Engineering pathways for malate degradation in *Saccharomyces cerevisiae*

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Deacidification of grape musts is crucial for the production of well-balanced wines, especially in colder regions of the world. The major acids in wine are tartaric and malic acid. *Saccharomyces cerevisiae* cannot degrade malic acid efficiently due to the lack of a malate transporter and the low substrate affinity of its malic enzyme. We have introduced efficient pathways for malate degradation in *S. cerevisiae* by cloning and expressing the *Schizosaccharomyces pombe* malate permease (*mae1*) gene with either the *S. pombe* malic enzyme (*mae2*) or *Lactococcus lactis* malolactic (*mleS*) gene in this yeast. Under aerobic conditions, the recombinant strain expressing the *mae1* and *mae2* genes efficiently degraded 8 g/L of malate in a glycerol-ethanol medium within 7 days. The recombinant malolactic strain of *S. cerevisiae* (*mae1* and *mle*S genes) fermented 4.5 g/L of malate in a synthetic grape must within 4 days.

Keywords: wine, malate, malolactic enzyme, malic enzyme, malate permease

Grape must and wine contain a variety of organic acids that significantly affect the quality and taste of wine. The amount of acid varies and depends on the climate and grape variety. Malic acid, together with tartaric acid, make up 70–90% of the total acidity of grape juice[1]. The production of well-balanced wines requires the reduction of excess acidity, especially in the colder viticultural regions of the world.

Wine yeast strains of *Saccharomyces cerevisiae* cannot metabolize malate in grape must efficiently, and only minor changes to the total acidity of wine are observed during fermentation[1]. Many wines are therefore subjected to the crucial malolactic fermentation after alcoholic fermentation. During malolactic fermentation, L-malic acid is decarboxylated to L(+)-lactic acid and CO_2 by lactic acid bacterial genera such as *Lactobacillus*, *Pediococcus*, and *Leuconostoc*[2]. Malolactic fermentation reduces the total acidity of wine, enhances microbiological stability and presumably improves the organoleptic quality of wine[3]. However, there are various complications in managing this process, and stuck or sluggish malolactic fermentation often leads to spoilage of wines[3,4].

Other winemaking practices are used to reduce the acidity of wine[1]. Physicochemical methods such as blending, chemical neutralization, and precipitation can successfully deacidify wine, but often reduce wine quality and require extensive labor or capital input. The possibility of using other yeasts has also been investigated. Fermentations using the fission yeast *Schizosaccharomyces pombe*, which efficiently degrades malate to ethanol through a malo-ethanolic fermentation, have been attempted. The malate was effectively degraded but off-flavors were produced[5]. Attempts to hybridize wine yeasts with malate-metabolizing yeast strains by cell fusion and conjugation failed[6]. The application of high density cell suspensions of several yeasts, including *S. cerevisiae*, did not increase the rate at which L-malate was degraded during fermentation[7].

S. cerevisiae contains its own constitutive NAD-dependent malic enzyme and the biochemical mechanism for malate degradation is the same as in *S. pombe*[6]. The malic enzyme is responsible for the conversion of L-malate to pyruvate, which, under anaerobic conditions, will be converted to ethanol and carbon dioxide. Aerobically, malic acid is decarboxylated into CO_2 and H_2O(ref. 8). However, the substrate affinity of the *S. cerevisiae* malic enzyme (K_m = 50 mM)[9] is much lower than that of the *S. pombe* malic enzyme (K_m = 3.2 mM)[10]. Furthermore, L-malate enters *S. cerevisiae* by simple diffusion, and it was therefore suggested that the absence of an active malate transport system combined with the low substrate affinity of the enzyme is responsible for this yeast's inefficient metabolism of malate[9–11].

Genetic engineering of *S. cerevisiae* strains to carry out alcoholic fermentation and malate degradation simultaneously has been explored for several years. In order to engineer a malolactic pathway in *S. cerevisiae*, the malolactic genes (*mle*S) from *Lactococcus lactis*[12,13] and *Lactobacillus delbrueckii*[14] have been cloned and characterized, and the genes have been introduced and expressed in *S. cerevisiae*[12–14]. The *mle*S gene encodes a NAD-dependent malolactic enzyme that converts L-malate to L-lactate and CO_2 (ref. 3). However, the absence of a transport system for malate in *S. cerevisiae* precluded *mle*S recombinant strains from degrading malate effectively.

