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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

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The development and characteristics of a bioreactor employing bacteria (*Streptococcus thermophilus*) encapsulated in Ca-alginate beads coupled with an L-lactate biosensor are reported. The biosensor comprises a carbon paste electrode modified with enzymes HRP (horseradish peroxidase), LOD (lactate oxidase), and FcH (ferrocene) as redox mediator. The measurement of L-lactate is based on the signal produced by H_2O_2, the product of the enzymatic oxidation of L-lactate by LOD. The detection of H_2O_2 is performed at the electrode surface via HRP/FcH at low operating potential (−100mV vs Ag/AgCl). Optimization studies were performed using the bioreactor in conjunction with an L-lactate electrode operating in a flow injection system to assess the ability of encapsulated bacteria to ferment carbohydrate solutions. The possibility of using the developed method to assess the fermentation capability of milk samples was evaluated. Bronopol (2-bromo-2-nitro propane-1,3-diol) was chosen to simulate the effect of an inhibitory agent of milk fermentation. The obtained results indicated that the evaluation of the amount of L-lactate amount produced through the bioreactor could be used as a measure of inhibition of lactic acid production in milk samples.

KEYWORDS: Biosensor; L-lactate; milk; FIA; inhibition detection

INTRODUCTION

During the past decade, the demand for rapid devices for food analysis has increased and is still of great interest today. Various methods have been studied to develop simple and alternative approaches to food analysis. Much effort has been focused on developing enzymatic or microbial sensors (*1*−*3*), and a range of strategies had been reported for the determination of various analytes (*4*−*6*). The estimation of biological oxygen demand (BOD) was the best known example of a biosensor based on viable microorganisms using a Clark electrode as an electrochemical transducer (*7*−*8*). Because of their ability to degrade or produce several analytes, microorganisms have usually been used as biological sensing materials, combining biospecific recognition with physical transducers (*9*). The sensors based on viable microorganisms enable the metabolic activity of cells to be exploited directly for an analytical purpose. Different types of both microbial biosensors and bioreactors have thus been explored and studied for the determination of other analytes related to the metabolic activity of microorganisms (*10*, *11*). Since then, attention has been focused on ways to retain cells or microorganisms to prepare appropriate biosensors or bioreactors. With this aim, several techniques have been used: membranes, chemical reactions, electrostatic binding or encapsulation (*12*, *13*). Cell immobilization is known to constitute probably one of the most attractive methods for retaining high cell density per unit volume, thus leading to higher metabolic activity rates.

Alginate beads with entrapped *Lactococcus lactis* have already been used as a biocatalyst in continuous fermentation to study and measure the kinetic behavior of this bacterium (*14*). *Lactobacillus* cells coupled to a pH electrode have also been developed and studied (*15*) to evaluate various carbohydrates in relation to fermentation activity. In addition, it has been reported that the immobilization of *Kluyveromyces lactis* in calcium alginate beads (*16*) offers an efficient milk whey conversion and could easily be used in industrial applications. To assess the metabolic activity, the bioreactor could be coupled to a pH meter to quantify the change by using the acidification (lactic acid) of the medium (*15*). We report here a bioreactor coupled to an L-lactate biosensor for the assessment of the lactic fermentation of the encapsulated bacteria. Various enzymatic electrodes for L-lactate monitoring have been described in the literature (*17*−*19*) and different configurations have been

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proposed based on lactate oxidase (*20−24*) or lactate dehydrogenase (*25−26*). The aim of this investigation was to develop a rapid method for the detection of nonspecific inhibitory agents toward L-lactic acid fermentation. A bioreactor containing encapsulated bacteria in Ca-alginate beads coupled with an L-lactate biosensor were used in this study. Bronopol was used as an example of an inhibitory agent to simulate the inhibition of lactic fermentation in the bioreactor. The capability of the bioreactor to metabolize various carbohydrates such as glucose, lactose, and galactose was examined in the presence and absence of a bactericidal agent by using an L-lactate electrode operating in flow injection analysis mode.

