Proteases and protein instability – finally happening!

SAWWV SASEV

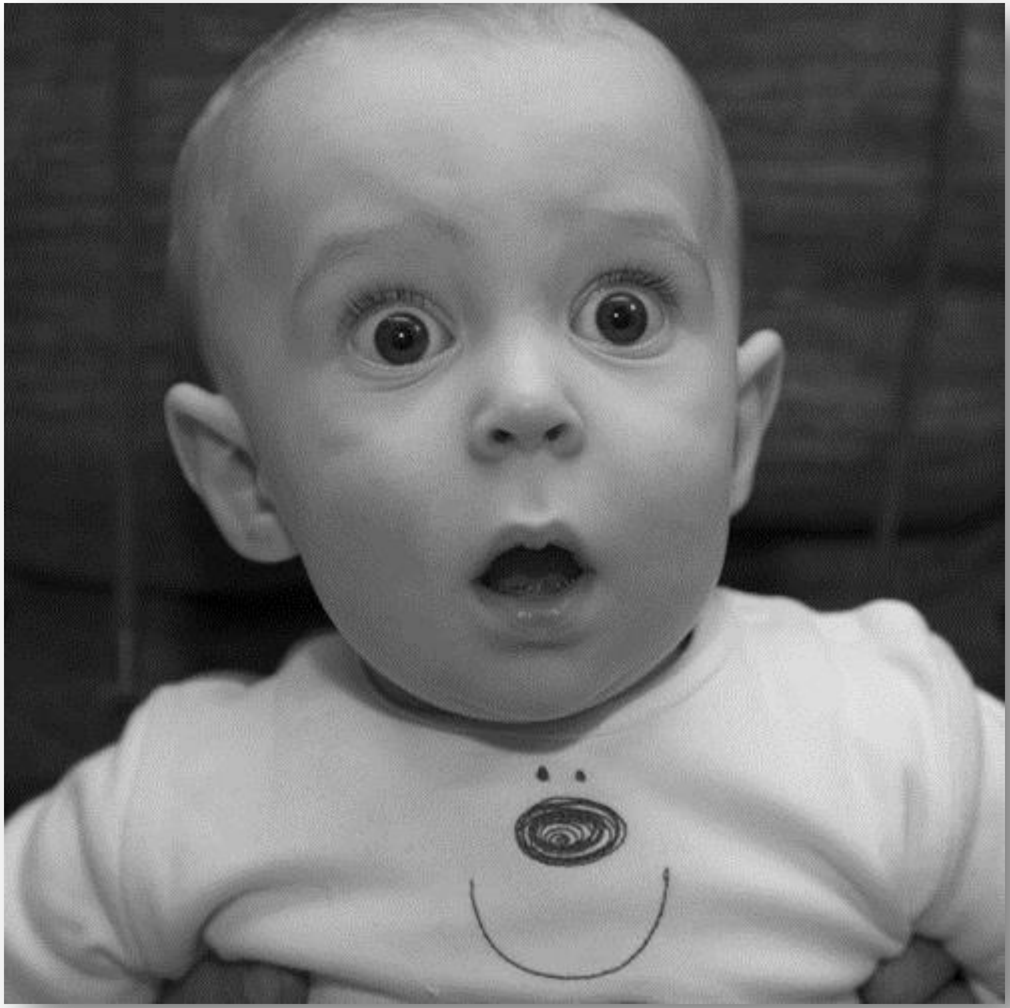
IGWS INSTITUTE FOR GRAPE AND WINE SCIENCES

INDUSTRY WORKSHOP
6 May 2015

Elda Lerm
Technical Consultant
Anchor Yeast

Anchor WINE YEAST
THE LEADING NEW WORLD WINE YEAST BRAND


AT FIRST I WAS LIKE
BUT THEN I WAS LIKE
made on imgur




GEEK IS
The New Sexy


GEEK


KEEP
CALM
AND
GEEK
ON


I WAS
A GEEK
BEFORE
IT WAS COOL


DIAGNOSIS
DIAGNOSIS
DIAGNOSIS
DIAGNOSIS
IN PROGRESS
IN PROGRESS
IN PROGRESS

Enzymes in winemaking

Enological enzymes:

