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## **REDUCTION OF ACIDITY IN NORTHERN REGION BERRY JUICES**

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**Key words:** Northern region berries, organic acids, soluble sugars, acid reduction, malolactic fermentation, *Oenococcus oeni*, yeast uptake, alcoholic fermentation, berry wine, sensory quality.

**ABSTRACT**

Northern region berries may be used for wide variety of product alternatives as well as serving as a supply of many nutritionally valuable components. However, there are only rare, fragmentary and inconsistent scientific data available on the chemical composition of northern region berries. Therefore, comprehensive information was gathered on organic acid and soluble sugar concentrations of juices of six wild berries (bilberry, lingonberry, cranberry, cloudberry, red raspberry, black crowberry) and five cultivated berries (black currant, white currant, red currant, gooseberry (red), strawberry) all grown in Finland. The main acids of the berry juices were invariably citric and malic acid even though their concentrations varied widely from one berry variety to another (2.9 – 16.2 g/l and 3.3 – 24.7 g/l, respectively). In addition, juices of lingonberry, cranberry, cloudberry and black crowberry contained benzoic acid (0.1 – 0.7 g/l). The main sugars of the investigated berry juices were fructose (18.0 – 57.2 g/l) and glucose (22.2 – 50.0 g/l). Most of the berries contained also sucrose (0.2 – 5.1 g/l). The data enable direct comparison of northern region berries and underline the wide variation in their organic acid and soluble sugar content, which offers possibilities for the production of numerous sensory profiles. Accordingly, the selection of the right berry for individual purposes is enhanced.

Due to their acid and sugar composition, fermentation of northern region berry juices into wines faces challenges that are not normally met when using grape juices. In berry juices the fermentations should maintain or alleviate the often rich berry aroma under conditions where the content of organic acids is high and that of fermentable sugars low. Prior to fermentation the juices have to be diluted and sugar has to be added. This causes significant weakening of the aroma and body of the wine. To reduce the acidic mouthfeel of grape wines malolactic fermentation (MLF) is widely used. However, it is not known whether MLF is applicable to modifying the acid composition of berry juices. Therefore, acid conversion by MLF by *Oenococcus oeni* was studied to improve the usability of northern region berry juices. During MLF at low pH values (pH < 4.5), malic acid was always degraded first to completion without consumption of sugars or citric acid. After the exhaustion of malic acid the degradation of both citric acid and glucose were initiated simultaneously. Thus, it is concluded that by MLF, selective conversion of malic acid to lactic acid can be achieved without loss of sugar, also in berry juices. Sequential utilization of substrates by MLF thus enables a multitude of compositional changes in acidic juices. Control of duration

of the fermentation is essential when acid reduction without loss of sugar should occur.

The most problematic compound with reference to winemaking from lingonberry is benzoic acid, which contributes to the acidity of the berry. As a microbicidal compound, benzoic acid also prevents fermentation of lingonberry juice. Thus, the known pH-dependent ability of *Saccharomyces cerevisiae* yeast to uptake benzoic acid from solutions was applied. By suspending 15 – 20 % (w/w) of the yeast for 10 min in undiluted lingonberry juice, the benzoic acid concentration was reduced by 75 – 91 %, titratable acids by about 14 % and pH increased by 0.1 units. The resulting undiluted juice was successfully fermented with a new yeast inoculum. Thus, yeast may be used as a selective absorbent to remove a certain fermentation-hindering component from the juice. These results offer new insights into berry juice fermentation.

Accordingly, MLF represents a new, promising means for acidity reduction of northern region berry juices and berry wines without a significant loss of their natural sugar content. Also, the benzoic acid uptake by the yeast was proven to be effective. By these new methods, the critical inhibitors of the further processing of the juices can be eliminated and thus it is possible to facilitate the development of various berry products of northern regions.

## PREFACE

This work was carried out at the Laboratory of Biochemistry and Microbiology of Helsinki University of Technology during the years 1997 – 1998, 2000 and 2002. The financial support of the Graduate School “Electrochemical Science and Technology of Polymers and Membranes including Biomembranes” (ESPOM), Foundation of Technology, Finnish Food Research Foundation, Finnish Cultural Foundation, Ella and Georg Ehrnrooth Foundation, Jenny and Antti Wihuri Foundation, Emil Aaltonen Foundation, The Finnish Chemical Congress Foundation (Kemian Päivien Säätiö) and The Research Foundation of Economic and Technical Sciences (Kaute Foundation) are gratefully acknowledged.

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Espoo, 15.4.2003      Sanna Viljakainen

## ORIGINAL PUBLICATIONS

This work is based on the following publications (Appendices I – V), which are referred to in the text by their Roman numerals.

- I Viljakainen, S., Visti, A. & Laakso, S. (2002). Concentrations of organic acids and soluble sugars in juices from Nordic berries. *Acta Agriculturae Scandinavica B* **52**, 101-109.
- II Viljakainen, S. & Laakso, S. (2000). The use of malolactic *Oenococcus oeni* (ATCC 39401) for deacidification of media containing glucose, malic acid and citric acid. *European Food Research and Technology* **211**, 438-442.
- III Viljakainen, S. & Laakso, S. (2002). Acidity reduction in northern region berry juices by the malolactic bacterium *Oenococcus oeni*. *European Food Research and Technology* **214**, 412-417.
- IV Viljakainen, S., Visti, A. & Laakso, S. (2003). Malolactic and alcoholic fermentations in black currant juice. *Die Wein-Wissenschaft*. In press.
- V Visti, A., Viljakainen, S. & Laakso, S. (2003). Preparation of fermentable lingonberry juice through removal of benzoic acid by *Saccharomyces cerevisiae* yeast. *Food Research International* **36**. In press.

In addition, some unpublished data are presented. The author of the present thesis had the main responsibility for planning the research, carrying out the experiments and interpretation of the results in all publications except for the experimental design in publication V, which was carried out by M. Sc. Tech. Arto Visti.

## ABBREVIATIONS

6-PG/PK	heterolactic pathway of sugar metabolism
AF	alcoholic fermentation
ATP	adenosine triphosphate
CFU	colony forming unit
Da	Dalton (a non-SI unit of mass)
DSA	descriptive sensory analysis
HPLC	high performance liquid chromatography
$K_m$	Michaelis-Menten constant
LAB	lactic acid bacteria
MLE	malolactic enzyme
MLF	malolactic fermentation
MRS	Man-Rogosa-Sharpe medium
NAD	nicotinamide adenine dinucleotide
PFK	phosphofructokinase
$pK_a$	the negative logarithm of acidity constant
PMF	proton motive force
$\Delta p$	electrochemical gradient
$\Delta pH$	chemical potential of protons
$\Delta\Psi$	electrical potential

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# 1 INTRODUCTION

## 1.1 NORTHERN REGION BERRIES

Berries – wild or cultivated – are traditionally a part of the natural diet among northern Europeans, since the northern location favours the cultivation of berries instead of fruits. Berries of the northern region are valuable in many respects. Both cultivated and wild berries are unpolluted and their nutritional value is high. Berries have a high content of fibre, vitamins and minerals and, particularly wild berries, are rich in flavonoids (Viberg & Sjöholm 1996, Törrönen *et al.* 1997, Häkkinen *et al.* 1998, Häkkinen *et al.* 1999, Vuorinen *et al.* 2000).

However, due to the short growing season, only a small portion of the berry harvest is consumed as fresh berries, and the rest is processed to various products. The main problem in development of products from the northern region berries is that they are rich in organic acids. The juices have very low pH (pH 2.7 – 3.6) and due to low sugar content the flavour is also very acidic. The main acids are citric acid and malic acid and the main sugars are glucose and fructose (Haila 1990, Souci *et al.* 1994). Fermentation of the juices commonly requires dilution and sugar addition. This causes significant weakening of the aroma and body of the wine. Consequently, the use of northern region berries could be markedly enhanced if the acidity of the juices were controlled.

### *1.1.1 Organic acid and soluble sugar composition of the berries*

Sugar and acid composition together with different aromatic compounds in berries are important features that affect the sensory properties of the product. Individual berries have a characteristic sugar and acid composition that is influenced by e.g. cultivar, season, and geographic origin (Wrolstad & Shallenberger 1981, Boccorch *et al.* 1998). Each acid conveys a characteristic flavour, aroma or taste.

Not only composition, but also concentration, of each acid is essential in the quality of the product (Herrero *et al.* 1999). The post harvest changes in flavour are usually due to an increase in sugars and aromatic constituents and a decrease in acidity. From the industrial point of view, the characteristic acid and sugar composition significantly affects the processing properties and is essential when developing various processes using berries as the raw material. Furthermore, the contents of sugars will be of interest to nutritionists and processors of foods for consumers with special dietary requirements (Charley 1977, Wrolstad & Shallenberger 1981, Haila *et al.* 1992, Boccorh *et al.* 1998, Herrero *et al.* 1999).

Many of the physical properties of the two main acids in berries, citric and malic acid, resemble each other. This might be expected from their similar chemical structure. There is also a resemblance in the pH of solutions of malic acid and citric acid. Equal weights of each acid will lower the pH of a solution in like proportions. On the other hand, although several similarities are evident in the physical properties of malic acid and citric acid, malic acid differs in its effects on taste when used in various types of foods and beverages. On an equal weight basis, malic acid generally has a stronger acidic taste than citric acid. In addition to a stronger acidic taste, malic acid stimulates the taste buds for a longer duration, carrying with it the inherent flavour of the product (Sansville 1969, Peynaud 1984). From a taste and flavour point of view, malic acid is considered undesirable in wines because the taste is quite tart. Instead, lactic acid is not as sour tasting as malic acid (Prahl & Nielsen 1993, Russell 1998). Thus, although malic acid plays a minor part of the total acid, it is known to cause noticeable acidity in berry juices and thus cannot be ignored.

Despite the importance of the organic acids and soluble sugars in the berries, there are only rare, fragmentary and inconsistent data available on their contents in northern region berries. The methods of analysis vary from one study to another and often only total acids, titratable acids or total sugars have been determined. Moreover, the reports concern only a limited number of berries (Salo & Suomi

1972, Kallio & Markela 1982, Varo *et al.* 1984, Haila 1990, Haila *et al.* 1992, Huopalahti *et al.* 2000, Kallio *et al.* 2000).

### 1.1.2 Benzoic acid in berries

One problematic compound for many microbial processes from berries is benzoic acid ( $pK_a$  4.19). It occurs naturally, e.g. in lingonberry, cranberries, cloudberries and cinnamon (Archer 1980, Busta & Foegeding 1983, Chipley 1983, Heimhuber *et al.* 1990, Davidson 1997). Benzoic acid is one of the oldest and still one of the most widely used chemical preservative (Lueck 1980, Chipley 1983, Davidson 1997, Brul & Coote 1999). According to Chipley (1983), besides inhibition of yeast fermentation, benzoic acid functions effectively also against bacteria if the concentration is 0.1 % and the pH is low, as in northern region berry juices. Therefore, berries containing high amounts of benzoic acid are nonfermentable by many microorganisms and can normally be preserved without addition of preservatives.

The growth inhibitory effect of benzoic acid is strongly pH-dependent and most effective under acidic conditions. Under these conditions benzoic acid exists predominantly in the undissociated state ( $C_6H_5COOH$ ), a form in which it is a potent growth inhibitor. The undissociated acid, being uncharged, readily diffuses across the plasma membrane of the cell only to dissociate in the higher pH environment of the cytosol. Such dissociation generates protons and the acid anion ( $C_6H_5COO^-$ ). The benzoic acid anions and protons will tend to accumulate intracellularly to very high levels as, being charged, they cannot very readily diffuse from the cell (Krebs *et al.* 1983, Pearce *et al.* 2001, Piper *et al.* 2001).

Therefore, inhibition of growth by such a weak acid preservative has been proposed to be a due to a number of actions including e.g. inhibition of essential metabolic reactions such as glycolysis by inhibition or deactivation of the phosphofructokinase (PFK) reaction (Krebs *et al.* 1983, Pearce *et al.* 2001, Piper

*et al.* 2001). In yeasts, it has also been proposed that the actual inhibitory action could be due to the induction of an energetically expensive stress response that attempts to restore homeostasis and results in the reduction of available energy pools for growth and other essential metabolic functions (Macris 1975, François *et al.* 1986, Warth 1988, Warth 1991, Pelczar *et al.* 1993, Holyoak *et al.* 1996).

### 1.1.3 Economic importance of black currant and lingonberry

Black currant (*Ribes nigrum*) is an important and widely utilized berry crop in northern countries. It is popular for its characteristic strong flavour, which is highly esteemed. Black currant possesses the highest flavonoid content of any soft fruit. The juice also has a high concentration of vitamin C (150 – 300 mg/100 g) and a high content of carotenes, B-vitamins and pectine. Black currant is also rich in minerals such as magnesium (Mg), potassium (K), calcium (Ca) and phosphorous (P) (Green 1971, Koivistoinen *et al.* 1974, Wathen 1977, Matala 1999, Viola *et al.* 2000, Hummer & Barney 2002). The stability of the strong colour is an important quality parameter and a major concern of e.g. the winemaking industry (Eiro *et al.* 2000).

