

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Abstract A complete malolactic fermentation was achieved using *Saccharomyces cerevisiae* strains coexpressing the genes *mleS* and *mae1* coding for the *Lactococcus lactis* malolactic enzyme and the *Schizosaccharomyces pombe* malate permease under the control of yeast promoters. The expression level of *mae1* greatly influences the kinetics of the reaction by controlling the rate of malate uptake meanwhile a high expression level of *mleS* induces a partial consumption of malate derived from glucose by the malolactic enzyme. A strain expressing several copies of *mae1* and one copy of *mleS* degrades 3 g/l of malate almost exclusively through the malolactic pathway in 4 days under enological conditions, without metabolic side effects.

Key words: Malate metabolism; (*Saccharomyces cerevisiae*); Malate transport; Malolactic enzyme; Malolactic fermentation; Enology

1. Introduction

In winemaking alcoholic fermentation, performed by the yeast *Saccharomyces cerevisiae*, is followed by malolactic fermentation (MLF) for most red and some white wines. This secondary fermentation, carried out by several genera of lactic acid bacteria (LAB, *Lactobacillus*, *Leuconostoc* and *Pediococcus*), results in the decarboxylation of malate into lactate. This reaction is essential for the quality of wine, since the resulting deacidification improves the taste of wine and because the removal of malate is of major importance for wine stability. MLF is difficult to control owing to a poor growth of lactic acid bacteria in wine. Their development can be prevented by a combination of different factors (nutrient limitation, low temperature, acidic pH, high alcohol and sulFur dioxide concentrations). Sluggish or stuck fermentations are frequent and often lead to microbial alteration. MLF can be obtained by using starter cultures of LAB, but adaptation to wine conditions may fail [1,2].

Rapid onset of MLF could be ensured by using a single wine yeast strain engineered to perform simultaneously alcoholic and malolactic fermentation. The responsible enzyme for MLF is malolactic enzyme (MLE), a bi-functional enzyme which needs NAD^+ and $Mg2^+$ and does not generate intermediate nor cofactor reduction. The gene *mleS* coding for MLE has been recently isolated from *Lactococcus lactis* [3,4] and from *Leuconostoc Ïnos* [5]. *S. cerevisiae* strains expressing *L. lactis mleS* under the control of alcohol dehydrogenase regulatory elements on a multicopy vector were only slightly improved for malate degradation [3,6,7]. At the same time a large amount of L-lactate was produced from the endogenous L-malate pool, demonstrating that MLE was functional. The assumption that malate transport, a simple diffusion step in *S. cerevisiae* was the limiting step to achieve a complete malolactic fermentation in this yeast was further supported by the demonstration that a full malolactic fermentation was achieved in *Schizosaccharomyces pombe* expressing *mleS* [6]. In this yeast, a carrier-mediated transport for malic acid has been characterized [8,9]. Recently, the *mae1* gene of *S. pombe* encoding a malate permease was cloned [10], and preliminary data on its functional expression in *S. cerevisiae* were reported [11]. In this paper, we assessed the ability of *S. cerevisiae* to perform malolactic fermentation by coexpression of *L. lactis mleS* and *S. pombe mae1* genes. *MleS* and *mae1* genes were placed under the control of respectively alcohol dehydrogenase and phosphoglycerate kinase regulation elements, and introduced in *S. cerevisiae* either on monocopy or multicopy plasmids. The recombinant strains were studied for their ability to transport L-malate and to convert L-malate into L-lactate under fermentative conditions on sugar-rich medium (180 g/l) and acidic pH (enological-like conditions). The influence of *mae1* and *mleS* expression level on the kinetics of malolactic fermentation was also investigated. Isotopic filiations were used to identify the pathways by which malate was metabolized and to assess possible metabolic side effects of the introduction of this new malate degradation pathway.

