ARGININE DEGRADATION BY MALOLACTIC WINE LACTIC ACID BACTERIA AND ITS OENOLOGICAL AND TOXICOLOGICAL IMPLICATIONS

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ABSTRACT

Malolactic fermentation (MLF) in wines is a secondary fermentation carried out by lactic acid bacteria (LAB), mostly encouraged for decreasing acidity by degradation of malic acid and for modifying flavour. During MLF, wine LAB may also degrade arginine, leading to the formation of ATP, ammonia and citrulline, among others. This is of concern to the winemaker and the consumer alike because ammonia increases the pH and thus the risk of growth by spoilage micro-organisms, and citrulline is a precursor in the formation of carcinogenic ethyl carbamate (EC).

The degradation of arginine and the excretion of citrulline was studied with resting and growing cells of selected wine LAB in synthetic buffer and wine. All wine LAB tested degraded arginine and excreted citrulline, and also degraded citrulline as a sole amino acid. However, reutilization of excreted citrulline depended on the biomass condition. Arginine degradation and citrulline excretion rates had a linear, positive correlation. Arginine to citrulline conversion ratios ranged between 0.8 and 4.6% (w/w) and can be used to estimate the potential formation of citrulline from a given amount of arginine. Combining these ratios with literature data, an approximate arginine to EC conversion ratio of 0.006% (w/w) can be calculated. In *Lactobacillus buchneri*, arginine degradation occurred during growth and malic acid degradation, and this stimulated growth. In contrast, arginine degradation in oenococci occurred in late exponential or stationary phase and after malic acid degradation, and this did not stimulate growth.

Citrulline excretion results from an unfavourable equilibrium of the citrulline degrading reaction. The pH (3.5 - 6.5) did not have a direct effect on citrulline excretion. However, the excretion of citrulline was influenced by the growth phase in which arginine degradation occurred and can be partially regarded as an overflow metabolism caused by insufficient coupling of energy formed from arginine degradation to growth.

To control citrulline formation and pH increase in wine from bacterial arginine degradation, the results suggest carrying out MLF with pure oenococcal cultures and inhibiting bacterial metabolism after malic acid depletion. This is especially important in wines with high arginine concentrations and high pH values. Malic acid itself should be measured since pH increase or CO_2 formation may also result from arginine degradation. Alternatively, arginine negative *Oenococcus* strains could be isolated and used for MLF. The excessive addition of ammonia as yeast nutrient during alcoholic fermentation and storage of wines on yeast sediments (lees) increase the potential for citrulline formation. A simple enzymatic assay for the determination of arginine was developed and this method enables a fast "risk-assessment" of must and wines.

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ABBREVIATIONS

All abbreviations and units used in this thesis and not specified in this list are standard SI-units. Note that the anion names of acids have been used synonymously.

ADI ..... arginine deiminase
AF ..... alcoholic fermentation
A_λ ..... absorbance at specified wavelength (λ) in nanometers
Arg, arginine ..... L-arginine (174.25 g mol^{-1})
c ..... concentration
cfu ..... colony forming unit (cp. 2.4.3.2)
Citr, citrulline ..... L-citrulline (175.25 g mol^{-1})
CK ..... carbamate kinase
Ctrl ..... Control (experimental control)
CV(%) ..... coefficient of variation or relative standard error of the mean (=SE/mean × 100)
DAP ..... di-ammonium hydrogen orthophosphate (a common yeast nutrient)
dry wine ..... wine with no or low sugar concentration (0-8 g l^{-1} of reducing sugars)
EC ..... ethyl carbamate (urethane)
FMOC ..... 9-fluorenylmethyl chloroformate (for amino acid derivatization)
Fructose ..... D(-)-fructose
Galactose ..... D(+)-galactose
g ..... acceleration equivalent to the earth's gravity (9.806 m s^{-2})
Gl-DH ..... glutamate dehydrogenase
Glucose ..... D(+)-glucose
HPLC ..... high performance liquid chromatography
ID ..... inner diameter (HPLC column parameter)
α-KG ..... α-ketoglutaric acid
LAB ..... lactic acid bacteria
L. buchneri ..... *Lactobacillus buchneri*
Malic acid or malate ..... L(-)-malic acid
Mannitol ..... D-mannitol
MLF ..... malolactic fermentation
n ..... number of measurements
OD_λ ..... optical density at specified wavelength (λ) in nanometers
O. oeni ..... *Oenococcus oeni*
OPA/3-MPA ..... o-phtaldialdehyde/3-mercaptopropionic acid (for amino acid derivatization)
Orn, ornithine ..... L-ornithine (132.16 g mol^{-1})
OTC ..... ornithine transcarbamylase

(abbreviations, cont'd)

PC ..... *Saccharomyces bayanus* Première Cuvée
PCR..... polymerase chain reaction
PVPP.....polyvinylpolypyrrolidone
r.....coefficient of correlation
Rce ..... resting cell experiment (cp. 2.3.2.1)
Rhamnose ..... L(+)-rhamnose
Ribose ..... D(-)-ribose
RP-HPLC.....Reverse Phase HPLC
S. (*cerevisiae* or *bayanus*).....*Saccharomyces cerevisiae* or *bayanus*
SD .....standard deviation
SE.....standard error (= SD/$\sqrt{n}$)
TEA ..... triethanolamine
Trehalose .....D(+)-trehalose
TRIS..... 2-amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloride
U ..... enzymatic unit (enzyme quantity leading to formation of 1 µmol product min^{-1})
UV ..... ultraviolet (referring to the wavelength range of a spectrophotometer)
VIS ..... visible (referring to the wavelength range of a spectrophotometer)
v/v ..... volume/volume
w/v ..... weight/volume
w/w ..... weight/weight

PREFACE

Please pay attention to Figure 1 inside the back cover of this thesis. It contains a graphical representation of arginine degradation pathways in heterofermentative LAB and their consequences. The arginine deiminase pathway is essential for this thesis and Figure 1 allows the reader to view this pathway in relation to arginine degradation by yeasts and the formation of biogenic amines.

Whereas the experimental conditions of every experiment are outlined in the respective chapters, the detailed methods and media used are described in the general Materials & Methods section, simplifying the search for a specific method or medium.

To increase the readability of the thesis and to avoid unnecessary length of the main part, some figures have been cited in the text only and shown in the Appendix. This is consistent with the move of some scientific journals in providing "Supplementary Information" to facilitate the assessment of the work without increasing the length of manuscripts.

All chapters are intended to be independent and are fully discussed; cross-references are made where appropriate. Chapter 11 contains a synopsis that examines this work in the wider contexts of oenology and microbial metabolism.