First, we prepared and examined the analytical performance of the L-lactate electrode. This latter is based on a generic model that we had previously described to prepare biosensors based on the measurement of hydrogen peroxide (*27*). In these biosensors, HRP (horseradish peroxidase) and FcH (ferrocene) were employed to detect H_2O_2 produced by oxidase reactions. We then studied the feasibility of developing a bioreactor coupled with an enzyme electrode for the detection of a nonspecific inhibition of L-lactic fermentation. Finally, we examined the suitability of operating the biosensor in conjunction with the bioreactor to detect an inhibition of lactic fermentation in milk samples.

MATERIALS AND METHODS

Materials. The strain *Streptococcus thermophilus* ATCC 19258 was obtained from the Belgian Coordinated Collections of Microorganisms BCCM (Laboratorium voor Microbiologie Universiteit Gent, Belgium). Tryptone (Code A1401HA), peptone (Code A1708HA), yeast extract (Code A1202HA), and M17 agar (Code BK088HA) were obtained from Biokar Diagnostics, Beauvais, France. Glycerophosphate hydrate (Code 1.04164.5000) came from Merck, Nogent sur Marne, France. Anhydrous magnesium sulfate (Code 5833.1000), ascorbic acid (Code A-7506), lactose (Code L-3750), polyethylenimine/PEI (Code P-3143), sodium m-periodate (Code S-1147), sodium alginate (Code A-22033), and calcium chloride (Code C-3881) were supplied by Sigma, Saint Quentin Fallavier, France. Ferrocene (Code 46260) was obtained from Fluka, France. Carbon graphite (Code 9901/16855) came from Carbon Lorraine, Gennevilliers, France. HRP (Code 815 462) and LOD (Code 1 798 197) were supplied by Boehringer Mannheim, Mannheim, Germany.

Strains and Media. *Streptococcus thermophilus* ATCC 19258 was used in the preparation of the microorganism encapsulated in Ca-alginate beads. The MRS growth medium contained (per L) 2.5 g of tryptone, 2.5 g of peptic digest meat USP, 5 g peptone from soymeal papain-digested, 2.5 g of yeast extract, 5 g of meat extract, 19 g of sodium glycerophosphate hydrate, 0.25 g of anhydrous magnesium sulfate, 0.5 g of ascorbic acid and 50 g of lactose. The medium was pre-warmed to 37 °C before use. Bacterial viable counts (cfu · mL^{-1}) were determined from serial decimal dilutions and plated on M17 agar. Biomass concentration was measured by absorbance at 600 nm (Milton Roy-Spectronic 401 from Bioblock Scientific, France).

Encapsulation Procedure in Calcium Alginate. *Streptococcus thermophilus* was grown in 100 mL of M17 medium, pH 6.5 shaken at 37 °C, and then harvested at the beginning of the exponential phase of growth (DO_{600} = 1.2). The suspension was centrifuged for 10 min at 5500 rpm at 4 °C. Cell pellets were suspended in 25 mL of sodium alginate. The resulting suspension was dropped with a 10 mL syringe into a gelling bath containing 2 g/L of $CaCl_2$ in bis-tris buffer under continuous agitation. The rounded beads obtained were similar in size (average external diameter 2.7 ± 0.2 mm). Encapsulated bacteria in Ca-alginate beads were then introduced into a thermostated column, 2-cm internal diameter, 15-cm in length, to provide a dead volume of 2 mL.

Preparation of Electrodes. The HRP/LOD/FcH electrode was prepared according to the procedure described for the glucose biosensor (*27−28*). HRP (9 mg or 7900 U) was dissolved in 3 mL of $NaIO_4$ solution (1.6 mg/L prepared in distilled water) and stirred for 20 min at room temperature. The treated HRP was ultrafiltered, using an Amicon Cell with a membrane of 30-kDa cutoff, and dissolved in 800 μL of 0.05 mol/L of phosphate buffer pH 8.0. The graphite powder (400 mg) was then added to the resulting HRP mixture and stirred again for 20 min. LOD (16 mg) was dissolved in 800 μL of phosphate buffer containing 0.05% (w/w) PEI and added to the HRP/graphite mixture then stirred for an additional 30 min. The enzyme/graphite mixture was freeze-dried in order to obtain bi-enzymatic modified graphite powder. Finally, the carbon paste electrode modified with HRP/LOD/FcH was obtained by mixing, in a mortar, the resulting active powder with a given amount of paraffin oil in which FcH was already dissolved (1.3% w/w). The final carbon paste contained 24.3% of oil (w/w). The electrode comprised a plastic cartridge filled with the resulting active graphite paste. One end of the cartridge was in contact with the solution and the other was connected through a metallic piston to the potentiostat.