2. Materials and methods

2.1. Strains and medias and growth conditions

E. coli DH5α was used for cloning experiments. Expression studies of *mleS* and *mae1* genes were performed in the *S. cerevisiae* strain OL1 (*MAT* α *leu2 his3 ura3*). *E. coli* cultivation and media were as described previously [12]. The *S. pombe* strain (*leu1-32h+*) [6] was used as source of *mae1* gene. Yeasts were maintained on YPD medium (10 g/l yeast extract, 20 g/l Bacto peptone, 20 g/l glucose). Batch fermentation experiments were carried out on YNB minimal synthetic medium (6.7 g/l yeast nitrogen base without amino acids, 180 g/l glucose, 3 g/l L-malate, required amino acids, 6 g/l phthalic acid, pH 3.5). Precultured cells were inoculated at a density of 10^6 cells/ml in fermentors with a working volume of 1.1 l, equipped with fermentation locks. Fermentations were carried out at 28°C with permanent stirring (500 rpm). CO_2 release was determined by automatic measurement of fermentor weight loss each 20 min. The CO_2 production rate was automatically calculated by polynomial smoothing of CO_2 evolved. This method gives fermentation kinetics similar to that of industrial scale wine making [13].

2.2. Recombinant DNA techniques and plasmid constructions

DNA manipulations were carried out using standard methods [12]. *E. coli* and *S. cerevisiae* were transformed as described previously [14,15]. *S. pombe* genomic DNA was isolated using standard methods [16]. Oligonucleotides were synthetized by Eurogentec. The plasmids used and constructed in this study are listed in Table 1. The gene *mae1* was amplified from *S. pombe* genomic DNA by polymerase chain reaction (PCR) using oligonucleotides AAAGATCTTGGCCA-

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CTATTTTTTTTTTTAATT and AAAGATCTCATATGCAAGACGCATACATA based on the sequence of *mae1* [10]. The primers introduced a *Bgl*II site at 5Õ and 3Õ ends of *mae1*. The amplified fragment was cut with *Bgl*II and cloned into the plasmid pMA91 [17]. The expression cassette containing *mae1* gene under the control of *S. cerevisiae PGK* regulatory elements was amplified by PCR using pMA91*mae1* as template and oligonucleotides TTCTAGATCTATCCAAAACTGAAAAT and TATCTAGAGCAGAATTTTCGAGTTATTAA based on the sequence of *PGK* promotor and terminator. *Xba*I sites were introduced at the 5Õ ends of the primers. The 3.4 kb fragment obtained was digested by *Xba*I and ligated into pRS316 [18] and YEp352 [19] yielding respectively p6*mae1* and YEp*mae1*. Plasmids p5*mleS* and pRS*mleS* were obtained as follow. The expression cassette containing *mleS* gene under the control of *S. cerevisiae ADH1* regulatory elements was isolated by PCR from the plasmid pM1 [3] using oligonucleotides GCAGGCCTCAACTTCTTTTCTTTTTTTT and CGAGGCCTCATGCCGGTAGAGGT based on the sequence of *ADH1* promoter and terminator. StuI sites were introduced at the 5′ ends of the primers. The 2.4 kb fragment amplified from pM1 was digested by *Stu*I and ligated into pRS315 [18] or pRS425 [20] digested by *Sma*I, giving p5*mleS* and pRS*mleS*.

2.3. Analytical methods

Growth was monitored by optical density (660 nm). L-Lactate and L-malate and glucose were analysed by HPLC on an HPX-87H Aminex column (BioRad). Elution was performed at 45°C with 8 mM H_2SO_4 at a flow rate of 0.6 ml/min. Dual detection was performed by means of refractometer (Shimidazu) and UV detector (Shimidazu SDD-2A, λ 214 nm). Quantification was done using external standards (Sigma) and an HP 3365 integration system.

2.4. Radioactivity determination

[^{14}C]Malic acid and [^{14}C]lactic acid contained in culture supernatants were separated by paper chromatography, collected and assayed as described previously [6].

2.5. Transport assays and intracellular malate determinations

Yeast were cultivated in the same conditions as described for batch fermentation, except that malic acid was omitted. Cells were harvested at the end of exponential phase, washed twice with buffer A (0.1 M potassium phosphate, 5 mM glucose, pH 3.5) and suspended in the same buffer to a final concentration of about 2 mg dry weight/ml. After 2 min pre-incubation at 28°C of 170 μl of yeast suspension, malic acid uptake was initiated by addition of 30 ml of 13.3 mM ^{14}C-labelled malic acid, 133.3 mM glucose in 0.1 M potassium phosphate buffer, pH 3.5. After each incubation time (28°C) the corresponding sample was diluted in buffer A (5 ml), quickly filtered through glass microfibre filters (Whatmann GF/C) and washed with 10 ml of buffer A. Scintillation fluid (10 ml) was added to dried filters (IR lamp) and the radioactivity was measured with a liquid scintillation counter (Beckman). Assays of 30 s duration were used for the determination of the initial rate of malate uptake.