We recently cloned and characterized the *mae1* gene that encodes a permease which is responsible for the active transport of L-malate in *S. pombe*[15]. In addition to the *mae1* gene we cloned and characterized the malic enzyme (*mae2*) gene from *S. pombe*[16]

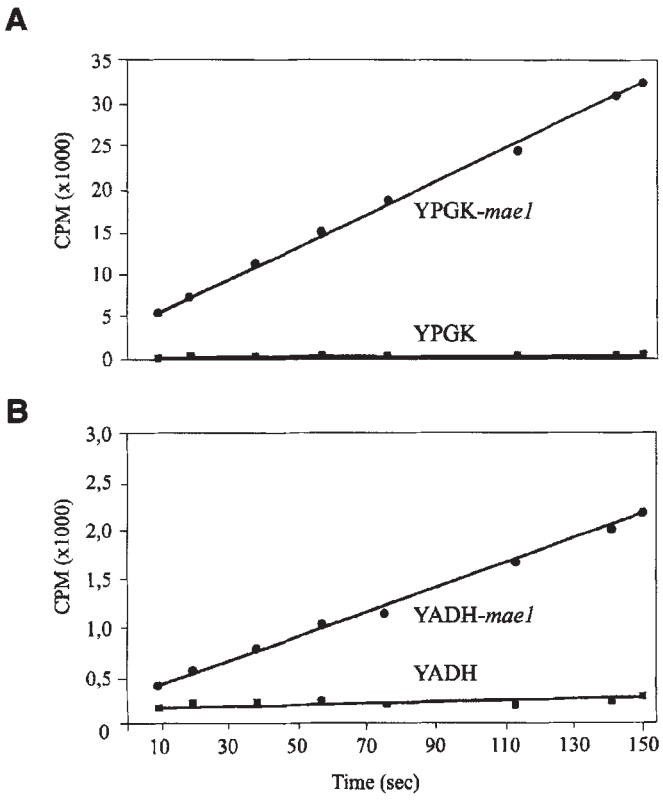

Figure 1. Uptake of ^{14}C L-malate by recombinant strains of *S. cerevisiae* containing the *S. pombe mae1* gene under the regulation of (A) the *PGK1* and (B) the *ADH1* promoter and terminator sequences. The cells were cultured to OD_{600nm} of 0.6 in a 2% glucose medium.

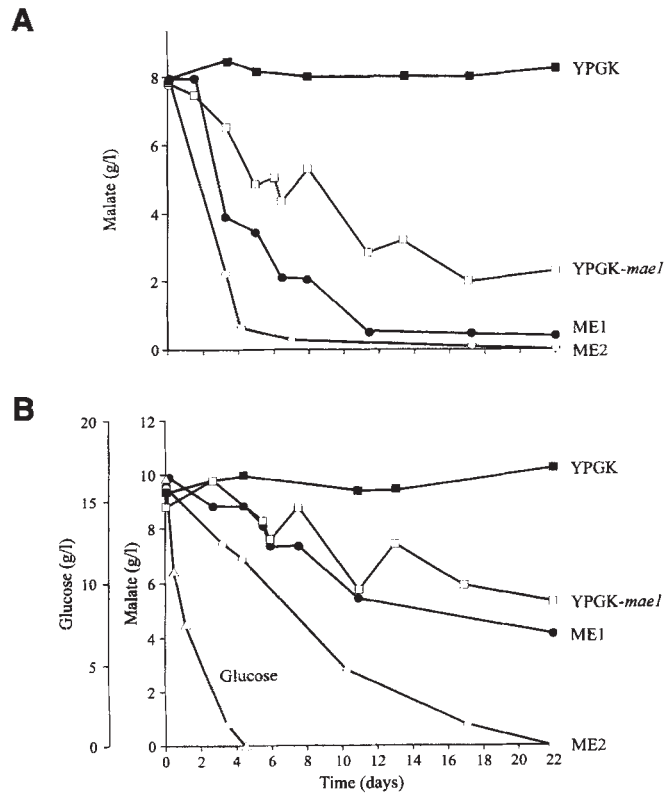

Figure 2. Malate degradation by recombinant strains of *S. cerevisiae* containing the *mae1* and/ or *mae2* genes of *S. pombe* in (A) 2% glycerol-ethanol and (B) 2% glucose medium containing 8 g/L of L-malate. Malate degradation was regarded as complete when the malate concentration reached 0.3 g/L L-malate (malolactic fermentation is considered to be complete at this point during vinification)[30].

