Application of biosensors in early detection of contamination with lactic acid bacteria during apple juice and concentrate production

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A B S T R A C T

The aim of Collective Research Project QUALI-JUICE (COLL-CT-2005, Co Nr 012461) was to introduce the biosensors in control of microbiologically pure apple juice production. Three commercial lactate biosensors were used and compared with enzyme kits assays. Parallel the microbiological analysis of the production process at one juice producing enterprise was done. The results of lactic acid assay with biosensors were in good correlation with those obtained by enzyme kits. The main benefit of biosensor use was shortening of measurement time as compared with assay by enzyme kit and possibility to measure at line. The concentration of l-lactic acid in apple pulp could be correlated with the number of lactic acid bacteria. Pasteurization process was eliminating lactic acid bacteria and the concentration of lactic acid was at this stage not exceeding 0.1 g L−1. The final product (apple concentrate) contained in some cases very high amounts of lactic acid indicating secondary microbiological contamination after pasteurization step. Parallel microbiological analysis of production process and lactate assay indicated that the critical point during production was prolonged vacuum filtration after pasteurization.

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1. Introduction

Europe is one of the world’s largest markets for fruit products and fruit juice and the demand is still on rise. The fruit juice industry is very important for the economy of the European Union counting more than 20,000 companies, of which about 90% are small- or medium-sized enterprises. The undesired microbial contamination is a problem in fruit juice industry. The origin and source of infection is often unknown and the beginning of juice spoilage is difficult to be detected. The infections of juices in storage tanks by alcohol producing microorganisms can be detected by exclusion chromatography, colorimetric test strips or enzymatic test kits. All these methods are time consuming, expensive and demanding trained laboratory stuff. Only enzymatic kit methods can distinguish the stereoisomers of lactic acid, the colorimetric test strips measure only l-lactate and chemical methods the sum of both forms. The detection methods for LABs (microbial cultivation or DNA fingerprinting) are time and work consuming and expensive. The increase of l-lactic acid concentration in juice can be a marker for fermentation by LABs (Trifirò et al., 1997) and in some cases it could be a measure of index of Escherichia coli number (Casimiri and Burnstein, 1998). Lactic acid can be assayed using HPLC, ion exclusion chromatography, colorimetric test strips or enzymatic test kits. All these methods are time consuming, expensive and demanding trained laboratory stuff. Only enzymatic kit methods can distinguish the stereoisomers of lactic acid, the colorimetric test strips measure only l-lactate and chemical methods the sum of both forms.

The alternative method for monitoring lactic acid is the use of biosensors, mainly amperometric enzyme electrodes. The state of art of amperometric lactate biosensors was reviewed recently (Nikolaus and Strehlitz, 2008). The use of biosensors can shorten the assay time and lower the cost of analysis.

The objective of Collective Research Project QUALI-JUICE (COLL-CT-2005 Co Nr 012461) is to develop a biosensor based detection and early warning system for juice spoilage by LABs for the European fruit juice industry. For purpose of the project four commercial lactate biosensors were chosen and tested for measurements of lactate concentration in apple juices during their production and in final products. This work presents testing of three of them – BIOSEN C line sport (EKF, Germany), LactatProfi 3000 (ABT, Germany) and Sensytec 1 (Tectronik, Italy) and application of them in control
of apple juice concentrate production. The second goal of the research was to show how the biosensors with parallel microbiological analysis can be used to find the origin of secondary contamination by LABs on the production line.

2. Experimental

2.1. Reagents

Polyamide (nylon) 6 (for column chromatography) was purchased from Fluka (Germany), polyvinylpolypyrrolidone powder (PVPP) was purchased from Sigma (Germany). As the standards l-lactic acid, approx. 98% (Sigma, Germany) or lithium l-lactate, approx. 97% (Sigma, Germany) were used dissolved in distilled water or 0.1 M phosphate buffer, pH 7. For enzymatic assay of l- and d-lactate, ethanol and acetic acid in juice samples the enzyme kits produced by Megazyme (Ireland) were used. All other chemicals were of analytical grade. The samples of apple pulp (crushed apples), must and juice were signed by the producer with date of the production. The samples of concentrates were signed by the producer symbols depending on the production stock or storage tank from which they were taken. These symbols are used throughout the text.

2.2. Measurements

The apple juice concentrates were dissolved with distilled water to 11.18°Brix. The samples of apple must or pulp were centrifuged at 14 000 rpm for 15 min. For enzymatic assay (spectrophotometric) of lactate 1 mL of sample was added by 100 mg of PVPP, shaken vigorously, left for 5 min and then centrifuged at 14 000 rpm for 15 min. The assay was done according to the procedure given by the producer of kits. Each assay was triplicated. The absorbance was measured at 340 nm in disposable PMMA Plastibrand® cuvettes (Sigma, Germany) with UV spectrophotometer Nicolet Evolution 300 from Thermo Electron Corporation (MA, USA).