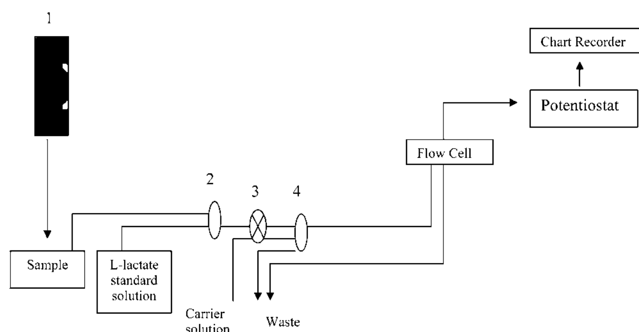

Figure 1. Schematic diagram of combined bioreactor with L-lactate biosensor system: (1) bioreactor containing bacteria encapsulated in Ca-alginate beads (2) two-way valve (3) peristaltic pump (4) injector with six-way valve.

Analysis. L-lactate measurements were performed by using a flow injection analysis system (**Figure 1**). This comprises a potentiostat (BAS model Petit Ampère CV-1B), a homemade flow cell equipped with a three-electrode system, a peristaltic pump (Ismatec), and a six-way automatic injection valve (Rheodyne). The enzyme-modified carbon paste electrode was used as the working electrode, and Ag/AgCl and stainless steel wire were used as the reference and counter-electrode, respectively. An x/t recorder was connected to the potentiostat to record the output current. The surface of the HRP/LOD/FcH-modified carbon paste electrode was polished and smoothed on clean paper before use. The working potential was fixed at −100 mV vs Ag/AgCl. A loop of 100 μL was used for the injection of samples and standard solutions. All measurements were carried out with a flow rate of 0.8 mL/min.

For the determination of the activity of the microorganisms encapsulated in the Ca-alginate beads, three injections were achieved after the incubation of carbohydrate solutions through the bioreactor. The L-lactate concentration determination in the incubated carbohydrate solutions was then performed using a calibration curve I_p (peak intensity) vs [L-lactate].

RESULTS AND DISCUSSION

Analytical Performances of LOD/HRP/FcH-Modified CPE. Biosensors offer a real possibility for real-time monitoring of metabolites. In our study, an L-lactate electrode was presented as an example of a biosensor to monitor the production of L-lactate by *S. thermophilus* through the bioreactor. A variety of biosensors have been reported for L-lactate determination and have generally used either lactate dehydrogenase (LDH) (*25−26*) or lactate oxidase (LOD) (*20−23*). However, the biosensors based on LDH have been found less sensitive for the determination of L-lactate in the μmolar range (*29−31*). For this reason, we decided to use a biosensor based on LOD, which appeared both more sensitive (μmolar range) and accurate