and the malolactic gene (*mle*S) from *Lactococcus lactis*[13]. A functional malolactic strain of *S. cerevisiae* could replace the unreliable bacterial malolactic fermentation. A malo-ethanolic yeast strain will be useful for the production of fruity floral wines, as malolactic fermentation is considered to be undesirable in these wines. Moreover, application of malo-ethanolic yeasts for the production of distilled beverages will lead to enhanced ethanol yields.

We have functionally expressed the *mae1* and *mae2*, as well as the *mae1* and *mle*S genes in *S. cerevisiae*. A recombinant strain of *S. cerevisiae* containing the *mae1* and *mae2* genes degraded 8 g/L of L-malate within 7 days under aerobic conditions. The *S. cerevisiae* strain transformed with the *mae1* and *mle*S genes fermented 4.5 g/L of L-malate in a synthetic grape must to lactate and CO_2 in 4 days.

Results and discussion

Functional expression of the *S. pombe mae1* gene in *S. cerevisiae*. Expression of the *lacZ* gene fused to the *S. pombe mae2* promoter revealed that this promoter is not functional in *S. cerevisiae*. (data not shown). To express the *mae1* and *mae2* genes in *S. cerevisiae*, we therefore subcloned both open reading frames (ORFs) into expression cassettes containing the *S. cerevisiae* alcohol dehydrogenase (*ADH1*) and 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences leading to the pHV1, 2, 3, and 4 constructs (Table 1).

The recombinant strains of *S. cerevisiae* transformed with the pHV1 (YADH-*mae1*) and pHV3 (YPGK-*mae1*) constructs were both able to actively transport L-malate (Fig. 1). This suggests correct synthesis, posttranslational modification and insertion of the

Table 1. Constructs used to engineer malate degrading pathways in *S. cerevisiae* YPH259 (ref. 23).

Construct	Description	Recombinant strain
pHVX1	Shuttle vector YEplac181 (ref. 24), containing the $ADH1_p$-$ADH1_t$ expression cassette	YADH
pHVX2	Shuttle vector YEplac181, containing the $PGK1_p$-$PGK1_t$ expression cassette	YPGK
pHV1	pHVX1 with *mae1* ORF ($ADH1_p$-*mae1*-$ADH1_t$)	YADH-*mae1*
pHV2	pHVX1 with *mae2* ORF ($ADH1_p$-*mae2*-$ADH1_t$)	YADH-*mae2*
pHV3	pHVX2 with *mae1* ORF ($PGK1_p$-*mae1*-$PGK1_t$)	YPGK-*mae1*
pHV4	pHVX2 with *mae2* ORF ($PGK1_p$-*mae2*-$PGK1_t$)	YPGK-*mae2*
pHV5	YEplac181-based vector containing the $ADH1_p$-*mae1*-$ADH1_t$: $PGK1_p$-*mae2*-$PGK1_t$ expression system	ME1
pHV6	YEplac181-based vector containing the $ADH1_p$-*mae2*-$ADH1_t$: $PGK1_p$-*mae1*-$PGK1_t$ expression system	ME2
pMDMALO[13]	Multicopy episomal plasmid containing the *mle*S ORF inserted between the *PGK1* promoter and terminator sequences, as well as the *URA3* marker gene.	YPGK-*mle*S

S. pombe mae1 protein (mae1p) into the plasma membrane of *S. cerevisiae*. Malate transport by the recombinant strain of *S. cerevisiae* containing the *mae1* gene under the control of the *ADH1* promoter was slower than in the recombinant strain containing the *mae1* gene under the *PGK1* promoter. Western blot analysis showed that the reduced transport of malate in the YADH-*mae1* recombinant strain correlated with a lower concentration of the transport protein, suggesting that the *ADH1* promoter used was weaker than the *PGK1* promoter (data not shown).