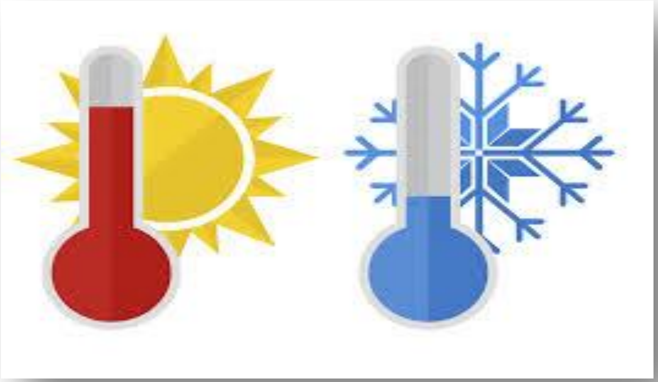
- used at several stages in processing: grape/must/wine
- complex cocktails of several enzymes
- varied concentration and activity; primary and secondary

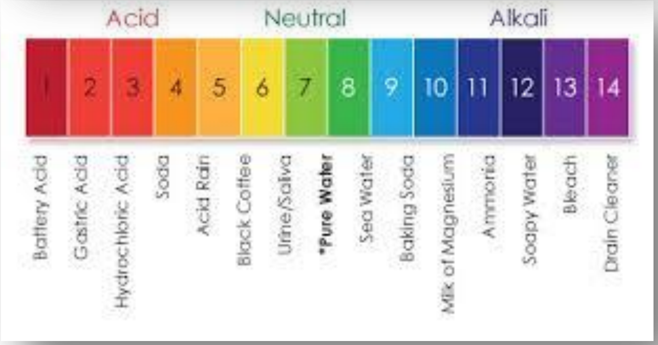
- supplement/complement endogenous enzymes

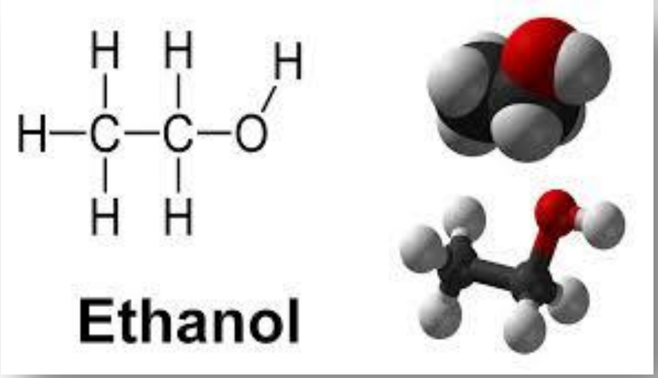
    - effective
    - specific
    - convenient

Keep in mind:






Acid
Neutral
Alkali
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Battery Acid
Gastric Acid
Hydrochloric Acid
Soda
Acid Rain
Black Coffee
Urine/Saliva
*Pure Water
Sea Water
Baking Soda
Milk of Magnesium
Ammonia
Soapy Water
Bleach
Drain Cleaner


H H H
H–C–C–O
H H
Ethanol

Protein stability in wine

Protein Stability = ability of a protein to retain its structural conformation or its activity when subjected to physical/chemical manipulations

Protein sources in wine:

- synthesis during berry development $_{50\%}$
- yeast protein synthesis $_{\text{fermentation}}$
- yeast autolysis

Protein levels in wine influenced by:

Protein haze = visual defect / no flavour impact
unacceptable to consumers

Problematic	***Less problematic***
white wines	red wines*
lower phenol red varietals	wines fermented/stored in barrels
stainless steel fermented wines	*phenolic compounds react with proteins during fermentation

Cause of protein haze = grape pathogenesis-related (PR) proteins
- infection/damage
- 10 – 500 mg/L

Challenges of PR proteins:

Acidic pH			
	6.0	Eggs Beans Cucumber	Asparagus Corn Green beans
	5.0	Coffee Tea Bananas	Pears Potatoes Squash
	4.0	Apples Yogurt Bell pepper	Raspberries Beer Basil pesto
	3.0	Orange juice Tomato juice Pineapple	Peaches Cherries Ketchup
	2.0	Vinegar Colas Lemon	Lime Pomegranate