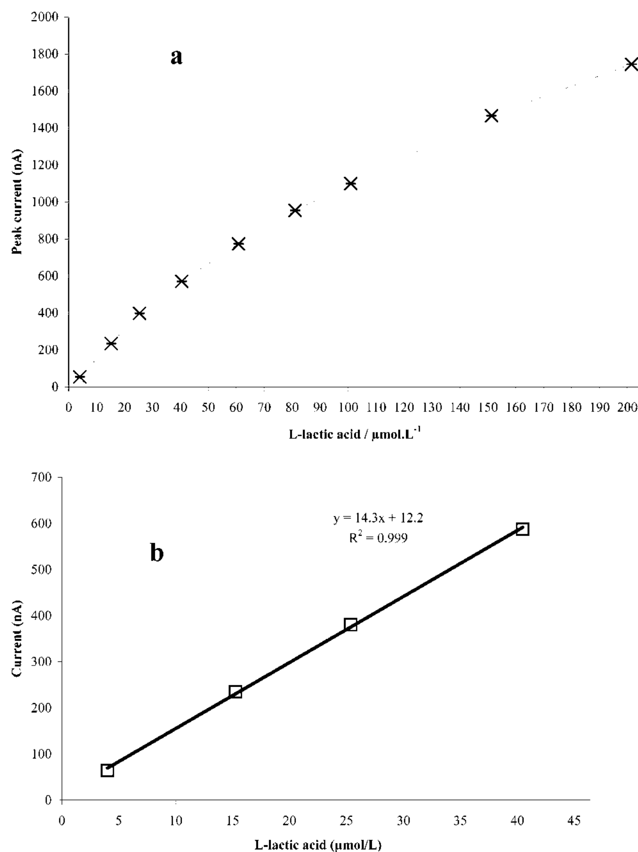

Figure 2. (a) L-lactate response of the modified electrode to various concentrations of L-lactate in standard solutions. (b) Calibration curve of the modified electrode from 4 to 40 μmoles/L.

to detect a low lactic fermentation activity. The reactions involved in the L-lactate detection can be summarized as follows: hydrogen peroxide, produced enzymatically by LOD in the presence of L-lactate, is oxidized at the electrode surface via HRP/FcH at low operating potential. The analytical performance of the resulting carbon paste electrode (CPE), modified with HRP/LOD/FcH, was examined including its repeatability, sensitivity, linear range, shelf life stability, and operational stability. The optimization of the resulting electrode was achieved by taking into account both the sensitivity and low volumes of sample (V < 2 mL). **Figure 2a** shows the amperometric response of the modified electrode to various concentrations of L-lactate in standard solutions. The electrode was calibrated using different values of flow rates. A flow rate of 0.8 mL · min^{-1} (linear velocity of 2.8 cm · s^{-1}) constituted a good compromise between sensitivity and measurement time. This flow rate was selected for further experiments. The calibration curves obtained at −100 mV vs Ag/AgCl were used to evaluate both the sensitivity (slope) and linear range. **Figure 2b** shows a linear dependence of the amperometric response to L-lactate concentration over the range from 4 to 40 μmole/L. The parameters of the calibration curve in terms of slope, intercept, and correlation coefficient were respectively 14.31 nA · μmol^{-1} · L, $b = 12.59$ nA, and $r^2 = 0.999$. The lowest quantifiable concentration of L-lactate with our electrode was determined to be around 4 μmol/L.

The repeatability was thereafter examined for the L-lactate standard solution by calculating the relative standard deviation (rsd). The obtained rsd was less than 5% for 25 successive injections of L-lactate solution (25 μmol/L). The shelf lifetime has also been examined for the HRP/LOD/FcH-modified CPE; the results of our experiments showed that the modified electrode

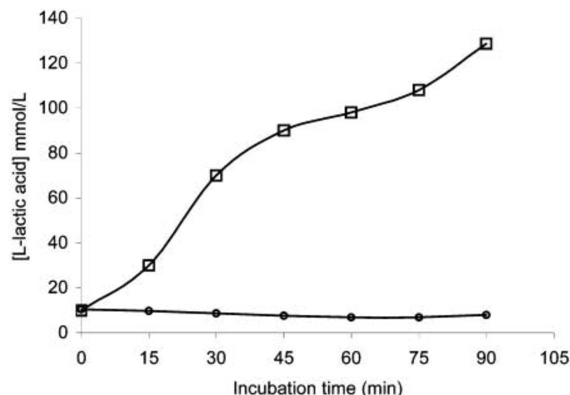

Figure 3. L-lactic acid production through Ca-alginate beads with immobilized *S. thermophilus* ATCC 19258 cells (12 × 10^6 fcu/mL) as a function of incubation time at 37 °C in tris-buffer (○) and (□) tris buffer containing lactose (49 g · L^{-1}).

