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Effect of temperature on the sensitivity of cascaded lactose biosensors

Delia Peedel and Toonika Rinken*

Institute of Chemistry, University of Tartu, Ravila 14a, 50411 Tartu, Estonia

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Abstract. The sensitivity and application options of cascaded lactose biosensors along with the catalytic activity of three enzymes (β-galactosidase, glucose oxidase, and galactose oxidase) used for bio-recognition in these biosensors were studied at different temperatures ranging from 4 to 38.6 °C, the usual temperature span in the milk processing. Although at 4 °C, which is the common temperature of raw milk storage, the apparent activity of these enzymes was quite low and the resulting biosensor sensitivity decreased nearly 100 times in comparison with its sensitivity at 38.6 °C, it was possible to carry out lactose measurements with a biosensor comprising β-galactosidase and glucose oxidase within 10 min.

Key words: temperature, glucose oxidase, galactose oxidase, β-galactosidase, activity, lactose biosensor sensitivity.

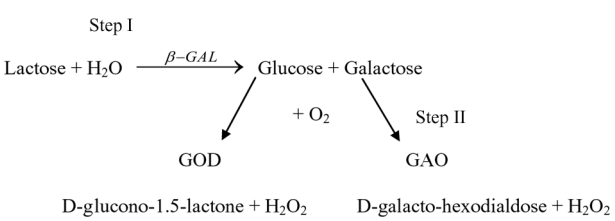
1. INTRODUCTION

One of the most important physical factors influencing the speed of chemical reactions is temperature. The effect of temperature applies equally to the speed of elementary and complex enzyme-catalysed chemical reactions, widely used for selective bio-recognition in various bio-sensing systems. The speed of an ongoing bio-recognition reaction determines the biosensor's sensitivity and the quality of the obtained results.

Lactose or milk sugar is an important component of bovine milk and milk products. As lactose contributes to the sensory and functional properties of milk, its controlled levels enable to ensure process and product control in the dairy industry [1]. The detection of lactose in milk is even more important for health reasons, as around 70% of the world's adult population suffers from either lactose maldigestion or intolerance, caused by the deficiency of the enzyme β-galactosidase in their organism [2]. At present, routine analyses of lactose are commonly carried out by using spectrophotometric [3–5], polarimetric [6], titrimetric or chromatographic [7,8] methods. The replacement of these traditional analytical methods, which are time-consuming and expensive, with biosensors that enable carring out rapid on-line analysis is a goal worth focusing on.

The principle of lactose biosensing is based on measuring the decrease of the dissolved oxygen concentration in the solution in the course of several cascaded enzyme-catalysed reactions, as there is no enzyme for a direct selective oxidation of lactose. In the first step, lactose is enzymatically hydrolysed into glucose and galactose catalysed by β-galactosidase (β-GAL, EC 3.2.1.23). This step is followed by the oxidation of the produced glucose or galactose catalysed by glucose oxidase (GOD, EC 1.1.3.4) or galactose oxidase (GAO, EC 1.1.3.9), respectively (Scheme 1).

The resulting oxygen consumption rate in the solution is directly related to the lactose concentration, as it is proportional to the products of lactose hydrolysis



Scheme 1. Processes in the cascaded lactose biosensor.

* Corresponding author, toonika.rinken@ut.ee

(glucose and/or galactose). At present there are lactose biosensors that are based either on β-GAL and GOD [9–14], β-GAL and GAO [9,15], or all these three enzymes [9]. Due to the relatively high activity of GAO towards lactose, problems of selectivity may arise in biosensors where this enzyme has been used [16,17]. In some lactose biosensing devices where hydrogen peroxide production resulting from the enzyme-catalysed oxidation is measured, a fourth enzyme, horseradish peroxidase (EC 1.11.1.6), has been additionally used to increase the system's selectivity [18–20].

