Food Chemistry 169 (2015) 197–202

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A microbial biosensor based on *Lactobacillus delbruecki* sp. bacterial cells for simultaneous determination of lactic and pyruvic acid

Erhan Canbay *, Alper Habip, Goknur Kara, Zeynep Eren, Erol Akyilmaz

Department of Biochemistry, Faculty of Science, Ege University, 35100 Bornova-İzmir, Turkey

ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form 26 June 2014
Accepted 30 July 2014
Available online 7 August 2014

Keywords:
Microbial biosensor
Lactate
Pyruvate
Polypyrrole
Lactobacillus delbruecki sp.

ABSTRACT

The aim of this study was mainly to develop a microbial biosensor for the simultaneous determination of lactic acid and pyruvic acid. In developing biosensor, lyophilised *Lactobacillus delbruecki* sp. bacterial cells were immobilised with polypyrrole on a platin electrode surface using electropolymerization method. Lactate concentration was determined based on the differences in amperometric responses at cathodic peak (+0.2 V) of potassium ferricyanide, whereas pyruvate concentration was determined using the differences at anodic peak (+0.1 V). The response of biosensor showed linearity between 0.1 and 1.0 mM for both of two substrates. Optimisation studies were carried out for amount of microorganism, pyrrole concentration, pH and temperature. In the characterisation studies, substrate specificity, interference effect of some substances on the biosensor response, and storage stability were established.

1. Introduction

In the development of biosensors biologically active materials such as enzymes, cells, plant and animal tissue have been mostly used. Because of their high specific activities and analyte sensitivities, enzymes are mostly used in the biosensor construction. But most of enzymes used in the microbial biosensor construction are unstable and cost affair for routine analysis of the specific substances (Akyılmaz & Dinçkaya, 2005). On the other hand, whole cell microbial biosensors have received recent attention; because enzyme purification is unnecessary, whole cell microbial sensors are simple and inexpensive systems to construct the biosensors and enzymes are usually more stable in their natural environment in the cell (Reshetilov et al., 1997). In addition their short responsive time makes them suitable for online and in time field monitoring (D'Souza, 2001; Lei, Chen, & Mulchandani, 2006).

Lactic acid is one of the most studied analytes since its concentration is an important parameter for many applications in food (Zaydan, Dion, & Boujtita, 2004) and beverages industry (De Luca et al., 2005), as well as clinical diagnostics (Burmeister, Palmer, & Gerhardt, 2005) and sports medicine (Volpe, Moscone, Compagnone, & Palleschi, 1995). Pyruvate is a key intermediate in the glycolytic and pyruvate dehydrogenase pathways, which are involved in biological energy production. It may also have cardiac and skeletal muscle inotropic activity as well as bariatric activity, and also antioxidant activity (Stanko, Tietze, & Arch, 1992). Pyruvate may help some obese individuals lose weight. There is also the suggestion in current research that it might help some overweight individuals lower their blood pressure, and it may favourably modify lipid profiles (Kalman, Colker, Wilets, Roufs, & Antonio, 1999; Stanko, Reynolds, Hoyson, Janosky, & Wolf, 1994). Many methods have been reported for lactate and pyruvate determination, such as chromatographic and spectrometric analysis (Barıskaner et al., 2003; Fernandes, Relva, Da Silva, & Freitas, 2003; Posner, Li, Bethell, Tsuji, & Benkovic, 1996; Wulkan, Verwers, Neele, & Mantel, 2001). However, these methods are relatively expensive, time consuming, complex to perform and require laborious sample pre-treatment. Thus, there is an increasing demand for inexpensive, rapid and reliable methods for lactate and pyruvate determination.

Lactate dehydrogenase (LDH) is an enzyme present in a wide variety of organisms, including plants and animals. It catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. Lactate biosensors can be based on two enzymes, either lactate oxidase or lactate dehydrogenase (Laval, Bourdillon, & Moiroux, 1984; Wang, Xu, & Chen, 2006; Yang, Atanasov, & Wilkins, 1999). On the other hand, there are only a few microbial biosensors for lactic acid based on baker's yeast *Saccharomyces cerevisiae* (Garjonyte & Malinauskas, 2003; Garjonyte, Melvydas, & Malinauskas, 2006, 2008) genetically modified *Hansenula polymorpha* yeast cells (Shkil et al., 2009), *Lactobacillus bulgaricus* and *Streptococcus thermophilus* mixed culture (Chen & Jin, 2011). There is not any study based on microbial biosensor for pyruvate determination.