The intracellular malate concentration was determined on cells harvested at the end of exponential growth phase, and treated as described above. After 1 h incubation with 16.6 mM L-malate at 28°C, cells were collected on glass microfibre filters (Whatmann GF/C) and washed with 10 ml of cold isoton–methanol (v/v). The filter was frozen and cells were disrupted 10 min at 100°C in 2 ml of Tris-HCl 0.5 M, pH 7. Cellular debris were eliminated by centrifugation and malate was enzymatically determined in the supernatant using Boehringer kit. The intracellular volume used for calculation of malate concentration was measured with a channalyser C256 (Coultronics).

3. Results

3.1. Malate degradation of OL1 strains coexpressing mleS, mae1, or both genes

S. cerevisiae strains expressing malolactic enzyme were previously shown to be scarcely improved for malate degradation, owing to a limitation of malate transport [6]. To investigate the possibility of increasing L-malate influx, a set of *S. cerevisiae* strains expressing *mleS*, *mae1* or both genes on monocopy or multicopy vectors was constructed (Table 1). The ability of the recombinant strains to degrade malate was tested on YNB medium (glucose 180 g/l, L-malate 3 g/l, pH 3.5) simulating enological conditions (Table 1). The control strain OL1p degraded a small amount of malate (12%) in these experimental conditions. Malate degradation level was similar or slightly increased in the strain expressing *mleS* alone on monocopy (OL1r) or multicopy (OL1q) vectors. On the other hand, the installation of a malate transport system in *S. cerevisiae* significantly improved malate degradation (OL1u and OL1v). Nevertheless, more than half the amount of malate was not metabolized by these strains. In contrast, malate was completely or almost totally degraded by the strain containing both *mleS* and *mae1* genes (OL1e and OL1f). However, a different level of malate degradation was achieved in the strains OL1a and OL1b expressing *mae1* on a multicopy vector (100%) in comparison with the strains OL1e and Ol1f containing one copy of this gene (83–86%), whereas no difference was observed depending on the copy number of *mleS* gene. This suggests that the ability of a strain to perform a complete malolactic conversion depends mainly on its ability to transport malate.

3.2. Kinetics of L-malate degradation and L-lactate production

The ability of the strains coexpressing both genes (OL1a, -b, -e and -f) to perform malolactic fermentation was studied under enological conditions. Growth, glucose and L-malate degradation and L-lactate production are shown on Fig. 1. A rapid and total degradation of L-malate (Fig. 1A) was achieved with the strains containing *mae1* on multicopy vectors (OL1a and OL1b), leading to the assimilation of 3 g/l L-malate during the first 100 h of fermentation. In contrast, the

Table 1
Yeast transformants

Strain	Plasmids	Copy number		L-Malate degraded
		mae1	*mleS*	(%)[a]
a	YEP *mae1*+pRS *mleS*	multicopy	multicopy	100
b	YEP *mae1*+p5 *mleS*	multicopy	monocopy	100
e	p6 *mae1*+pRS *mleS*	monocopy	multicopy	83
f	p6 *mae1*+p5 *mleS*	monocopy	monocopy	86
p	pRS 316+pRS 315	–	–	12
q	pRS *mleS*	–	multicopy	20.2
r	p5 *mleS*	–	monocopy	14
u	YEp *mae1*	multicopy	–	47.6
v	p6 *mae1*	monocopy	–	43.1

[a]The percentage of L-malate degraded was calculated from residual L-malate determined in growth medium after sugar exhaustion.