Every enzyme has an optimal temperature range that enables to achieve the maximum efficacy of the catalysed reaction. Usually this range coincides with the physiological one, as enzymes are the catalysts that regulate processes in living organisms. GOD has been found to be satisfactorily active between 10 and 45 °C [21], with its activity maximum between 25 and 30 °C [21,22]. The activity of GAO is the highest around 30 °C [16] and 35 °C [23,24]. The temperature optimum for β-GAL (*Aspergillus oryzae*) lies between 35 and 50 °C [25,26]. At higher temperatures the tertiary structure of enzymes is often ruptured, leading to denaturation and loss of their catalytic activity.

The catalytic activity of the bio-recognizing compound in a bio-sensing system determines the system's sensitivity. For the studied lactose biosensors, the optimal working temperature lies in the range from 25 to 40 °C (Table 1). Studies at lower temperatures are scarce, only Logoglu et al. [12] studied a lactose biosensor at 8 °C and found that at this temperature the biosensor signal is less than 10% of the maximum. Results of measurements at lower temperatures than this are not available, although due to their consistency milk and dairy products should be kept at low temperatures to ensure the maintenance of their colloidal nature and properties. After milking, milk is quickly cooled down to 4 from 38.6 °C, the normal bovine body temperature [27]. Therefore it is necessary to carry out the determination of lactose in milk at temperatures around 4 °C.

The aim of the present study was to examine the catalytic properties of enzymes used in lactose cascaded biosensors and the sensitivity of these biosensors at temperatures suitable for the storage of milk and dairy products.

Table 1. Lactose biosensing systems

Enzyme	Temperature range	Best working temperature	Detection limits	Sensitivity	Working principle	Source
GOD + β-GAL	32–42 °C	37 °C	>1.0 mM	–	Amperometric	[9]
	15–55 °C	40 °C	>10 ng mL^{-1}	–	Clark electrode	[10]
	20–45 °C	30 °C	0.1–3.5 mM	–	Chromoamperometric	[11]
	8–45 °C	25 °C	0.1–15 mM	–	Amperometric	[12]
	25–40 °C	30 °C	<0.1 mM	111 nA mM^{-1} mm^{-2}	Electrophoretic deposition (AC-EPD)	[13]
	Room temperature	–	0.0035–2 mM	1 μA mM^{-1}	Amperometric	[14]
GAO + β-GAL	32–42 °C	37 °C	>0.5 mM	–	Amperometric	[9]
	25–40 °C	40 °C	1–6 g dL^{-1}	–	Amperometric	[15]
GOD + GAO + β-GAL	32–42 °C	37 °C	>0.5 mM	–	Amperometric	[9]
GOD + β-GAL + Horseradish peroxidase	Room temperature	–	10–340 μg mL^{-1}	–	Amperometric	[28]
Hybrid biosensor: β-GAL production of genetically manipulated bacteria *E. coli* K-12 recombinant PQ-37 + GOD	37–50 °C	37 °C	–	–	Clark electrode	[29]
Cellobiose dehydrogenase from *Phanerochaete sordida*	–	–	1–100 μM	1100 μA mM^{-1} cm^{-2}	Amperometric	[30]

– No numerical data in the source.

2. MATERIALS AND METHODS

Glucose oxidase (from *Aspergillus niger*), galactose oxidase (from *Dactylium dendroides*), β-galactosidase (from *Aspergillus oryzae*), D-glucose, D-galactose, and D-lactose were obtained from SigmaAldrich. All reagents used were of analytical grade.

To follow the change of dissolved oxygen concentration (DOC) in the course of oxidase-catalysed reactions, we used a fibre-optic oxygen sensor constructed in the Institute of Physics, the University of Tartu. A fibre-optic oxygen sensor was used in order to minimize the influence of external electromagnetic interferences and to avoid the consumption of oxygen by the oxygen transducer itself [31,32].

This sensor is based on measuring the oxygen-induced phosphorescence quenching of Pd-tetraphenyl-porphyrin, encapsulated into thermally aged polymethyl methacrylate (PMMA) film, which covers a 30 mm long PMMA optical fibre with a diameter of 1 mm [32]. The oxygen-sensitive film was additionally covered with a thin black silicone coating to eliminate the effect of divergent light. The concentration of DOC was calculated according to the Stern–Volmer law from the output signal, recorded with an interval of 1 s.