* Corresponding author. Tel./fax: +90 232 3115485.
E-mail address: erhancanbay87@gmail.com (E. Canbay).

http://dx.doi.org/10.1016/j.foodchem.2014.07.140
0308-8146/

In this study, a novel microbial biosensor method has been developed based on *Lactobacillus delbruecki* bacteria for simultaneous determination of lactate and pyruvate. For this purpose, *L. delbruecki* was used as a biomaterial because it has either lactate or pyruvate metabolism and also lactate dehydrogenase enzyme, providing lactate–pyruvate conversion.

2. Experimental

2.1. Chemicals

Potassium ferricyanide, polypyrrole, glutaraldehyde (2.5%), sodium lactate, sodium pyruvate and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions used in the experiments were prepared just before their use.

2.2. Apparatus

In the experiments PalmSens potentiostat (Palm Instruments, Houten, Netherlands), a three-electrodes system from CH Instruments (USA) that contains a CHI 102 model platinum working electrode, a CHI 111 model Ag/AgCl reference electrode and a CHI 115 model platinum wire counter electrode, Gilson P100 and P1000 automatic pipettes (France), Yellow-Line magnetic stirrer (Germany) and Nuve model thermostat (Turkey) were used.

2.3. Culture medium of L. delbruecki cells

L. delbruecki is a gram-positive rod that may appear long and filamentous. This bacterium is regarded as aciduric or acidophilic, since it requires a low pH (around 4.6–5.4) to grow effectively (Axelsson, 1998).

L. delbruecki was grown in potato dextrose broth (PDB) at 30 °C for 18 h. The bacteria strain was cultured in 250 ml of erlenmeyer flasks containing of 50 ml fermentation medium (PDB) consisting of (g/l): 4 potato extract and 20 g dextrose, at pH 5.5. Lactic acid was added to the fermentation medium at the concentration of 10 μg mL^{-1} after membrane filter sterilisation. The erlenmeyer flasks were inoculated with 1% (v/v) of 18 h old *L. delbruecki* cells and flasks were kept on a rotary incubator shaker at 30 °C with agitation (1500 rpm) for 12 h. The culture medium contains agar extract (3.0 g L^{-1}), $(NH_4)_2SO_4$ (10.0 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g L^{-1}), $CaCl_2$ (0.1 g L^{-1}), peptone (0.5 g L^{-1}), KCl (1.0 g L^{-1}) and lactic acid (20 mg L^{-1}). After 12 h of fermentation period that is the log phase of cell growth, culture medium was centrifuged at 5000*g* for 15 min. The cells were resuspended with physiological saline water for two times and recentrifuged with the same condition. At the end of the process the cells of *L. delbruecki* were lyophilised and used for the preparation of amperometric microbial biosensor for simultaneous determination of lactic acid and pyruvic acid.

2.4. Preparation of the biosensor

Before the immobilisation of microorganism on the surface of the electrode, Pt electrode was polished with Gamma alumina 1.0 and 0.1 μm, respectively on microfiber cloth. Then Pt electrode was rinsed with double distilled water and it was sonicated to remove adsorbed particles first in ethanol solution (96%) and then in double distilled water for 10 min. After that, for the electrochemical cleaning the surface was cleaned by fifteen successive cyclic voltammetric sweeps between −1.0 and +1.0 V potentials in the 0.1 M HCl.

0.4 M pyrrole solution was prepared in 0.1 M KCl solution for the electropolymerization experiment. 2.5 mg microorganism, 300 μl (50 mM, pH: 5.5) citrate buffer and 30 μl 2.5% glutaraldehyde solution were added and mixed in an eppendorf tube. This mixture was added to the pyrrole solution. The triplet electrode system was submerged into this solution found in reaction cell and it was provided to occur a layer on the surface of the electrode with amperometric screening for 45 s at 0.7 V (Guerrieri, De Benedetto, Palmisano, & Zambonin, 1998).