remained constant for more than two months when kept in a refrigerator at 4 °C without any other specific care. After a period of two months, the sensitivity of the electrode was found to have dropped below 20%. During our experiments, a new batch of modified CPE was prepared every two weeks. The reproducibility was assessed by comparing the sensitivities obtained from the slopes of various calibration curves and was found to be about 5% ($n = 10$) intra-batches and 15% ($n = 10$) inter-batches. A reproducibility of 15% did not affect the accuracy of our results and was considered as acceptable, because a calibration step was required before each series of experiments. The low detection limit of the electrode makes the L-lactate electrode advantageous for a rapid detection of L-lactic fermentation.

Microbial Immobilization and Characterization of Bioreactor. A preliminary study of the bioreactor preparation was focused on the $CaCl_2$/Na-alginate ratio to optimize both the mechanical stability and homogeneity of the Ca-alginate beads modified with the bacteria. Spherical and homogeneous beads with a good mechanical stability were obtained by extruding the Na-alginate/cell mixture through a stainless steel needle using a manual syringe. The Na-alginate and $CaCl_2$ solutions were kept at concentrations of 2 and 0.2%, respectively, at (*32*−*33*). To avoid the swelling of Ca-alginate beads, as already described in the literature (*33*−*34*), bis-tris buffer solution with added calcium chloride was used in our experiments. The resulting beads presented an average diameter of 2.7 ± 0.1 mm with a good hardness and mechanical stability.

Evaluation of the Fermentation Activity of the Encapsulated Bacteria. The metabolic activity of encapsulated bacteria in Ca-alginate beads was examined by measuring the produced L-lactic acid through the bioreactor. First, the L-lactic fermentation activity of the encapsulated bacteria was examined by varying the incubation duration with lactose solution. Thereby, the bioreactor was washed with a continuous flow of bis-tris buffer solution with added with $CaCl_2$ without carbohydrate; this step permitted the residual L-lactate initially present in the beads to be eliminated. The bioreactor was considered washed once the L-lactic acid level in the buffer solution collected from the bioreactor became less than 4−5 μM. Then, the production of L-lactic acid was quantified in lactose solution after its incubation with the encapsulated beads. A significant amount of L-lactic acid was produced by bacteria through the bioreactor even for the short period of incubation (**Figure 3**). As was expected, the amount of L-lactic acid produced increased with the incubation period time and the L-lactic acid electrode was

found sensitive for this purpose. A duration of 30 min constituted a good compromise between the amount of L-lactic acid produced and the measurement time. No significant production of L-lactic acid was obtained when the same experiment was performed using buffer without lactose. On the other a hand, as it revealed by several authors, the presence of L-lactic acid in contact with beads could cause dissolution of Ca-alginate (*35*), but in our study, no apparent swelling of Ca-alginate beads was observed even after 1 day of experiment. This could be explained by the fact that the level of L-lactic acid was low (<0.2 mM) and would not compete significantly with alginate to complex Ca^{2+}.

Viability of the Encapsulated Cells Under the Experimental Conditions. The capability of microorganisms to grow in the beads was also examined. The number of viable cells was determined after dissolving the encapsulated bacteria in 10 mL of sterile phosphate buffer solution 0.1 M; pH = 7.0 (*38*−*38*). After total dissolution of the beads, 1 cm^3 was plated, and cell concentrations were expressed as cfu/cm^3 of the dissolution medium. Our results showed that the number of viable cells was (1.02 ± 0.21) 10^6 and (1.20 ± 0.24) 10^6 cfu/beads before and after the experiment respectively, showing a poor growth of cells inside the Ca-alginate beads. This result is in agreement with the literature (*15*)

The operational stability of the bioreactor was also evaluated. The effect of bronopol on the fermentation activity of the beads was found irreversible, so the resulting bioreactor had to be considered disposable. Concerning the storage stability, the L-lactic fermentation activity of the beads was determined over a one-week period of storage during which encapsulated bacteria activity was tested daily. The beads, stored in buffer solution at 4 °C without any other specific care, showed a continuous decrease in activity. Less than 30% of the activity remained after one week. Therefore, we decided to store the beads at 4 °C for up to 3 days. Beyond this time, a new batch of beads was prepared.