2.3. Biosensors

The assay of lactate concentration with the use of biosensors was done according to the producer manual with standards and buffers purchased from them. Each measurement was triplicated (except Sensytec 1).

BIOSEN_C line sport (EKF, Germany) and LactatProfi 3000 (ABT, Germany) devices use the biosensors in form of screen printed electrodes (biochips) with immobilized l-lactate oxidase (LOD). l-lactate is oxidized to pyruvate according to the reaction:

\[
\text{l-lactate} + \text{O}_2 \xrightarrow{\text{L-lactate oxidase}} \text{pyruvate} + \text{H}_2\text{O}_2
\]

H₂O₂ produced in reaction (1) is oxidized on the electrode surface producing current which is proportional to l-lactate concentration. The concentration of l-lactate in the sample is calculated by comparison of current produced with standard solution of known concentration. BIOSEN_C line sport and LactatProfi 3000 are dedicated for assay of l-lactate in blood and serum and their analytical performance is optimized for these matrices. In case of BIOSEN_C line sport 20 μL of sample is added to 1 mL of buffer in test tube purchased by the producer. Fifteen test tubes are placed in the apparatus and the measurement is done automatically. Time of one measurement is about 15 s. One biochip can be used at least for 50 days or 6000 measurements. In case of LactatProfi 3000 5 μL of sample is sucked by a capillary which is then placed in the apparatus. The sample is sucked from capillary to chamber where the biochip is placed and the sample is analyzed. Time of measurement is about 2 min. One biochip can be used for 15 days or 400 measurements.

Sensytec 1 (Tectronik, Italy) use the screen printed electrodes with l- or d-lactate dehydrogenase. Lactates are oxidized by them according to the reactions:

\[
\text{l-lactate} + \text{NAD} \xrightarrow{\text{l-lactate dehydrogenase}} \text{pyruvate} + \text{NADH} \quad (2)
\]

\[
\text{d-lactate} + \text{NAD} \xrightarrow{\text{d-lactate dehydrogenase}} \text{pyruvate} + \text{NADH} \quad (3)
\]

The details of electrochemical reaction cannot be given while they are secret due to patent. The device is dedicated to assay lactates in wines. The biochip is dipped in 10 mL of buffer solution. After equilibration of the biochip (240 s) 200 μL of standard solution is added, after waiting 60 s sample (200 μL) is added, and after next 60 s standard solution once again. After next 60 s the result of measurement is given. The biosensors used with Sensytec 1 are disposable and can be used only for three measurements.

The samples were used as received in case of juices or centrifuged in case of pulp and must. For comparison the measurements were done for samples purified by adsorption with PVPP or polyamide 6. Amount of 100 mg of the was added to 1 mL of sample, shaken vigorously, left for 5 min and then centrifuged at 14 000 rpm for 15 min.

2.4. Microbiological analysis

Enumeration of microorganisms was done according to Polish standards: aerobic mesophilic and psychrophilic microorganisms (PN-90/A-75052/05); yeasts and moulds (PN-90/A-75052/08); lactic acid bacteria (PN-90/A-75052/07) and aerobic thermophilic bacteria (PN-90/A-75052/06, 2006).

2.5. Fermentations

Raw apple must was obtained from apples bought on local market by crushing them and pressing. Then 200 mL must was left for fermentation for 168 h at different temperatures (20 and 30 °C). For model LABs fermentations two types of bacteria were chosen: Lactobacillus plantarum and Lactobacillus brevis. Reconstructed apple juice was obtained by dissolving apple concentrate to 11.18°Brix and sterilized. 699 mL of the juice was inoculated with 1 mL of bacteria suspension. Fermentations were done in closed bottles at 20 and 30 °C for 168 h. For comparison the apple juice was inoculated with wild strains using fermented apple must as inoculum.

3. Results and discussion

Performance characteristic of BIOSEN_C line sport and LactatProfi 3000 is reported elsewhere (Przybyt and Biernasiai, 2008). The results presented there indicated that the two biosensors can be used to assay l-lactate in apple juice after some modifications. The results are more accurate when samples are purified with polyamide 6 as compared with typical adsorbent is PVPP which is used in wine and juice industry (Spagna et al., 1996; Borneman et al., 2001) and in enzymatic assay of lactate to decolorize samples. Also the disturbing influence of vitamin C which is oxidized at the electrode at the same potential as H₂O₂ (García Armada et al., 2003; Leduc and Boujaita, 2004; Wang et al., 2006) causing the apparent increase of current leading to underestimated results of measurements is partially eliminated by this adsorbent. The
application of PVPP is leading to significant increase of measured L-lactate results for both devices. PVPP dissolves to some extent in juice and most probably is electroactive. Additionally PVPP is strongly swelling in water and sedimenting slowly. Polyamide 6 has advantage as the purifying agent in practical use while it is not swelling and is sedimenting quickly so there is no need of centrifugation of samples. Important modification in measurement procedure for LactatProfi 3000 was the calibration of the device before each measurement instead every 8 h as it is indicated by the manual.