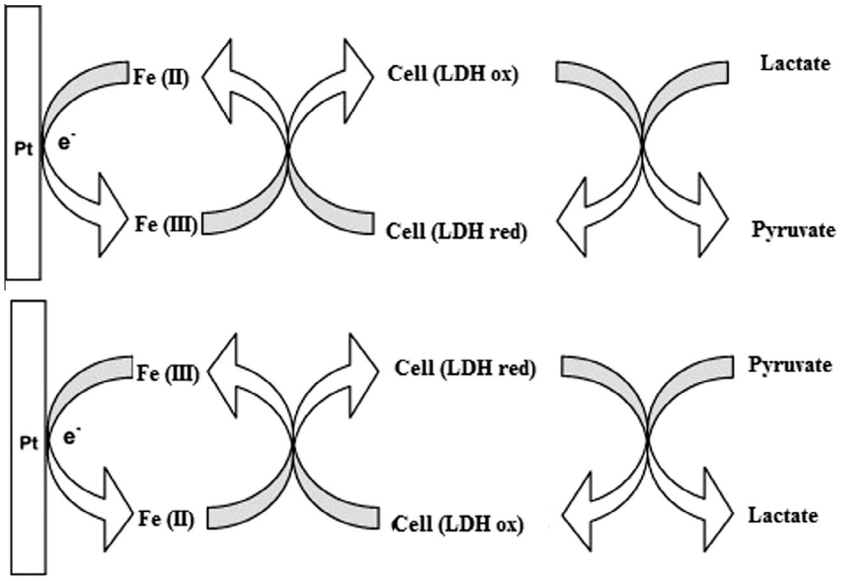
2.5. Principle of measurements

Principle of measurements was based on monitoring of amperometric responses which occurs during the reaction on the cathodic peak (+0.2 V) for determination of lactate and on the anodic peak (+0.1 V) for determination of pyruvate in the presence of potassium ferricyanide (1.0 mM) as a mediator (Scheme 1).

3. Results and discussion

3.1. Cyclic voltammograms and amperometric measurements

Cyclic voltammogram (CV) is an important method that could show electrochemical interaction and also differences. So, the bio-



Scheme 1. Suggested mechanism of the microbial biosensor.

sensor was characterised by using this method during preparation period in the absence and presence of lactate and pyruvate. CV measurements were made between −0.6 V and +0.6 V potential range. CV responses of different electrodes that are a bare Pt electrode, Pt/PPy and Pt/PPy-MO (microorganism) electrodes were examined in presence and absence of substrates in 0.05 M citrate buffer (pH 5.5) containing 1.0 mM potassium ferricyanide at a scan rate of 50 mV s^{-1}(Supporting information Figs. S1A and S1B). Where as the bare Pt electrode shows redox peak with lower background current, Pt/PPy and Pt/PPy-MO electrodes show also redox peaks of potassium ferricyanide with higher background current. Here, it can be clearly seen that the formation of polypyrrole on the electrode surface has greatly enhanced the current with defined oxidation and reduction peaks. In the presence of the substrates, the cyclic voltammogram of Pt/PPy-MO showed detectable anodic current response for lactate and cathodic current response for pyruvate whereas no detectable signal was observed for bare Pt and Pt/PPy electrodes. The results indicated clearly the enzymatic reaction according to *L. delbruecki* which immobilised on the electrode surface. A pair of well defined redox peaks' responses were observed at 0.1 V and −0.1 V potentials for lactate and +0.2 V and −0.15 V potentials for pyruvate, respectively. Peak potential separation (ΔEp) was nearly 100 mV and 200 mV lactate and pyruvate biosensors, respectively. These results strongly indicated a surface controlled electrochemical process for lactate but a diffusion controlled electrochemical process for pyruvate.

In order to detect the most suitable applied potential for amperometric measurements some experiments were made at different applied potentials between 0.0 V and 0.5 V potentials region for both substrates (Supporting information Fig. S2). The most suitable and highest responses were obtained at +0.2 V potential for lactate and +0.1 V potential for pyruvate. Fig. 1 shows the results obtained from the experiments. According to the figure, the microbial biosensor gave more clear response to lactic acid than pyruvate. It was expected because of the nature of microorganism, *L. delbruecki* sp. It is a lactic acid bacteria and its adaptation to lactic acid is more preferred level than pyruvate. In addition, diffusion controlled electrochemical process for pyruvate can be effective in obtaining this result.

3.2. Optimisation of bioactive layer

3.2.1. Detection of the effect of the microorganism amount on the biosensor response

To detect the effect of microorganism amount on the biosensor response, different amount of the microorganism were used in the biosensor preparation. For this purpose three different amount of microorganism were selected (0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml). Results obtained from the experiments are given in Supporting information Fig. S3. According to the results, the highest biosensor responses and the most suitable calibration curves were obtained when 0.25 mg/ml microorganism amount was used in the biosensor preparation for both substrates. 0.125 mg/ml was not so efficient to obtain good results because at this concentration the biosensor response time is longer than the others. In addition when the biosensor that prepared with a 0.5 mg/ml microorganism concentration was used, responses were decreased for both lactate and pyruvate. Because, the increase in the amount of microorganism on the biosensor surface caused a diffusion barrier.