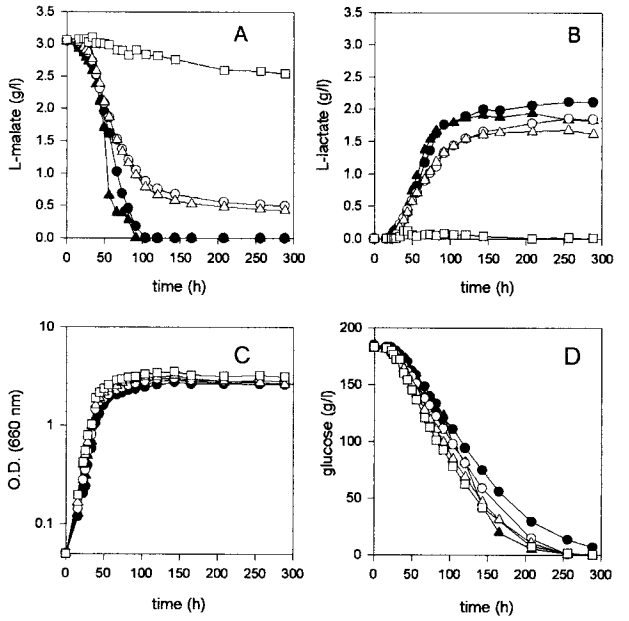

Fig. 1. Malate degradation (A), lactate production (B), growth (C) and glucose degradation (D) during fermentation on YNB glucose 180 g/l, pH 3.5. OL1a (●, *mae1* multicopy, *mleS* multicopy); OL1b (▲, *mae1* multicopy, *mleS* monocopy); OL1e (○, *mae1* monocopy, *mleS* multicopy), OL1f (△, *mae1* monocopy, *mleS* monocopy), OL1p (□, control strain).

strains containing *mae1* in monocopy (OL1e and OL1f) exhibited a slower and incomplete malate degradation. The rate of malate degradation of these strains was similar to that of the strains expressing several copies of *mae1* during the first 60 h of fermentation, which corresponds to the cellular growth phase (Fig. 1C), then slowed down during the transition phase (60 to 120 h, Fig. 1C). In any case, no difference was observed between the strains expressing one or several copies of *mleS*, demonstrating that a low expression level of *mleS* was sufficient to drive a full malate degradation. Simultaneously to malate degradation, a large amount of L-lactate was produced by the strains coexpressing both genes (Fig. 1B), whereas only traces were detected for the strains expressing only *mae1* and a small amount for OL1q and OL1r transformed with *mleS* alone (0.5 g/l and 0.3 g/l, respectively). The growth characteristics were not affected (Fig. 1C) by the introduction of a new malate degradation pathway in *S. cerevisiae*; the same final biomass was reached for the five strains. In the same way, the degradation kinetics of the four strains were closed to that of the control strain (Fig. 1D).

3.3. Malate transport assays and intracellular malate determination

Depending on the copy number of the *mae1* gene (mono or multicopy) present in the strains transformed with *mleS*, different rate and levels of malate degradation were obtained (Table 1 and Fig. 1). This suggests that the ability of *S. cerevisiae* to perform a complete malolactic fermentation depends on their efficiency to transport malate and on the level of available intracellular malate. These aspects were further studied by determining the kinetics of malate transport in the strains OL1a, -b, -e, -f and -p (Fig. 2). A very slow malate influx (0.013 nmol/min/mg dry weight) was determined in OL1p (control strain), in agreement with simple diffusion of the undissociated form of the acid (representing 43% of total malic acid at pH 3.5). Heterologous expression of the *mae1* gene resulted in a great improvement of the kinetics of malate transport. In the strains OL1e and OL1f, containing *mae1* in monocopy, the initial rate of malate uptake increased to 0.94 and 0.76 nmol/min/mg dry weight, respectively, when this rate reached 1.9 and 2.1 nmol/min/mg dry weight in the strains transformed with *mae1* in multicopy (OL1a and OL1b, respectively).

The consequences of the expression of *mleS* or *mae1* on malate pool were investigated by determination, for all the strains described in Table 1, of the intracellular malate concentration on late exponential phase cells. A lower intracellular malate concentration was found in the strains expressing *mleS* gene alone (OL1q and -r, 3 mM) when compared to that of the control strain (OL1p, 6 mM), in agreement with a depletion of malate through the malolactic pathway. On the other hand, expression of *mae1* resulted in increased malate concentration (11 mM) regardless the copy number of *mae1* (OL1u and -v). When both *mae1* and *mleS* were expressed, this level dropped to that of the control strain (6 mM) or below this value (4 mM) depending on the expression level of *mleS* (monocopy or multicopy, respectively). Since the intracellular malate concentration was not enhanced when *mae1* expression was increased, no difference in the rate of malate degradation could be expected between the strains expressing one or several copies of this gene, that was actually the case during growth phase (Fig. 1).