Interestingly, the recombinant strains of *S. cerevisiae* containing only the permease showed a highly improved ability to degrade L-malate in the glycerol-ethanol-based medium (Fig. 2). However, in the glucose containing medium, degradation of malate only became significant once glucose had been exhausted. The recombinant yeast containing the *mae1* gene under the *PGK1* promoter reduced L-malate content by 70% in the glycerol-ethanol medium and by 50% in the glucose medium after 22 days (Fig. 2). This reduction was probably accomplished by the native malic enzyme of *S. cerevisiae*.

Coexpression of the *S. pombe mae1* and *mae2* genes in *S. cerevisiae*. The ability of *S. cerevisiae* strains transformed with plasmids pHV5 (ME1) or pHV6 (ME2) (Table 1), containing both the *mae1* and *mae2* genes under control of *S. cerevisiae* promoter and terminator signals, to degrade 8 g/L of L-malate in glycerol-ethanol-based and glucose-based media, were investigated (Fig. 2).

The control yeast strains degraded only insignificant amounts of L-malate after 22 days. Degradation of L-malate by recombinant strains containing only the *S. pombe* malic enzyme (YADH-*mae2* and YPGK-*mae2*) was not significantly different from that of the control yeasts (data not shown). However, when both the malate permease *mae1* and the *S. pombe mae2* genes were introduced, complete degradation of L-malate occurred.

In a 2% glycerol-ethanol and a 2% glucose medium the recombinant strain ME2 was able to degrade L-malate completely within 7 and 19 days, respectively (Fig. 2). Compared to ME2, the ME1 recombinant strain degraded malate less efficiently in all the conditions used. This could be the result of the lower transport rate for malate in the recombinant yeast containing the *mae1* gene under control of the *ADH1* promoter (Fig. 1).

The ability of both the ME1 and ME2 strains to metabolize L-malate differed considerably in the glycerol-ethanol and the glucose containing media. Both strains performed much more efficiently in glycerol-ethanol than in glucose medium. Under aerobic conditions, 92% (7 g/L) of L-malate in glycerol-ethanol medium was rapidly degraded to CO_2 and H_2O in 4 days by the ME2 strain (Fig. 2A). In glucose medium (Fig. 2B) this strain degraded L-malate much slower; after 4 days only 27% of the malate was degraded. Complete degradation of L-malate in the 2% glucose medium was achieved only 18 days after glucose was depleted. In a medium containing 10% glucose and 10% fructose, no degradation of L-malate was observed after 19 days (data not shown). Western blot analysis confirmed that the *PGK1* promoter is not subject to glucose regulation, but indicated that the modified *ADH1* promoter[17] is still to some extent sensitive to glucose (data not shown). However, the Western blots also showed that both mae1p and mae2p were present in glucose grown recombinant cells (Fig. 3). The absence of malate degradation by recombinant strains in a 10% glucose, 10% fructose medium is therefore intriguing. It could be due to glucose-mediated regulation of the malic enzyme activity in *S. cerevisiae* cells, a phenomenon apparently not present in *S. pombe* where glucose (or other carbon

