

Isolation and Identification of Microorganisms Including Lactic Acid Bacteria and Their Use in Microbial Deacidification of Wines from Domestic Vineyards

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Abstract

The aim of this study was to identify various bacteria isolated from grapes and their wines. Additionally we investigated the capacity of lactic acid bacteria for microbiological deacidification of wines produced in Poland. We have identified *Oenococcus oeni*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*. During the microbial deacidification process, we observed decreases of total acidity and increases of volatile acidity, with statistically significant changes noted for *O. oeni* in Marechal Foch and Seyval Blanc, and for *Lb. acidophilus* in Frontenac. On the other hand, a statistically significant increase in pH was observed in Marechal Foch and Seyval Blanc following deacidification by *O. oeni*.

Key words: lactic acid bacteria, malolactic fermentation, wine

Wine is the final product of a complex process in which different groups of microorganisms are involved: yeasts, bacteria and molds. The wines obtained from national raw materials are characterized by high acidity. High concentrations of organic acids in wines can be a problem when introducing the final product for sale. Reduction of the total acidity of wine can be achieved by chemical or biological deacidification. An appropriate choice of strains for malolactic fermentation (MLF) can reduce the acidity of the wine (Henick-Kling, 1995). The microorganisms which reduce the acidity of wines are mainly species naturally occurring on the surface of the fruit, this process is often performed by lactic acid bacteria (LAB).

The most common bacteria present on grapes are *Oenococcus oeni*, *Leuconostoc mesenteroides*, *Pediococcus parvulus*, *P. pentosaceus*, *P. damnosus* and different species of *Lactobacillus* (*L. brevis*, *L. plantarum*, *L. fermentum*, *L. buchneri*, *L. hilgardii* and *L. trichodes*) (Fleet, 2007; Du Toit *et al.*, 2011). Malolactic fermentation, in addition to deacidification, affects the flavor characteristics of wine and has a fully positive role in microbial stabilization (Pozo-Bayon *et al.*, 2005).

The process of deacidification and starter cultures of LAB (*O. oeni* and *Lactobacillus* spp.) (Du Toit *et al.*, 2011) are well known and used in southern Europe. Poland is among the countries with a cool climate for

wine. The study of microflora of grapes and the control process of wine production are still in progress. It would be ideal to isolate the microflora responsible for deacidification process and obtain stable starter cultures for microbial deacidification of cool climate wine. The authors believe that this would be the most effective process of deacidification of wines, which will result in the preferred sensory properties of beverages, giving them a specific and regional character. The aim of this study was to identify various bacteria, including lactic acid bacteria, present on grapes. We also studied the capacity of lactic acid bacteria for malolactic fermentation and their use in microbial deacidification of cold climate wine.

The tested grapes were washed in 10 ml of Ringer's solution. 1 ml of the eluate was plated onto agar. Each culture was taken in duplicate. Microorganisms were plated on nutrient dextrose agar (AO) and MRS agar. The plates with AO were incubated for 24 h and those with MRS for 72 h at 32°C. Pure cultures were incubated at 32°C for 24 h and then stored at +4°C. Bacterial cultures were analyzed macroscopically and microscopically (Gram stain, Nikon Eclipse 1000x magnification microscope, with a Moticam 2000 digital camera and the program Tomic Live Imaging). Biochemical test for catalase and microtests of fermentation by Kocwa (Burbianka *et al.*, 1971) were performed. For testing

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of fermentation we used glucose, galactose, maltose, mannose, fructose, ribose, arabinose, and lactose, with CaCO_3 as an indicator. After incubation at 32°C for 24 h, the appearance of a halo around the colony indicates the ability to ferment the tested sugar.

Microorganisms from wines were plated on AO and GYC medium (for acetic acid bacteria), or MRS and substrate for *Leuconostoc*. The cultures were incubated for 24 h at 32°C. Samples of wine were diluted to 10^0 , 10^{-1} and 10^{-2} in sterile physiological saline. Pure cultures were derived for further research, and stored at 4°C. Microorganisms were analyzed macroscopically and microscopically (Gram stain, Schaeffer-Fulton (microscope magnification 1000x)). The following biochemical tests were performed: test of catalase and microtests of fermentation by Kocwa (Burbianka *et al.*, 1971), to determine the capacity of bacterial strains for degradation of fructose, sucrose, maltose, lactose, L-arabinose, glycerol, mannitol and raffinose. Lactic acid bacteria, acetic acid bacteria and *Enterobacteriaceae* were identified. The pure cultures of bacteria were identified with the key of Dworkin *et al.* (2006).