Differences in:

- molecular weights
- unfolding temp's
- structures
- stability

(greatly impacted by other grape components)

<u>Cause of protein haze</u> = grape pathogenesis-related (PR) proteins

1. **thaumatin-like proteins (TLP)**
2. **chitinases**
3. **invertases**
4. ***β*-glucanases**

unstable over time
(storage conditions)
(wine matrix composition)

↓

1. **denaturation/unfold**
2. **aggregate**
3. **flocculate**

slow process; normal storage

↓

visible haziness

Cause of protein haze = grape pathogenesis-related (PR) proteins

1. **thaumatin-like proteins (TLP)**
2. **chitinases**
3. **invertases**
4. ***β*-glucanases**

reversible unfolding
less likely to aggregate over
short time periods

↓

1. **denaturation/unfold**
2. **aggregate**
3. **flocculate**

↓

light-dispersing particles and visible haziness

Cause of protein haze = grape pathogenesis-related (PR) proteins

1. **thaumatin-like proteins (TLP)**
2. **chitinases**
3. **invertases**
4. ***β*-glucanases**

unfold irreversibly
aggregate more aggressively

↓

1. **denaturation/unfold**
2. **aggregate**
3. **flocculate**

↓

light-dispersing particles and visible haziness

Cause of protein haze = grape pathogenesis-related (PR) proteins

1. **thaumatin-like proteins (TLP)**
2. **chitinases**
3. **invertases**
4. ***β*-glucanases**

↓

2 mechanisms

1. **denaturation/unfold**
2. **aggregate**
3. **flocculate**

↓

visible haziness

__Cause of protein haze__ = grape pathogenesis-related (PR) proteins

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↓

1. **denaturation/unfold**
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↓

light-dispersing particles and visible haziness

__native to non-native state:__

a) pH
b) temp
c) salt
d) co-solutes
e) preservatives
f) intrinsic protein properties

pH:

low pH → smooth and homogenous haze
high pH → coarse and flocculated haze

salt and co-solutes:

sulphate anions	→ denaturation + aggregation
organic acids (-)	→ prevents proteins (+) form sulphate interaction
	→ stabilising effect on haze potential of proteins
polysaccharides	→ inhibit aggregation
	→ stabilising effect on haze potential of proteins

During fermentation:

- ↑ soluble proteins
- ↑ protein instability
- Δ proportion of protein fractions

After fermentation:

- protein stability increases (stabilizing yeast polysaccharides)

1. Measure protein stability/haze potential

↓

2. Adjust protein content before bottling

↓

3. No haze formation during transport/storage

INDUCE haze
MEASURE haze
Protein stable – no treatment
Protein unstable – treatment

Protein stability evaluation = just before bottling

acidification/MLF/fortification/cold stab: precipitation of protein complexes

INDUCE haze

MEASURE haze

stable – no treatment

unstable – treatment

Heat test

Bentotest

all proteins
false unstable

✓

- unreliable
- colour

- difficult
- observer variance

Removal of proteins:

- adsorption (Bentonite)
- precipitation

Bentonite

✓ effective

ˣ efficiency
ˣ cost
ˣ impact on environment: waste disposal
ˣ impact on wine characteristics: quality
ˣ protein removal not selective
ˣ increased tank time

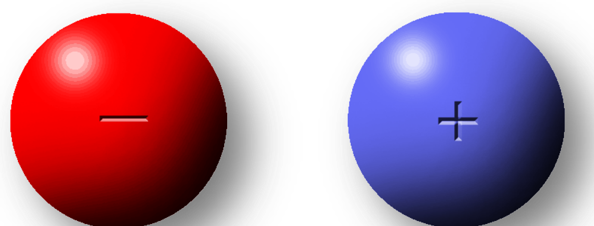
Na

- more reactive
- very fluffy lees
- higher % wine loss

Ca

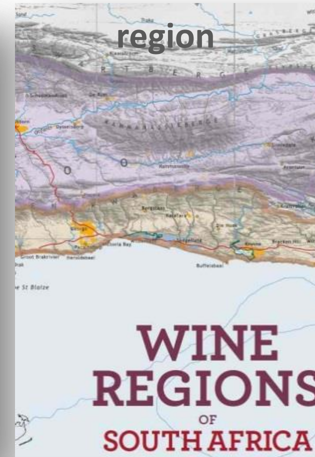
- more compact lees
- lower % wine loss
- less effective, so use more