Biosensor Selectivity Toward Incubated Samples Through the Bioreactor. From an analytical point of view, the selectivity of the L-lactate electrode had to be examined toward L-lactate present in the collected sample already incubated with the beads through the bioreactors. Indeed, nonspecific metabolites could be generated during the fermentation process through the reactor, and could also interfere with the electro-enzymatic response of L-lactate. The biosensor employed in these experiments is based on a carbon paste electrode modified with HRP, LOD, and FcH. Because of the presence of both HRP and ferrocene at the electrode surface, neither hydrogen peroxide nor electro-active species (at the value of the potential used) has to be present at a significant level in the sample. The selectivity of the L-lactate electrode was achieved by examining the electrochemical response on a series of different electrodes. No electrochemical response was obtained when the incubated lactose solution was injected at a CPE modified with only HRP/FcH without LOD, while the presence of LOD at the electrode surface led to a sharp and well-defined peak (not shown) when the same incubated sample was injected. Thus, the presence of LOD in the carbon paste with both HRP and FcH was found necessary to detect L-lactate, showing the high selectivity of the biosensor toward L-lactate. In an additional experiment, the selective elimination of L-lactic acid by treating the same incubated lactose solution with a mixture of catalase and lactate oxidase showed a complete disappearance of the electrochemical signal. On the basis of these results, we concluded that L-lactate was measured selectively in collected samples from the bioreactor. These results constitute the first step in the development of a rapid method using a biosensor coupled with a bioreactor for the detection of nonspecific inhibitors toward lactic fermentation.

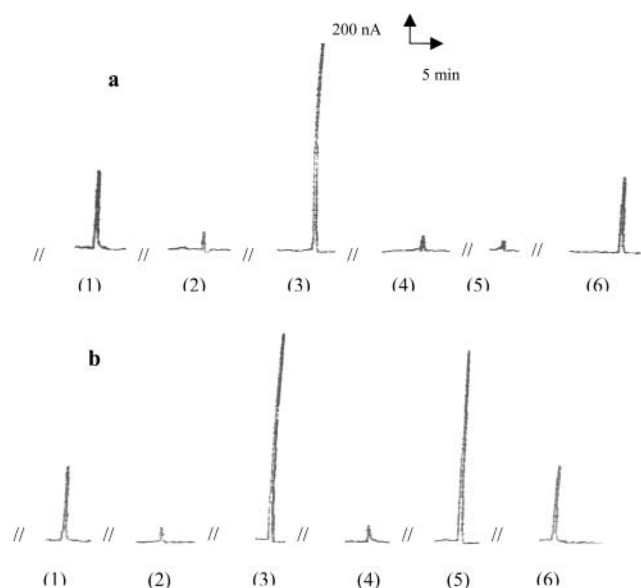

Figure 4. Typical response of electrode/bioreactor system toward an inhibition of lactic fermentation. Inhibition step was achieved using lactose solution in the presence (a) and absence (b) of bronopol. Peaks (1) and (6): amperometric response after injecting standard L-lactate solution 40 $\mu mol \cdot L^{-1}$. Peak (2), checking of washing step. Peak (3), evaluation of the L-lactic fermentation (incubation of beads with lactose solution 49 g $\cdot L^{-1}$) before the inhibition step. Peak (4), checking of washing step after inhibition step. Peak (5), evaluation of L-lactic fermentation after the inhibition step (incubation of the beads with lactose solution 49 g $\cdot L^{-1}$).

