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Preparation of fermentable lingonberry juice through removal of benzoic acid by *Saccharomyces cerevisiae* yeast

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Abstract

Lingonberry (*Vaccinium vitis-idaea*) is a commercially important wild, uncultivated berry in northern regions of the world. It contains high amounts of benzoic acid, which contributes to the acidity of the berry and, as a microbisidic compound, prevents fermentation of lingonberry juice. Therefore a method was developed utilizing the pH-dependent ability of *Saccharomyces cerevisiae* to remove benzoic acid from solutions. By suspending 15–20% (w/w) of the yeast for 10 min in undiluted lingonberry juice, benzoic acid concentration was reduced by 75–91%, titratable acids by about 14% and pH raised by 0.1 units. The resulting undiluted juice was readily fermented with a new yeast inoculum. Applicability of the method for benzoic acid removal from other food material is discussed.

Keywords: Lingonberry; *Vaccinium vitis-idaea*; Benzoic acid; Acidity reduction; Yeast; *Saccharomyces cerevisiae*; Fermentation

1. Introduction

Benzoic acid is one of the oldest and still one of the most widely used chemical preservatives (Chipley, 1983; Davidson, 1997; Lueck, 1980). Its growth inhibitory effect is strongly pH-dependent and most effective under acidic conditions, where the protonated form of the acid predominates (Macris, 1975). Under these conditions, the lipophilic character of protonated benzoic acid enables its penetration through the cytoplasmic membrane. Within the cell, benzoic acid is ionized and the released proton is excreted from the cell by an energy requiring process. Benzoate ions are also driven out of the cell by an electrochemical gradient, but once outside, they are protonated again and re-enter the cell. This energy requiring cycling of protons and benzoate ions starvates the cells. In addition, benzoate ions inhibit glycolysis especially by deactivation of phosphofructokinase (François, van Schaftingen, & Hers, 1986; Pelczar, Chan, & Krieg, 1993; Warth, 1988, 1991a).

Benzoic acid occurs naturally, e.g. in lingonberry, cranberries, cloudberries and cinnamon (Archer, 1980; Busta & Foegeding, 1983; Chipley, 1983; Davidson, 1997; Heimhuber, Wray, Galensa, & Herrmann, 1990; Viljakainen, Visti, & Laakso, 2002). Especially in lingonberry, benzoic acid concentration is high (0.6–1.3 g/l free benzoic acid) and the pH is low (pH 2.6–2.9). The concentration of benzoic acid is increased further if the juice is stored at warm conditions (Solberg, 1980; Souci, Fachmann, & Kraut, 1994; Viljakainen, Visti, & Laakso, 2002). Therefore, processed lingonberries are nonfermentable by many microorganisms and can normally be conserved without addition of preservatives.

According to Warth (1988), benzoic acid concentrations of 0.2–0.3 g/l are sufficient to prevent growth of *S. cerevisiae*. Therefore, 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. Elevating the pH until benzoate ions become dominating would be another approach for obtaining fermentable juices. Addition of calcium carbonate or

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usage of ion exchange resins have been suggested for this purpose (Macris, 1975). However, dramatic changes in colour and organoleptic qualities can be expected together with increased risk of microbial contamination. Benzoic acid resistant fermenting strains, such as *Zycosaccharomyces bailii*, can also be used to ferment lingonberry juice. However, organisms resistant to benzoic acid are often known to form foams and unpleasant aroma compounds, and are considered as spoiling organisms in food and alcoholic beverages industries (Boulton, Singleton, Bisson, & Kunkee, 1996; Warth, 1988).

In order to avoid disadvantages associated to the above methods, an alternative procedure is described here. The method is based on the ability of *S. cerevisiae* yeast to remove benzoic acid from acidic solutions. Essential in the process is incubation of the yeast for a short period in the juice followed by its removal. The juice so obtained could be readily fermented by a new inoculum of yeast. Recovery of benzoic acid from the yeast is also described to enable its reutilization.

2. Materials and methods

2.1. Outline of the method

Fig. 1 shows the main stages of the method used. They included 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 was inoculated with the desired fermenting organism. The used yeast mass could be regenerated by suspending it in a large volume of a buffered solution able to maintain the pH at a higher level than the pKa of benzoic acid (pKa = 4.19).