The crop of black currant varies every year due to the annual climate conditions. The average crop in Finland was about 1.6 million kg in 2000, in 1999 only 1.0 million kg and in 1998 2.5 million kg. Only about 30 % of the crop was sold to retailers and the rest was transported directly to industry. The buying price for black currant was 1.07 euro/kg in 2001 and the selling price was 1.33 euro/kg. The whole crofting income has been estimated to be 1.87 million euro. Fresh black currants, 2 thousand kg, were exported in 2001 mainly to Estonia and some also to Russia. Imported black currant equalled altogether 139.1 thousand kg, from which only 5 thousand kg was fresh and the rest was frozen. The imported black currant came almost exclusively from Poland (Matala 1999, Tullihallitus 1999, Malin 2000, Malin 2002).

Lingonberry (*Vaccinium vitis-idaea*) is a commercially important berry crop harvested from the wild throughout its distribution in northern regions of the world. In Finland lingonberry is perhaps the most important wild berry. The most problematic compound concerning winemaking from lingonberry is benzoic acid, which contributes to the acidity of the berry and, as a microbicidal compound, prevents fermentation of lingonberry juice. In fact, lingonberries contain more sugar than the sweeter-tasting bilberries, but the sweetness of lingonberry is masked by acids. In addition, lingonberries are rich in dietary fibres, flavonoids and also tannic substances and contain K, Ca, Mg, P, iron (Fe), manganese (Mn), vitamins A and C. Lingonberries also have antibacterial and dehydrative properties (Hansell & Rautavaara 1971, Koivistoinen *et al.* 1974, Wathen 1977, Souci *et al.* 1994).

The annual crop of lingonberries has been estimated to be as large as 500 million kg, from which only a small part is harvested (Rantala 1995). In 2001 only 4.5 million kg was sold with a price of 1.86 euro/kg. The average price for the berry pickers was 0.76 euro/kg and the whole crofting income was about 3.18 million euro. Fresh lingonberries were exported in 2001 altogether 1.2 million kg mainly to Sweden. Other export countries were in order of importance Germany, Norway, Estonia and Denmark. Fresh lingonberries, 346 thousand kg, were imported in 2001 mainly from Russia and Estonia. The amounts of frozen lingonberries cannot be calculated, because they are included in statistics in a larger group of frozen berries (Tullihallitus 1999, Malin 2002).

## 1.2 BERRY WINEMAKING PROCESS

The berry winemaking process differs from the traditional winemaking process in certain aspects. Northern region berries have high acidity, which causes difficulties in berry wine production. In contrast to grapes, the main acids are citric and malic acid. On the other hand, the sugar concentration is relatively low and northern region berries contain high amounts of pectine. Therefore, dilution

with water and addition of sugar is necessary for fermenting berry juice into wine (Jarczyk & Wzorek 1977, Röhrig 1994, Puputti 1997, Vuorinen *et al.* 2000). This causes significant weakening of the aroma and body of the wine. Figure 1 represents a typical berry winemaking process, which, however, is used with many variations by different berry winemakers.

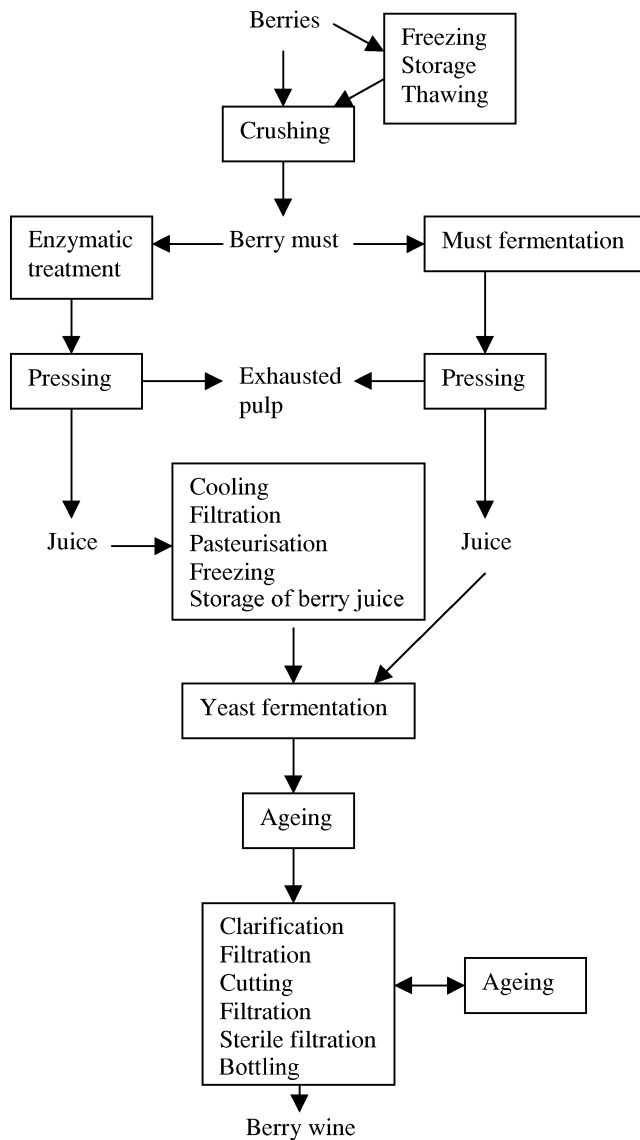


Figure 1. General scheme of the berry winemaking process (Modified from Kaukovirta-Norja & Lehtinen 1997).

In Finland, berries such as black, red, and white currants, gooseberry, strawberry, red raspberry, bilberry, bog whortleberry, and crowberry are used for the production of wines (Vuorinen *et al.* 2000). However, winemaking from berries has not been studied in much detail and the control of the process is not well understood.

### 1.3 METHODS FOR REMOVAL OF ORGANIC ACIDS

#### 1.3.1 *Biological methods*

Malolactic fermentation (MLF) is widely used to reduce the acidity of grape juices in wine production. The malolactic enzyme of lactic acid bacteria deacidifies the juice or wine by converting L-malic acid to L-lactic acid and carbon dioxide. Malolactic fermentation is discussed in more detail in section 1.4.

Some yeast species are able to cause complete degradation of malic acid and the use of these species for wine deacidification has been suggested. *Schizosaccharomyces pombe* has been studied extensively with respect to this property, which is not a true MLF. In this deacidification reaction malic acid is first decarboxylated to pyruvic acid, which is then further metabolised to ethanol. Although *Schizosaccharomyces* spp. can completely degrade L-malic acid in wine, these yeasts may also produce undesirable flavours, including hydrogen sulphide. The major drawbacks in the use of *Schiz. pombe* is its variable degree of degradation of malic acid and a flavour change that subtracts from the varietal character of the wine. Off-flavour problems associated with using wine yeasts for deacidification may be overcome in the future by genetic engineering (Munyon & Nagel 1977, Snow & Gallander 1979, Taillandier & Strehaiano 1991, Rosini & Ciani 1993, Sousa *et al.* 1993, Taillandier *et al.* 1995, Camarasa *et al.* 2001).



*Saccharomyces cerevisiae* cannot degrade malic acid efficiently due to lack of malic acid transporter and the low substrate affinity of its malic enzyme. The incomplete consumption of L-malic acid may also be due to limited malate uptake (Camarasa *et al.* 2001). The genetic engineering of *S. cerevisiae* to achieve MLF of wine has been only partially successful, due to e.g. difficulty in expressing the malolactic gene in the host cell (Volschenk *et al.* 1997, Versari *et al.* 1999). Other yeast species that are able to use L-malic acid as a sole carbon and energy source are e.g. *Hansenula anomala*, *Schizosaccharomyces malidevorans* and *Saccharomyces bailii* (Côrte-Real & Leão 1992, Henschke 1993, Thornton & Rodriguez 1996).

### 1.3.2 Physical methods

Ion-exchange resins were first used for treating wines in the late 1950s. Many countries have limitations and regulations on the use of ion-exchange systems and some may prohibit their use. Anion exchangers are primarily designed for reducing wine acidity. These resins are weakly basic and usually in the hydroxyl form. As the wine passes through the resin, the various anions are replaced with the hydroxyl ions thus reducing acidity. There is conflicting information concerning the merits of treating juices and wines with anion exchange resins. Treated juices and wines have generally less colour and bouquet when compared to the untreated ones. Also the degree of deacidification varies among resins used. Although, ion exchange is reported to have less undesirable effects on wine composition and sensory quality than treatment with potassium carbonate (Beelman & Gallander 1979, Lue & Chiang 1989, Couture & Rouseff 1992, Walker *et al.* 2002).

Electrodialysis is a membrane unit operation used for the separation or concentration of ions in solution. Under the influence of a direct current, ionic species with their positive and negative charges are driven in opposite directions through selective permeable ion exchange membranes. Electrodialysis has been

operated in the conventional and ion substitution modes for deacidification (Girard & Fukumoto 2000). Deacidification of fruit musts, juices and wines by means of electrodialysis is possible. The process is technically and economically feasible (Adhikary *et al.* 1983, Adhikary *et al.* 1987, López-Leiva 1988, Calle *et al.* 2002).

### 1.3.3 Chemical methods

In some winemaking regions where grapes or berries commonly contain excess acidity and insufficient sugar to make balanced wines, a common practice is to add both sugar and water to the must or fermenting wine. This practice is referred to as amelioration. Thus, amelioration does not necessarily imply a process to reduce acidity, but can simply involve the addition of sugar to grapes or berries to increase potential alcohol content of the resultant wine. However, amelioration is usually employed to simultaneously decrease acidity and increase sugar in the must. On the other hand, dilution of aroma, flavour, body and colour intensity results from excessive amelioration (Jarczyk & Wzorek 1977, Beelman & Gallander 1979, Röhrig 1994, Puputti 1997, Vuorinen *et al.* 2000).

A significant reduction in acidity occurs during the intracellular fermentation known as the carbonic maceration process. Intact grapes, berries or other fruits are detained in tanks filled with carbon dioxide before crushing and alcoholic fermentation (AF). The malic acid in the intact berries is degraded by intracellular enzymes, and the main end-product is ethanol. Up to 50 % of the initial malic acid can be degraded by this process; the remainder is usually metabolised by yeasts and bacteria in the AF and MLF. Besides acid reduction, carbonic maceration is accompanied by an increase in glycerol, acetaldehyde and succinic acid (Beelman & Gallander 1979, Gadek *et al.* 1980, Miller & Howell 1989, Sun *et al.* 2001).

The neutralization of must or wine with calcium carbonate ( $\text{CaCO}_3$ ) is a commonly used method to reduce acidity. However, a major problem with

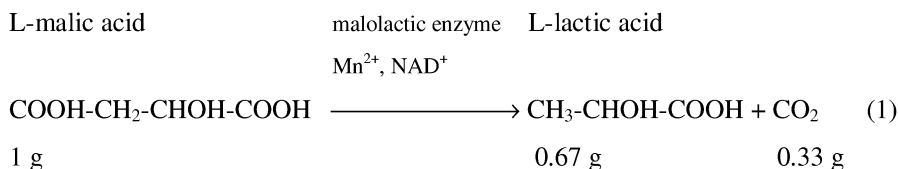
neutralization with calcium carbonate is that most of the deacidification is due to precipitation of calcium tartrate. Most of the calcium malate formed remains in solution and can cause the wine to taste salty if the concentration is too high. Neutralization with carbonates often increases the pH of the wine excessively which can lead to problems with colour and stability (Beelman & Gallander 1979, Gadek *et al.* 1980, Rapp *et al.* 1985, Lue & Chiang 1989). Besides calcium carbonate also potassium hydrogen carbonate ( $\text{KHCO}_3$ ) or potassium carbonate ( $\text{K}_2\text{CO}_3$ ) is used. This treatment is appealing because nothing foreign to wine needs to be added. All that results is an increase in  $\text{K}^+$  concentration and a decrease in both acid and tartrate. Furthermore, the treatment can be accomplished without the pH ever rising above 3.6, avoiding complications of high pH reactions. A major disadvantage of the method is that it may not be effective in all cases (Mattick *et al.* 1980).

Many of the problems associated with chemical neutralization are prevented with a process called double-salt (calcium malate-tartrate) deacidification. The major significance of this method is that malic acid and tartaric acid are removed by a single chemical means in a semiquantitative fashion. Calcium carbonate is added to only a portion of the must until the pH is increased to 4.5, or above, whereby double-salt is formed. The treated must is then settled overnight and filtered before blending it back with the untreated portion (Steele & Kunkee 1978, Beelman & Gallander 1979, Enkelmann 1989).