3.2.2. Investigation of polypyrrole concentration on biosensor responses

In electropolymerization methods monomer concentration is an important factor which influences the development of the polymer on the electrode surface. A constant potential of 0.7 V vs Ag/AgCl was applied in the electropolymerization of pyrrole. Three biosensors were prepared by using different pyrrole concentrations (0.2, 0.4 and 0.8 M) for the investigation of the effect of pyrrole concentration on the biosensor responses (Supporting information Fig. S4). The most suitable calibration curves were obtained by the microbial biosensor prepared with electropolymerization of 0.4 M pyrrole for both of two substrates. There were not any significant signals neither lactate nor pyruvate when using the biosensor prepared by using below and above this concentration. Lower pyrrole concentration did not allow sufficient polymer formation and cells entrapment on to the electrode surface. Using of higher concentration of pyrrole resulted in very low responses by the biosensor.

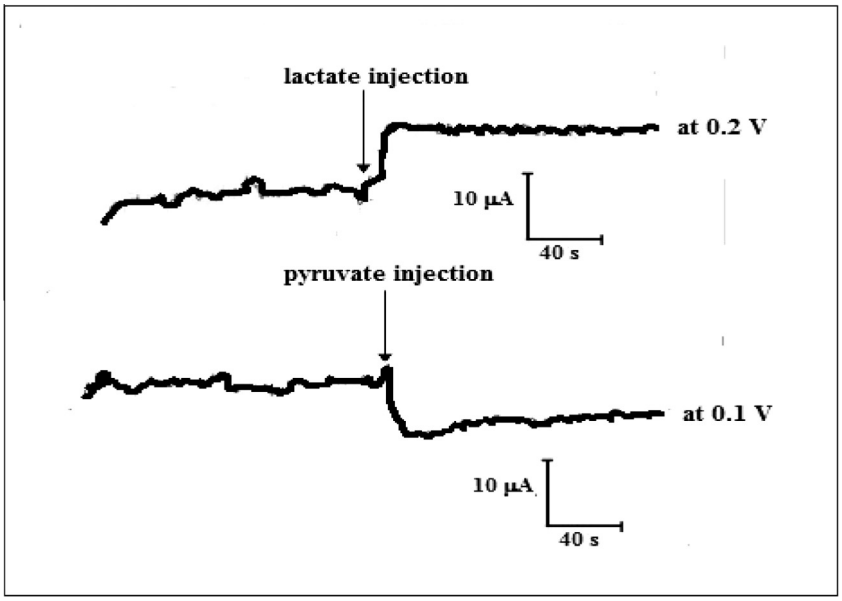


Fig. 1. Amperometric responses for 0.5 mM lactate and pyruvate added in 50 mM pH: 5.5 citrate buffer containing 1.0 mM potassium ferricyanide. Applied potential is +0.2 V for lactate and +0.1 V for pyruvate vs Ag/AgCl.

3.3. Optimisation of working conditions

3.3.1. Working temperature and pH effect on the biosensor responses

In order to investigate the working temperature, different biosensor systems were prepared and used at various temperatures such as 20, 25, 30, 35 and 40 °C. From the results, maximum biosensor responses were observed at 35 °C for pyruvate and 40 °C for lactate. Thermal stability of the enzymes decreases at high temperatures and also the bacteria used in the construction of the microbial biosensor is not a thermophilic bacteria so optimum temperature was chosen as 35 °C for both substrates.

Optimisation of pH level is the one of the most important parameter for biosensor studies. Some performance factors of the biosensor generally are affected with pH. To detect this effect different buffer solutions at various pH values (4.5, 5, 5.5, 6 and 6.5) were prepared and used in the experiments. According to the results, optimum pH value was found to be 5.5 for both lactate and pyruvate. This result was expected and arised from the nature of the acidic bacteria, *L. delbruecki*. In addition pH value of the culture medium of the bacteria is 5.5.

3.4. Analytical characteristics of biosensor

3.4.1. Linear range for lactate and pyruvate

After optimisation of the bioactive surface and working conditions of the biosensor we obtained a calibration curve for pyruvate and lactate under optimised conditions (Fig. 2). From the figures it can be said that responses of the biosensor depend linearly on both pyruvate and lactate concentration between 0.1 and 1.0 mM. Detection limits of the biosensor were calculated to be 0.012 mM for lactate and 0.018 mM for pyruvate.