The results of performance characteristics showed that BIOSEN_C line sport is more accurate, less work and time consuming and more user friendly.

The optimization of Sensytec 1 for assay of lactates in apple juice was done by the producer of the device (Tectronik) and is not presented in this paper.

3.1. L- and D-lactate assay in apple juice concentrates

Table 1 summarizes the results of lactic acid assay by enzyme kits and biosensors at different analytical conditions. The results of L-lactate assay with enzyme kit and biosensor are in good agreement. Purification of samples with PVPP is leading to great errors in the results (Przybyt and Biernasiak, 2008). Application of polyamide 6 as purifying agent is leading to more accurate results. The greatest differences between results of enzyme kit assay and measurements with biosensors are observed for concentrates signed by the producer as 65/VII, 66/VII and A1/FB/07. These concentrates had very dark brown colour as compared with others. Probably the products of enzymatic and non-enzymatic browning could interfere with the biosensors. For D-lactate values of concentrations assayed by enzyme kits and measured with Sensytec 1 are also in good agreement. Characteristic is that concentrates with high concentration of L-lactate have also elevated level of D-lactate.

3.2. Laboratory fermentations

To check if the increase of L-lactate concentration can be connected with the growth of lactic acid bacteria the fermentation of apple must and apple juice reconstructed from concentrate were carried on. During spontaneous fermentation of apple must the growth of LABs is causing the increase of L-lactate especially at temperature 20 °C (Fig. 1) in the 4th day. The concentration of D-lactate was unchanged indicating that in this case the wild LABs are not producing this isomer of lactic acid. This could not be truth always while populations of LABs from different apple stocks could be different. Some preliminary experiments with spontaneous fermentations of samples of apple pulp and unpasteurized must indicated that sometimes also the D-lactic acid could be produced.

Also the growth of yeast during wild fermentation was observed leading to alcoholic fermentation which was predominant at temperature 30 °C. In this case after 7 days the concentration of ethanol was 17.6 g L−1. Also the production of acetic acid was observed, once again more significant at 30 °C.

The inoculation of apple juice with L. plantarum and L. brevis caused no increase of lactic acid when the initial inoculum was about 10^5 CFU mL−1. The microbiological analysis confirmed these results showing no increase of bacteria even decrease of their numbers. Only in case of L. brevis when the initial inoculum was 2.6 × 10^6 CFU mL−1 some production of L-lactic acid was observed. L. brevis was growing during first 2 days and then the number of them was decreasing. These results indicate that laboratory strains of LABs cannot adapt to apple juice environment.

The results of apple juice inoculation with wild bacteria strains were very similar to those of spontaneous fermentation of unpasteurized apple must. The increase of L-lactate concentration and

<table>
<thead>
<tr>
<th>Concentrate (symbols of the producer)</th>
<th>L-Lactate (g L−1)</th>
<th>D-Lactate (g L−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme kit</td>
<td>BIOSEN_C line sport</td>
</tr>
<tr>
<td>46/VII</td>
<td>0.052 ± 0.019</td>
<td>0.046 ± 0.002</td>
</tr>
<tr>
<td>47/VII</td>
<td>0.096 ± 0.019</td>
<td>0.080 ± 0.002</td>
</tr>
<tr>
<td>48/VII</td>
<td>0.119 ± 0.035</td>
<td>0.081 ± 0.004</td>
</tr>
<tr>
<td>64/VII</td>
<td>0.248 ± 0.013</td>
<td>0.254 ± 0.006</td>
</tr>
<tr>
<td>65/VII</td>
<td>0.884 ± 0.093</td>
<td>0.718 ± 0.021</td>
</tr>
<tr>
<td>66/VII</td>
<td>0.915 ± 0.106</td>
<td>0.652 ± 0.016</td>
</tr>
<tr>
<td>71/VII</td>
<td>0.044 ± 0.020</td>
<td>0.039 ± 0.003</td>
</tr>
<tr>
<td>T 154</td>
<td>0.039 ± 0.015</td>
<td>0.021 ± 0.004</td>
</tr>
<tr>
<td>T 156</td>
<td>0.041 ± 0.005</td>
<td>0.054 ± 0.005</td>
</tr>
<tr>
<td>T 262</td>
<td>0.033 ± 0.005</td>
<td>0.013 ± 0.001</td>
</tr>
<tr>
<td>T 348</td>
<td>0.048 ± 0.008</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>A1/FB/07</td>
<td>1.068 ± 0.018</td>
<td>0.986 ± 0.025</td>
</tr>
</tbody>
</table>

— Not detectable.