#### 1.4 MALOLACTIC FERMENTATION

Malolactic fermentation (MLF) is an important part of the traditional winemaking techniques for red and white grape wines and was discovered at the end of the 19<sup>th</sup> century by Müller-Thurgau (Wibowo *et al.* 1985). The term MLF refers to the most noticeable activity of the malolactic enzyme (MLE) of the lactic acid bacteria (LAB), that is their conversion of L-(-)-malic acid (a dicarboxylic acid) to L-(+)-lactic acid (a monocarboxylic acid) and  $\text{CO}_2$  as shown in equation 1. This

decarboxylation reduces the acidity of the wine because lactic acid is a weaker acid than malic acid and can produce a better balanced wine from a highly acidic wine (Kunkee 1974, Radler 1975, Berk 1976, Dittrich 1977, Peynaud 1984, Edwards & Beelman 1989, Stanier *et al.* 1992, Axelsson 1993, Henick-Kling 1993, Prahll & Nielsen 1993, Cogan & Accolas 1996, Versari *et al.* 1999).



LAB capable of MLF consist of the genera *Leuconostoc*, *Oenococcus*, *Lactobacillus* or *Pediococcus*. The principal malolactic-fermenting organism is *Oenococcus oeni* (formerly *Leuconostoc oenos*). Others include e.g. *Lactobacillus plantarum*, *Lb. brevis*, *Lb. hilgardii*, *Lb. casei*, and *Lb. fructivorans* (Sharpe 1981, Lafon-Lafourcade 1983, Sharpe & Pettipher 1983, Peynaud 1984, Wibowo *et al.* 1985, Davis *et al.* 1986, Wibowo *et al.* 1988, Edwards & Beelman 1989, Axelsson 1993, Buckenhüskes 1993, Prahll & Nielsen 1993, Cogan & Accolas 1996).

MLF occurs naturally in many wines after completion of the alcoholic fermentation (AF) and it has considered as being one of the most difficult steps to control in winemaking. The rate of MLF is directly linked to cell density and secondly to the specific malolactic activity of the cell (Henick-Kling 1993, Nielsen & Richelieu 1999). The density of malolactic bacteria in wine must exceed  $10^6$  colony-forming units (CFU) per ml. Also, the composition of the wine directly affects the ability of strains to carry out acid degradation. The malolactic activity of bacteria and their growth depend on various factors; in particular pH, ethanol and SO<sub>2</sub> content and temperature (Wibowo *et al.* 1985, Davis *et al.* 1986, Wibowo *et al.* 1988, Edwards & Beelman 1989, Asmundson & Kelly 1990, Colagrande *et al.* 1994, Vaillant *et al.* 1995). Other factors also interfere, such as amino acids (Fourcassie *et al.* 1992, Saguir & de Nadra 2002), sugars (Pilone &

Kunkee 1972) and organic acids (Naouri *et al.* 1990) or even phages (Lonvaud-Funel 1995, Gindreau *et al.* 1997). Retarded bacterial growth may also be due to the lack of certain nutrients.

#### 1.4.1 *Beneficial effects of malolactic fermentation*

MLF is not only a process of acid reduction. The metabolic potential of wine LAB is diverse and complex. The main advantages of MLF are following (Davis *et al.* 1985, Buckenhüskes 1993, Liu 2002):

1. reduction of acidity;
2. contribution to the biological stability;
3. flavour modification;
4. contribution to complexity (structure, body).

One of the main reasons, in addition to deacidification, which are in favour of MLF, is the added microbiological stability, which may be achieved by using MLF. Wines, which have not undergone MLF, are not as stable. MLF offers microbiological stability by ensuring that the degradation of malic acid does not occur in the bottle, where the growth of LAB and the formation of CO<sub>2</sub> are considered spoilage (Kunkee 1974, Kunkee & Goswell 1977, Beelman & Gallander 1979, Peynaud 1984, Davis *et al.* 1985, Henick-Kling *et al.* 1989, Henick-Kling 1993). Also wine spoilage by other bacteria is less frequent when a LAB flora has already developed. This may be explained not only by the deprivation of nutrients, but also probably by the synthesis of antimicrobial compounds (Henschke 1993, Lonvaud-Funel 1995). However, it needs to be emphasized that microbiological stability after MLF is not absolute; wine still contains nutrients that can support significant bacterial growth. Bacterial growth in wine does not stop after the completion of MLF, instead, growth of various LAB and various spoilage yeast can continue until the winemaker interferes and clarifies the wine and adds sulphur dioxide (Davis *et al.* 1986).

The LAB are well known for their ability to produce flavour compounds, such as acetaldehyde, acetic acid (acetate), ethanol, diacetyl, acetoin, and 2,3-butanediol, in a variety of fermented products. Particularly, diacetyl, acetoin, and 2,3-butanediol are compounds that are of considerable importance to the flavour profile of wine, and their production is intimately associated with the growth of yeasts and LAB. Concentration of 1 – 4 mg/l of diacetyl adds complexity to wine, but higher concentrations are undesirable (Kunkee 1974, Berk 1976, Sharpe 1981, Davis *et al.* 1985, Wibowo *et al.* 1985, Henschke 1993, Laurent *et al.* 1994, Patarata *et al.* 1994, Henick-Kling *et al.* 1995, Martineau & Henick-Kling 1995, Martineau *et al.* 1995, Versari *et al.* 1999). The flavour consequences of MLF appear to depend on the strain of LAB and wine type and style wanted (Henschke 1993). Wine taste and colour are also modified due to the metabolic activity of bacteria on phenolic compounds (tannins, anthocyanins), which are basic components of wines (Lonvaud-Funel 1995). In considering wine as a substrate for the growth of LAB, it is necessary to understand how the sensory quality of wine is affected by the compounds that are either removed from or added to the wine by bacterial growth. Moreover, the ability to distinguish between sensory changes caused by such growth and those arising from mere acidity reduction becomes a challenging task (Davis *et al.* 1985).

A persistent characteristic of MLF is that it enhances the body and gives a longer aftertaste. The qualities describing the texture of the wine are very difficult to quantify because their chemical basis is not known. The improved mouthfeel (body) of the wines which have undergone MLF is likely due to the bacterial production of other flavour enhancing substrates, which as yeast have not been determined. The fact that the aromas of a young wine become more complex (excessive vegetative and simple fruit characters are modified; yeasty, buttery, nutty, earthy aromas are added) also contributes to a fuller, rounder taste impression (Henick-Kling *et al.* 1995, de Revel *et al.* 1999). Also, the degradation of acetaldehyde by *O. oeni* is significant, since SO<sub>2</sub>, which is strongly bound to

this component, is freed to become active against further bacterial growth (Davis *et al.* 1986).

#### *1.4.2 Undesirable effects of malolactic fermentation*

MLF is not always beneficial and can lead to the excessive reduction in acidity of high pH wines leading to risk of spoilage. It also can be responsible for undesirable changes to wine sensory properties. For example, species of *Lactobacillus* and *Pediococcus* may conduct MLF in wines of higher pH, but it is a general view that such species lead to less acceptable wines than those in which *O. oeni* has grown. Besides, MLF may alter wine colour, and may even lead to generation of amines, e.g. histamine (Kunkee 1974, Davis *et al.* 1985, Kunkee 1991, Henick-Kling 1993, Henick-Kling 1995). In addition, LAB can spoil the wine when the multiplication of the bacterium takes place at the end of AF, not after, as it should do. In such a case the bacteria ferment carbohydrates, particularly hexoses, which have not been completely exhausted by yeast fermentation (Lonvaud-Funel 1999).

#### *1.4.3 Mechanism of malolactic fermentation*

Although the decarboxylation of L-malic acid to L-lactic acid by MLE is a non-energy-yielding reaction and it does not liberate biologically available energy (e.g., ATP, NADH), the growth rate of many malolactic bacteria is greatly enhanced after MLF. The deacidification itself, by increase in intracellular and extracellular pH, is recognized to be advantageous to the cell, but this cannot fully account for the stimulatory effect. The exact benefit of the malolactic fermentation reaction to the bacterium has been a matter of discussion among researchers for years (Pilone & Kunkee 1972, Radler 1975, Pilone & Kunkee 1976, Dittrich 1977, Renault *et al.* 1988, Kunkee 1991, Stanier *et al.* 1992).

Since substrate-level phosphorylation or direct ion extrusion by a membrane bound decarboxylase does not occur during MLF, the generation of metabolic energy must originate from the uptake of L-malic acid and/or excretion of L-lactic acid and/or carbon dioxide across the membrane. Additionally, the cell could take advantage of the fact that a proton is consumed during the intracellular decarboxylation of L-malic acid. Assuming that carbon dioxide diffuses out of the cell without affecting the pH gradient, three distinct mechanisms of metabolic energy generation during MLF can be operative: 1) electrogenic malic acid/lactic acid antiport, 2) electrogenic malic acid uptake, and 3) electrogenic lactic acid efflux. For each of the proposed mechanisms, the overall transport process is electrogenic; i.e., a membrane potential is generated either by the antiport reaction, malic acid uptake, or lactic acid efflux, and a pH gradient is generated as a result of proton consumption in the cytoplasm (Cox & Henick-Kling 1989, Poolman *et al.* 1991).

In the electrogenic transport mechanism the electrochemical energy can be conserved via an indirect electrical potential or gradient ( $\Delta\Psi$ ). As a proton is consumed in the decarboxylation reaction the internal pH increases. Alkalinisation of the cytoplasm results in creation of chemical potential of protons across the membrane ( $\Delta\text{pH}$ ) that, together with the  $\Delta\Psi$ , forms the electrochemical gradient ( $\Delta\mu$ ) or the proton motive force (PMF) across the cytoplasmic membrane (chemiosmotic mechanism). The relative contribution of  $\Delta\Psi$  and  $\Delta\text{pH}$  depends on the mechanism of transport systems: uniporters (transit of one solute across the cytoplasmic membrane), symporters (combined translocation of two or more solutes in the same direction) and antiporters (associative transport of a solute in one direction to the translocation of another solute in the opposite direction). The electrochemical proton gradient can then be used to drive tasks such as ATP (adenosine triphosphate) synthesis by the membrane bound ATPase (or ATP synthase or ATP phosphohydrolase or  $F_0F_1$ -ATPase or  $H^+$ -ATPase), which is active at low pH (Renault *et al.* 1988, Stryer 1988, Cox & Henick-Kling 1989, Cox & Henick-Kling 1990, Henick-Kling *et al.* 1991).



Since the decarboxylation of L-malic acid by the LAB is analogous to the decarboxylation of oxalate by *Oxalobacter formigenes*, it has been suggested that the metabolic energy may be gained from electrogenic malic acid/lactic acid antiport analogous to the energy generation by oxalate/formate antiport. However, because L-lactic acid efflux is unable to drive L-malic acid transport in the absence of a  $\Delta\text{pH}$ , it does not appear that the carrier is a malic acid-lactic acid exchanger (Olsen *et al.* 1991, Poolman *et al.* 1991).

Many researchers claim that monoprotonated L-malic acid (L-malate<sup>-</sup>) is taken up by an electrogenic uniport in which a net negative charge is moved inwards, thereby generating an electrical potential,  $\Delta\Psi$  (inside negative relative to outside) (London 1990, Henick-Kling *et al.* 1991, Kunkee 1991, Olsen *et al.* 1991, Poolman *et al.* 1991, Loubiere *et al.* 1992, Axelsson 1993, Henick-Kling 1993, Salema *et al.* 1994, Cox & Henick-Kling 1995, Salema *et al.* 1996, Konings *et al.* 1997, Versari *et al.* 1999). Once inside the cell, L-malic acid is decarboxylated to L-lactic acid plus carbon dioxide in a reaction that requires one proton. This creates a pH gradient that, together with the  $\Delta\Psi$ , forms the PMF. The symport of lactate<sup>-1</sup> and 1 or 2 protons is responsible for generating a proton potential during malic acid catabolism. Thus, ATP, the principal carrier of biological energy can be generated by electron transport phosphorylation. A model of the ATP-generating chemiosmotic mechanism for the malolactic conversion is shown in figure 2.

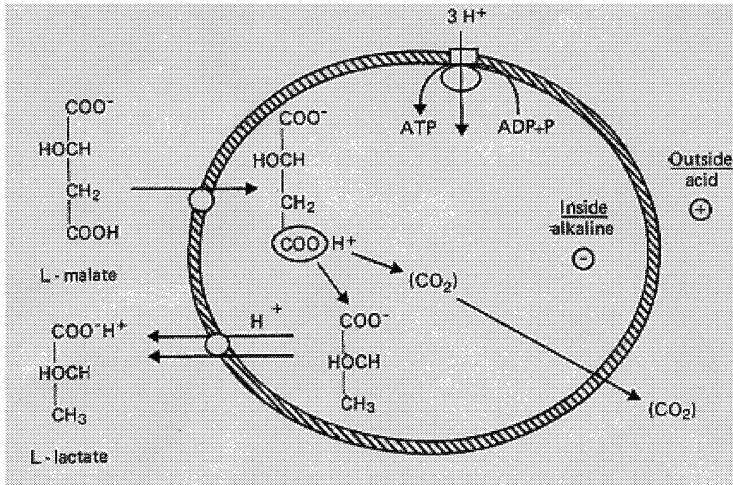


Figure 2. Energy conversion during malolactic fermentation (Henick-Kling *et al.* 1991, courtesy of Dr. Henick-Kling).