3.4.2. Reproducibility

The reproducibility experiments of the biosensor were made for 0.5 mM of pyruvate and lactate ($n = 10$). From the experiments, average value, standard deviation (SD) and coefficients of variation (CV%) were calculated as 0.506 mM, ±0.015, and 3.07% for pyruvate and 0.493 mM, ±0.013, and 2.59% for lactate. It is obvious that both of two substrates can be determined simultaneously and sensitively by the microbial biosensor.

Table 1
Substrate selectivity of the microbial biosensor.

Substrate[a]	Biosensor response (%)	Substrate[b]	Biosensor response (%)
Pyruvate	100	Lactate	100
Ascorbate	60.6	Ascorbate	62.7
Aspartate	18.7	Aspartate	10.9
Glucose	15.7	Glucose	12.3
Lactate	13.2	Pyruvate	13.6

Averages of three measurements.
[a] For pyruvate.
[b] For lactate.

3.4.3. Substrate selectivity

In order to detect the substrate selectivity of the microbial biosensor, some experiments were made by using 0.5 mM pyruvate, lactate and various substances such as L-ascorbic acid, L-aspartic acid, and glucose. Experiments were done at +0.1 V potential for pyruvate and +0.2 V potential for lactate. The microbial biosensor responses obtained for pyruvate (at +0.1 V) and lactate (at +0.2 V potential) were accepted as %100 and compared to the biosensor obtained for the other substances. Results obtained from the experiments were given in Table 1.

The results indicated that the microbial biosensor responded to all substances because of inclusion of various metabolic process and enzymes of the microorganism (Axelsson, 1998). But compared with the substrates the maximum responses were obtained at +0.1 V potential for pyruvate and at 0.2 V potential for lactate.

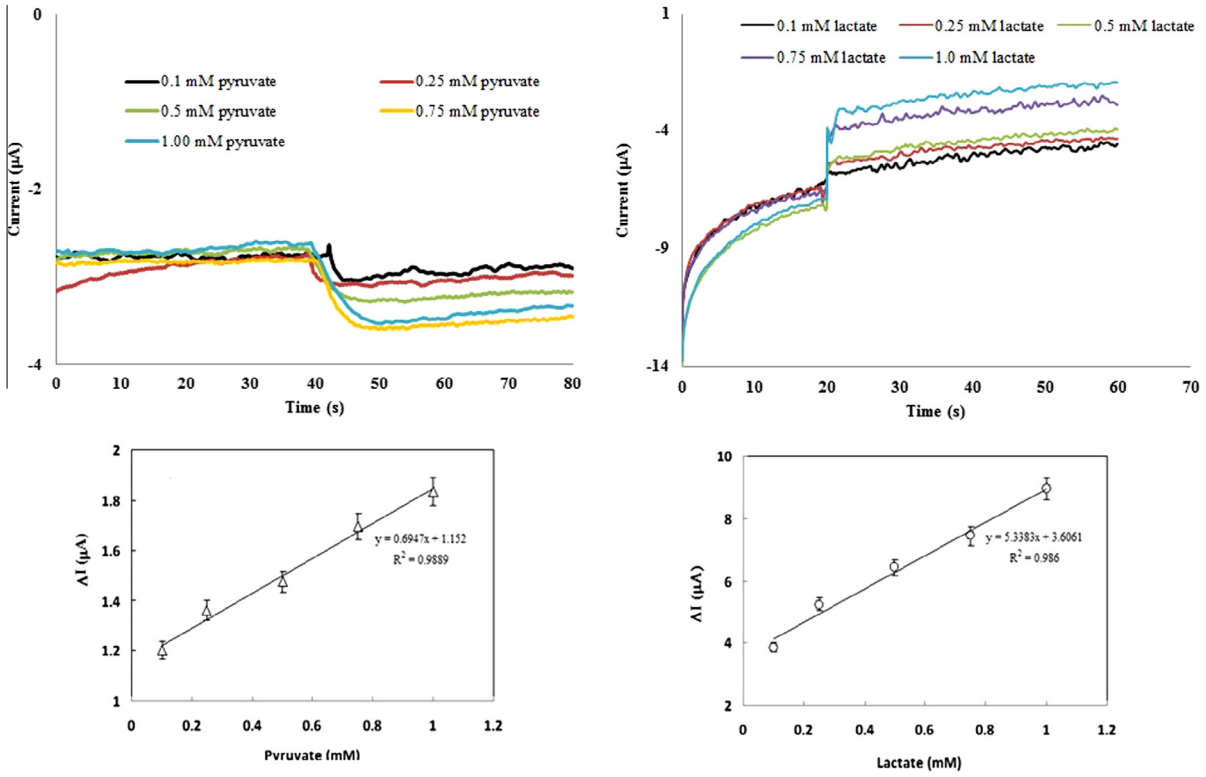


Fig. 2. Amperometric responses and calibration curves for linear range experiment (50 mM pH 5.5 citrate buffer containing 1.0 mM potassium ferricyanide, $T = 35$ °C).

