THE EFFECT OF PECTOLYTIC ENZYME TREATMENTS ON RED GRAPE MASHES OF *VRANEC* ON THE MICROBIOLOGICAL QUALITY OF WINES

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Abstract: The paper investigates effects of pectolytic enzyme treatments on red grape mashes on the microbiological quality of wines and wine stability with counting of the presence yeast cells (Saccharomyces cerevisiae), after incubation on Sabourald-maltose agar. Also wine samples were investigates of the presence moulds and other bacteria, as and dangerous bacteria Salmonella and Shigella, Staphylococus aureus, Proteus spp., Sulphite-reducing clostridy and Escherichia coli.
In all wine samples the results showed that have yeasts Saccharomyces cerevisiae. Saccharomyces is not regard as spoilage organism only if it is found in the wrong place at the wrong time (e.g. in a bottle of semi-sweet wine) causing re-fermentation, meanwhile the better is to have less. Pectinolytic enzyme preparation Trenolin Rot DF showed the best results.
Key words: pectolytic enzymes, red grape *Vranec*, microbiological quality of wines, microbial spoilage in wine

INTRODUCTION

Vranec is a variety of red grape cultivated in Republic of Macedonia. It is capable of producing high quality red table wines in this country. Although the composition of the grape depends on its variety, the soil and the climatic conditions, there is little variation in the actual cell structure of the plant.
Enzymes play a definite role in the ancient and complex process of winemaking. From the pre-fermentation stage, through fermentation, post-fermentation and aging, enzymes are the major driving forces catalysing various biotransformation reactions [9]. Pectolytic enzyme preparations have been used for over 60 years in fruit juice production. These enzymes play a mayor role in fruit juice technologies. Protopectinases, polygalacturonases, lyases and pectin esterases are among the extensively studied pectinolytic enzymes. Protopectinases catalyze the solubilization of protopectin. Polygalacturonases hydrolyze the polygalacturonic acid chain by addition of water and are the most abundant among all the pectinolytic enzymes. Lyases catalyze the trans-eliminative cleavage of the galacturonic acid polymer. Pectinesterases liberate pectins and methanol by de-esterifying the methyl ester linkages of the pectin backbone. The largest industrial application of pectinases is in fruit juice extraction and clarification. Pectinolytic enzymes added to macerated fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristics (colour and turbidity) as compared to the untreated wines. Enzymatically treated red wines presented chromatic characteristics, which are considered better than the control wines. These wines also showed greater stability as compared to the control [7].
The winemaking process is a complex ecological niche where the biochemistry and interaction of yeasts, bacteria, fungi and the viruses play a pivotal role in the final product. These microorganisms involved are at the core of the winemaking process, whether for good or ill [5]. The main microorganisms associated with wine spoilage are yeasts, acetic acid bacteria and lactic acid bacteria. Winemaking processes include multiple stages at which microbial spoilage is likely to occur. One must attemt to reduce the numbers of microbes in the juice and on the equipment. This is achieved through processing the pulp by applying food hygiene practices and following the hazard analysis critical control point (HACCP) system. The second stage of microbial spoilage may occur during fermentation because at this stage, the fruit juice contains both the natural flora of the fruit and fora that may be harboured by the wine cellar and its equipment. This may render the wine unacceptable, since the spoilage can include bitterness and off-flavours (mousiness, ester taint, phenolic, vinegary, buttery, etc.), as well as cosmetic problems such as turbidity, viscosity, sediment and film formation. The major spoilage organisms of the yeast genera include *Brettanomyces, Candida, Hanseniaspora, Pichia* and *Zygosaccharomyces*. The genera of lactic acid bacteria include *Lactobacillus, Leuconostoc* and *Pediococcus*, while the acetic acid bacteria genera are *Acetobacter* and *Gluconobacter* [6].

The spoilage caused by yeasts is important because they cause refermentation, ester formation, hydrogen sulphide and volatile sulphur compounds, volatile acidity, the formation of volatile phenols, mousiness, film formation, deacidification and the formation of ethyl carbamate. *Saccharomyces* is regarded as spoilage organism only if it is found in the wrong place at the wrong time (e.g. in a bottle of semi-sweet wine) causing re-fermentation. Schizosaccharomyces pombe has been associated with wine spoilage when growing in bottled wine and forming a sediment at the bottom of the bottle [3].
The spoilage caused in wine by lactic acid bacteria is associated particularly with acetification of the wine through the production of acetic acid, mousy taints, bitterness, ropiness, buttery flavour and increased viscosity of the wine [8], [10].
The main spoilage caused by acetic acid bacteria is associated with oxidation of the ethanol to acetaldehyde and eventually acetic acid. Gram-negative acetic acid bacteria require oxygen for growth. They carry out incomplete oxidation of alcohols, leading to the accumulation of organic acids as end products [1], [2].
Yeasts play a central role in the spoilage of beverages. A few species are capable of spoiling beverages. These can survive and grow under stress conditionswhere other microorganisms are not competitive. This investigates uses the wine industry as a case study where serious microbiological problems are caused by yeasts. The effect of pectolytic enzyme treatments on red grape mashes are discussed on the microbiological quality of wines, the susceptibility of wine to spoilage yeasts and wine stability based on scientific knowledge and industrial practices for monitoring yeast contamination i.e. for monitoring the presence of yeast.