Colonies identified using the API 50CH (Bio-Merieux) for identification of lactic acid bacterial species (Gotcheva *et al.*, 2000). The cultures of LAB were passaged on slants and liquid MRS medium, with limited access of oxygen at 28°C. Two of the isolated strains of LAB were used for microbiological deacidification of wine.

Malolactic fermentation was carried out on three varieties of wine, that came from the vineyards Garlica Murowana (UR in Krakow). Red wines were made from Marechal Foch and Frontenac strains and white wine from Seyval Blanc strain. All three wines were fermented by MLF using two strains of lactic acid bacteria, *O. oeni* (10^9 cfu/cm³) and *Lactobacillus acidophilus* (10^9 cfu/cm³). The cell density was determined using a McFarland densitometer (Den-1B, Biosan). The wine, in quantities of 300 ml, was subjected to deacidification for 2–3 weeks at 20°C. During the process, wine acidity (PN-90/A-79120/07), volatile acidity (PN-90/A-79120/08), alcohol content (PN-90/A-79120/04) and pH were measured.

All experiments were performed in three replicates, for each sample. To determine the significance of differences between mean values, multivariate analysis of variance with *post hoc* Dunn's multiple comparisons test was performed. Distribution of normality was determined by Friedman test using the program InStat3 (Graph Pad Software, Inc., San Diego, CA).

We identified 30 bacterial isolates from different varieties of grapes. Six tested grapes are used for the production of red wine (Marechal Foch, Leon Millet, Alden, Frontenac, Rondo, Swenson Red) and five for white wines (Prim, Muskat Odessa, Aurora, Perle von

Alzey, Bianca). The grapes were obtained from the vineyard Golesz (Podkarpacie province). Gram positive bacilli (*Bacillus* spp., 56.5%), and cocci (*Micrococcus* spp., 10%), Gram positive and catalase negative lactic acid bacteria (*Lactobacillus* spp., 7%) and Gram-negative bacilli (26.5%) were identified. The identified bacteria were not typical representatives for these grapes. According to current knowledge, we expected mainly LAB and acetic acid bacteria (Du Plessis *et al.*, 2004; Du Toit *et al.*, 2011), which constitute the dominant microflora. However, other species of bacteria such as *Micrococcus* spp. and *Bacillus* spp. (Ponce, 2003), *Alicyclobacillus* spp. (Parish and Goodrich, 2005) and anaerobic *Clostridium* spp. (Irudayaraj *et al.*, 2002) were reported.

We analyzed 42 colonies of bacteria isolated from the wines and identified cocci (7%), *Bacillus* (35%), lactic acid bacteria (5%), acetic acid bacteria (17%) and Gram-negative *Enterobacteriaceae* (36%). Among the isolated strains of LAB we detected *Lactobacillus delbrueckii* and *Oenococcus oeni*, acetic bacteria *Acetobacter aceti*, *A. pasteurianus*, *Gluconacetobacter liquefaciens*, *G. hansenii* and *G. xylinus*. Wine is a specific ecosystem and therefore it is not always possible to create laboratory conditions for breeding these microorganisms. Studies on isolation of microorganisms from a variety of wines allow one to characterize the microflora of grapes and interactions between bacteria, yeasts and molds (Fleet, 2007).

The lactic acid bacteria were tested for their biological suitability for the process of deacidification of wine. Wines produced from grapes grown in Poland and in other regions with colder climates are characterized by higher contents of organic acids (Liu, 2002). At present, consumers around the world prefer wine with a fruity character and moderate acidity, which means that biological deacidification has become an important issue in the production of wines. Therefore, searching for appropriate strains to serve as new starter cultures, able to reduce the acidity of the wine and increase its quality, is an important direction of research in Poland.

Our test results confirmed the presence of *O. oeni*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*, although one species could not be identified. Under laboratory conditions, only *O. oeni* and *L. acidophilus* was able to grow and was used for microbiological wine deacidification.