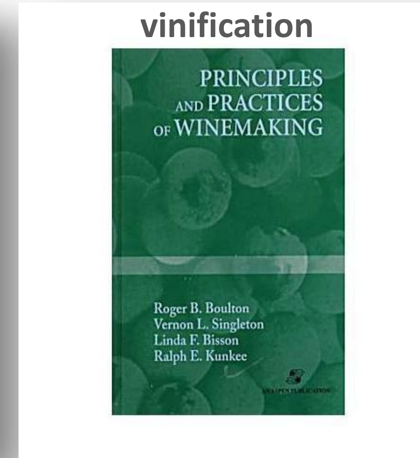
wine pH:



bentonite protein

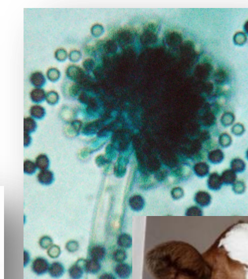
Amount of bentonite required:





Best time to fine and remove largest portion of proteins = juice

- no loss of aromatic quality
- reduced amount of proteins for removal later
- less bentonite
- wine less altered by stabilisation process




DIAGNOSIS
DIAGNOSIS
DIAGNOSIS


IN PROGRESS
IN PROGRESS
IN PROGRESS

Protease

- hydrolases
- cleavage of peptide bonds (links between amino acids) in proteins
- synthesis and degrading properties

= food / diary / detergent / leather industries etc.

Preparation, Properties and Possible Application of Coimmobilized Biocatalysts

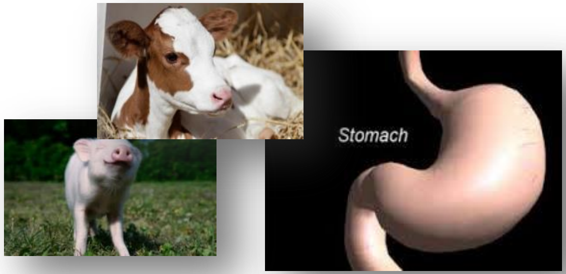
W. Hartmeier

Summary

Recently, coimmobilizates have been developed which combine the biocatalytic properties of whole cells or parts of the cells and additional enzymes. The new method presented leads to very small immobilized particles with extraordinarily high specific activities and neglegible diffusion barriers. Enzymatic properties

Endogenous proteases associated with grape and wine:

pepsin
chief digestive enzyme
proteins → polypeptides



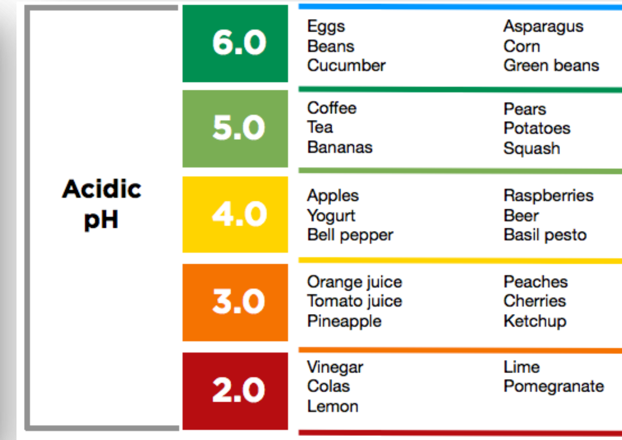
trypsin
digestive enzyme

bromelain
anti-inflammotory

ficin
proteins → amino acids

papain

Challenges of PR proteins:



Differences in:

- molecular weights
- unfolding temp's
- structures
- stability

(greatly impacted by other grape components)

Right now?