All kinetic measurements were carried out under constant stirring at different substrate concentrations in an airtight and thermostatted glass cell (volume 28 mL) covered with black silicone. Injection of the enzyme solution into the reaction medium started the process. Final enzyme concentrations were the following: 3.60 IU mL^{-1} for β-GAL, 0.036 IU mL^{-1} for GOD, and 0.036 IU mL^{-1} for GAO. Measurements with GOD and β-GAL were carried out in 0.1 M acetate buffer (pH 5.60) and GAO in 0.1 M phosphate buffer (pH 7.00). For the analysis of milk, 1 : 1 solutions by volume of raw milk and acetate buffer (pH 5.60) were prepared.

At all studied temperatures, the initial DOC was 6.6 mg L^{-1}, which corresponds to the oxygen concentration in 100% air-saturated water at 38.6 °C. Below 38.6 °C, DOC values of 6.6 mg L^{-1} were generated by bubbling gaseous nitrogen through air-saturated solutions and determining the DOC value with an oxygen sensor. The bubbling was carried out at a definite temperature in a closed glass cell with only a small break in its upper part to provide an outlet for gas to eliminate the possible oxygen transfer from the surrounding air.

The studies of the hydrolytic activity of β-GAL and of the sensitivity of the lactose bio-sensing system were carried out with GOD (glucose concentration was measured after 10 min incubation with β-GAL) because of the low selectivity of GAO towards galactose in the presence of lactose. Thus the measured decrease in the oxygen concentration catalysed by GAO was the result of both the oxidation of the remaining lactose and of the produced galactose. To achieve the maximum activity of β-GAL, the initial lactose concentration 0.14 M was chosen, which is similar to the lactose concentration in milk and exceeds 8 times the K_M value of 1.8×10^{-2} M with lactose for β-galactosidase from *A. oryzae* [33].

The decrease of DOC in time was described with an earlier proposed biosensor dynamic model, taking into account the kinetics of the enzyme reaction based on the ping-pong mechanism, diffusion phenomena of substrates, and inertia of the measuring system, and enabling the calculation of reaction parameters from the transient phase data [34,35]. All results are shown as the mean value of at least three parallel measurements.

3. CALCULATION OF CHARACTERISTIC PARAMETERS OF THE REACTION AND BIOSENSOR SENSITIVITY: THEORETICAL BACKGROUND

The decrease of DOC in time due to oxidase-catalysed reactions followed an exponential pathway with a short lag period in the initial phase; the speed and scale of these reactions depended on the concentration of the substrate oxidized (Fig. 1). Each output curve was characterized with the normalized maximum signal change parameter A, describing the catalytic properties of different enzymes in various conditions.

This parameter A was calculated with the help of the biosensor model, enabling the calculation of steady-state parameters from the biosensor transient response with errors less than 3% and no need for additional determination of the system's geometrical, diffusion,

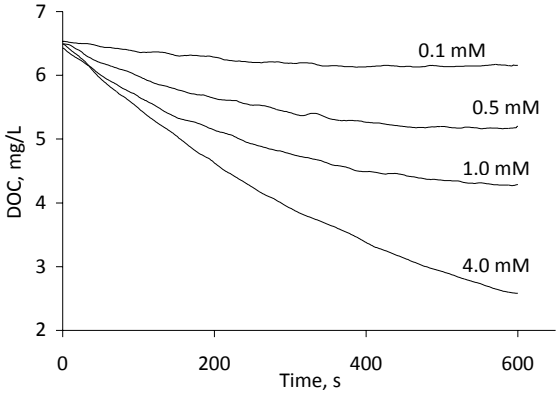


Fig. 1. An example of the decrease of dissolved oxygen concentration (DOC) in time at different substrate concentrations. Measurements were carried out with a fibre-optical oxygen sensor in stirred 0.1 M acetate buffer solution (pH = 5.60) at 25 °C, $[O_2]_{t=0}$ = 6.6 mg L^{-1}, final concentration of GOD 0.036 IU mL^{-1} at different glucose concentrations.