The net energy gain would be equivalent to one proton translocated from the inside to the outside per L-malic acid metabolised (Cox & Henick-Kling 1989, Poolman *et al.* 1991). This mechanism potentially can produce one mole of ATP for every one mole of malic acid converted to lactic acid. The actual amount of ATP produced from the reaction is somewhat lower and depends on the external pH, the external lactic acid concentration, and the energy status of the cell. Maximum amounts of ATP from the reaction are produced at pH values at and below 4.5. At pH values above 5.5 MLF does not contribute significant amounts of ATP to the growth of the bacteria (Henick-Kling *et al.* 1991).

#### 1.4.4 Malolactic enzyme

The decarboxylation of L-malic acid to L-lactic acid is catalysed by a single enzyme, called malolactic enzyme (MLE), which is  $\text{NAD}^+$  and  $\text{Mn}^{2+}$  dependent. The proper name of the enzyme is L-malate: $\text{NAD}^+$  carboxylase (EC 1.1.1.38) (Lonvaud & Ribereau-Gayon 1975, Radler 1975, Naouri *et al.* 1990, Kunkee 1991, Gao & Fleet 1994, Cogan & Accolas 1996, Salema *et al.* 1996, Miranda *et*

*al.* 1997). The pathway of MLF includes the uptake of L-malic acid, the conversion of L-malic acid to L-lactic acid and carbon dioxide, and the excretion of the end products, including a proton. NADH<sub>2</sub> and pyruvate are not produced in the reaction (Salema *et al.* 1996).

The MLE has been purified from various LAB species including *Lactobacillus* sp. and *Leuconostoc* sp. as well as *Lactococcus lactis*. It is a single protein with a molecular weight of about 130 000 – 150 000. The enzymes studied so far are homodimers or tetramers of 60 to 70 kDa subunits and have a K<sub>m</sub> for malic acid ranging from 3 to 17 mM. The intracellular enzyme has an optimal pH of 4.9 – 6.0. Optimum temperature of the purified material is 35 – 48 °C (Radler 1975, Dittrich 1977, Naouri *et al.* 1990, Gao & Fleet 1994, Cogan & Accolas 1996, Lonvaud-Funel 1995, Miranda *et al.* 1997, Arthurs & Lloyd 1999). The enzyme is inactive at wine pH and the required cofactor, NAD, is particularly unstable in wine. It might exist in an inactive or repressed form and become active only after some inhibitory repressor substance is removed from the wine by earlier growth of LAB (Wibowo *et al.* 1985).

#### 1.4.5 *Oenococcus oeni*

*Leuconostoc oenos* is the main species of LAB present in wine and one of the best-adapted organisms to perform MLF at the low pH of wine. It was originally isolated from wine must (Dundee, OR) and it carried out MLF in wines at relatively low temperatures and in wines having relatively high acidity. The species is phenotypically very different from other leuconostocs and it has been later reclassified as *Oenococcus oeni*. It is distinguished from other *Leuconostoc* spp. by its growth in acidic media, by its requirement for a growth factor in tomato juice and by a number of carbohydrate fermentation characteristics (Sandine & Heatherbell 1985, Dicks *et al.* 1995, Guzzo *et al.* 2002).

*O. oeni* is a heterofermentative, non-motile, Gram-positive coccus. The cells are ellipsoidal to spherical, but lenticular when grown on agar and usually occur in pairs or short chains. Cell morphology varies from strain to strain and is influenced by the growth medium. Growth on surface plates is poor and the smooth, round, greyish white colonies are seldom larger than 1 mm in diameter. Growth in broth cultures is uniform, except when cells in long chains sediment. The growth of this organism is slow and takes five to seven days at 22 °C. The optimum growth temperature is 20 °C to 30 °C. *O. oeni* is a facultative anaerobe and grows best in low pH media (Garvie 1986, Asmundson & Kelly 1990, Degre 1993, van Vuuren & Dicks 1993, Dicks *et al.* 1995).

The pH is one of the main limiting factors of bacterial growth. The pH threshold of growth is a property of the strain and of the composition of the culture medium. The pH threshold of L-malic acid attack differs from the pH threshold of attack on sugars and it is generally lower. The growth of *O. oeni* is extremely slow at pH 3.0 even in a suitable medium. The optimal pH for growth of *O. oeni* is in the region of pH 4.2 – 4.8. The malic activity of resting cells is almost identical from pH 3.0 to pH 4.0 and then decreases as the pH rises; at pH 4.5 it is slightly inhibited. The pH factor therefore influences bacterial growth and MLF at different levels; malic acid metabolism is brought about easily at relative low pH values at which all growth is inhibited (Lafon-Lafourcade 1975, van Vuuren & Dicks 1993, Dicks *et al.* 1995).

*O. oeni* has high malolactic activity, high tolerance to low pH and high concentrations of ethanol. It also has the ability to proliferate in the pH of wine, capacity to produce small quantities of acetic acid, and capacity to not alter organoleptic characteristics (Sharpe 1981, Sandine & Heatherbell 1985, Wibowo *et al.* 1985, Henick-Kling *et al.* 1989, Asmundson & Kelly 1990, Axelsson 1993, Buckenhüskes 1993, van Vuuren & Dicks 1993, Henick-Kling 1995). Under non-growing conditions *O. oeni* first degrades malic acid instead of utilizing glucose. In addition to malic acid, some other organic acids (Radler 1975, Nielsen & Richelieu 1999) and sugars (Firme *et al.* 1994, Pimentel *et al.* 1994) may be

utilized by this bacterium. This can lead to significant changes in the concentration of wine constituents and to the appearance of new metabolites that affect the sensory quality of wines (Davis *et al.* 1985, Radler 1986, Henick-Kling 1995).

The metabolism of sugars by *Oenococcus oeni* is reported to follow the heterolactic pathway (6-PG/PK pathway) (Garvie 1986, Axelsson 1993, Henick-Kling 1993). Thus, the bacterium converts glucose into carbon dioxide, lactic acid, acetic acid and ethanol. The acetic acid/ethanol ratio depends on the redox potential of the system (Kandler 1983, Gottschalk 1988, Coccagn-Bousquet *et al.* 1996). Diacetyl (2,3-butanedione) can also be formed by lactic acid bacteria in the presence of high concentrations of sugar, which has a negative effect on affects wine quality (Kunkee 1991). Sugar metabolism also has a positive influence on wine quality via the formation of aroma components such as acetaldehyde, acetic acid, diacetyl, acetoin, 2,3-butanediol, ethyl lactate, and higher alcohols (Lafon-Lafoucade 1983, Postel & Meier 1983, Martineau *et al.* 1995). The heterofermentative lactic acid bacteria can metabolise citric acid mainly to acetic acid, lactic acid and carbon dioxide (Dittrich 1977, Subramanian & SivaRaman 1984, Martineau & Henick-Kling 1995). The co-fermentation of citric acid and glucose in *O. oeni* is physiologically important for this bacterium. This co-metabolism has been shown to enhance the growth rate and biomass yield of this bacterium, which result from increased ATP synthesis both by substrate-level phosphorylation via acetate kinase and by osmotic mechanism (proton motive force) (Salou *et al.* 1994, Ramos & Santos 1996, Liu 2002).

#### 1.4.6 *New technologies to carry out malolactic fermentation*

Traditionally, the MLF has been allowed to develop spontaneously towards the end of or after AF by the growth of LAB naturally present in wine (Wibowo *et al.* 1985, Krieger 1991, Buckenhüskes 1993, Prahil & Nielsen 1993). However, this natural process is slow and unreliable and it can take many weeks or months for

the fermentation to be completed. Also the flavour characteristics are unpredictable due to the different microorganisms, which may be present in the must or in the cellar. Anyway, traditional fermentations are still used worldwide, although delay or failure is not an unusual outcome.

The use of starter cultures in winemaking ensures achievement of MLF in a more rapid and predictable manner, and also provides uniformity to the final product (Wibowo *et al.* 1985, Herrero *et al.* 1999, Maicas 2001). Since the beginning of the 1980s, commercial starter cultures for MLF have been available. With a few exceptions, all of the successful commercial cultures are strains of *Oenococcus oeni*. Others are *Pediococcus damnosus* and *Lactobacillus plantarum* (Kunkee 1991). The advantages of starting MLF by inoculation include better control over timing and speed of MLF and the effect of MLF on wine aroma and flavour. It also includes the better control over the strain of LAB that carries out the process (Davis *et al.* 1985, Kunkee 1991, Henick-Kling 1993, Prahil & Nielsen 1993, Henick-Kling *et al.* 1995, Maicas *et al.* 2000). Problems with carrying out MLF by using starter cultures include e.g the expensiveness due to difficulty in propagation of large amounts of malolactic bacteria (Davis *et al.* 1985, Edwards & Beelman 1989, Henick-Kling 1993).

During recent years several other technologies have been proposed for carrying out biological deacidification of wines by using malolactic bacteria. Besides the use of high densities of cells, these alternative technologies usually involve the use of high densities of enzymes, free or immobilized onto different matrices (Maicas 2001). Because resting (or non-proliferating) cells of, for example *O. oeni*, at high concentration ( $10^6$  to  $10^7$  CFU/ml) can bring about rapid degradation of malic acid, the use of immobilized cells is possible (Davis *et al.* 1985, McCord & Ryu 1985, Henschke 1993). The LAB can be entrapped for example within cubes of polyacrylamide, carrageenan or alginate beads, which can be supported in columns or in a stirred reactor (McCord & Ryu 1985, Crapisi *et al.* 1987, Naouri *et al.* 1991, Gao & Fleet 1994). Wine or juice is continuously passed through the reactors where the malolactic conversion occurs. Residence

time of the wine in the columns depends on several factors, but, essentially, the reaction is completed in a matter of hours compared with the days or weeks required by the conventional fermentation process (Diviès *et al.* 1994).

Immobilized cells can offer several advantages for the deacidification of wine, e.g., continuous operation, greater tolerance to wines of high alcohol and SO<sub>2</sub> concentration and low pH, better control over the timing and extent of deacidification, and absence of effects of bacterial growth, such as flavour modification, in the wine. However, even though immobilized cells may offer practical advantages in the deacidification of wines, ultimate acceptance of the technology will depend on its impact on the sensory quality of the final product, which have not been adequately studied (Crapisi *et al.* 1987). Disadvantages of the technique include also the possibility of microbial contamination of the reactors, transfer of taints from the reactor to the wine, loss of activity on prolonged operation, and leakage of cells or immobilization substrate into the wine (Maicas 2001).

Attempts to deacidify wine and malic acid solutions using the MLE immobilized on gels have not been successful, probably because the enzyme loses activity at wine pH and the required cofactor, NAD, is particularly unstable in wine. Complete and rapid consumption of the L-malic acid has not yet efficiently been achieved (Davis *et al.* 1985, Edwards & Beelman 1989, Diviès *et al.* 1994, Maicas 2001).

## **2 AIMS OF THE PRESENT STUDY**

The aim of the present study was to create new processing techniques for northern region berry juices in order to reduce their natural acidity and thereby enhance their usability for wider food and beverage applications. This aim was divided into specific subsections as follows.

- I Development of malolactic fermentation applicable to berry juices and berry wines.
- II Development of a process for specific removal of benzoic acid naturally present in juices of e.g. lingonberry.
- III Gathering of comparative data on organic acid and soluble sugar contents in northern region berries to assist in evaluating the needs for the developed deacidification processes.



### 3 MATERIALS AND METHODS

A summary of the main materials and methods used is presented below. Detailed information is included in the original publications I – V.

#### 3.1 PREPARATION OF BERRY JUICES (I, III, IV, V)

Six wild and five cultivated berries were used. The wild berries were bilberry (*Vaccinium myrtillus* L.), lingonberry (*Vaccinium vitis-idaea* L.), cranberry (*Vaccinium oxycoccos* L.), cloudberry (*Rubus chamaemorus* L.), red raspberry (*Rubus idaeus* L.) and black crowberry (*Empetrum nigrum* ssp. *hermaphroditum* (Hagerup) Böcher). The cultivated berries were black currant (*Ribes nigrum* L.) (15 different cultivars), white currant (*Ribes x pallidum* Otto & F. Dietr.), red currant (*Ribes rubrum* L.), gooseberry (red) (*Ribes uva-crispa* L.) and strawberry (*Fragaria x ananassa* Duch.).

