Use of directed evolution to generate multiple-stress tolerant *Oenococcus oeni* for enhanced malolactic fermentation

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Declaration of authorship

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Abbreviations

∆p	proton motive force
ABC	ATP binding cassette
ACE	Australian Centre for Ecogenomics
ADI	arginine deiminase pathway
AF	alcoholic fermentation
AGRF	Australian Genome Research Facility
AJ	apple juice
AP	antagonistic pleiotropy
ATP	adenosine-5'-triphosphate
BLAST	Basic Local Alignment Search Tool
C8	octanoic acid
C10	decanoic acid
C12	dodecanoic acid
CDGJM	Chemically Defined Grape Juice Medium
CFA	cyclopropane fatty acid
CFU	colony forming unit
DAVID	Database for Annotation, Visualization and Integrated Discovery
DE	directed evolution
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GMOs	genetically modified organisms
GOT	glutamate-oxaloacetate transaminase
HGT	horizontal gene transfer
INDELs	small insertions and deletions
IS	insertion sequence
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LAB	lactic acid bacteria
L-LDH	L-lactate dehydrogenase
L-MDH	L-malate dehydrogenase
MA	mutation accumulation
MCFA	medium chain fatty acids

MDR	multidrug resistance
ME	malic enzyme
MLE	malolactic enzyme
MLF	malolactic fermentation
MMR	methylated mismatch repair
MRS	De Man, Rogosa and Sharpe
MRSAJ	MRS supplemented with 20% (v/v) apple juice
N/A	not applicable
NAD^+	nicotinamide adenine dinucleotide, oxidised
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidised
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NCBI	National Center for Biotechnology Information
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PPP	pentose phosphate pathway
PSI	Position-specific Iterated
RFCDGJM	Red Fermented Chemically Defined Grape Juice Medium
RNA	ribonucleic acid
SAM	S-adenosyl methionine
SIFT	Sorting Intolerant from Tolerant
SNP	Single Nucleotide Polymorphism
SS	Sanger sequencing
TA	titratable acid
TAE	tris-acetate EDTA
TDP	thymidine-5'-diphosphate
tRNA	transfer RNA
UDP	uridine-5'-diphosphate
UniProt	universal protein resource
WGS	whole genome sequencing
WIC	Wine Innovation Cluster

Abstract

This study aimed to optimise *Oenococcus oeni* for more efficient malolactic fermentation in wine with multiple stressors. First, a previously evolved ethanol tolerant strain, A90, was characterised for resistance to combined pH and ethanol stress in both MRSAJ and Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM). A90 showed a similar viability in RFCDGJM compared to its parent, SB3, indicating the need for further improvement. With the success of the previous proof-of-concept directed evolution (DE) in *O. oeni*, a new DE was carried out to determine 1) if DE can be applied to further improve A90 in a wine-like environment using combinations of stressors to generate more superior strains with better general stress resistance; 2) how much further can A90 be developed, and how stable the new phenotype would be; 3) possible new patterns of stress response through study of the genetic basis for the superior phenotype.

A continuous culture of A90 was established in a bioreactor and grown in a wine-like environment for approximately 350 generations with increasing ethanol and sulfur dioxide (SO_2), and decreasing pH over time. Samples of the population in the bioreactor were collected at three significant times during the DE to screen for improved isolates based on L-malic acid consumption and growth. Three strains, namely 1-161, 2-49 and 3-83, outperformed from a total of 378 isolates.

With a view to applying these strains to the industry, in-depth physiological characterisations were undertaken. Aspects examined included tolerance to various oenologically related stressors such as ethanol, pH, SO_2 and medium chain fatty acids, as well as phenotype stability and fermentation ability under more realistic winemaking conditions, i.e. un-filtered wine and winery scale fermentation. Overall, 2-49 and 3-83 constantly displayed better growth and

malolactic activity than the parent strain A90 in either lab-scale or winery-scale trials.

Finally, whole genome sequencing of strains SB3, A90, 2-49 and 3-83 and genetic characterisation were utilised to investigate changes during DE in *O. oeni*. A total of 19 single nucleotide polymorphisms (SNPs) were found in 2-49 and 3-83 strains compared to A90. The SNPs identified may affect cell envelope and fatty acids biosynthesis, DNA translation and homeostasis of internal pH, leading to the improved performance of DE strains. Sequences were also compared to the available sequence for commercial strain VP41. Several mutations were identified in stress response genes, indicating VP41 and SB3-related strains might have different responses to stressors. SNPs in the predicted *mleA* promoter sequence may suggest a new mechanism of MLF activation. Additionally, Nucleotide BLAST was used to analyse the presence of genes with oenological traits in SB3-related strains. Genes associated with the release of desirable aromas were found, whilst genes involved in the formation of biogenic amines were absent.

This study expands the knowledge regarding optimisation of *O. oeni*, and may be helpful for the further improvement of food-related microbes with enhanced performance.