3.4. Filiation of ^{14}C-labelling from L-malate to L-lactate

Although malolactic strains produced L-lactate through the malolactic pathway (Fig. 1), a significant part of L-malate could be metabolized via *S. cerevisiae* malate degradation pathways (malic enzyme, fumarase and malate dehydrogenase). Furthermore, lactate could be produced from intracellular malate generated from sugar. To assess the amount of

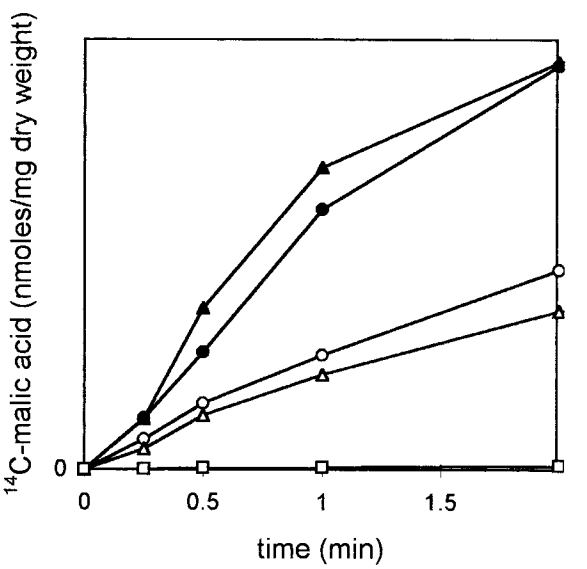

Fig. 2. [^{14}C]malate uptake by *S. cerevisiae* strains cotransformed by *mleS* and *mae1* genes under the control of ADHI or PGK promoters, respectively. OL1a (●, *mae1* multicopy, *mleS* multicopy); OL1b (▲, *mae1* multicopy, *mleS* monocopy); OL1e (○, *mae1* monocopy, *mleS* multicopy), OL1f (△, *mae1* monocopy, *mleS* monocopy), OL1p (□, control strain).

Table 2
Filiation of [^{14}C]malate during fermentation

Strain	Ratio of [^{14}C]lactate (mM) produced to [^{14}C]malate (mM) assimilated (%)[a]	Ratio of ^{14}C lactate (mM) to total lactate (mM) produced (%)[a]	Total lactate (mM)
OL1a	95 ± 3	84 ± 3	25.2 ± 0.5
OL1b	92 ± 1	97 ± 2	20.3 ± 0.4
OL1e	104 ± 4	83 ± 1	25.3 ± 0.9
OL1f	98 ± 3	102 ± 3	18.9 ± 0.3

[a]All values are expressed as means derived from determinations on three or four different samples obtained after stabilization of the reaction.

malate metabolized via MLE and to determine the origin of L-lactate produced, the strains a, b, e, f and q were grown in minimal medium (glucose 180 g/l) containing 3 g/l [U^{14}C]L-malate (specific radioactivity 0.004 mCi/mmol). Total L-malate and L-lactate amounts and ^{14}C filiation from L-malate to L-lactate were kinetically monitored during fermentation. The ratio of labelled lactate produced to labelled malate metabolized reflects the utilisation of malate through the malolactic pathway, while the origin of L-lactate produced through this pathway (from endogenous or exogenous L-malate) was estimated by means of the ratio of labelled lactate to total lactate (Table 2). For the four recombinant strains, malate was mainly metabolized via malolactic enzyme (> 92%), demonstrating that the heterologous enzyme competes efficiently with *S. cerevisiae* enzymes involved in malate utilization. Moreover, a low expression level of *mleS* was shown to be enough to ensure this competition, since no significant difference was observed between the four strains. On the other hand, the expression level of *mleS* influences lactate production. When *mleS* was expressed on a monocopy vector (OL1b and OL1f), lactate originates exclusively from exogenous L-malate. In contrast, a significant part of lactate (15%) produced by the strains containing several copies of *mleS* corresponded to the degradation of endogenous malate.