The frozen berries were allowed to thaw 2 – 3 days at +4 °C before use. The thawed berries for laboratory scale studies (I, III) were minced manually and treated with pectinase (Panzym Super E, Novo Nordisk Ferment, Ltd., Switzerland) for 2 h in 37 °C, if necessary. The mash was extracted to juice in a hydraulic press (Hafico, Germany) with a maximum pressure of 300 kp/cm<sup>2</sup>. For the pilot scale studies (IV, V) the thawed berries were first minced (ENOL OM 10 – 0976, G. Wein GmbH + Co., Germany) and treated with 4 ml/10 kg pectinase (Panzym Super E, Novo Nordisk Ferment, Ltd., Switzerland) for 3 hours at +30 °C. The mash was extracted to juice in a hydraulic press (ENOL OP 20 – 442, G. Wein GmbH + Co., Germany).

## 3.2 MICROORGANISMS

### 3.2.1 *Oenococcus oeni* (II, III, IV)

The malolactic bacterium used was a commercial strain *Oenococcus oeni* (formerly *Leuconostoc oenos*) (ATCC 39401) (Oregon State University, Er1a) obtained from the American Type Culture Collection (Maryland, USA). The strain was stored in 10 % (v/v) glycerol at -60 °C until used.

### 3.2.2 *Saccharomyces cerevisiae* (IV, V)

The yeast used for the alcoholic fermentation was dried *Saccharomyces cerevisiae* obtained from Lallemand Inc. (Lalvin V1116, Danmark) (IV) or freeze-dried yeast *Saccharomyces cerevisiae* (strain DF 639, Siha 6, Begerow, Germany) (V). The yeast used to remove benzoic acid was fresh baker's yeast *Saccharomyces cerevisiae* (Suomen Hiiva Oy, Finland) (V).

## 3.3 EXPERIMENTAL CONDITIONS

### 3.3.1 *Malolactic fermentation in modified MRS media* (II)

The inoculum for the studies was prepared by first growing the thawed bacteria in de Man-Rogosa-Sharpe (MRS) medium. The pH was preadjusted to 5.0 with 2 M HCl. The strain was grown at 30 °C without shaking for 4 to 6 days or to the stationary phase of growth.

The experiments were carried out in a modified MRS medium and, depending on the experiment, various concentrations of citric acid, L(-)malic acid and glucose were added. The pH-adjustments were made with 1 M NaOH prior to sterilisation.

Klett flasks with a volume of 250 ml were used. After inoculation the flasks were closed with aluminium foils and incubated without shaking to obtain microaerophilic conditions. The experiments were carried out at 25 °C. Two or three parallel inoculated samples and two uninoculated samples (as control) were used for each combination.

### 3.3.2 *Malolactic fermentation in berry juices (III, IV)*

The thawed bacterium was first cultivated in a MRS medium supplemented with 2 g/l citric and 2 g/l malic acid. The tubes were incubated at 30 °C without stirring for 3 – 4 days, so that the final cell density was  $10^8 - 10^9$  CFU/ml.

To avoid reduction of the viable cells during the fermentations the bacteria were adapted to the different juices by cultivating in a second medium (adaptation medium) containing distilled water and the respective juice in a 1:1 ratio, supplemented with 5 g/l yeast extract. The pH was adjusted with NaOH to 3.6. From the supplemented MRS medium a 10 % aliquot was transferred to the adaptation medium and incubated at 27 °C for 2 – 4 days, so that the final cell density was  $10^7 - 10^9$  CFU/ml.

In the laboratory scale experiments (150 ml) (III), the autoclaved or non-autoclaved 1:1 diluted berry juice media were inoculated by 10 % of the fully developed adaptation medium. The inoculated media were plugged with aluminium foil and incubated without stirring in an incubator at 25 °C. Each bottle was inoculated in duplicate and an uninoculated bottle was used as a comparison.

The pilot scale experiments (4 – 10 l) (IV) were also carried out in diluted (1:1) berry juice. The juice was filtered with a disc filter (ENOL F20Z, G. Wein GmbH + Co., Germany) equipped first with Seitz-800 filter (cut size 7.0 µm) and sterilized with Seitz EK sterile filter (cut size 0.45 µm). Fermentations were carried out in pre-sterilized steel fermentation tanks (Tankki Oy, Finland) equipped with a water seal and stirring (77 rpm/min). The MLF were carried out at 25 °C for 48 hours. Two parallel-inoculated fermentation tanks were used.

### 3.3.3 Removal of benzoic acid (V)

For benzoic acid uptake, the yeast was prepared by inoculating 10 % (w/w) of it to a medium containing 10 % (w/w) glucose (Merck, Germany) and 0.1 % (w/w) Vitamon Combi -yeast nutrient (Erbslöh, Germany) in distilled water. The yeast was incubated with shaking (200 rpm, Certomat R, B.Braun, Germany) at 30 °C for 3 hours, and then centrifuged (5860 x g, 10 min). The cell mass was washed with sterile 0.9 % (w/v) NaCl.

*S. cerevisiae* cell paste prepared was suspended into the undiluted lingonberry juice either as a single batch (15 – 20 %, w/w) or as several 1 – 3 % (w/w) consecutive batches and the mixture was vigorously stirred for 10 min at room temperature. Finally, the juice suspension was centrifuged (5860 x g, 10 min). All the experiments were performed at least in duplicates.

The method developed for removal of benzoic acid from solutions has been explained in more detail in section 4.3.

### 3.3.4 Yeast fermentation (IV, V)

For alcoholic fermentations 10 % of dry *S. cerevisiae* -yeast was rehydrated 10 – 25 min in physiological saline before inoculation. The concentration of living

cells in the inoculum was  $3 \times 10^6$  CFU/ml. The alcohol fermentations were carried out at 20 °C for 7 – 14 days. The fermentation experiments were performed at least in duplicates.

## 3.4 ANALYTICAL PROCEDURES

### 3.4.1 HPLC-analyses

#### 3.4.1.1 Acid, sugar and ethanol concentrations (I, II, III, IV, V)

Organic acids (citric, malic, acetic and lactic acids), sugars (sucrose, fructose and glucose), and ethanol were analysed by using a Hewlett Packard (HP Series 1100) high performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87 H<sup>+</sup> column (300 x 7.8 mm, 9 µm) (Bio-Rad Laboratories, USA). Column temperature was 35 °C and elution was carried out with 5 mM H<sub>2</sub>SO<sub>4</sub>. The flow rate was 0.6 ml/min. Acids were detected with an UV-detector (Hewlett Packard, USA) at 214 nm and the sugars with a RI-detector (Hewlett Packard, USA). Prior to sugar and ethanol analyses the samples were exposed to a strong anion exchange column (Varian, USA) to remove the acidic compounds (Varian 2000). Before analysing, the samples were centrifuged if necessary and always filtered through a membrane filter (pore size 0.2 µm).

The acid and sugar determinations of the experiments in modified MRS (II) were determined with a Waters (Waters Corporation, USA) chromatograph equipped with an Aminex HPX-87 H<sup>+</sup> column (300 x 7.8 mm) (Bio-Rad Laboratories, USA), Waters 410 refractometer, and 486 UV-detector in series. Soluble sugars and ethanol of black currant juices and wines (IV) were determined by a HPLC (Series 200, Perkin Elmer, USA), equipped with a RI-detector (1047A, Hewlett Packard, USA). An Aminex HPX-87C column (300 x 7.8 mm, Bio-Rad

Laboratories, USA) was used. The ethanol concentration was alternatively analysed by using a HP 6890 gas chromatograph (Hewlett Packard, USA) equipped with a HP-INNOWax column (30 m, 0.25 mm, 0.25  $\mu$ m) (Hewlett Packard, USA) (V).

#### 3.4.1.2 Benzoic acid concentration (I, III, V)

For benzoic acid analyses a Hypercil BDS C8 reverse phase column (250 x 4.6 mm, 5  $\mu$ m) (Hypercil, UK) was used. The column temperature was 30 °C, the eluent was 0.05 M phosphate buffer and methanol (1:1) and the flow rate was 1.0 ml/min. Acids were detected with an UV-detector (Hewlett Packard, USA) at 214 nm.

#### 3.4.2 pH measurements (I, II, III, IV, V)

pH determinations and adjustments were made in room temperature with the use of PHM 80 portable pH meter (Radiometer, Denmark) (I, II, III, IV) or RL 150 pH/mV device (Russel, USA) (V). Before determinations the pH meter was calibrated with standard buffer solutions of pH 4.00 and 7.00 (Reagecon, Ireland).

#### 3.4.3 Viable cell counts (II, III, IV, V)

Viable counts of *O. oeni* (II, III, IV) were expressed in colony-forming units (CFU) per ml of solution. For colony counting, MRS medium was used at pH 5.0 supplemented with agar (15 g/l). The plates were left for 6 to 9 days in an incubator at 30 °C.

The yeast count (IV, V) was performed with YM-agar supplemented with 0.1 g/l streptomycin and 0.06 g/l ampicillin. The plates were left for 2 days in an incubator at 30 °C.

#### 3.4.4 Sensory analyses

Sensory evaluation is essential when introducing new food products onto the market. Descriptive sensory analysis (DSA) is one of the most comprehensive and informative tools used in sensory analysis. These techniques can provide complete sensory descriptions of products, determine how ingredient or process changes affect product characteristics, and identify key sensory attributes that promote product acceptance (Meilgard *et al.* 1991, Lawless & Heyman 1999). The optimisation of MLF necessitates that the quality of the end product can be characterized, not only in chemical, but also in sensory terms. Thus, descriptive analysis was proposed in order to obtain an objective characterization of malolactic fermented black currant juices by means of selected sensory descriptors.

The tests were done in normal white light in individual booths in a sensory laboratory. In all experiments the samples were presented to the panellists coded and in random order, and water was provided for cleansing the palate between the juice samples. The samples were analysed on two sequential weeks in three sensory replicates. The panel of 10 members consisted of trained assessors, the ages ranging from 25 to 55 years. All assessors had previous experience in the sensory evaluation of black currants. The assessors were familiarized with the sensory descriptors and the attribute intensities prior to the evaluations. Hence, 3 odour and 5 taste descriptors were generated. The sensory attributes evaluated were odours such as fermented, black currant and fusty, tastes such as fermented/winy, black currant, sourness/acidity, bitterness together with a beery aftertaste. In addition, the lightness of the colour was also evaluated. Attribute intensities were rated on continuous, unstructured, graphical intensity scales. The scales were verbally anchored at the end, and the left side of the scale

corresponded to the lowest or opposite intensity (value 0) and the right side to the highest intensity (value 10).

In accordance to the sensory evaluations, the transmitted color of the juices was measured by a Minolta Chroma Meter CT-210 (Minolta, Japan) at room temperature. A cell with an optical path length of 10 mm was used.

#### *3.4.5 Data analyses*

The mean values and standard deviations of the results were calculated with the use of Microsoft Excel. Statistical analyses of sensory evaluations were carried out by using SPSS (Chicago, USA) with significance at  $P \leq 0.05$ . Results were submitted to univariate analysis of variance (ANOVA). The model included four fixed main factors (assessors, cultivars, treatment and replications) and one interaction term (cultivar x treatment). Turkey's test was used for determining the differences among the means at  $P < 0.05$ .



## 4 RESULTS AND DISCUSSION

### 4.1 NONFERMENTED BERRY JUICES (I, III, IV, V)

The main organic acids of the berry juices were invariably citric and malic acid. In addition, lingonberry, cranberry, cloudberry and black crowberry contained benzoic acid. The main soluble sugars of the investigated berry juices were fructose and glucose. Most of the berries contained also sucrose.

As a whole, the available literature data on the pH, sugar and acid concentrations of the northern region berry juices has been rare and scattered (Kuusi 1969, Salo & Suomi 1972, Solberg 1980, Kallio & Markela 1982, Skrede 1982, Skrede 1983, Varo *et al.* 1984, Haila 1990, Fuchs & Wretling 1991, Haila *et al.* 1990, Livsmedelstabelle 1993, Huopalahti *et al.* 2000, Kallio *et al.* 2000). Tables 1 – 4 in I summarize available literature data with comparison to the present results. The wide variations in previous literature on sugar and acid concentrations of the same berry can partly be due to different methods of analysis but also to the geographical origin of the investigated berry. In addition, the berry cultivar and the climate conditions of the crop year are seldom mentioned. Variables such as post harvest storage and degree of ripeness are difficult to eliminate in a comparative study including several berries. In the present study variables such as geographical origin, storage conditions, juice preparation, sample treatment and analytical procedures were excluded and thus valuable and comparable data was obtained to be used for a wide field of applications.