2.2. Preparation of yeast

The yeast used to remove benzoic acid was fresh *S. cerevisiae* (Suomen Hiiva Oy, Finland). The yeast was prepared by inoculating 10% (w/w) yeast to a medium containing 10% (w/w) glucose (Merck, Germany) and

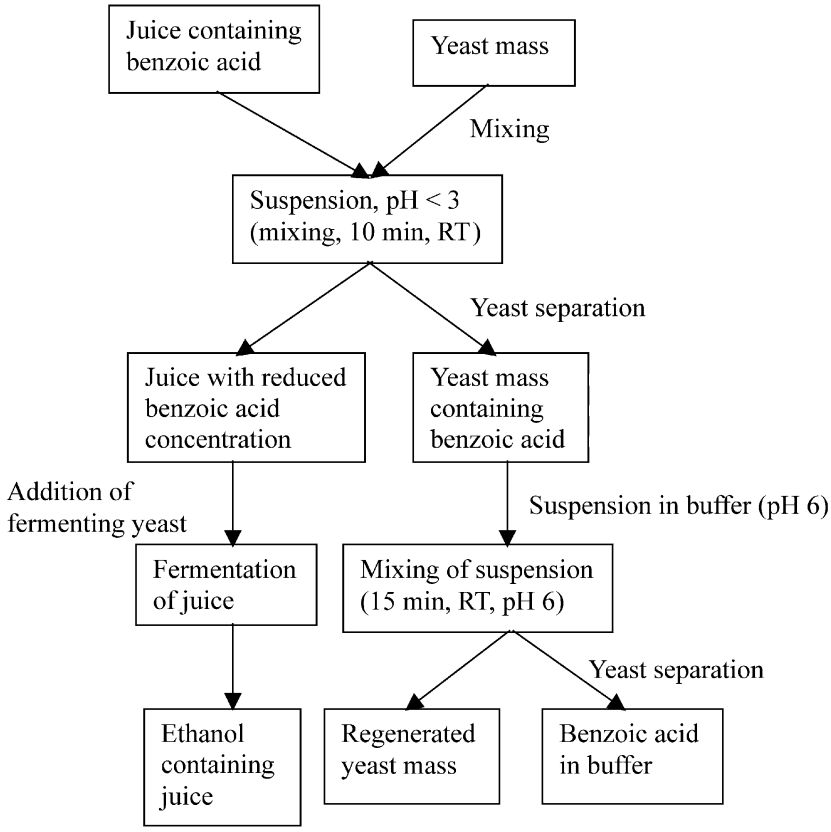

Fig. 1. Schematic diagram of the method.

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 h, and then centrifuged (5860×*g*, 10 min). The cell mass was washed with sterile 0.9% (w/v) NaCl.

2.3. Lingonberries and preparation of juice

Ripe Finnish lingonberries were obtained frozen from Suomen Marjat Oy (Finland) (season 1997) and from Marjamestarit—Berry Masters Oy (Finland) (season 1999) and stored at −20 °C until used. The berries were allowed to thaw at +4 °C before use. The thawed berries were pressed with a hydraulic press (Hafico 6-72, Germany) at a maximum pressure of 2500 N/cm^2 to obtain the juice.

2.4. Removal of benzoic acid

S. cerevisiae cell paste prepared (see Section 2.2) 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 stirred strongly for 10 min at room temperature. Then, the juice suspension was centrifuged (5860×*g*, 10 min) and the remaining juice was assayed for benzoic acid, sugar and citric acid concentrations, as well as for changes in colour and pH. All the experiments were performed as at least in duplicates. Variation of data was negligible.

2.5. Regeneration of yeast

The yeast used for removal of benzoic acid from the lingonberry juice was regenerated by suspending it (20%, w/w) in citric acid buffer containing 11.5% (v/v) of 0.1 M citric acid and 88.5% (v/v) of 0.1 M trisodiumcitrate (Merck, Germany). The pH was adjusted to 6.0 with 0.1 M NaOH. The suspension was shaken for 15 min at 200 rpm (Certomat R, B.Braun, Germany) at room temperature and thereafter centrifuged (5860×*g*, 10 min). Samples for benzoic acid analyses were taken from the centrifuged solution. The procedure was repeated four times with the fresh buffer. The final buffer-yeast suspension was incubated for one hour. The five-step regeneration process was performed fourfold.

2.6. Fermentation

Alcoholic fermentation was performed with a freeze-dried yeast *Saccharomyces cerevisiae* (strain DF 639, Siha 6, Begerow, Germany), which was hydrated in water for 10 min before use. The effectiveness of the removal of benzoic acid was tested by inoculating both the treated and untreated juices with 0.06% (w/w) of the prehydrated dry yeast. The flasks were sealed with a water seal and shaken at 100 rpm (Certomat R, B. Braun, Germany) at room temperature for 7 days. Ethanol formation and changes in benzoic acid concentration were measured. The fermentation experiments were performed in triplicates.