MATERIAL AND METHODS

Commercial pectolytic enzyme preparations

- Vinozym Vintage FCE, Novozymes A/S, Bagsvaerd, Denmark; 2, 3, 4, and 5 g/100 kg grapes
- Rohapect VR-C, AB Enzymes GmbH, Darmstadt, Germany; 2, 3, 4, and 5 g/100 kg grapes
- Trenolin Rot DF, Erbslöh Geisenheim AG, Geisenheim, Germany); 10, 15, 20, and 25 ml/100 kg grapes

These enzyme preparations are derived from cultures of Aspergillus niger which is a species accepted as G.R.A.S. (Generally Recognized As Safe) [4].

Grape samples for laboratory trials

The grape cultivar Vranec (*Vitis vinifera*), cultivated in the Ovce pole vineyard, the Povardarie region, was harvested at optimal maturity (2009 vintage), at 200-220 g l^{-1} sugar, 6.5-7.5 g l^{-1} total acids, and pH from 3.1 to 3.3, and transported to the private winery "Imako Vino" Stip, Republic of Macedonia.

Wine samples. Microvinification

Wines were prepared in the laboratory of winery "Imako Vino" Stip.Grapes was weighed, crushed/destemmed and divided in 5 liters plastic fermentation tanks. Red grape mashes were macerated for 6 hours (18-20 °C), with addition on one commercial pectolytic enzyme preparation. After addition of SO_2 (50 ppm) and yeast (*Saccharomyces cerevisiae*) NEUTRE SC (Lallemand)(200 mg kg^{-1} grape), was applied maceration time of 5 days (~25 °C). After the maceration, the pomace was removed. All wines were plunged twice daily to completion of fermentation. Control trial was in all same with experimental trials only no added pectolytic enzyme preparation. All treatments were performed in duplicate.
The bottled wines (0.5 l) were stored at 4–6°C.

Determination of the total yeast cells

The effect of pectolytic enzyme treatments on red grape mashes on the microbiological quality of wines and wine stability were investigated with counting of the presence yeast cells (*Saccharomyces cerevisiae*), after incubation on Sabourald-maltose agar.
1 ml wine sample was added to Sabourald-maltose agar base for yeasts and moulds in petri dish. After keeping the samples at room temperature (thermostat) for 3-5 days were counted the presence yeast cells (cells/ml wine).
Sabouraud-maltose agar: peptone (1.0%), maltose (4.0%), agar (2.0%).

Determination of bacteria

Salmonella and Shigella, Staphylococus aureus, Proteus spp., Sulphite-reducing clostridy and Escherichia coli.

***Salmonella and Shigella*in 25 ml.** 25 ml wine sample was added in Erlenmeyer with 225 ml Selenite broth. After keeping the samples at 37 °C (thermostat) for 24 hours with eza were transplanted at SS base for *Salmonella and Shigella* and placed 24-48 hours at 37 °C.

Selenite broth: peptone (o.5%), lactose (0.4%), Na-selenite (0.4%), Na-phosphate (1.0%).

***Staphylococus aureus*in 0.1 ml.** 1 ml wine sample + 9 ml physiological solution=10 ml wine solution

1 ml from wine solution are put at BAIRD PARKER base for *Staphylococus aureus,* and are keep in thermostat 24 hours at 37 °C.

BAIRD PARKER AGAR: tryptone (1.0%), meat extract (0.5%), LiCl (0.5%), yeast extract (0.1%), agar (2.0%).

***Proteus spp.* in 0.1 ml.** 1 ml from wine solution are put at SS base for *Proteus spp.,* and are keep in thermostat 24-48 hours at 37 °C.

SS agar: peptone (5.0 g), meat extract (5.0 g), lactose (10.0 g), egg salts (8.5 g), natrium citrate (8.5 g), natrium thiosulphate (3.5 g), ferric citrate (1.0 g), agar (13.0 g), neutral red (0.023 g), brilliant green (0.00033 g).