#### 4.1.1 *Organic acid composition and pH of juice*

All the berry juices studied contained large amounts of organic acids; 2.3 – 29.0 g/l citric acid and 2.9 – 16.2 g/l malic acid, respectively. In addition, benzoic acid (0.1 – 0.9 g/l) was found in some berries. Thus, the acid composition is

notably different from that of grape juices, which contain on average 2 – 10 g/l tartaric acid, 1 – 8 g/l malic acid, 0.1 – 0.7 g/l citric acid and no benzoic acid. The composition varies widely depending on e.g. the grape variety, maturity and quality of soil (Lafon-Lafourcade 1983, Peynaud 1984, Henick-Kling 1993). Table 1 summarizes the data on pH-values and organic acid concentrations of the berry juices investigated.

Table 1. Mean acid concentrations and pH of the berry juices.

<b>Berry</b>	<b>Citric acid/ g/l</b>	<b>Malic acid/ g/l</b>	<b>Benzoic acid/ g/l</b>	<b>pH</b>
<b>Wild berries</b>				
Bilberry	8.4±0.2	4.2±0.1	0	3.0±0.05
Lingonberry	18.2±0.7	4.2±0.5	0.87±0.05	2.7±0.05
Cranberry	14.8±0.5	16.2±0.4	0.18±0.02	2.4±0.05
Cloudberry	3.7±0.1	7.7±0.1	0.48±0.05	3.2±0.05
Red raspberry	15.2±0.3	2.9±0.1	0	3.3±0.05
Black crowberry	2.3±0.3	4.3±0.2	0.06±0.01	3.5±0.05
<b>Cultivated berries</b>				
Black currant	29.0±0.3	4.1±0.1	0	3.0±0.05
White currant	24.0±0.5	4.1±0.1	0	3.0±0.05
Red currant	24.7±0.8	4.9±0.3	0	2.9±0.05
Gooseberry (red)	11.1±0.1	10.8±0.2	0	3.0±0.05
Strawberry	6.8±0.3	4.5±0.2	0	3.5±0.05

The acid concentrations of the juice of wild berries appeared to be mostly higher than has previously been reported for the northern region berry juices (table 1 in I). For example, Huopalahti *et al.* (2000) reported citric and malic acid concentrations for cranberry to be as low as 9.4 g/l and 2.8 g/l. Instead, the present data on organic acid concentrations of juices of cultivated berries agree quite well with the few available reports found in the literature (table 2 in I). This can be partly due to the wide variation range given in the literature or to more constant growing conditions when compared to those of wild berries.

According to the studies of 15 different black currant cultivars from 2 different seasons (unpublished data), the variations in acid concentrations were characteristic for each cultivar. Black currant cultivars of different growing seasons showed only small differences in total acid concentration.

The pH of the berry juices was in all cases very low (2.4 – 3.5) and did not correlate with total amounts of acids. This is perhaps due to the fact that the buffer system of the berry juice keeps the pH quite constant. For comparison, the pH of grape juice has reported to be 2.8 – 3.9 (Beelman & Gallander 1979, Lafon-Lafourcade 1983). The pH values of wild berries appeared mostly to be lower than reported earlier (table 1 in I) and those of cultivated berries agree quite well with the available literature (table 2 in I). The low pH of the berry juice is advantageous as it prevents microbial contaminations. On the other hand, in e.g. berry wine production, even a small increase in pH values enables more effective yeast fermentation.

#### 4.1.2 Soluble sugar composition

Variations in total and individual soluble sugars between different berry juices were wide. Raspberry, white currant, gooseberry and black currant juices contained highest amounts of sugars (96.1 – 107.7 g/l). The lowest sugar contents were in juices from black crowberry, red currant and cranberry (43.1 – 48.7 g/l). As comparison, total sugar concentration of grape juices has reported to be from 70 g/l up to 500 g/l. The concentration of fructose, in particular, has been reported to increase in grapes during ripening (Southgate *et al.* 1978, Lafon-Lafourcade 1983, Peynaud 1984, Henick-Kling 1993, Livsmedelstabell 1993). Table 2 summarizes the data on soluble sugar concentrations of the berry juices investigated in the present study.

Studies on black currant show that the berry cultivar in particular has a marked effect on sugar concentrations (unpublished data). For example, total sugar concentrations among 15 black currant cultivars varied between 49.6 and 107.7 g/l. Seasonal differences were also evident, but these may be explained by differences in climate conditions (Wrolstad & Shallenberger 1981). The exact ripeness of the berries is difficult to determine but the present results from

different seasons showed that the sugars, especially fructose and glucose accumulate during ripening (unpublished data). These results were in agreement also with other studies (Haila 1990, Heiberg *et al.* 1992, Toldam-Andersen & Hansen 1997, Matala 1999).

Table 2. Mean sugar concentrations of the berry juices.

Berry	Fructose/ g/l	Glucose/ g/l	Sucrose/ g/l	Total sugars/ g/l
<b>Wild berries</b>				
Bilberry	40.9±0.2	31.5±0.3	0.6±0.1	73.0±0.5
Lingonberry	42.3±0.3	42.4±0.4	1.2±0.1	85.9±0.7
Cranberry	20.7±0.1	27.8±0.1	0.2±0.1	48.7±0.3
Cloudberry	27.0±0.1	25.2±0.1	0	52.2±0.1
Red raspberry	57.5±0.1	47.2±0.1	0.5±0.1	105.2±0.2
Black crowberry	18.0±0.1	24.8±0.1	0.4±0.1	43.2±0.2
<b>Cultivated berries</b>				
Black currant	36.2±0.1	31.9±0.3	2.1±0.1	70.2±0.4
White currant	48.1±0.3	50.0±0.3	0	98.1±0.5
Red currant	24.2±0.2	22.2±0.3	0	46.4±0.5
Gooseberry (red)	46.7±0.3	44.3±0.3	5.1±0.1	96.1±0.6
Strawberry	34.4±1.7	33.1±1.7	0.6±0.1	68.1±3.4

As shown in table 3 in I, the measured values in wild berry juices were in general higher than reported in the literature. For example, according to Varo *et al.* (1984) the fructose content of lingonberry is only 2 g/l, glucose content 36 g/l and sucrose content 2 g/l and for cranberry 12 g/l, 22 g/l and 0 g/l, respectively. The present results on the sugar concentrations in juices from the representative cultivated berries were in fairly good agreement with available literature reports (table 4 in I). The comparison between the present data and the literature is difficult as the latter reports no berry variety and different analytical methods were also used. Nevertheless, differences between the present and existing data can at least partially be explained by seasonal and variety differences and differences in geographical origin of the berries as also Wrolstad & Shallenberger (1981) reported.

Sucrose concentration varied widely from one juice to another and the proportion of sucrose was low, only 0 – 8.0 % of the total sugars. This can be explained by

the fact that the pH of the juices is sufficiently low to promote sucrose hydrolysis. The enzymatic or chemical hydrolysis of sucrose to glucose and fructose occurs due to disruption of the cellular structure during thawing of the berries and during juice extraction (Plowman *et al.* 1989, Plowman 1991). Skrede (1983) reported that during thawing as much as 70 % of sucrose is degraded by invertase. As a whole, the sugar concentration can be greatly influenced by the ripeness and post harvest processes of the berry as also other studies have showed (Haila 1990, Heiberg *et al.* 1992, Toldam-Andersen & Hansen 1997, Matala 1999).

The fructose/glucose ratio varied among the berry juices from 0.73 to 1.40 (unpublished data) probably due to the post harvest storage. During this period, glucose and fructose participate in the initial stages of Maillard browning reactions. In such reactions glucose is the preferred substrate and influence its content (Boccorh *et al.* 1998).

#### 4.1.3 Juice yield

The juice yield of the berries is an economically significant criterion that should be taken into account when choosing the cultivar for different purposes. Considerable variation in juice yield of the different black currant cultivars were noticed (unpublished data). The juice yield was from 49.9 % to 78.8 %, depending on the cultivar. Juice yields were higher, up to 20 %, in the 2001 season than in 2002. Summer 2002 was particularly dry in Ilomantsi (Ilmatieteen laitos 2002, Mekrijärven tutkimusasema 2002) and thus also the berries contained less water. The influence of seasonal variations on juice yield of the black currant has also been reported by Toldam-Andersen & Hansen (1997). Despite the seasonal changes the low and high yield berry cultivars remained the same (unpublished data).

## 4.2 MALOLACTIC FERMENTATION

### 4.2.1 *Malolactic fermentation in synthetic media (II)*

Information concerning the degradation of malic acid and citric acid by malolactic bacteria and the requirement for sugar and acid co-metabolism has led to many different hypotheses (Subramanian & SivaRaman 1984, McCord & Ryu 1985, Cogan 1987, Naouri *et al.* 1990, Henick-Kling *et al.* 1991, Axelsson 1993, Colagrande *et al.* 1994, Pimentel *et al.* 1994, Dicks *et al.* 1995, Henick-Kling 1995, Martineau & Henick-Kling 1995, Vaillant & Formisyn 1996, Saguir & Nadra 1996, Miranda *et al.* 1997). Thus, in order to obtain consistent data, the co-metabolism of glucose, citric acid and malic acid by the lactic acid bacterium *Oenococcus oeni* was studied at laboratory scale in a modified MRS medium. All the experiments were carried out at low pH values.

As shown in figure 3, malic acid degradation is always favoured over glucose and citric acid utilization at low pH values. The organism used was able to degrade selectively malic acid and arrest the degradation of both glucose and citric acid at least for as long as the malolactic process was ongoing. Further, malic acid degradation always proceeded to completion and the pH increased during the MLF by about 0.2 units. Similarly, Henick-Kling *et al.* (1991) and Henick-Kling (1995) noticed that at low pH malic acid catabolism inhibits glucose utilization. On the other hand, Martineau & Henick-Kling (1995) reported, contradictory to present results, that the same organism metabolised simultaneously both malic acid and citric acid. Further, they observed that the highest rate of citric acid utilization was observed in the presence of malic acid. The pH in their studies was also low (pH 3.0 – 3.5).

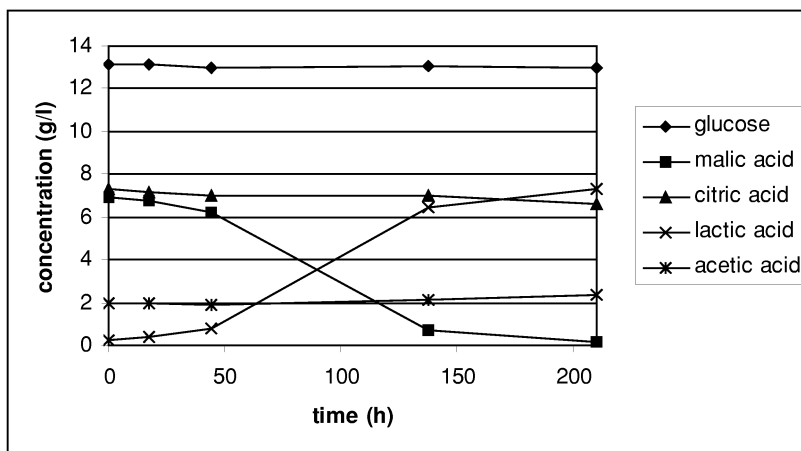


Figure 3. Degradation of L-malic acid in MRS medium containing malic acid, citric acid and glucose (pH 3.5) (figure 2 in II).

Even in the absence of sugar L-malic acid was readily fermented into lactic acid while citric acid remained unattacked (figure 1 in II). This result disagrees with previous results of Axelsson (1993), Colagrande *et al.* (1994) and Dicks *et al.* (1995). These reports conclude that malic acid degradation occurs only by co-fermentation with sufficient concentration of a fermentable carbohydrate. This result, conflicting with the literature, may indicate that the strain, pH of the medium or cultural conditions are not the same. However, no indication of differences in experimental conditions was given in the above-mentioned literature.

According to results of the present study, only after the exhaustion of malic acid both citric acid and glucose degradations were initiated simultaneously. Thus, in complex highly acidic substrate mixtures undergoing MLF, sugar and citric acid degradation can be inhibited only by the presence of malic acid. Consequently, *O. oeni* seems promising for use in low-pH media, such as berry juices and wines, to reduce selectively the acidity by degradation of malic acid while maintaining the sugar content. However, the present results point to the importance of process monitoring during MLF. For deacidification purposes the critical monitoring points are the initial concentrations of different acids and sugar, pH of the medium

and the progress of degradation of malic acid and to a lesser extent that of citric acid.

#### 4.2.2 Malolactic fermentation in berry juices

In berry wine production the reduction of acid concentration by MLF before AF is advantageous, because the natural pH of the berry juices is very low for the fermenting yeast. A wider range of malolactic strains may also be considered, giving greater options for flavour development. For example, for low ethanol tolerant strains (e.g. *Lactobacillus plantarum*) inoculation is recommended before yeasting. An additional advantage of the MLF of native juice is that the resulting juice with lower acidity offers many alternatives for further use and product development. A disadvantage, however, is the possible increase in volatility due to the bacterial fermentation of sugars (Henschke 1993), as was also the case in the present study. Thus, the main objective is to time the duration of the MLF properly. In general it can be concluded that far greater vigilance is required with pre-fermentation inoculation in order to recognize problems before rapid and serious spoilage occurs (Kunkee 1991).