and partition parameters [34]. According to this model, the normalized dissolved oxygen concentration $c_{O_2}(t)/c_{O_2}(0)$ is expressed as a 3-parameter function of time t:

$$\frac{c_{O_2}(t)}{c_{O_2}(0)} = A\exp(-Bt) + (1-A) - 2A\sum_{n=1}^{\infty}(-1)^n \frac{\tau_S}{\frac{n^2}{B-\tau_S}} \times\left[\exp(-Bt) - \exp\left(-n^2\frac{t}{\tau_S}\right)\right], \quad (1)$$

where $c_{O_2}(t)$ is the dissolved oxygen concentration at time moment t, $c_{O_2}(0)$ is the dissolved oxygen concentration at the start of the reaction, t is time, and τ_S is the time constant of the inertia of the response. The parameter A corresponds to the maximum possible biosensor signal change in case time $t \to \infty$ (normalized signal change or the signal decrease percentage from its maximum to the steady-state value) and is a complex parameter depending hyperbolically on substrate concentration:

$$A = \frac{k_{cat}^{*}[E]_{total}c_S^{bulk}}{k_{diff}^{O_2}K_S K_{O_2} + (k_{cat}^{*}[E]_{total} + k_{diff}^{O_2}K_{O_2})c_S^{bulk}}, \quad (2)$$

where k_{cat}^{*} is the catalytic constant of the reaction, $[E]_{total}$ is the overall concentration of enzyme in the biosensor enzyme-containing matrix, $k_{diff}^{O_2}$ is the diffusion constant of oxygen, K_S is the dissociation constant for the enzyme–substrate complex, K_{O_2} is the dissociation constant for the enzyme–oxygen complex, and c_S^{bulk} is the substrate concentration in the solution. As this parameter is calculated from the transient phase signal, the influence of side reactions in the output signal is minimized and the time of measurements diminished. According to definition, the parameter A corresponds to the whole biosensor working range and its maximum possible value is 1.

For individual reactions, the limiting maximum value of this parameter at high substrate concentrations can be expressed as

$$A_{max} = \frac{k_{cat}^{*}[E]_{total}}{k_{cat}^{*}[E]_{total} + k_{diff}^{O_2}K_{O_2}} \quad (3)$$

and the value of the half-effect coefficient $K_{1/2}$ of the dependence of the parameter A on substrate concentration as

$$K_{1/2} = \frac{k_{diff}^{O_2}K_{O_2}K_S}{k_{cat}^{*}[E]_{total} + k_{diff}^{O_2}K_{O_2}}. \quad (4)$$

The coefficients A_{max} and $K_{1/2}$ are formally similar to the ones used in Michaelis–Menten kinetics, but their absolute values include also diffusion effects of the measuring system [35].

The ratio $S = A_{max}/K_{1/2}$ at fixed conditions serves as a measure of sensitivity of a specific biosensor and can be expressed as

$$S = \frac{A_{max}}{K_{1/2}} = \frac{k_{cat}^{*}[E]_{total}}{k_{diff}^{O_2}K_{O_2}K_S}. \quad (5)$$

As we used the same oxygen sensor and operated with relative values, it was possible to compare the catalytic activity of enzymes and the sensitivity of biosensors based on particular enzymes in different conditions. The ratio of A_{max} values and the ratio of S values at different temperatures were used to characterize the relative catalytic activity of an enzyme and the relative sensitivity of a specific biosensor, respectively. These relative values were not dependent on the total concentration of an enzyme, which was also tested experimentally at different GAO and GOD concentrations (data not shown).

4. RESULTS AND DISCUSSION

4.1. Temperature effects on glucose oxidase

As expected, the value of the calculated maximum signal change parameters increased along with the rise of temperature from 4 to 38.6 °C at all studied substrate concentrations for both glucose and galactose oxidases. Within this temperature range, we did not detect any effect of reaction slowdown of any of the studied reactions due to enzyme denaturation.