Inhibitory Effect of Bronopol Toward the Fermentation Activity of the Encapsulated Bacteria. Owing to its bactericidal effect toward *S. thermophilus* (*39*), bronopol has been widely added (0.01 and 0.5% w/w) as a preservative to stabilize milk and dairy samples during storage. In this study, bronopol was chosen as an example of an inhibitory agent to simulate the inhibition of the L-lactic fermentation. The capability of beads to produce L-lactic acid was then examined in the presence and absence of bronopol. **Figure 4a** shows the typical response of the electrode/bioreactor system toward an inhibition of lactic fermentation. Standard L-lactate solution (40 μmol/L) was injected at the beginning (peak 1), and at the end of the experiment (peak 6) to control the repeatability of the biosensor. To initiate the measurement of the degree of inhibition of bronopol on the encapsulated microorganisms, the bioreactor was washed with 25 mL of buffer solution to eliminate residual L-lactate. This washing step also permitted the microorganisms to be starved, leading to a higher yield of lactose fermentation (*15*). The bioreactor was considered as washed when the level of L-lactic acid became less than 4−5 μM in the collected sample (peak 2). The initial metabolic activity of the beads was assessed by injecting lactose solution after their incubation with the beads for 30 min into the bioreactor (peak 3). The amount of L-lactic acid in the collected sample was found to be about 200 $\mu mol \cdot L^{-1}$. To examine the inhibition effect on the L-lactic fermentation, the second washing step of the bioreactor was carried out (peak 4), and the inhibition step was then performed by incubating the beads with lactose solution added with bronopol. The degree of inhibition was calculated by comparing (eq 1) the L-lactic production in the bioreactor before (peak 3)

and after (peak 5) the inhibition step

$$\% I = [(I_3 - I_5)/I_3] \times 100 \quad (1)$$

where I_3 and I_5 are the peak intensities of peak 3 and peak 5, respectively.

The relative decrease in the peak intensities was about 95% in the case of the bioreactor exposed to the lactose solution containing bronopol, as shown in **Figure 4a**. The inhibition of the metabolic activity toward the L-lactic acid production was found irreversible, so the resulting bioreactor was considered a disposable bioreactor. The experiment was repeated more than 20 times, and on average, the degree of inhibition was found to be 100 ± 15% ($p<0.05$). We considered the production of L-lactic acid to be inhibited if the degree of inhibition is higher than 15%. There was no significant difference between the L-lactic acid production through the reactor before and after the incubation step when beads were incubated with lactose solution prepared without bronopol (**Figure 4b**). These results showed an interesting method to detect an inhibition of L-lactic production during lactose fermentation.

It should also be mentioned that no inhibitory effect of bronopol was observed on the enzymatic process of the LOD/HRP/FcH-modified CPE. However, in our study, the biosensor was not in contact with bronopol, because the inhibitory effect was indirectly detected without any contact between the sample and the electrode surface.

The effect of the amount of bronopol on the metabolic activity of the beads was also examined. The comparison of the production of L-lactic acid before and after exposing beads to increased amounts of the inhibitor showed a total inhibition when the concentration of bronopol was used at 0.05 and 0.10% respectively in the lactose solution. With a concentration of bronopol lower than 0.05% in the lactose solution, a smaller but significant inhibition (% I = 42) was obtained.

Bronopol Effect on Fermentation Activity of Encapsulated Bacteria Toward Lactose, Glucose, and Galactose. In our study, lactose, galactose, and glucose were catabolized through the bioreactor used. This result is in agreement with the literature (*40*). However, the degree of the inhibitory effect of bronopol on the fermentation activity of the modified Ca-alginate beads with *S. thermophilus* in the presence of various carbohydrates has not been previously reported. In the present work, we decided to compare the inhibitory effect of bronopol on lactic acid production when glucose, galactose, and lactose were used as a carbon source. The procedure was identical to the one used before, including washing the beads to eliminate L-lactic acid and starving the bacteria, incubation with carbohydrate solution to prove the fermentation activity, incubation with bronopol and elimination of all traces of bronopol, and finally re-incubation with the same carbohydrate to assess the inhibitory effect. A significant inhibitory effect of bronopol toward L-lactic fermentation was obtained when the beads were incubated with lactose or galactose in solution. The relative decrease in the production of acidity before and after the inhibition phase was found to be higher than 90%. In contrast, no inhibitory effect of bronopol on L-lactic production by the beads was obtained when glucose solution was used instead of lactose or galactose, even at a higher concentration of inhibitor (0.5%). In additional experiments, the use of lactose or galactose solution in the presence of glucose did not show significant inhibition of lactic acid production with bronopol (data not shown).