##### 4.2.2.1 Laboratory scale fermentation (III)

The MLF and selectivity of substrate utilization were clearly noticed in both autoclaved and non-autoclaved black and white currant juices. Thus, as seen in figure 4, malic acid was completely metabolised to lactic acid during the first day following inoculation without degradation of citric acid. The pH increased by about 0.1 – 0.2 units. Removal of malic acid occurred also without a significant loss of sugars. However, when malic acid was exhausted, citric acid and sugar degradations were initiated simultaneously. During the fermentations the viable cell density did not change. Overall, white and black currant juices proved to be applicable for MLF by *O. oeni*. Thus, the sequential malolactic utilization of



substrates, which were described in synthetic media (see section 4.2.1), applies also to currant juices.

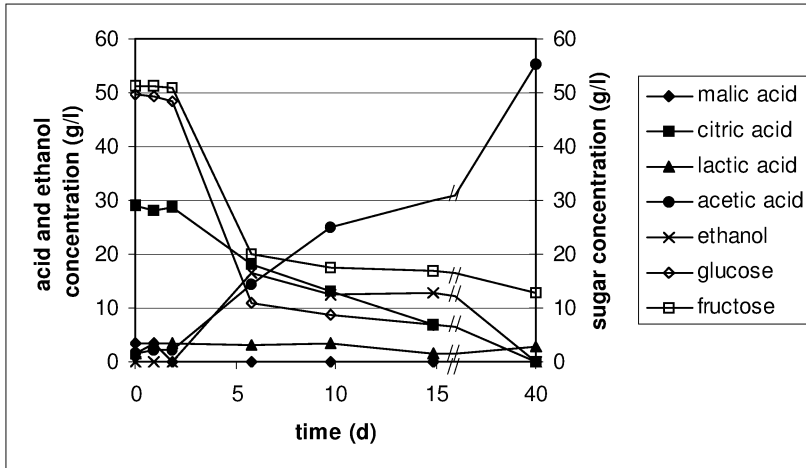


Figure 4. Malolactic fermentation of non-autoclaved white currant juice (figure 2 in III).

The MLF proceeded also in bilberry juice similarly with the exception that malic acid degradation did not proceed to completion. It was observed that in bilberry juice the initial cell content (over  $10^8$  CFU/ml) was reduced to fewer than  $10^6$  CFU/ml within 6 days. This amount is not sufficient to maintain a MLF (King 1985, Prah & Nielsen 1993). The very slow degradation of the acids and sugar is probably a reflection of both the weak residual activity of the malolactic bacterium and the inhibitory effect of the residual L-malic acid on the degradation of citric acid and sugars. Several reasons may account for the poor viability of *O. oeni* in bilberry juices. Amongst them may be natural antimicrobial compounds, low pH (3.0) or lack of essential nutrients.

The fermentation of lingonberry juice proceeded differently. The viable cell density of the inoculum decreased as early as during the second adaptation cultivation, although the decrease was not drastic. However, during the MLF of the autoclaved lingonberry juices the cells apparently suffered and after 14 days

lost all viability. The low pH of the juice could not be solely responsible for this. The presence of benzoic acid is the most probable explanation for the cell death and for the observation that neither acids nor sugars were degraded. Thus, the pH did not change either. According to Chipley (1983), benzoic acid inhibits the yeast fermentation and also functions effectively against bacteria if the concentration is 0.1 % and the pH is low, as it is in lingonberry juice. Only in the non-autoclaved and uninoculated juice trace degradation of sugars could be detected due to other natural microorganisms probably more adapted to benzoic acid.

#### 4.2.2.2 Pilot scale fermentation (IV)

MLF was carried out in the native 1:1-diluted black currant juice without any glucose supplementation. During MLF malic acid concentration decreased and lactic acid concentration increased, while the sugar concentrations remained practically unchanged. Malic acid concentration decreased from 2.3 to 1.1 g/l and lactic acid concentration increased from 0.3 to 2.1 g/l and the pH increased by approximately 0.1 pH-units. Simultaneously, citric acid concentration decreased only from 13.9 g/l to 13.2 g/l and acetic acid concentration increased from 0.1 g/l to 0.5 g/l. Towards the end of the MLF, slight decreases in sugar concentrations were noticed indicating an initiating shift of MLF from malic acid utilization to sugar fermentation and pointing to the importance of accurately timed cessation of MLF (see figure 8). As already discussed in section 4.2.2.1, malolactic fermentation proceeded successfully in black currant juice also in larger scale.

#### 4.2.3 Malolactic fermentation in berry wines (IV)

After the primary AF of black currant juice in pilot scale, the wine was filter sterilised and inoculated with *O. oeni* to carry out the MLF. Figure 5 indicates the changes in sugar, acid and ethanol concentrations in such an experiment. During 40 hours of MLF the concentration of malic acid decreased from 2.1 to 1.0 g/l,

that of lactic acid increased from 0.3 to 1.8 g/l and the pH increased by about 0.1 pH-units. Slight increase in acetic acid concentration was also noticed. MLF did not alter the sugars remaining after AF of the native juice. The ethanol already produced during AF did not disturb the malolactic reactions. Consequently, the traditional order of fermentations (AF – MLF) used for grape juices (Edwards & Beelman 1989, Krieger *et al.* 1992, Henschke 1993) is applicable also to black currant juice. The MLF can be successfully performed to berry wine and the ethanol concentration does not disturb the malolactic reactions.

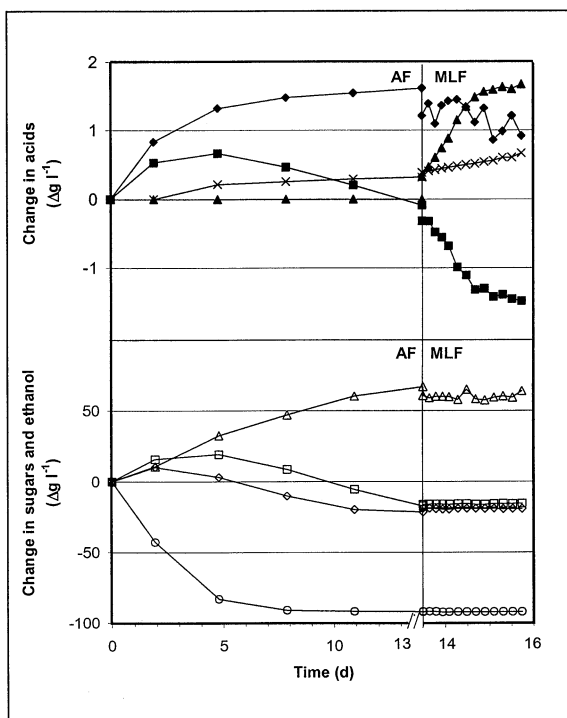


Figure 5. Changes in acid, sugar and ethanol concentrations in alcoholic fermentation and in the following malolactic fermentation of black currant juice. The vertical line on the time scale axis indicates the point at which filtration was performed. ◆, citric acid; ■, malic acid; ×, acetic acid; ▲, lactic acid; ◇, glucose; □, fructose; o, sucrose; Δ, ethanol (figure 1 in IV).

As the malolactic *O. oeni* are inoculated after completion of the AF, the sugar metabolism of these heterofermentative bacteria can be avoided and no increase in

the volatile acidity occurs (Garvie 1986, Henschke 1993, Lonvaud-Funel 1995). The yeast strain used in the AF may be stimulatory, neutral or inhibitory to bacterial growth. As an added benefit, the decline in yeast growth and autolysis release nutrients into the wine, particularly vitamins, amino acids and peptides that stimulate bacterial growth. On the other hand, some strains of wine yeasts may be inhibitory to bacterial growth under certain conditions by competing for nutrients or elaborating toxic compounds, including aldehydes, sulphite and medium chain length fatty acids. MLF may also be inhibited by the presence of bacteriophage or parasitic molds of the berries (Wibowo *et al.* 1985, Lonvaud-Funel *et al.* 1988, Edwards & Beelman 1989, Henschke 1993). According to results of the present study, the yeast fermentation had an inhibitory effect to the malolactic reactions. The MLF did not proceed to completion possibly due to lack of essential nutrients.

#### *4.2.4 Sensory effects and colour changes*

The variations between assessors should be minimized. However, it is very difficult to eliminate individual differences in the use of scale and in sensitivity, motivation and culture, which do exist (Carlucci & Monteleone 2001). Consequently, also sensory evaluation of the malolactic-fermented black currant juices showed definite inconsistencies between assessors (unpublished data).

The assessors generated taste attributes fermented/winy, black currant, sourness/acidity and bitterness together with a beery aftertaste. Generated odor attributes were fermented, black currant and fusty. The effect of the treatment, that is the malolactic fermentation, caused a significant difference in every taste attribute, except bitterness (Figure 6). The fermented/winy taste increased significantly ( $P < 0.001$ ) partially also due to the spontaneous yeast-like fermentation, which occurred in the juices. The black currant taste decreased ( $P < 0.001$ ), however the characteristic flavour of the berry was still noticed. Instead, the taste of acidity slightly increased ( $P < 0.01$ ). Besides the evaluated sensory

attributes, some other terms were used to describe the taste or odor of malolactic-fermented black currant juices. These include tastes such as astringent and nutty or odors like red winy and fresh.

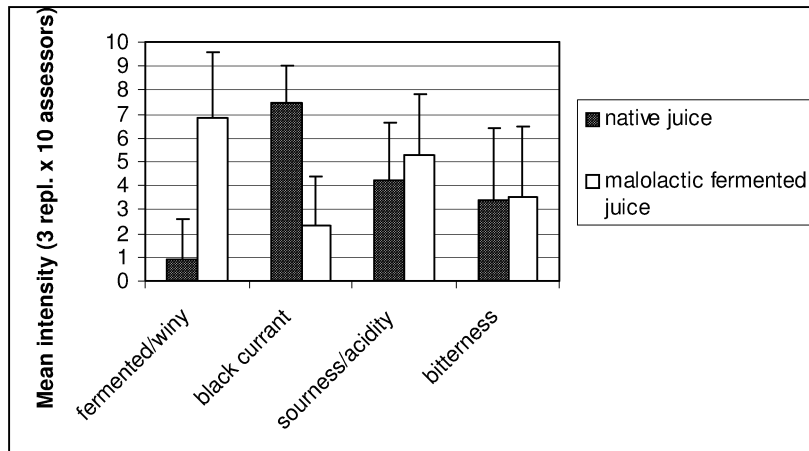


Figure 6. The comparison between black currant juice (variety ‘Öjebyn’) and malolactic fermented juice: Mean intensities of taste characteristic (columns) and mean deviations (lines).

Thus, MLF had a definite effect on the sensory qualities of berry juices as has also been showed by other studies on grape juices (Davis *et al.* 1985, Henick-Kling 1993, Henick-Kling *et al.* 1994, Laurent *et al.* 1994, Sauvageot & Vivier 1997, Delaquis *et al.* 2000, Gámbaro *et al.* 2001). However, the ability to distinguish between sensory changes caused by mere malolactic fermentation, acidity reduction, or other microbial metabolism, becomes a challenging task.

The results showed no significant differences ( $p \leq 0.05$ ) amongst the three black currant cultivars studied for any odor attribute. For black currant taste and beery aftertaste the cultivar difference was also not significant. On the other hand, cultivar differences were significant amongst the attributes such as fermented/winy taste ( $P < 0.01$ ), sourness/acidity ( $P < 0.001$ ), bitterness/astringency ( $P = 0.001$ ) and color ( $P = 0.001$ ) (unpublished data). Also

Brennan *et al.* (1997) have reported significant variation between different black currant varieties in all investigated sensory characters.

MLF reduced the color of black currant according to sensory analysis by 32 – 63 % and measured by Minolta Chroma Meter by 37 – 60 % (unpublished data) depending on the cultivar. The very high color reduction rates can be partly explained by the simultaneous yeast fermentations in the natural juices. Also Vetsch & Lüthi (1964) noted that color could be reduced by 30 % during MLF of red wines. They suggested that dehydration of citric acid supplied the hydrogen for reduction of wine colour, which could be partially regenerated after MLF.

#### 4.3 UPTAKE OF BENZOIC ACID BY *SACCHAROMYCES CEREVISIAE* (V)

When reprocessing of the product containing benzoic acid with e.g. fermentation is desired, the elimination of this acid becomes a main problem. For example, lingonberry juice has to be diluted with water in a ratio of 1:6 for performing the alcoholic fermentation. However, concentrations of sugar and aromatic compounds would be diluted, as well. Thus, a method was developed which utilizes the pH-dependent ability of *Saccharomyces cerevisiae* to absorb benzoic acid from solutions. Especially under acidic conditions, the lipophilic character of protonated benzoic acid enables its penetration through the cytoplasmic membrane and benzoic acid accumulates into the yeast cell (Macris 1975). Thus, essential to the method is the incubation of the yeast in the juice for a short period followed by the removal of the yeast mass. The addition of baker's yeast is a known and safe method and no extra chemicals are required. This process can also be applied for the removal of other preservatives, such as sorbic acid, which have similar mechanism of entry into the yeast cell (Macris 1975, Archer 1980, Pelczar *et al.* 1993).