GOD is the enzyme used for glucose bio-recognition in glucose and lactose cascaded biosensors. The dependence of the maximum signal change parameter A on the concentration of D-glucose in the GOD-catalysed reaction at different temperatures is shown in Fig. 2. From these curves we calculated the values of A_{max}, $K_{1/2}$, and S at different temperatures. These values for GOD at different temperatures are shown in Table 2. It can be seen that the relative catalytic activity of GOD at 4 °C is only 9% of the enzyme's activity at 25 °C. At higher temperatures, the system reached its limits as all available dissolved oxygen was used in oxidation and diffusion became the limiting process of signal rising.

The sensitivity S of the GOD-based glucose biosensor increased over 40 times in the studied temperature range. The rise of the sensitivity accelerated at higher temperatures (Fig. 3). The detection limit of glucose at the studied GOD concentration at signal: noise ratio 3 was 0.15 mmol L^{-1}. In case lower detection limits are required for practical applications, this detection limit can be lowered by increasing the GOD concentration.

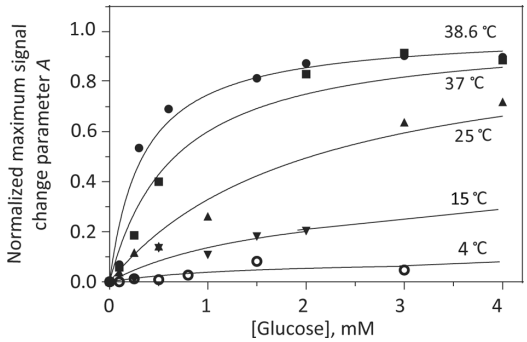


Fig. 2. Dependence of the normalized maximum signal change parameter A on glucose concentration at different temperatures. Measurements were carried out in stirred 0.1 M acetate buffer solution (pH = 5.60), $[O_2]_{t=0}$ = 6.6 mg L^{-1}, final concentration of GOD 0.036 IU mL^{-1}.

Table 2. Catalytic properties of GOD and GAO and the sensitivity ($S = A_{max}/K_{1/2}$) of biosensors based on these enzymes at different temperatures

Temperature, °C	GOD		GAO	
	A_{max}	Sensitivity	A_{max}	Sensitivity
4	0.091	0.066	0.163	0.032
15	0.383	0.212	0.307	0.332
25	1.000	0.490	1.000	0.596
37	1.000	1.503	1.000	0.898
38.6	1.000	2.964	1.000	2.718

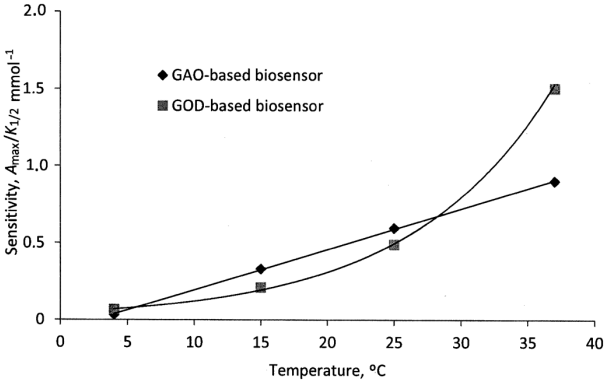


Fig. 3. Sensitivity of GOD-based glucose and GAO-based galactose biosensors at different temperatures. Measurements with GOD were carried out in 0.1 M acetate buffer solution (pH = 5.60), final concentration of GOD 0.036 IU mL^{-1} and with GAO in 0.1 M phosphate buffer solution (pH = 7.0), final concentration of GAO 0.036 IU mL^{-1} under constant stirring; $[O_2]_{t=0}$ = 6.6 mg L^{-1}.