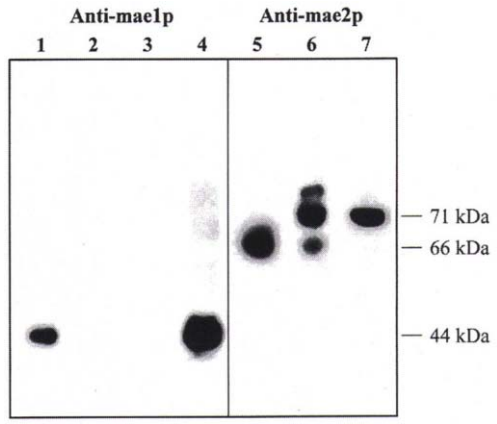

Figure 3. Western blot analysis of the *mae1* and *mae2* gene products. Strains of *S. pombe* and *S. cerevisiae* were grown in a 2% glucose medium. Proteins of both yeast species were extracted when the cultures had reached an OD_{600nm} of 0.5. Forty µg of either hydrophobic (lanes 1-4) or total (lanes 5-7) protein was loaded. Lanes 1-4 were incubated in the presence of anti-mae1p antibodies, while anti-mae2p antibodies were used for lanes 5-7. The apparent molecular weight of each protein is indicated on the right. Lanes 1 and 5: *S. pombe* wild-type; lane 2: *S. pombe mae1*⁻ transport mutant[15]; lanes 3 and 7: *S. cerevisiae* YPH259; lane 4: *S. cerevisiae* containing the *mae1* gene under the control of the *PGK1* promoter (YPGK-*mae1*); lane 6: *S. cerevisiae* containing the *mae2* gene under the control of the *ADH1* promoter (YADH-*mae2*). The antibodies raised against the *S. pombe* malic enzyme were able to recognize the native *S. cerevisiae* malic enzyme. The apparent molecular weight of the proteins (60 kDa for the *S. pombe* mae2p, 71 kDa for the *S. cerevisiae* malic enzyme and 44 kDa for the *S. pombe* mae1p) corresponds closely to their theoretical molecular weight (62.5, 74, and 49 kDa, respectively).

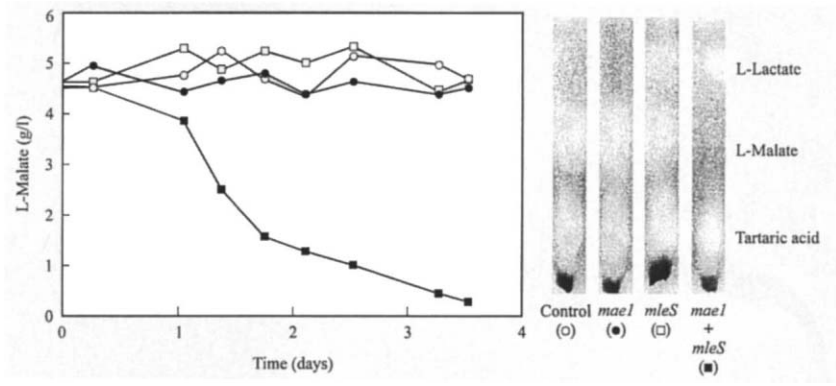

Figure 4. Conversion of L-malate to L-lactate by recombinant strains of *S. cerevisiae* in synthetic grape must[19]. Malate degradation was regarded as complete when the concentration reached 0.3 g/L L-malate. The production of lactate was visualized with paper chromatography (panel on the right)[20]. The malolactic strain of *S. cerevisiae* (■) containing both the *mae1* and *mleS* genes rapidly degraded 4.5 g/L L-malate within 4 days. No significant degradation of L-malate was observed by the yeasts containing the *PGK1* expression cassette (YPGK, [○]), the *mleS* gene (YPGK-*mleS*, [□]) or the *mae1* gene (YPGK-*mae1*, [●]).