- proteases not allowed for use in winemaking
- resolution for their approval in certain conditions
- “not approved for 2 more years”
- more applications for proteases will be investigated

MATERIALS AUTHORIZED FOR TREATMENT OF WINE AND JUICE	
Materials and use	***Reference or limitation***
Protease (general)*	*Aspergillus niger* *Bacillus subtilisper* *Bacillus licheniformis*
Protease (Bromelain)*	*Ananus comosus* *Ananus bracteatus*
Protease (Ficin)*	*Ficus* spp.
Protease (Papain)*	Carica papaya
Protease (Pepsin)*	Porcine or bovine stomachs
Protease (Trypsin)*	Porcine or bovine pancreas
* To reduce or to remove heat labile proteins	

Proctase

- Aspergillopepsins I and II
- food grade
- well characterised
- active at wine pH and unfolding temp of PR proteins

Treatment	*Reduction in protein*
+ Proctase	20%
+ 1 min @ 75°C	40%
+ Proctase + 1 min @ 75°C	85-91%
→ ***NO BENTONITE NEEDED***	

heat treatment $_{\text{1 min @ 75°C}}$

↓

unfolding

↓

aggregation

Proctase $_{\text{15 mg/L}}$

protein most susceptible to attack

juice treatment **=** no negative sensory impact

Costing implications

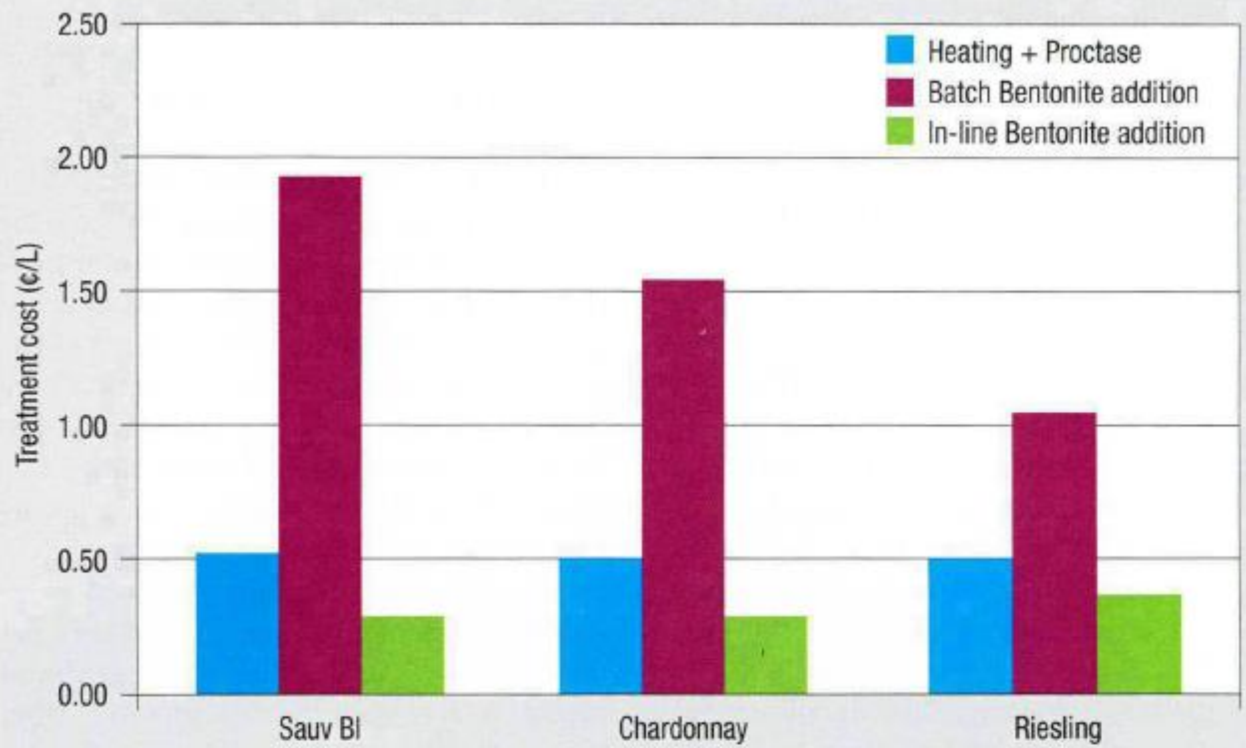


Figure 3. Results of economic analysis of heating plus Proctase addition, compared with batch and in-line bentonite addition for Sauvignon Blanc, Chardonnay and Riesling juices (treatment cost in cents per L).