4. Discussion

Data presented in this work demonstrate that a complete malolactic conversion by *S. cerevisiae* can be achieved by installation of an efficient system of malate uptake and introduction of a malolactic activity in *S. cerevisiae*. Coexpression of *mae1* and *mleS* was shown to be absolutely required to achieve a complete malate degradation, since a strain expressing only *mae1* degraded no more than 45% of malate. This can be explained by a much higher affinity of malolactic enzyme for malate (K_m 12 mM, [21]) than that of malic enzyme (K_m 50 mM [22]), the main malate degradation pathway in anaerobiosis [23]. Actually, we demonstrated by isotopic filiation of [^{14}C]malate that more than 92% of malate was metabolized via malolactic enzyme, confirming that competition for malate utilization is in favour of malolactic enzyme.

The study of a set of strains expressing both *mae1* and *mleS* genes with different expression levels allowed us to characterized in further details the kinetics of the reaction and the metabolic effects of the introduction of a new malate degradation pathway in *S. cerevisiae*. The malate transport rate was shown to be greatly influenced by the copy number of *mae1* gene. Strains expressing several copies of *mae1* (a, b) exhibited high rate of malate influx and were shown to degrade completely and rapidly malate. On the other hand, a low rate of malate influx such as triggered by one copy of *mae1* (e, f) was sufficient for achieving a rapid malate degradation during the first 60 h of fermentation. The similar intracellular malate concentration found in those strains is consistent with this observation. However, malate degradation stops in these strains when the cells reached the stationary phase before complete malate exhaustion. This might suggest the existence of in vivo limiting steps, such as decrease in transport uptake or in malolactic activity at this stage. Since a PEST region has been identified at the C-terminal end of the mae1 protein [10] the stop in malate degradation in the strains expressing a low level of malate permease could be related to a degradation of this protein. Further studies on the regulation of *mae1* gene and product are required to lighten this point. On the other hand, the hypothesis that malolactic enzyme would be limiting at this stage of fermentation is unlikely since the same malate degradation kinetics were observed whatever the copy number of *mleS* gene.

The expression of one copy of *mleS* was sufficient to trigger wholely malate through MLE, as shown by isotopic filiation studies. This is consistent with the fact that the intracellular malate concentration is below the K_m for malate of malolactic enzyme. Increasing the copy number of *mleS* had no effect on the uptake of malate and on malate degradation kinetics. On the other hand, a high level of malolactic activity resulted in the utilization of endogenous malate pool, independently of the expression level of the transporter, and lead to a slight decrease in intracellular malate concentration. However, these side effects do not affect growth and fermentation characteristics. In a similar way, we have not previously detected any growth default associated with a decrease of intracellular malate concentration resulting of a high utilisation level of malate endogenous pool, in the strain V5 expressing *mleS* alone [6].

S. cerevisiae strains performing malolactic fermentation are of crucial interest for wine making. The removal of malic acid, one of the main organic acid of the grape must is essential for deacidification and stabilisation of wine. Utilization of malolactic yeast will lead to a control of this essential step. The results presented show that a complete malate degradation via malolactic reaction was achieved in 4 days. Similar results were very recently reported [24]. In contrast to bacterial MLF which usually starts after alcoholic fermentation, the conversion of malate into lactate and CO_2 by yeast occurs simultaneously to alcoholic fermentation. Under our experimental conditions, malate was exhausted when a high sugar amount was remaining (75%), as a result of the high rate of the malolactic reaction. The construction of an industrial strain stabilized by integration of genes encoding a malolactic enzyme and of a malate transporter is in progress. High constraints exist for the construction of a commercial genetically modified industrial strain. Besides the fact that the final strain must satisfy several requirements to obtain the authorizations of deliberate release and commercialization by the advisory

committes, special care must be paid to preserve the technological characteristics of the strain [25]. The data presented here show that a high level of expression of the gene *mleS* results in a decreased malate pool. Although growth and fermentation kinetics were unchanged, we cannot exclude that a decrease in malate intracellular concentration would affect the formation of other by-products of organoleptic interest. We have shown that these eventual side effects can be overcomed by expressing *mleS* at a low level. Finally, bacterial MLF was described in some instances to contribute to wine flavour [1], althought the perceived influence by MLF depends on the prevailing characteristics of the wine [2,26]. The comparison of the consequences of bacterial and yeast MLF on the organoleptic characteristics of wine will enable to clarify this much debated point.