***Sulphite-reducing clostridy*in 0.1 ml.** 1 ml from wine solution are put at sulphite agar base for *Sulphite-reducing clostridy,* and are keep in thermostat 24-48 hours at 37 °C.

Sulphite-reducing bacteria usually produce black colonies as a result of the reduction of sulphite to sulphide, which reacts with the iron(III)salt.

Sulphite agar: tryptone (15.0 g), yeast extract (10.0 g), distilled water (750 ml), water (aqua fontis) (250 ml).

***Escherichia coli*in 10 ml**. 10 ml from wine solution are put at liquid MAC CONKEY base (5 ml) for *Escherichia coli,* and are keep in thermostat 24-48 hours at 44 °C. After this, with eza are transplants at pink red egg yolk agar, and are keep in thermostat 34-48 hours at 44 °C. *Escherichia coli* grow as red or pink colonies.

MAC CONKEY AGAR: peptone (20.0 g), synthetic detergent (5.0 g), sodium chloride (5.0 g), lactose (10.0 g), neutral red (0.07 g), agar (12.0 g).

RESULTS AND DISCUSSION

Yeasts and bacteria are part of the natural microbial ecosystem of wine and play an important role in winemakingby reducing wine acidity and contributing to aroma and flavour. They can cause numerous unwelcome wine spoilage problems, which reduce wine quality and value.

Enzymes play an important role in winemaking. The application of industrial enzyme preparations in the wine industry is a common practice.They have been used to increase juice yield, filtration rate, rate of settling, and clarity of wines besides some microbiological implications.

The effect of pectolytic enzyme treatments on red grape mashes on the microbiological quality of wines and wine stability were investigated with counting of the presence yeast cells (*Saccharomyces cerevisiae*), after incubation on Sabourald-maltose agar, as shown in Table 1.

In all wine samples the results showed that have yeasts *Saccharomyces cerevisiae* from 400 to 2000 cells/ml. *Saccharomyces* is not regard as spoilage organismonly if it is found in the wrong place at the wrong time (e.g. in a bottle of semi-sweet wine) causing re-fermentation, meanwhile the better is to have less. Pectinolytic enzyme preparation Vinozym Vintage FCE showed yeasts *Saccharomyces cerevisiae* from 487 to 953 cells/ml wine, Rohapect VR-C from 1900 to 1953 cells/ml, and Trenolin Rot DF from 240 to 567 cells/ml depend of used doses and contol trial “no-enzyme addition” (433 cells/ml). Pectinolytic enzyme preparation Trenolin Rot DF showed the best results.

In all wine samples the results showed that have not the growth of unwanted bacteria as *Salmonella and Shigella, Staphylococus aureus, Proteus spp., Sulphite-reducing clostridy and Escherichia coli.*

Table 1. The effect of pectolytic enzyme treatments on red grape mashes of *Vranec* on the microbiological quality of wines

Enzyme preparations	Dose (g or ml/100kg grape)	[a]Yeasts, *Saccharomyces cerevisiae* (cells/ml)	[a]Bacteria, *Salmonella and Shigella; Staphylococus aureus; Escherichia coli; Sulphite-reducing clostridy;Moulds and other bacteria,* (cells/ml)
Vinozym Vintage FCE	2 g	953 ± 41	0
	3 g	933 ± 47	0
	4 g	533 ± 47	0
	5 g	487 ± 19	0
Rohapect VR-C	2 g	1907 ± 82	0
	3 g	1913 ± 66	0
	4 g	1900 ± 82	0
	5 g	1953 ± 52	0
Trenolin Rot DF	10 ml	240± 33	0
	15 ml	417 ± 23	0
	20 ml	513 ± 34	0
	25 ml	567 ± 34	0
Control-no added enzyme	0	433 ± 47	0

Note: [a]The values are average from 3 replicates ±SD

The largest industrial application of pectinases is in fruit juice extraction and clarification. Pectinolytic enzymes added to macerated fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristics (colour and turbidity) as compared to the untreated wines. These wines also showed greater stability as compared to the control (Revilla and Gonzalez-san jose, 2003). The concepts of the susceptibility of wine to spoilage yeasts and wine stability are based on scientific knowledge and industrial practices for monitoring yeast contamination. A discussion on acceptable levels of yeasts and microbiological criteria in the wine industry is supported by data obtained from wineries, wholesalers, and the scientific literature.

CONCLUSION

Significance and impact of the study is that pectolytic enzyme treatments on red grape mashes had a pronounced effect on the microbiological quality of wines and wine stability. Results from comparison of effects of pectolytic enzyme preparations in winemaking on the microbiological quality of wines can contribute to a better orientation in the choice of suitable enzyme preparations in wine industry.

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