Table 2
Determination of interference effects of some compounds.

Substrate[a]	Biosensor response (%)	Substrate[b]	Biosensor response (%)
Pyruvate	100	Lactate	0
Pyruvate + ascorbate	144.9	Lactate + ascorbate	140.5
Pyruvate + aspartate	101.8	Lactate + aspartate	104.8
Pyruvate + glucose	104.7	Lactate + glucose	103.5
Pyruvate + lactate	108.3	Lactate + pyruvate	108.2

Averages of three measurements.
[a] For pyruvate.
[b] For lactate.

Table 3
Determination results of L-lactate in real samples using two methods.

Sample	Determined by reference method (mM) (Sigma–Aldrich Assay kit)	Determined by biosensor (mM)	Recovery rate (%)
Buttermilk 1	1.2	1.19	99.2
Buttermilk 2	0.9	0.92	102.2
Kefir 1	2.11	2.13	101.4
Kefir 2	1.96	1.98	101.02
Milk 1	0.13	0.12	92.3
Milk 2	0.16	0.15	93.7

Averages of three measurements.

3.4.4. Interference effects of some compounds on the microbial biosensor responses

To investigate the interference effects of L-ascorbic acid, L-aspartic acid and glucose on the microbial biosensor response 0.5 mM of the substances were added to 0.5 mM of lactate or pyruvate standards and the effects of these substances in the presence of pyruvate (at +0.1 V potential) and lactate (at +0.2 V potential) were investigated by the microbial biosensor. Responses obtained for pyruvate (at +0.1 V) and lactate (at +0.2 V) potentials were accepted to be %100 and compared with the responses in the presence of other substances. From the experiments it can be said that substances showed much less interference effects when they are together with pyruvate and lactate. A negligible effect of lactate was observed in biosensor responses at 0.1 V potential for pyruvate determination. In addition a little effect of pyruvate was detected in for lactate determination responses at +0.2 V potential. The highest interference effects were observed with L-ascorbic acid for both of two substrates but it was expected because of its universal interference capacity. The results obtained from the experiments were given in Table 2.

3.5. Application

In this section of the study the lactate content of some drinks were detected by using the biosensor. The results obtained from the biosensor were compared to results obtained using a reference procedure for the same samples in order to complete the validation of the new method. For this goal, some drinks such as milk, buttermilk and kefir which contains different quantity of lactate, were used. Results obtained from the experiments were given in Table 3. From the experiments when we compare the results of two methods it can be said that lactate in the drinks can be determined sensitively by using the biosensor.

3.6. Comparison of the microbial biosensor

As can be seen from Table 4 it is obvious that there is not any biosensor developed for simultaneous determination of lactate and pyruvate so it can be said that the microbial biosensor developed is novel and original. By using the microbial biosensor, sensitive and fast determination of lactate and pyruvate can be done. Table 4 shows some analytical parameters of various biosensors developed for lactate or pyruvate determination.

4. Conclusions

The aim of this study is mainly to develop a microbial biosensor intended for simultaneous determination of lactate and pyruvate. Preparation of the microbial biosensor is very easy and the response time is only 3s. Using the cells as a biocomponent which has metabolic activity for lactate and pyruvate in biosensor construction give an advantage for simultaneous determination of lactate and pyruvate. Consequently, the simplicity in the bioactive material immobilisation demonstrates a considerable economic advantage because of low cost, which is desirable in routine analysis. Response time (3s) supports that there is not any diffusion resistance for the microbial biosensor and this means that the diffusion of substrates into the cell and also assimilation of substrate is not a rate – limiting process. When we consider detection limits (0.012 mM for lactate and 0.018 mM for pyruvate), substrate selectivity and reproducibility of the microbial biosensor, it can be said the microbial biosensor system developed is a simple, suitable and fast method for simultaneous determination of lactate and pyruvate.

Acknowledgements

We acknowledge the financial support from the The Scientific and Technological Research Council of Turkey (TUBITAK) 2209 – Scientific research program for college students.

