Enhanced Wine-Making Efficiency Through Fool-Proof Malolactic Fermentation: Evolution of Superior Lactic Acid Bacteria

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Summary

Malolactic fermentation (MLF), also known as the secondary fermentation in winemaking, involves the enzymatic decarboxylation of L-malic to L-lactic acid, by lactic acid bacteria, usually *Oenococcus oeni*. This conversion improves the organoleptic properties of wine and also decreases the risk of microbial spoilage. *O. oeni* is a notoriously fastidious microbe prone to slow growth, especially in the harsh physiochemical environment of wine: high ethanol, presence of sulfur dioxide, low pH and low temperature. Each of these factors influences the growth rate and metabolism, including MLF, of this organism.

This study aimed to generate an improved strain of *O. oeni* with the ability to withstand the environmental pressures of wine, particularly high ethanol, using directed evolution (DE). Directed evolution is a non-recombinant method of generating improved strains. The process involves an organism mutating and potentially adapting to a high stress environment, in this case a high ethanol environment, over several hundred generations. This method has been used successfully to generate improved strains of other lactic acid bacteria and its efficacy as a method for the production of bacterial strains for the wine industry is detailed here.

A continuous culture of *O. oeni* was established in MRS supplemented with 20 % (v/v) apple juice medium at 30 °C and 5 % (v/v) ethanol. Over the next 290 days and approximately 260 generations the ethanol concentration in the medium was gradually increased to 15 % (v/v) ethanol. A sample of this culture was screened for malic acid consumption (MLF) compared to the original parent.

With proof of concept achieved, individual isolates from the DE culture were obtained in order to identify clones that demonstrated the ethanol tolerant phenotype to the greatest degree. An individual isolate, strain 90, was selected and its fermentation

performance was characterised under a range of different ethanol (13, 15, 17 and 19 % (v/v)) concentrations and temperatures (15, 22 and 30 °C). This strain also retained viability for 48 hours in a medium supplemented with 22 % (v/v) ethanol, a condition that lead to an almost total loss of viability in the parent strain after only one hour.

Finally the parent and two evolved strains (90 and 89) were sequenced using whole genome sequencing. 32 single nucleotide polymorphisms (SNPs) were discovered in the evolved strains compared to the parent. Twenty of these are non-synonymous mutations located in nineteen different genes; five of these are located in both strains. None of the mutations appear in known *O. oeni* ethanol stress response genes. GO analysis, BLAST and current literature were used to analyse these changes and propose possible reasons for the new phenotype.

This study is the first known use of DE for *O. oeni* strain improvement and results have confirmed DE can be successfully used as a technique for developing new strains. Furthermore these findings form the basis of exciting new studies further exploring the genetic basis for tolerance to ethanol stress.

Declaration of authorship

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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Alice Betteridge Date

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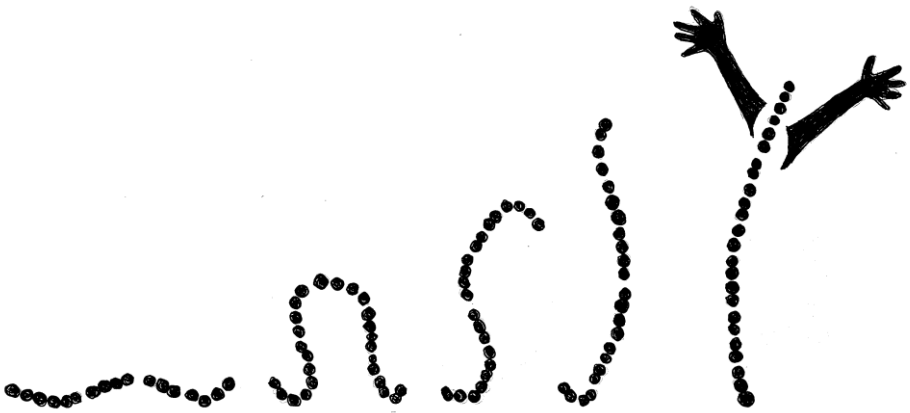
Onwards now to the next great adventure.



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Abbreviations and Symbols

#	number
%	percent
×	times
°C	degrees Celsius
α	alpha
β	beta
Δ	change
Δp	proton motive force
µ	maximum specific growth rate
µL	microlitre
µM	micro molar
3'	three prime, of nucleic acid sequence
5'	five prime, of nucleic acid sequence
A	absorbance
ABC	ATP binding cassette
ATP	adenosine triphosphate
BC	Before Christ
BLAST	Basic local alignment search tool
bp	base pair
Cat.	catalogue
CDD	Conserved domain database (Marchler-Bauer *et al.*, 2013)
CCR	carbon catabolite repression
CFA	cyclopropane fatty acid
CFU	colony forming unit
CTP	cytidine triphosphate
DAVID	Database for annotation, visualization and integrated discovery (Huang *et al.*, 2008; 2009)
DE	directed evolution
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
g	gram
gDNA	genomic DNA

GMOs	Genetically modified organisms
GO	gene ontology
GOT	glutamate-oxaloacetate transaminase
GTP	guanosine triphosphate
H^+	hydrogen ion
HCl	hydrochloric acid
HGT	horizontal gene transfer
hrs	hours
HSP	heat shock protein
IS	insertion sequence
kb	kilobase
KEGG	Kyoto encyclopedia of genes and genomes (Kanehisa and Goto, 2000; Kanehisa *et al*., 2014)
L	litre
LAB	lactic acid bacteria
LB	lysogeny broth
L-MDH	L-malate dehydrogenase
M	molar
max	maximum
MDR	multidrug resistance
mg	milligram
min	minimum
mL	millilitre
MLF	malolactic fermentation
MMR	methylated mismatch repair
Mn^{2+}	manganese ion
MQ	sterile ultra-pure water
MRS	de Man, Rogosa and Sharpe
MRSA	MRS agar
MRSAJ	MRS supplemented with 20% apple juice
N/A	not applicable
NAD^+	nicotinamide adenine dinucleotide, oxidised
NADH	nicotinamide adenine dinucleotide, reduced
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidised
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NCBI	National Center for Biotechnology Information
ng	nanogram

nm	nanometres
NSW	New South Wales
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
OD_{600}	optical density measured at 600 nm
PBS	phosphate buffered saline
pH_i	intracellular pH
PCR	polymerase chain reaction
PEG	polyethylene glycol
PRPP	phosphoribosylpyrophosphate synthetase
RNA	ribonucleic acid
rpm	revolutions per minute
SA	South Australia
SAM	S-adenosyl methionine
SNP	single nucleotide polymorphism
SO_2	sulfur dioxide
TAE	tris-acetate EDTA
tRNA	transfer RNA
U	unit
USA	United States of America
USD	USA dollars
UTP	uridine triphospahte
UV	ultra violet
v/v	volume per volume
VIC	Victoria
WMMBT	Wine Microbiology and Microbial Biotechnology Laboratory