4.2. Temperature effects on galactose oxidase

GAO is the enzyme used in lactose cascaded biosensors along with or instead of GOD for the detection of the products of lactose hydrolysis (galactose). The dependence of the maximum signal change parameter A on galactose concentration at different temperatures is similar to that obtained for GOD. The values of A_{max} and $K_{1/2}$, characterizing GAO activity and the sensitivity S of the GAO-based biosensor for galactose at different temperatures, is shown in Table 2. The relative catalytic activity of GAO at 4 °C is slightly higher than the activity of GOD at that temperature: 16.3% of the enzyme's activity at 25 °C. At 4 °C the limit of detection of galactose at the studied GAO concentration at the signal: noise ratio 3 was 0.08 mmol L^{-1}.

The sensitivity of the GAO-based galactose biosensor increased over 80 times in the studied temperature range (Fig. 3). This big rise of sensitivity resulted from the fact that the sensitivity of this biosensor at 4 °C was even lower than that of the GOD-based biosensor, leading also to a higher impact of experimental noise.

The sensitivities of GOD-based and GAO-based biosensors were in the same range. At 4 °C, the conventional milk storage temperature, lactose biosensors comprising these enzymes are applicable only if the concentrations of the products of lactose hydrolysis in the solution are above the detection limits.

The natural levels of galactose and glucose in bovine milk are commonly around 0.1 mmol L^{-1} [36]. The common lactose concentration in milk is approximately 0.14 mol L^{-1}, so for an acceptable analysis with a lactose cascaded biosensor at 4 °C, at least 0.15% of the whole lactose in milk should be hydrolysed by β-GAL in step I to produce distinguishable from natural levels concentrations of glucose and galactose at given enzyme concentrations. The constructing cascaded lactose biosensors, it is also important to keep in mind that the selectivity of GAO towards galactose in comparison with lactose is quite poor and lactose produces a similar signal in the GAO-based biosensor output [16,17]. Accordingly, the use of GOD in step II of the cascaded lactose biosensor is preferable.

4.3. Temperature effects on β-galactosidase

The effect of temperature on the catalytic activity of β-galactosidase, catalysing the hydrolysis of lactose, was studied by following the GOD-catalysed oxidation of glucose, produced within 10 min in 0.14 M lactose solution with β-GAL (3.60 IU mL^{-1}). Based on the amount of glucose produced, we calculated the percentage of lactose hydrolysed at different temperatures (Fig. 4).

The efficiency of the β-GAL-catalysed lactose hydrolysis was quite low: even at 38.6 °C it was only 1.43% , which is in good correlation with earlier studies [9]. At 4 °C it was 0.15%, being in the same range with the minimal required efficiency of hydrolysis for the studied system. Along with the rise of temperature, the efficiency of hydrolysis rose hyperbolically (Fig. 4).

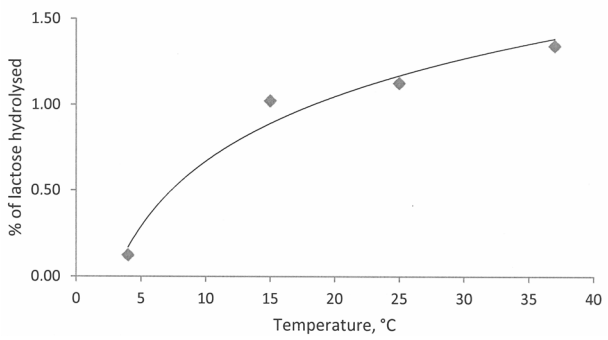


Fig. 4. The efficiency of lactose β-GAL-catalysed hydrolysis at different temperatures. Measurements were carried out in stirred 0.1 M acetate buffer solution (pH = 5.60), $[O_2]_{t=0}$ = 6.6 mg L^{-1}, final concentration of GOD 0.036 IU mL^{-1} and β-GAL 3.60 IU mL^{-1}, $[lactose]_{t=0}$ = 0.14 mol L^{-1}.

Overall, combining the decline of biosensor sensitivity, caused by the fallen activities of GOD and β-GAL at lower temperatures, the lactose biosensor sensitivity at every fixed enzyme concentration was about 100 times lower at 4 °C than its sensitivity at 38.6 °C in lactose solutions.