Table 4
Analytical parameters of some biosensors developed for the determination of lactate or pyruvate.

Biosensor type	Modification	Linear range	Analyte	Detection limit	Refs.
Lactate dehydrogenase based amperometric	Multiwall carbon nanotubes	0.1–10 mmol/L	Lactate	7.5×10^{-6} mol/L	Pereira, Aguiar, Kisner, Macedo, and Kubota (2007)
Lactate oxidase based amperometric	Conducting copolymer bound lactate oxidase	0.1–5.5 mmol/L	Lactate	1×10^{-3} mol/L	Suman, Singhal, Sharma, Malthotra, and Pundir (2005)
Lactate oxidase based amperometric	Immobilisation on sol–gel matrixes	0.05–5 mmol/L	Lactate	1×10^{-5} mol/L	Gomes et al. (2007)
Pyruvate oxidase based amperometric	Immobilised in gelatin	0.0025–0.05 μmol/L	Pyruvate	2.5×10^{-5} mol/L	Akyilmaz and Yorganci (2007)
Lactobacillus delbruecki sp. based microbial	Microorganism immobilised with electropolymerization of polypyrrole	0.1–1.0 mmol/L	Lactate and pyruvate	1.2×10^{-5} mol/L for lactate and 1.8×10^{-5} mol/L for pyruvate	This work

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.07.140.

References

Akyılmaz, E., & Dinçkaya, E. (2005). An amperometric microbial biosensor development based on *Candida tropicalis* yeast cells for sensitive determination of ethanol. *Biosensors & Bioelectronics, 20*, 1263–1269.

Akyilmaz, E., & Yorganci, E. (2007). Construction of an amperometric pyruvate oxidase enzyme electrode for determination of pyruvate and phosphate. *Electrochimica Acta, 52*, 7972–7977.

Axelsson, L. (1998). *Lactic acid bacteria: Microbiology and functional aspects* (2nd ed.). New York: Marcel Dekker Inc.

Bariskaner, H., Ustun, M. E., Ak, A., Yosunkaya, A., Dogan, N., & Gurbilek, M. (2003). Effects of deferoxamine on tissue lactate and malondialdehyde levels in cerebral ischemia. *Methods and Findings in Experimental and Clinical Pharmacology, 25*, 371–376.

Burmeister, J. J., Palmer, M., & Gerhardt, G. A. (2005). L-lactate measures in brain tissue with ceramic-based multisite microelectrodes. *Biosensors & Bioelectronics, 20*, 1772–1779.

Chen, J., & Jin, Y. (2011). Sensitive lactate determination based on acclimated mixed bacteria and palygorskite co-modified oxygen electrode. *Bioelectrochemistry, 80*, 151–154.

De Luca, S., Florescu, M., Ghica, M. E., Lupu, A., Palleschi, G., Brett, C. M. A., et al. (2005). Carbon film electrodes for oxidase-based enzyme sensors in food analysis. *Talanta, 68*, 171–178.

D'Souza, S. F. (2001). Microbial biosensors. *Biosensors & Bioelectronics, 16*, 337–353.

Fernandes, L., Relva, A. M., Da Silva, M. D. R. G., & Freitas, A. M. C. (2003). Different multidimensional chromatographic approaches applied to the study of wine malolactic fermentation. *Journal of Chromatography A, 995*, 161–169.

Garjonyte, R., & Malinauskas, A. (2003). Investigation of baker's yeast *Saccharomyces cerevisiae*- and mediator-based carbon paste electrodes as amperometric biosensors for lactic acid. *Sensors and Actuators, B: Chemical, 96*, 509–515.

Garjonyte, R., Melvydas, V., & Malinauskas, A. (2006). Mediated amperometric biosensors for lactic acid based on carbon paste electrodes modified with baker's yeast *Saccharomyces cerevisiae*. *Bioelectrochemistry, 68*, 191–196.

Garjonyte, R., Melvydas, V., & Malinauskas, A. (2008). Effect of yeast pretreatment on the characteristics of yeast-modified electrodes as mediated amperometric biosensors for lactic acid. *Bioelectrochemistry, 74*, 188–194.

Gomes, S. P., Odložilíková, M., Almeida, M. G., Araújo, A. N., Couto, C. M. C. M., & Montenegro, M. C. B. S. M. (2007). Application of lactate amperometric sol–gel biosensor to sequential injection determination of L-lactate. *Journal of Pharmaceutical and Biomedical Analysis, 43*, 1376–1381.