This study represents a new application, where the yeast is used as a selective absorbent to remove a certain component from the liquid. Figure 7 shows the main stages of the method used. These include immersion of an optimized amount

of *Saccharomyces cerevisiae* yeast into the acidic juice, shaking of the suspension at room temperature and separation of the yeast. Thereafter, the juice is inoculated with the desired fermenting organism. The yeast mass used can be regenerated by suspending the mass in a large volume of a buffered solution able to maintain the pH at a higher level than the  $pK_a$  of benzoic acid ( $pK_a = 4.19$ ).

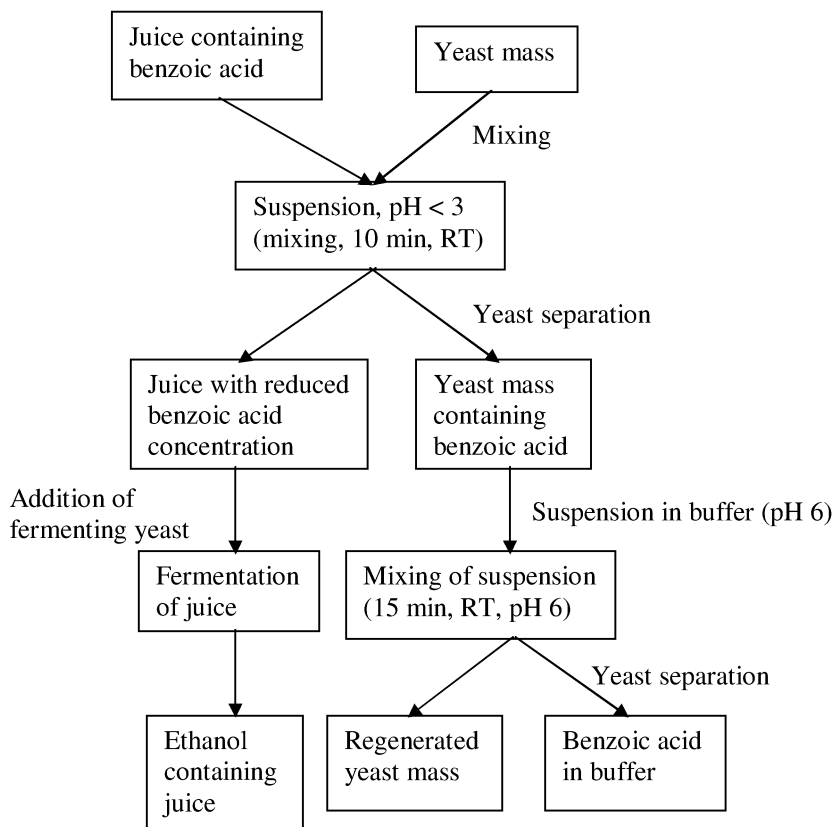


Figure 7. Schematic diagram of the method for selective absorption of benzoic acid from the liquids (figure 1 in V).

The concentration of benzoic acid in the lingonberry juices varied between 0.6 – 1.3 g/l depending on the berry batch analyzed. In order to make the juices from these batches fermentable, 50 – 80 % of the benzoic acid had to be removed. By using 15 – 20 % (w/w) yeast as a single batch, the benzoic acid concentration decreased by 75 – 91 %, depending on the juice used. Thus, the final benzoic acid

concentration after the yeast treatment was below 0.25 g/l in all juices, and thus, should not prevent alcoholic fermentation (Warth 1988). The same residual level of benzoic acid was achieved by adding yeast either in one single 15 % batch or in five sequential batches, at 3 % (w/w) each. Thus, the total amount of yeast required reached the same level in both cases. This is in accordance with the results of Warth (1991).

#### 4.4 ALCOHOLIC FERMENTATION

Little is known about how AF should be conducted in berry juices. Thus, *S. cerevisiae* was inoculated to native berry juices, and to juices, which had undergone the MLF. In addition, lingonberry juice, from which the benzoic acid had been removed, was fermented with the yeast.

##### 4.4.1 *Alcoholic fermentation in native juice (IV)*

Production of berry wines differs essentially from that of grape wines, particularly with regard to highly differentiated raw material, the need for sweetening and dilution of berry juices or musts (Jarczyk & Wzorek 1977). In this study, black currant juice (1:1 dilution) was supplemented with 100 g/l sucrose prior to fermentation. As expected, the sugars were fermented to ethanol. The changes in sugar, acid and ethanol concentrations are presented in figure 5. At the end of the AF, the total sugar concentration was approximately 10.0 g/l, the total acid concentration 18.1 g/l and the ethanol concentration 68.0 g/l. Thus, the product after the AF was red-coloured, low-alcoholic (6.8 %) black currant wine. Similar results of black currant winemaking procedures has been mentioned by e.g. Jarczyk & Wzorek (1977), who reached an alcohol content amounting between 8 – 9 %.



#### 4.4.2 Alcoholic fermentation after malolactic fermentation (IV)

After MLF the deacidified black currant juice was filter sterilised and inoculated with *S. cerevisiae*. At this point the juice was supplemented with 100 g/l sucrose. The conversion of sugars to ethanol was again clearly evident (figure 8). During the 14 days of AF total sugar concentration decreased from 106.3 g/l to 16.9 g/l while ethanol concentration increased from 4.4 g/l to 54.7 g/l. Degradation of sucrose to glucose and fructose was also evident. Changes in acid concentrations and pH were insignificant.

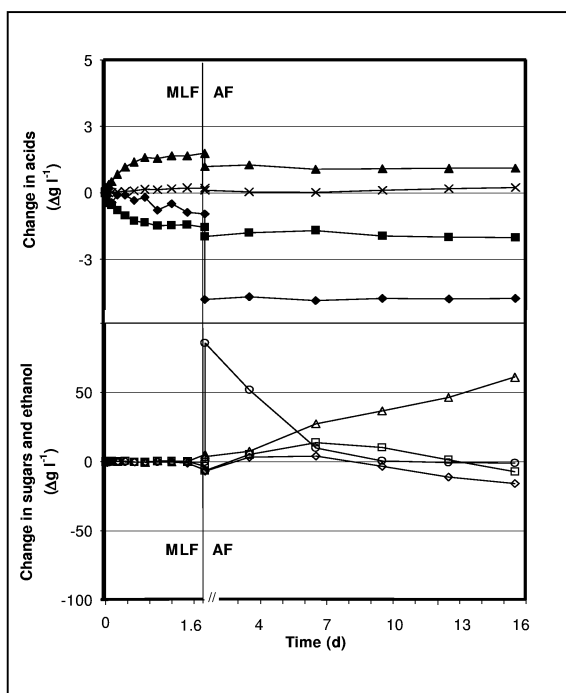


Figure 8. Changes in acid, sugar and ethanol concentrations in malolactic fermentation and in the following alcoholic fermentation of black currant juice. The vertical line on the time scale axis indicates the point at which filtration was performed. ◆, citric acid; ■, malic acid; ×, acetic acid; ▲, lactic acid; ◇, glucose; □, fructose; o, sucrose; Δ, ethanol (figure 2 in IV).

At the end of AF the final concentration of total acids was 12.6 g/l and the pH was 3.1. The end product of these consecutive fermentations was a deep red-coloured

low-alcoholic berry wine, where the acid composition was changed and the total acid concentration reduced and where some sugars remained. Due to the lack of nutrients after MLF the alcohol content of the end product may remain lower than when the AF is performed to the native juice. Similar results on the final products of alcoholic fermentation under nutrient limiting conditions, which impose a stress on the yeast cell, have been obtained by Kunkee (1991) and Prah1 & Nielsen (1993). Despite the potential problems concerning malolactic fermentation before alcoholic fermentation in grape juices, the present results showed that the AF can be successfully performed after the MLF at least in black currant juice.

#### 4.4.3 *Alcoholic fermentation after removal of benzoic acid (V)*

According to Warth (1988), benzoic acid concentrations of 0.2 – 0.3 g/l are sufficient to prevent growth of *Saccharomyces cerevisiae*. In the juice, where benzoic acid had been removed by *S. cerevisiae*, the residual benzoic acid concentration was below 0.1 g/l and the amount of fermentable sugars was about 78 g/l. During AF the ethanol concentration increased to 3.5 % (w/w) and benzoic acid concentration did increase slightly, perhaps due to release of benzoic acid from the benzoyl glucoside molecules (Heimhuber *et al.* 1990). Regardless, this slight increase of benzoic acid concentration did not inhibit the yeast fermentation and lingonberry juice could be fermented with yeast.

## 5 CONCLUSIONS

Northern region berry juices contain a large amount of acids and many processes, such as fermentation, face challenges that are not encountered when using e.g. grape or fruit juices. The results of this study provide new “natural” means to convert acid contents in berry juices and berry wines and therefore facilitate the development of a variety of new berry products. It is imperative that during these processes the resulting products retain their individual, characteristic colour and aroma. Further, the comparative study on the organic acid and soluble sugar concentrations of the berry juices offers means for the evaluation of the requirements for an acidity reduction process for various berries.

The efficient degradation of malic acid by *Oenococcus oeni* occurs by nongrowing cells and under conditions where glucose is not consumed. This gives a promising opportunity for deacidification of low-pH berry juices and berry wines without loss of sugars and possibly with minimal interference with other berry constituents. The typical malolactic reactions of *O. oeni* reported in grape products occur also in northern region berry juices and wines studied. Thus, malic acid can be eliminated with practically no consumption of sugar or citric acid and therefore the excess acidity typical for many berry juices can be attenuated. One interesting and malic acid containing berry is buckthorn (*Hippophaë rhamnoides*), which could be the raw material for further studies.

As the degradation of sugars and citric acid begins immediately after the exhaustion of malic acid, attention is called for continuous monitoring of the process. Monitoring of pH may not provide the needed accuracy, as may neither the assay of lactic acid, as it is a product of other fermentation substrates also. It seems evident that unwanted degradations can be avoided as long as malic acid degradation in the low-pH media is ongoing. Thus, proper timing for the fermentation can most reliably be achieved by measuring changes in the concentration of malic acid. Malolactic conversion of malic acid into lactic acid may occur rapidly in native juices especially when the content of malic acid is

low. Fermentation of sugars into volatile acids (acetic acid) may then be initiated. In general, MLF in sugar-containing juices requires greater vigilance than MLF after alcoholic fermentation to avoid rapid and serious spoilage.

MLF clearly affected the sensory qualities of berry juices. However, to distinguish between sensory changes caused by mere malolactic fermentation, acidity reduction or other microbial metabolism becomes a challenging task. As a whole, malolactic fermentation represents a promising means for acidity reduction of northern region berry juices and wines without a significant loss of their natural sugar content and retaining the characteristic sensory properties of the individual berry variety. The elimination of the natural microbial flora before MLF would ease the control of unwanted flavour development during the fermentations.

Benzoic acid can be eliminated from lingonberry juice by treatment with *Saccharomyces cerevisiae* yeast with minor changes in the physical and chemical properties of the raw material. Thus, after the treatment also lingonberry juice can be fermented by the yeast. The method is based on known and safe ingredients and no extra chemicals are required. The process itself is easy to perform and enables production of fermentation or distillation products that would otherwise be uneconomic or even impossible to produce. The same process principles can be used also to other liquid foodstuffs containing natural or added benzoic acid. In addition, this process can be applied for the removal of other preservatives, such as sorbic acid, which have similar mechanism of entry into the yeast.

To achieve the appropriate final level of benzoic acid in the solution, the yeast can be added in one or several consecutive batches. As the initial benzoic acid concentration varies from one raw material to another, the required amount of yeast should be optimized. Therefore, consecutive additions of yeast in small batches are advantageous to avoid adding unnecessary amounts of yeast. In addition, the choice may depend on availability of equipment for the removal of the yeast from the juice, the scale of the process and the type of yeast available. The present method requires relative high amounts of yeast. However, this

disadvantage is compensated by the possibility to regenerate and recycle the yeast cells.

The malolactic-fermented berry juice is well suited for AF. The present data suggests that the native juice has limiting concentrations of essential nutrients and thus if the first fermentation is malolactic, more efficient conversion of malic acid into lactic acid occurs. In addition, the reduced acidity is advantageous for the survival of the fermenting yeast and therefore compensates for possible nutrient limitation brought about the preceding malolactic process. The lingonberry juice treated with the yeast was also free of fermentation-hindering components and thus readily fermentable.

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