Acknowledgments

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1. Beelman, R.B. and Gallander, J.F. 1979. Wine deacidification. *Adv. Food Res.* **25:**1–53.
2. van Vuuren, H.J.J. and Dicks, L.M.T. 1993. *Leuconostoc oenos*: A review. *Am. J. Enol. Vitic.* **44:**99–112.
3. Davies, C.R., Wibowo, W., Eschenbruch, R., Lee, T.H., and Fleet, G.H. 1985. Practical implications of malolactic fermentation: A review. *Am. J. Enol. Vitic.* **36:**290–301.
4. Henick-Kling, T. 1995. Control of malolactic fermentation in wine: energetics, flavor modification and methods of starter culture preparation. *J. Appl. Bacteriol.* (Symp. Suppl.) **79:**29S–37S.
5. Gallander, J.F. 1977. Deacidification of Eastern table wines with *Schizosaccharomyces pombe*. *Am. J. Enol. Vitic.* **28:**65–68.
6. Radler, F. 1993. Yeast metabolism of organic acids, pp. 165–182 in *Wine Microbiology and Biotechnology*. Fleet (ed.), Harwood Academic Publishers, Switzerland.
7. Gao, C. and Fleet, G.H. 1995. Degradation of malic and tartaric acids by high density cell suspensions of wine yeasts. *Food Microbiol.* **12:**65–71.
8. Mayer, K and Temperli, A. 1963. The metabolism of L-malate and other compounds by *Schizosaccharomyces pombe*. *Arch. Mikrobiol.* **46:**321–328.
9. Fuck, E., Stark, G., and Radler, F. 1973. Äpfelsäurestoff-wechsel bei *Saccharomyces* II. Anreicherung und Eigenschaften eines Malatenzymes. *Arch. Mikrobiol.* **89:**223–231.
10. Temperli, A., Kunsch, V., Mayer, K., and Busch, I. 1965. Reinigung und Eigenschaften der Malatdehydrogenase (decarboxylierent) aus Hefe. *Biochem. Biophys. Acta.* **110:**630–632.
11. Ansanay, V., Dequin, S., Camarasa, C., Schaeffer, V., Grivet, J., Blondin, B., Salmon, J., and Barre, P. 1996. Malolactic fermentation by engineered *Saccharomyces cerevisiae* as compared with engineered *Schizosaccharomyces pombe*. *Yeast* **12:**215–225.
12. Ansanay, V., Dequin, S., Blondin, B., and Barre, P. 1993. Cloning, sequence and expression of the gene encoding the malolactic enzyme from *Lactococcus lactis*. *FEBS Lett.* **332:**74–80.
13. Denayrolles, M., Aigle, M., and Lonvaud-Funel, A. 1995. Functional expression in *Saccharomyces cerevisiae* of the *Lactococcus lactis mle*S gene encoding the malolactic enzyme. *FEMS Microbiol. Lett.* **125:**3744.
14. Williams, S.A., Hodges, R.A., Strike, T.L., Snow, R., and Kunkee, R.E. 1984. Cloning the gene for the malolactic fermentation of wine from *Lactobacillus delbrueckii* in *Escherichia coli* and yeasts. *Appl. Environ. Microbiol.* **47:**288–293.
15. Grobler, J., Bauer, F. Subden, R.E., and van Vuuren, H.J.J. 1995. The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C_4 dicarboxylic acids. *Yeast* **11:**1485–1491.
16. Viljoen, M., Subden, R.E., Krizus, A., and van Vuuren, H.J.J. 1994. Molecular analysis of the malic enzyme gene (*mae2*) of *Schizosaccharomyces pombe*. *Yeast* **10:**613–624.
17. Laing, E. and Pretorius, I.S. 1992. Synthesis and secretion of an *Erwinia chrysanthemi* pectate lyase in *Saccharomyces cerevisiae* regulated by different combinations of bacterial and yeast promoter and signal sequences. *Gene* **121:**35–45.
18. Maconi, E., Manachini, P., Aragozzini, F., Gennari, C., and Ricca, G. 1984. A study on the malo-alcoholic fermentation pathway in *Schizosaccharomyces pombe*. *Biochem. J.* **217:**585–588.
19. Murai, T., Tokushige, M., Nagai, J., and Katsuki, H. 1972. Studies on the regulatory functions of malic enzymes. *J. Biochem.* **71:**1015–1028.
20. Artus, N.N. and Edwards, G.E. 1985. NAD-malic enzyme from plants. *FEBS Lett.* **182:**225–233.
21. Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33:**103–119.
22. Sambrook, J., Fritsh, E.F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual* (2nd ed.). Cold Spring Harbor Laboratory Press, New York.
23. Sikorski, R.S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122:**19–27.
24. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds). 1995. *Current protocols in molecular biology*. John Wiley & Sons, New York.
25. Gietz, R. D. and Sugino, A. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:**527–534.
26. Kunkee, R.E. 1968. Simplified chromatographic procedure for detection of malolactic fermentation. *Wines and Vines* **49:**23–24.
27. Smith, D.B. and Johnson, K.S. 1988. Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67:**31–40.
28. Chirio, M.-C., Brethes, D., Napias, C., Grandier-Vazille, X., Rakotomanana, F., and Chevallier, J. 1990. Photoaffinity labelling of the purine-cytosine permease of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **194:**293–299.
29. Crous, J.M., Pretorius, I.S., and Van Zyl, W.H. 1995. Cloning and expression of an *Aspergillus kawachii* endo-1,4-β-xylanase gene in *Saccharomyces cerevisiae*. *Curr. Genet.* **28:**467–473.
30. Martineau, B., Henick-Kling, T., and Acree, T. 1995. Reassessment of the influence of malolactic fermentation on the concentration of diacetyl in wines. *Am. J. Enol. Vitic.* **46:**385–388.