Bronopol is known to oxidize the thiol groups (displayed by cysteines) contained in cytoplasmic proteins and membrane-bound enzymes (*41*−*44*). It has also been suggested that the mode of action of bronopol could be located within the membrane itself; bronopol could react with some constituents of the membrane of several bacteria and make the membrane impermeable to both lactose and galactose (*39*). This may mean that bronopol probably affects one or more of the enzymes involved in the transport catabolism of both lactose and galactose. Concerning lactose, it is now clear that cysteines are involved in the permease of *E. coli*, and this could also be the case for *S. thermophilus* (*45*). All these observations could support our results. In contrast, no inhibition of fermentation has been observed with glucose, which is probably internalized by the bronopol-resistant system. However, the main objective of this study was to develop a methodology to detect the inhibition of lactic fermentation in milk samples. To simulate this inhibition, bronopol was chosen, and its inhibitory effect could easily be detected, because a negligible amount of glucose is generally present in milk samples.

Table 1. Effect Detection of Inhibition of L-Lactic Fermentation. Evaluation of the Ability of Milk Samples to Ferment in the Presence (+) and Absence (–) of Bronopol (0.05% w/w)

	sample no.		L-lactic acid (μmol/L) before inhibition step	L-lactic acid (μmol/L) after inhibition step	inhibition degree(%)
skim milk	1	+	171.4	13.1	92.4
	2	+	210.2	15.8	92.5
	3	-	175.8	181.9	
92.5	4	-	181.9	179.1	
whole milk	5	+	222.9	36.9	83.4
	6	+	198.1	40.2	79.7
	7	-	180.4	198.2	
	8	-	198.7	224.5	

Detection of the Inhibition of L-Lactic Fermentation in Milk Samples. The present method was then used to detect an inhibition of lactic fermentation in milk samples, according to the protocol described in **Figure 4**. Because the bronopol inhibits the beads irreversibly, a new bioreactor was used for each milk sample. Inhibition of the beads was evaluated by comparing the L-lactic acid produced before and after incubation of milk samples through the bioreactor for 30 min. It was found that for 0.05% of bronopol added, the beads were significantly inhibited, as can be seen in **Table 1** by comparing the amount of L-lactic acid produced before and after the inhibition step (samples 1, 2, 5, and 6). The response of the biosensor to other milk samples without bronopol showed a constant production of L-lactic acid through the bioreactor (samples 3, 4;, 7, and 8). The samples containing bronopol were recognized and the decrease in the L-lactic acid production through the bioreactor proves that the inhibition was due only to the presence of bronopol.

No significant difference was obtained between semi-skimmed and whole milk. It was also noticed that no matrix effect was obtained on the beads, such as fouling of the Ca-alginate membrane. The response to L-lactic acid remained constant. The resulting methodology indirectly permitted the presence of bronopol to be detected without any direct contact between the electrode and the sample. This could be very interesting in the case of a complex medium where the matrix effect may not be negligible on the amperometric response of the biosensor.

In this paper, we have reported a methodology for the application of a disposable bioreactor used in conjunction with an L-lactic acid biosensor for milk quality control. This work presents the optimization of the experimental parameters for

both an L-lactate biosensor and a bioreactor. Rapid detection of the L-lactic fermentation activity was achieved due to the low detection limit of the L-lactate biosensor. The bioreactor allowed a high L-lactic fermentation activity. This is essential for the assessment of the ability of milk to ferment and the detection of the presence of nonspecific inhibitory agents. The applicability of the developed method was illustrated by detection of bronopol and its inhibitory effect on lactic production during fermentation. The approach presented here could be extended to other inhibitory agents and find application in a rapid assay in both the dairy industry and in environmental monitoring. Further work will include an evaluation of the L-lactate response toward various inhibitory agents, such as antibiotic compounds, which constitute one of the main inhibitor elements in dairy products. The sensitivity and specificity of the resulting method could be improved by using a specific recombinant microorganism in conjunction with specific biosensors.

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