2011

A promising enzyme for the stabilisation of white wines

New alternative to bentonite

2012

Beyond bentonite

By Ella Robinson, Neil Scrimgeour, Matteo Marangon,
Richard Muhlack, Paul Smith, Peter Godden and Dan Johnson
The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA 5064.

Managing director Dan Johnson

Until now, bentonite treatment has been the winemaker's best answer to troublesome haze-causing proteins. Breakthroughs in understanding the structure and properties of those proteins at the AWRI have led to the discovery of a potentially viable and practical alternative. Laboratory, pilot and industry-scale trials of proctase have now been successfully completed.

2013

Proctase as a bentonite alternative – what's the latest?

AWRI researchers have discovered information about the mechanisms of wine protein haze formation and have identified Proctase – with its ability to break down haze-forming grape proteins – as a potential alternative to bentonite.

working with OIV to gain approval for protease treated wines

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NEWS RELEASE

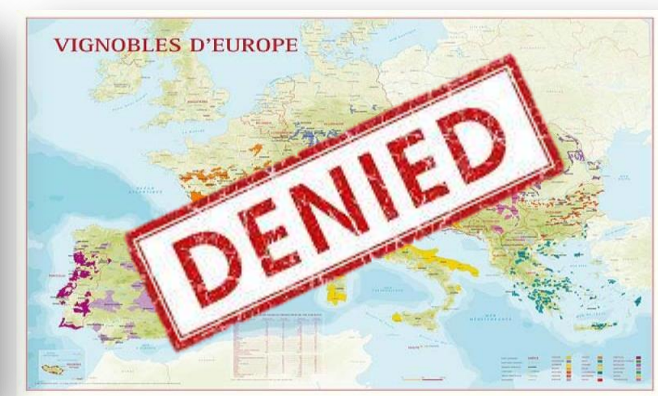
The Australian Wine Research Institute

For immediate release:
Thursday, 11 December 2014

Way now clear for haze-preventing enzymes in Australian winemaking

Proctase + flash past. = available for 2015 vintage

APPROVED: Food Standards Australian New Zealand (FSANZ)
Use in AUS winemaking: wines sold domestically and exported to NZ



Current Winetech funded research

IWBT

Applied and Environmental Microbiology

Identification and Partial Characterization of Extracellular Aspartic Protease Genes from Metschnikowia pulcherrima IWBT Y1123 and Candida apicola IWBT Y1384

Vernita J. Reid, Louwrens W. Theron, Maret du Toit and Benoit Divol

Appl. Environ. Microbiol. 2012, 78(19):6838. DOI: 10.1128/AEM.00505-12.
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produced and secreted by the yeast

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acid protease

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non-*Saccharomyces*

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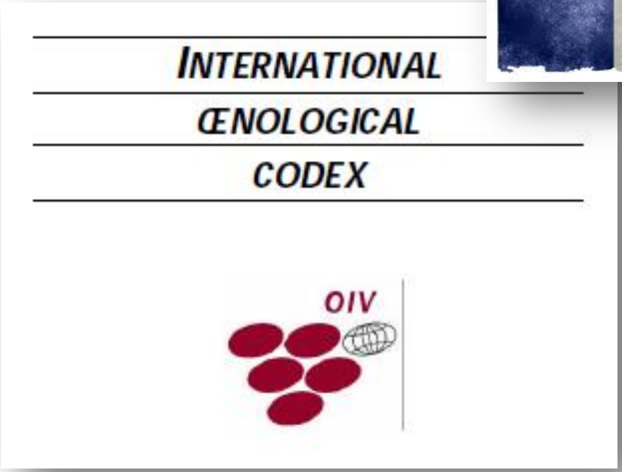
- enzyme is secreted in presence of grape juice proteins