All concentrates were dissolved to 11.18 °Brix. In case of D-lactate assay with Sensytec 1 all measurements were done by internal standard method.

* Measured by internal standard method.
LABs number was not so significant because lower initial concentration of them (one order of magnitude). Also the production of ethanol and acetic acid was observed. Fig. 2 presents the evolution of l-lactate during fermentation with wild strains measured with different methods. The results indicate that biosensors can be used to follow the lactic acid fermentation in apple juice.

3.3. Application of biosensors in juice production control

Lactic acid bacteria that can cause increase of lactate concentration during apple juice production can originate from the raw material – apples. Microbiological analysis of crushed apples (apple pulp) showed presence of bacteria (mainly mesophilic aerobes), lactic acid bacteria, yeasts and moulds. The values for different microorganisms were varying in limits:

Mesophilic aerobes – from $1.5 \times 10^2$ to $4.5 \times 10^4$ CFU g$^{-1}$

Thermophilic aerobes – from less than 1 to less than $10 \times 10^1$ CFU g$^{-1}$

Lactic acid bacteria – from $4 \times 10^1$ to $5 \times 10^5$ CFU g$^{-1}$

Yeasts from $-6.5 \times 10^1$ to $5.8 \times 10^5$ CFU g$^{-1}$

Moulds – from $5 \times 10^1$ to $2 \times 10^6$ CFU g$^{-1}$.

Highest number of microorganisms was found in samples from September 2007 (freshly harvested apples), lowest from January 2007 (stored apples). Bacteriological analysis of residual water at this production step. Concentration of D-lactate in all studied samples was very similar (between 0.03 and 0.05 g L$^{-1}$) and was independent on bacterial contamination of raw material and stage of production process. The values of lactate concentrations measured by biosensors were always similar to that assayed by enzyme kit method.

The bacteriological analysis of the production process showed that pasteurization was carried out properly. Yeasts, moulds and mesophilic aerobes among them LABs were practically completely eliminated but in some cases the thermophilic aerobes were still present after pasteurization (Table 2). This phenomenon is probably caused by thermal activation of dormant spores of thermophilic aerobes originating from soil (Iciek et al., 2006). The pasteurized must is then filtrated and directed to evaporator to obtain concentrate. Most of the concentrate samples had also low concentration of l- and d-lactate corresponding to values in the must after pasteurization. Surprisingly some concentrates had very high content of l-lactate with increased amount of d-lactate (Table 1). These results indicate indirectly that there must be a source of secondary microbiological contamination after pasteurization step.

<table>
<thead>
<tr>
<th>Tables</th>
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<tbody>
<tr>
<td><strong>Table 2</strong> Examples of the results of bacteriological analysis illustrating the effectiveness of pasteurization process.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Number of mesophilic aerobes</td>
</tr>
<tr>
<td>Number of thermophilic aerobes</td>
</tr>
<tr>
<td>Number of yeasts</td>
</tr>
<tr>
<td>Number of moulds</td>
</tr>
<tr>
<td>Number of lactic acid bacteria</td>
</tr>
<tr>
<td>D-Lactate (g L$^{-1}$)</td>
</tr>
<tr>
<td>L-Lactate (g L$^{-1}$)</td>
</tr>
</tbody>
</table>

**Fig. 2.** L-Lactate concentration during fermentation of apple juice inoculated by wild strains at temperature 20°C.

**Fig. 3.** Dependence of l-lactate concentration in apple pulp on number of lactic acid bacteria (l-lactate assayed by enzyme kit).
4. Conclusions

- The commercial lactate biosensors could be used to measure lactate concentration in juices during production of apple concentrate and in apple juices shortening the time of analysis and lowering costs.

- The apparent value of l-lactate measured by biosensors is influenced by interferents present in juices (vitamin C and polyphenols).

- The results of lactate measurements with commercial biosensors could be improved by purification of samples with polymamide 6.

- The l-lactate concentration in raw material (apple pulp) could be correlated with contamination with lactic acid bacteria.

- The process of pasteurization eliminates bacterial contamination with exception of thermophilic aerobes.

- The critical points in apple concentrate production are: washing of apples (important from soil bacteria contamination point of view) and filtering of juice after pasteurization. At this point on vacuum filter there is possibility of secondary bacterial contamination.

- The increase of l-lactate in apple juice during production process and storage could be used as indicator of juice spoilage by LABs and could be monitored with biosensors.

- The final choice of the device (biosensor) by the future user (juice producing company) would be defined by its particular demands (simplicity of the measurements, possibility of usage at line) and economical impact (price of the device and consumables).

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