Wine selection was not accidental. These were wines produced from fruit harvested during frequent rain, low temperatures and low sunlight. Both strains of bacteria caused a change in total acidity, volatile acidity, and pH. This research indicates that the degree of deacidification depends on the type of wine and grape varieties.

Total acidity determines the content of chemical substances of an acidic nature. It is a very important parameter influencing the flavor characteristics of the

Table I
The profile of total acidity, volatile, and pH of the wines tested
(the value are given as mean and standard deviation; * $p < 0.05$ vs. control)

Wine	Control	Deacidification by <i>Oenococcus oeni</i>	Deacidification by <i>Lb. acidophilus</i>
Total acidity [tartaric acid g/L]			
Marechal Foch – red	9.63 ± 0.04	7.55 ± 0.24*	8.47 ± 0.10
Frontenac – red	10.28 ± 0.06	10.04 ± 0.03	9.80 ± 0.04*
Seyval Blanc – white	8.87 ± 0.02	8.06 ± 0.09*	8.51 ± 0.00
Volatile acidity [acetic acid g/L]			
Marechal Foch – red	0.26 ± 0.01	0.42 ± 0.02*	0.27 ± 0.02
Frontenac – red	0.25 ± 0.01	0.28 ± 0.01	0.36 ± 0.02*
Seyval Blanc – white	0.20 ± 0.00	0.35 ± 0.05*	0.27 ± 0.02
pH			
Marechal Foch – red	3.37	3.64 ± 0.06*	3.49 ± 0.01
Frontenac – red	3.07	3.27 ± 0.02	3.15 ± 0.00
Seyval Blanc – white	2.43	3.27 ± 0.03*	3.28 ± 0.01

product. According to the Polish standard, acidity of the wine should be between 3.5 and 9 g/dm³ based on tartaric acid. On the other hand, the volatile acidity should be up to 1.2 g/dm³ in acetic acid for white wines and 1.4 g/dm³ for red wines. After MLF by tested microorganisms, a decrease of total acidity was observed, with statistically significant changes noted for *O. oeni* in Marechal Foch (red wine) and Seyval Blanc (white wine), and for *Lb. acidophilus* in Frontenac (red wine) (Table I). Similarly, a statistically significant change in the volatile acidity was observed for *O. oeni* in Marechal Foch and Seyval Blanc, and for *Lb. acidophilus* in Frontenac. Interestingly, a statistically significant change was noted in the pH of Marechal Foch and Seyval Blanc, following deacidification by *O. oeni*.

Increasingly, it is believed that in addition to *O. oeni* as well as various species of *Lactobacillus* spp. could be used for MLF (Dicks and Endo, 2009), as starter cultures. They influence the formation of taste and smell through their involvement in the metabolism of citrate, amino acids, polysaccharides, polyhydric alcohols, aldehydes catabolism, hydrolysis of glycosides, the synthesis and hydrolysis of esters, degradation of phenolic acids, lipolysis, proteolysis and peptidolysis (Liu, 2002). Our results are in concordance with other experiments (Du Toit *et al.*, 2011) and indicate the possibility of using various LAB species in the microbial process of wine deacidification. Interesting effects can be achieved using mixed starter cultures of LAB, which will require further studies. The use of starter cultures consisting of different species of homo- and heterofermenting LAB would provide numerous benefits for wine producers.

Microflora of different varieties of grapes grown in cool climates and finished wines are characterized by high species diversity. Isolated common types of

bacteria are lactic acid and acetic acid bacteria, cocci, aerobic bacilli and rods. Among the studied microflora, the lactic acid bacteria *O. oeni*, *L. acidophilus* and *L. delbrueckii* have been isolated; the first two have been used for microbiological deacidification of wines produced from national grape varieties. In the process of microbial deacidification of domestic wines, decreases of total acidity and increases of volatile acidity were observed, with statistically significant changes noted for *O. oeni* in Marechal Foch and Seyval Blanc, and for *Lb. acidophilus* in Frontenac. On the other hand, a statistically significant increase in pH was observed in Marechal Foch and Seyval Blanc after deacidification by *O. oeni*. Interesting results can be achieved using mixed starter cultures of lactic acid bacteria, which would provide control over the process, the specific flavor and aroma of wine, and microbiological stability.

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