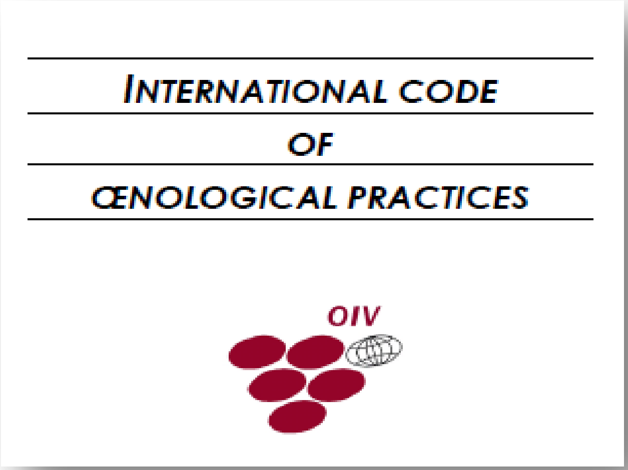
Still to determine:

- *optimum pH and temp. for enzyme activity?*
- *activity of enzyme in wine?*
- *ability to hydrolyze wine proteins and significantly reduce haze formation?*


INTERNATIONAL
ŒNOLOGICAL
CODEX
OIV

337 pages…0 mentions

description of the principal products used to make wine


INTERNATIONAL CODE
OF
ŒNOLOGICAL PRACTICES
OIV

708 pages…1 mention

conditions, instructions and limits for their use

2015

Annual work programme

In accordance with the axes of the

OIV 2015-2019 Strategic Plan

further information…
consideration…
opinion…
progression…

2015-2019 SP Reference	Start date	Actions	…pected in 2015	Lead (in bold) and other structures involved
2 c i	2014	Drafting of oenological practices: Use of proteases	Presentation … information, consideration of the opinion i… JAL, and progression of the draft resolutio… 5	CII, CIV

2015-2019 SP Reference	Start date	Actions	Outcomes expecte… 2015 according to the Impact study	Lead (in bold) and other structures involved
4 a i	2014	Evaluation and opinion on the new additives or processing aids proposed as new practices	Opinion given and pu… ished on: – Polydimethyls… oxane – Agar-agar – Potassium carbonate – Protease – Polyamino acids	CIV

In the future?

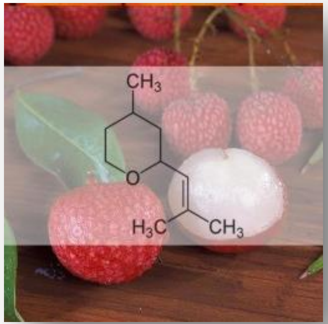
- inclusion of protease in the International Oenological Codex of the OIV

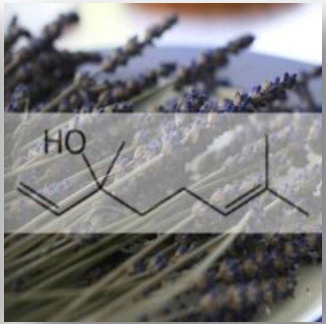
- yeast producing extracellular proteases during fermentation

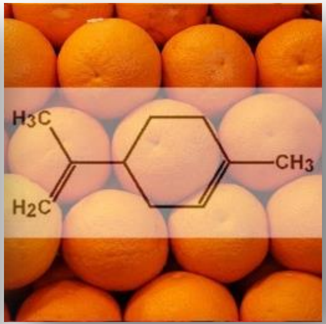
- other proteases under investigation:
    - Bcap8 (*B. cinerea*)
    - Antarctic fungi

- Carrageenan (red seaweeds)

Other issues to address with protease applications:







Alternative options to proteases for treatment of protein stability

Any alternative to bentonite

→ cheaper/more cost effective
→ better settling with less waste
→ more efficient
→ no flavour taint

Mannoproteins

- compete for non-protein compounds that contribute to instability
- positive impact on wine colour

Molecular imprinted polymers

- "designed" to selectively remove proteins

Proteases and protein instability – finally happening… ***but not just yet!***

INDUSTRY WORKSHOP
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