Guerrieri, A., De Benedetto, G. E., Palmisano, F., & Zambonin, P. G. (1998). Electrosynthesized non-conducting polymers as permselective membranes in amperometric enzyme electrodes: A glucose biosensor based on a co-crosslinked glucose oxidase/overoxidized polypyrrole bilayer. *Biosensors & Bioelectronics, 13*, 103–112.

Kalman, D., Colker, C. M., Wilets, I., Roufs, J. B., & Antonio, J. (1999). The effects of pyruvate supplementation on body composition in overweight individual. *Nutrition, 15*, 337–340.

Laval, J. M., Bourdillon, C., & Moiroux, J. (1984). Enzymic electrocatalysis: Electrochemical regeneration of NAD^+ with immobilized lactate dehydrogenase modified electrodes. *Journal of the American Chemical Society, 106*, 4701–4706.

Lei, Y., Chen, W., & Mulchandani, A. (2006). Microbial biosensors. *Analytica Chimica Acta, 568*, 200–210.

Pereira, A. C., Aguiar, M. R., Kisner, A., Macedo, D. V., & Kubota, L. T. (2007). Amperometric biosensor for lactate based on lactate dehydrogenase and Meldola Blue coimmobilized on multi-wall carbon-nanotube. *Sensors and Actuators, B: Chemical, 124*, 269–276.

Posner, B. A., Li, L. Y., Bethell, R., Tsuji, T., & Benkovic, S. J. (1996). Engineering specificity for folate into dihydrofolate reductase from *Escherichia coli*. *Biochemistry, 35*, 1653–1663.

Reshetilov, A. N., Iliasov, P. V., Donova, M. V., Dovbnya, D. V., Boronin, A. M., Leathers, T. D., et al. (1997). Evaluation of a *Gluconobacter oxydans* whole cell biosensor for amperometric detection of xylose. *Biosensors & Bioelectronics, 12*, 241–247.

Shkil, H., Stoica, L., Dmytruk, K., Smutok, O., Gonchar, M., Sibirny, A., et al. (2009). Bioelectrochemical detection of L-lactate respiration using genetically modified Hansenula polymorpha yeast cells overexpressing flavocytochrome b2. *Bioelectrochemistry, 76*, 175–179.

Stanko, R. T., Reynolds, H. R., Hoyson, R., Janosky, J. E., & Wolf, R. (1994). Pyruvate supplementation of a low-cholesterol, low-fat diet: Effects on plasma lipid concentrations and body composition in hyperlipidemic patients. *American Journal of Clinical Nutrition, 59*, 423–427.

Stanko, R. T., Tietze, D. L., & Arch, J. E. (1992). Body composition, energy utilization, and nitrogen metabolism with a 4.25-MJ/d low-energy diet supplemented with pyruvate. *American Journal of Clinical Nutrition, 56*, 630–635.

Suman, S., Singhal, R., Sharma, A. L., Malthotra, B. D., & Pundir, C. S. (2005). Development of a lactate biosensor based on conducting copolymer bound lactate oxidase. *Sensors and Actuators, B: Chemical, 107*, 768–772.

Volpe, G., Moscone, D., Compagnone, D., & Palleschi, G. (1995). In vivo continuous monitoring of L-lactate coupling subcutaneous microdialysis and an electrochemical biocell. *Sensors and Actuators, B: Chemical, 24*, 138–141.

Wang, K., Xu, J. J., & Chen, H. Y. (2006). Biocomposite of cobalt phthalocyanine and lactate oxidase for lactate biosensing with MnO_2 nanoparticles as an eliminator of ascorbic acid interference. *Sensors and Actuators, B: Chemical, 114*, 1052–1058.

Wulkan, R. W., Verwers, R., Neele, M., & Mantel, M. J. (2001). Phylogenic study of denaturation of lactate dehydrogenase isoenzymes from different species by high and low temperature. *Annals of Clinical Biochemistry, 38*, 554–558.

Yang, Q., Atanasov, P., & Wilkins, E. (1999). Needle-type lactate biosensor. *Biosensors & Bioelectronics, 14*, 203–210.

Zaydan, R., Dion, M. M., & Boujtita, M. M. (2004). Development of a new method, based on a bioreactor coupled with an L-lactate biosensor, toward the determination of a nonspecific inhibition of L-lactic acid production during milk fermentation. *Journal of Agriculture and Food Chemistry, 52*, 8–14. http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/1/mak064bul.pdf.