Enhancing yeast performance under oenological conditions by enabling proline utilisation

by

Kathryn Poole

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Department of Horticulture, Viticulture and Oenology
The University of Adelaide

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Thesis summary

Assimilable nitrogen, which is typically lacking in grape juice, is an important nutritional requirement of *Saccharomyces cerevisiae*. As such, fermentations frequently become protracted, terminate prematurely or develop undesirable aroma profiles. Amino acids and ammonium are the main sources of assimilable nitrogen in grape juice. The amino acid proline often predominates. Proline uptake is mediated by a high affinity, proline-specific permease, Put4p, and a low affinity general amino acid permease, Gap1p. The expression and activity of these transporters is subject to nitrogen catabolite repression (NCR) and nitrogen catabolite inactivation (NCI). That is, in the presence of a preferred nitrogen source, the expression of *PUT4* and *GAP1* is repressed and the permeases are inactivated. For yeast to fully exploit proline, its transport must be derepressed by depletion of other (preferred) amino acids and molecular oxygen must be present to allow proline catabolism by proline oxidase. Consequently, as oxygen is typically depleted well before the other amino acids in grape juice are reduced to non-repressive concentrations, proline is largely un-utilised by yeast during oenological fermentation. This study aims to overcome these metabolic restrictions on proline utilisation.

A preliminary study was conducted to determine the potential for proline transport-capable strains to utilise proline during the initial stages of fermentation when oxygen may be present, particularly in red grape must. Initially, the transcriptional regulation of the *PUT4* gene was targeted to generate strains capable of proline transport under normally repressive conditions. In the first case, the *URE2* gene, encoding a negative regulator involved in nitrogen discrimination, was deleted. In the second case, *PUT4* was expressed from the constitutive *TEF2* promoter. It was observed that both strains express *PUT4* in the presence of a preferred nitrogen source. This expression led to Put4p activity during the initial stages of growth and fermentation, with Put4p activity declining over the course of the growth phase. Proline removal from the media, however, was limited to the initial stages of fermentation while oxygen was available. It seems that the rapid depletion of oxygen limits the amount of proline transported into the yeast cell.

The two proline transport-capable mutants were analysed for growth and fermentation characteristics. It was found that the deletion of the *URE2* gene led to a slow initial growth and the formation of a larger biomass. The *ure2* delete strain also utilised significantly more nitrogen during fermentation than the wild type. Consequently, a *ure2* delete strain would not be suitable for industrial use. The expression of *PUT4* from a constitutive promoter did lead to an increase in nitrogen assimilation during fermentation when compared with the wild type. However, this observed increase was significantly less than that observed in the *ure2* delete strain. In an effort to produce a proline transport-capable strain with potential industrial benefit, strains constitutive for *PUT4* specifically were isolated using random, *in vitro* mutagenesis of the *PUT4* promoter region. Four point mutations were identified that, when introduced singly into the *PUT4* promoter, led to expression of *PUT4* in the presence of a preferred nitrogen source.

The rapid depletion of oxygen observed in the preliminary study will limit the potential usefulness of strains capable of proline transport. Micro-oxygenation is rapidly becoming an accepted practice during oenological fermentation. The potential benefit of the controlled addition of oxygen during fermentation is restricted by the timing of any oxygen addition. Oxygen additions made at the onset of the stationary phase are the most beneficial. During the preliminary study, it was noted that Put4p activity decreased during the growth phase to low levels at the onset of the stationary phase. To ensure that sufficient active Put4p is present at the onset of the stationary phase, the post-translational control of the Put4p was investigated.

Site-directed mutagenesis was used to target residues in the carboxy-terminal region of Put4p that are potentially involved in the ammonia-induced down-regulation of the permease. The substitution, S605A, lead to the amelioration of ammonia-induced down-regulation of Put4p. The activity of the Put4p S605A variant decreased over the course of the growth phase, but not to the same extent observed in the wild type. Furthermore, a recovery seen after down-regulation restored a greater percentage of the original activity compared with the wild type.

To determine whether such a strain proved better able to ferment media in the presence of micro-oxygenation, the fermentation kinetics of a strain constitutively expressing

PUT4(S605A) were compared with the wild type. Micro-oxygenation of ferments did not result in an increase in fermentation rate nor a decrease in fermentation time in the mutant. However, the cell viability of the strain capable of proline transport was increased in comparison with the wild type, suggesting a role for proline in stress responses within the yeast cell.