2.7. Determination of acids, sugars and ethanol

Benzoic acid was determined by high-pressure liquid chromatography analysis (HPLC), with a HP Series 1100 chromatograph (Hewlett Packard, USA) equipped with a Hypercil BDS C8 reverse phase column (250×4.6 mm, 5 μm) (Hypercil, UK), and UV-detector (254 nm) (Hewlett Packard, USA). The column was eluted with a 1:1 mixture of phosphate buffer (0.05 M, pH 3.5) and methanol at 30 °C and at flow rate of 1 ml/min.

Citric acid and sugars (sucrose, glucose and fructose) were determined with the same chromatograph equipped with an Aminex HPX-87 H+ column (300×7.8 mm, 9 μm) (Bio-Rad Laboratories, USA). The column was eluted with 0.005 M sulphuric acid at 35 °C, at a flow rate of 0.6 ml/min. Prior to sugar analyses, the samples were exposed to a strong anion exchange column (Bond Elut SAX, Varian, USA) to remove the acidic compounds. Citric acid was detected with a UV detector (Hewlett Packard, USA) at 214 nm and sugars with RI detector (Hewlett Packard, USA). For quantification, the internal standards formic acid or xylitol were used.

Ethanol was analyzed by using a HP 6890 gas chromatograph (Hewlett Packard, USA) equipped with a HP-INNOWax column (30 m, 0.25 mm, 0.25 μm) (Hewlett Packard, USA). The elution was carried out with helium at a flow rate of 1 ml/min. Ethanol was detected with a flame ionization detector (Hewlett Packard, USA).

Before injection, all the samples were filtered (0.2 μm pore size) (Supor 200, Gelman Laboratory, USA). Each assay was performed in triplicate.

2.8. Measurement of titratable acidity, pH and colour intensity

The juices were titrated with 0.1 M NaOH to the end point pH 8.1, the third pKa value of citric acid, and the total acidity was calculated as citric acid. pH determinations were made with a RL 150 pH/mV device (Russel, USA) calibrated with standard buffer solutions of pH 4.00 and 7.00 (Reagecon, Ireland).

Colour intensity was determined with a Hitachi U-2000 spectrophotometer (Hitachi, Japan) at 517 nm. The absorbance of untreated, 1:15 with water diluted lingonberry juice at this wavelength was defined as the reference and given absorbance index of 100. The absorbance of the processed lingonberry juice was compared to this reference. All experiments were done in triplicate.

3. Results and discussion

3.1. Removal of benzoic acid

The concentration of benzoic acid in the lingonberry juices varied between 0.6 and 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, benzoic acid concentration decreased by 75–91%, depending on the juice used (Fig. 2). 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 (Fig. 3). Thus, the total amount of yeast required raised in both processes to the same level. The same conclusion can be drawn from the results of Warth (1991a).

Other changes in the juice resulting from the removal of benzoic acid are shown in Table 1. In the juice containing initially 1.1 g/l benzoic acid, the total titratable acids decreased by about 16%. In addition, the pH raised approximately by 0.1 units, the absorbance index decreased from the initial 100 to 65.1, citric acid concentration decreased by 23.7% and total sugar concentration from 90.7 g/l to 78.3 g/l (13.7%).

3.2. Regeneration of yeast

Table 2 shows the release of benzoic acid from the yeast into the citrate buffer (pH 6.0) during four consecutive regeneration processes. The first 15-min process was the most effective, 46.6% of the benzoic acid absorbed by the yeast was released. The second process

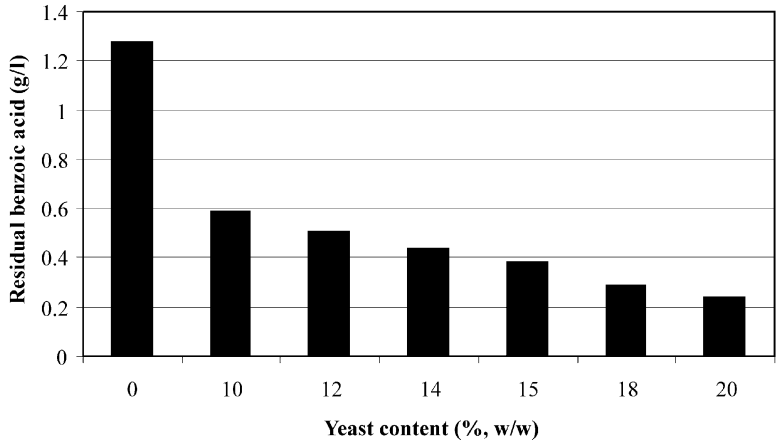

Fig. 2. Effect of yeast content on benzoic acid removal from lingonberry juice (initial benzoic acid concentration 1.3 g/l, pH 2.7).