4.4. Application of lactose biosensor in milk

The same β-GAL- and GOD-based lactose cascaded biosensing system was used for the analysis of raw milk in which the lactose concentration was 5% or 0.14 M. The decrease of DOC in milk at different temperatures followed a similar exponential pathway as in lactose solutions (Fig. 5). The dependence of the maximum signal change parameter A on temperature calculated from these curves was linear with a slope

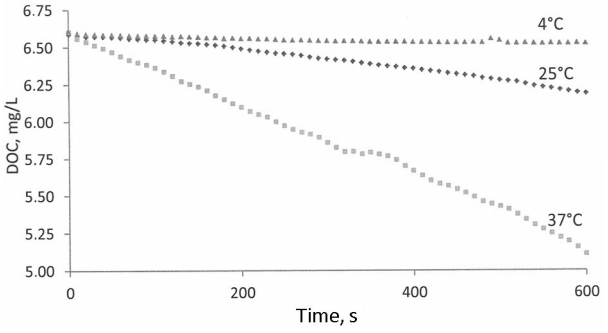


Fig. 5. Decrease of dissolved oxygen concentration (DOC) in time at different temperatures in milk–acetate buffer solutions (1 : 1, pH = 6.50) measured with a β-GAL- and GOD-based lactose cascaded biosensing system. Measurements were carried out with a fibre-optical oxygen sensor in a stirred 1 : 1 solution of raw milk and 0.1 M acetate buffer solution (pH = 5.60); final concentration of GOD 0.036 IU mL^{-1} and β-GAL 3.60 IU mL^{-1}.

(0.028 ± 0.001) deg^{-1} in milk and (0.016 ± 0.003) deg^{-1} in 5% lactose solution. The greater slope in milk was due to the increased respiration activity of bacteria present in raw milk at higher temperatures [37].

The absolute value of the parameter A at 4 °C was 0.045, making up 4.5% of the whole working range of the sensor. To increase the measurable biosensor signals at low temperatures, it is possible to incubate probes with β-GAL longer than 10 min, although this will lead to an increase of the analysis time and will make the system more vulnerable to experimental noise.

5. CONCLUSIONS

We studied the activity of two oxidases, glucose oxidase and galactose oxidase, along with the activity of β-galactosidase in the temperature range from 4 to 38.6 °C with the help of a fibreoptic oxygen sensor. The aim was to study the sensitivity of biosensors based on these enzymes and the possibilities of applying these enzymes for lactose determination at different temperatures, both in lactose solutions and in milk. The sensitivity of the lactose biosensor was relatively low at 4 °C, the common milk storage temperature, due to the low activity of β-galactosidase and the oxidases. Despite the relatively low activity of enzymes, the determination of lactose could be carried out at this temperature if lactose hydrolysis was going on with sufficient efficiency to detect the produced glucose/galactose. The sensitivity of the lactose biosensor grew rapidly along with the rise of temperature from 4 to 38.6 °C, which is the normal bovine body temperature. In case we use biosensors for the determination of lactose in milk processing, the quickest option is to carry out measurements right after milking before milk is cooled down to its storage temperature. As usually the analysis time is not so limited, the lactose concentration can be determined in milk storage tanks at 4 °C within 10 min.

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Temperatuuri mõju kaskaadsete laktoosi biosensorite tundlikkusele

Delia Peedel ja Toonika Rinken

Käesolevas töös uuriti kolmel ensüümil – β-galaktosidaasil, glükoosi oksüdaasil ja galaktoosi oksüdaasil – põhineva kaskaadse laktoosi biosensori tundlikkust ning kasutusvõimalusi ja nimetatud ensüümide katalüütilisi aktiivsusi piimatöötlemisprotsessis olulises temperatuurivahemikus 4–38,6 °C. Ehkki piima tavapärasel säilitustemperatuuril 4 °C juures olid ensüümide aktiivsused suhteliselt madalad ja nendel põhineva laktoosi biosensori tundlikkus ligikaudu 100 korda väiksem kui 38,6 °C juures, oli laktoosi biosensor toorpiimas siiski kasutatav, kui mõõtmise aeg oli vähemalt 10 minutit.