A control of acidity in enology is crucial to warrant wine quality. We have previously reported the construction of a yeast engineered for lactate production for acidification of wine in hot regions [27]. The availability of a malolactic yeast enlarges the range of new strains that could be used for a biological correction of lack or excess of acidity.

References

[1] Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. and Fleet, G.H. (1985) Am. J. Enol. Vitic. 36, 290–301.
[2] T. Henick-Kling in: G.H. Fleet (Ed.), Wine Microbiology and Biotechnology, Hardwood Academic Publ., Chur, Switzerland, 1993, pp. 289–326.
[3] Ansanay, V., Dequin, S., Blondin, B. and Barre, P. (1993) FEBS Lett. 332, 74–80.
[4] Denayrolles, M., Aigle, M. and Lonvaud-Funel, A. (1994) FEMS Microbiol. Lett. 116, 79–86.
[5] Labarre, C., Guzzo, J., Cavin, J.F. and Diviès, C. (1996) Appl. Environ. Microbiol. 62, 1274–1282.
[6] Ansanay, V., Dequin, S., Camarasa, C., Schaeffer, V., Grivet, J.P., Blondin, B., Salmon, J.M. and Barre, P. (1996) Yeast 12, 215–225.
[7] Denayrolles, M., Aigle, M. and Lonvaud-Funel, A. (1995) FEMS Microbiol. Lett. 125, 37–44.
[8] Osothsilp, C. and Subden, R.E. (1986) J. Bacteriol. 168, 1439–1443.
[9] Sousa, M.J., Mota, M. and Leao, C. (1992) Yeast 8, 1025–1031.
[10] Grobler, J., Bauer, F., Subden, R.E. and Van Vuuren, H.J.J. (1995) Yeast 11, 1485–1491.
[11] H.J.J. van Vuuren, M. Viljoen, T. Grobler, H. Volschenk, F. Bauer and R.E. Subden, in: A. Lonvaud (Ed.), Proceedings 5th Symposium International d'Oenologie, Bordeaux, France, 1995, pp. 195–197.
[12] J. Sambrook, E.F. Fritsh, T. Maniatis, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor laboratory, Cold Spring Harbor, NY, 1989.
[13] Bely, M., Sablayrolles, J.M. and Barre, P. (1990) Am. J. Enol. Vitic. 41, 319–324.
[14] D. Hanahan, in: D.M. Glover (Ed.), DNA Cloning, IRL Press, Washington, DC, 1985, pp. 109–135.
[15] Schiestl, R.H. and Gietz, R.D. (1989) Curr. Genet. 16, 339–446.
[16] Hoffman, C.S. and Winston, F. (1987) Gene 57, 267–272.
[17] Mellor, J., Dobson, M.J., Roberts, N.A., Tuite, M.F., Emtage, J.S., White, S., Lowe, P.A., Patel, T., Kingsman, A.J. and Kingsman, S.M. (1983) Gene 24, 1–4.
[18] Sirkoski, R.S. and Hieter, P. (1989) Genetics 122, 19–27.
[19] Hill, J.E., Myers, A.M., Koerner, T.J. and Tzagoloff, A. (1993) Yeast 2, 163–167.
[20] Christianson, T.W., Sirkoski, R.S., Dante, M., Shero, J.H. and Hieter, P. (1992) Gene 110, 119–122.
[21] Renault, P. and Heslot, H. (1987) Appl. Environ. Microbiol. 53, 320–324.
[22] Fuck, E., StŠrk, G. and Radler, F. (1973) Arch. Mikrobiol. 89, 223–231.
[23] Fuck, E. and Radler, F. (1972) Mikrobiology 87, 149–164.
[24] Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R.E., Young, R.A., Lonvaud, A., Denayrolles, M. and van Vuuren, H.J.J. (1997) Nat. Biotechnol. 15, 253–257.
[25] P. Barre, F. Vezhinet, S. Dequin, B. Blondin, in: G.H. Fleet (Ed.), Wine Microbiology and Biotechnology, Hardwood Academic Publ., Chur, Switzerland, 1993, pp. 265–287.
[26] Martineau, B., Henick-Kling, T. and Acree, T. (1995) Am. J. Enol. Vitic. 46, 385–388.
[27] Dequin, S. and Barre, P. (1994) Bio/Technology 12, 173–177.