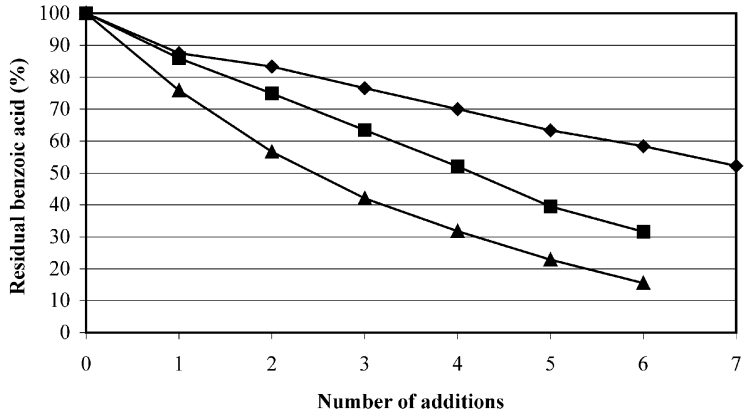

Fig. 3. Removal of benzoic acid from lingonberry juice by consecutive additions of yeast at different concentrations [◆, consecutive 1% (w/w) addition; ■, consecutive 2% (w/w) addition; ▲, consecutive 3% (w/w) addition].

Table 1
Changes in physical and chemical properties of lingonberry juice upon removal of benzoic acid by yeast added at 16% (w/w)[a]

	Initial	Final	Change (%)
pH	2.63±0.03	2.66±0.03	+1.0±0.3
Titratable acids[b] (g/l)	30.70±0.01	25.75±0.01	−16.1±0.1
Absorbance index (517 nm)	100±1.0	65.1±3.5	−34.9±3.5
Benzoic acid concentration (g/l)	1.06±0.003	0.27±0.03	−75.0±2.3
Citric acid concentration (g/l)	16.95±0.11	12.93±0.74	−23.7±4.1
Glucose concentration (g/l)	43.65±0.07	38.10±0.53	−12.1±1.1
Fructose concentration (g/l)	44.55±0.00	39.20±0.62	−11.3±1.3
Sucrose concentration (g/l)	2.51±0.01	1.02±0.02	−58.7±0.7

[a] Results are expressed as mean ± S.D., $n=3$.
[b] Expressed as citric acid.

Table 2
The regeneration of the yeast used for benzoic acid removal in lingonberry juice as carried out in citrate buffer (pH 6.0)[a]

Process no.	Time of influence (h)	Concentration of benzoic acid in buffer (g/l)	Benzoic acid concentration in yeast (mg)
	0	0±0	44.0±0.042
1	0.25	0.256±0.030	23.5±2.4
2	0.25	0.148±0.025	12.1±1.9
3	0.25	0.073±0.020	6.6±1.5
4	0.25	0.040±0.008	3.7±0.57
5	1	0.022±0.004	2.2±0.25

[a] Results are expressed as mean ± S.D., $n=4$.

Table 3
Fermentation of lingonberry juice after removal of benzoic acid[a]

	Treated juice	Untreated juice
Initial concentration of benzoic acid (g/l)	0.052±0.001	0.602±0.001
Final concentration of benzoic acid (g/l)	0.098±0.002	0.653±0.017
Initial ethanol concentration (%, w/w)	0.1±0.01	0.01±0.003
Final ethanol concentration (%, w/w)	3.5±0.03	0.01±0.003

[a] Results are expressed as mean ± S.D., $n=3$.

released 25.9% of the benzoic acid, the third 12.5% and the fourth 6.5%, correspondingly. During the final 1-h process still 3.4% of the uptaken benzoic acid was released into the buffer solution. Thus, with this regeneration method up to 95.5–98.1% of the benzoic acid could be removed from the yeast.

3.2.1. Fermentation

The data on alcoholic fermentation of untreated and treated lingonberry juice is presented in Table 3. In the treated juice the residual benzoic acid concentration was under 0.1 g/l, the initial amount of fermentable sugars was about 78 g/l and the ethanol concentration was raised to 3.5% (w/w) during alcoholic fermentation. During the fermentation benzoic acid concentration did slightly increase, 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.

4. Conclusion

The results show that benzoic acid can be eliminated from lingonberry juice by *S. cerevisiae* yeast with minor changes in the physical and chemical properties of the raw material. 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 make. 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 (Archer, 1980; Pelczar et al., 1993).

To achieve the wanted final level of benzoic acid in the solution, the yeast can be added in one or several consecutive batches. As the initial benzoic acid concen-

tration varies from one liquid to another, the required amount of yeast should be optimized. Therefore, consecutive additions of yeast in small batches are advantageous to avoid overdosing 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.

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