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Development of a sensor to quantify lactic acid in beer

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ABSTRACT

The determination of lactic acid in beer is a crucial quality parameter.Increase the product's shelf life and hinders the growth of spoilage bacteria such as salmonella. The analysis of this parameter is indispensable in production lines and the use of a biosensor could potentially simplify the process. An enzymatic sensor utilizing lactate oxidase (LacOx) was optimized for determining lactic acid content in beer. The sensor immobilized the enzyme with an oxygen electrode. LacOx from Pediococcus sp was used in this study, with a voltage of −600 mV. An amperometric signal was obtained, and the signal was recorded at 5 s due to oxygen depletion during lactic acid oxidation. The reaction rate was then correlated to the lactic acid content. A linear relationship was found between consumed as a function of time (mg O^2/L x s) and lactic acid concentration in the range of 10 and 2700 μM, with a coefficient of determination of $R^2 = 0.9947$. The immobilized enzyme sensor showed a high sensitivity of 0.01 mM and was stable enough to allow the reutilization of the membranes up to 25 times without loss of activity, were 90 % of its initial activity remained after for 50 days. This system showed good specificity (K_M = 450 μM). The correlation, validated through the use of the same linear range using reference method High -performance liquid chromatography (HPLC), establishes that is viable alternative to quantify lactic acid in various beer types.

1. Introduction

Craft beer should be analyzed from various perspectives to ensure quality. The absence of a pasteurization or filtration process leaves it vulnerable to the activity of microorganisms such as lactic acid bacteria (LAB). LAB produce exopolysaccharides which cause biofilm formation, resulting in higher beer viscosity, thin texture and increased turbidity (Fraunhofer et al., 2018). The presence of lactic acid bacteria (LAB) has been reported to have a negative impact on the quality of beer. Studies have found that American craft beers can experience up to a 15 % decrease in quality, while Australian craft beers can see a decrease of 27.5 % and Korean craft beers can be impacted by as much as 81 % (White, 2008; Menz et al., 2010; Jeon et al., 2015). The metabolism of LAB is the main source for the formation of biogenic amines, which can lead to their formation. (Romero et al., 2003; Loret et al., 2005).

The organic acids found in beer have a significant impact on its taste, particularly on the sour and sour-acid flavors that influence consumers' acceptance of the beverage. The major organic acids present are lactic,

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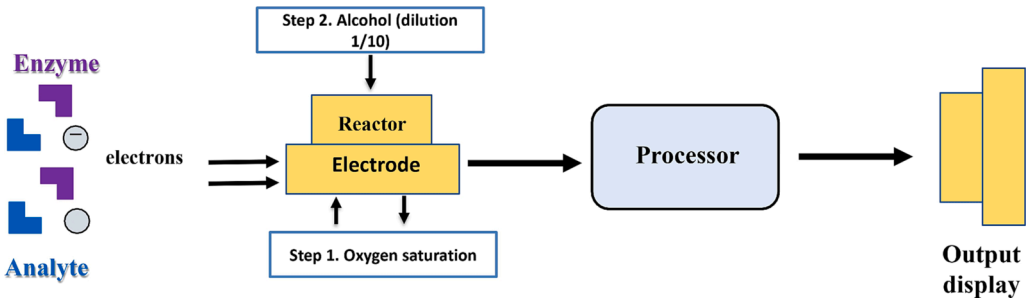

Fig. 1. Gaphical abastract of key principle of developing a biosensor.

tartaric, malic, acetic, citric, and succinic. Lactic acid is the most abundant, with concentrations ranging from 451 mg/L to 712 mg/L (Montanari et al., 1999; Das et al., 2014). Lactic acid, specifically, can inhibit bacterial growth that may spoil beer. Therefore, consuming beer safely may decrease the likelihood of developing coronary heart disease, diabetes, and overall mortality (Mukamal et al., 2008; Ferreira, 2008). Also, lactic acid has been identified as a potential raw material for synthesizing pyruvic acid through oxidative dehydrogenation (Jia et al., 2023). In addition, it can be used for the production of bio-propionic acid (Liu,et al., 2023). The positive effects of beer consumption can be attributed to its low energy and free sugar content, high levels of antioxidants including polyphenols and flavonoids, and essential vitamins and minerals that contribute to nutrition (Bamforth, 2002). Therefore, the development of biosensors for monitoring various parameters during beer manufacturing has become increasingly important. For instance, devices have been created to regulate the concentration of lactic acid and ethanol during alcoholic fermentation (Taurino et al., 2013; Vargas et al., 2016; Arivazhagan et al., 2020; Jadán et al., 2023). Recent studies show that research in the field of craft beers has increased exponentially ($R^2 = 0.8998$), reaching its peak in 2020 with 110 articles.

The United States leads with 30 % of the total documents, which can be attributed to being the second-largest beer producer after China, with 8,386 craft breweries. In terms of volume, the United States contributed 13.6 % to the total beer production. (Tirado-Kulieva et al 2023; Brewers Association, 2020).

The use of sensors is widespread, with research conducted in both environmental and security fields. For example, continuous monitoring allows for the detection of harmful compounds in the environment, such as bacterial spores and dichromate anions (Li et al., 2023). Additionally, biosensors have been developed to ensure human and environmental safety, capable of identifying buried explosive residues, such as 2,4-dinitrotoluene (Li et al., 2023; Wang et al., 2024).In the field of food safety, various methods have been developed to detect chemical contaminants (3-Chloropropane-1,2-diol) in hydrolyzed foods (Tang, et al., 2023). These non-destructive monitoring and dynamic control methods can allow for real-time stress monitoring in live fish in some cases (Wang et al., 2021). Enzymes can also be used in test kits to rapidly evaluate contaminants, such as aflatoxins, in different types of milk (Xiong et al., 2022).

This study detected lactic acid through electrochemical means by monitoring the oxygen consumption resulting from the reaction. The functioning principle of this sensor was based on Lactate oxidase (LacOx) catalyzing the oxidation of L-lactate in the presence of oxygen, forming pyruvate and hydrogen peroxide, according to the following scheme (1). This is possibly due to the selectivity exhibited by this enzyme (LacOx) towards the substrate. (Chandra, Vinay, and Bhawna (2016).

$$\text{L} - \text{lactate} + \text{LOx} \rightarrow \text{pyruvate} + \text{LOx red} \quad (1)$$

$$\text{LOx red} + \text{O}_2 \rightarrow \text{LOx ox} + \text{H}_2\text{O}_2$$

The schematic representation of the sensor operations can be seen in Fig. 1. It is possible to observe the process to quantify acid lactic during the reaction.

This reaction can be monitored by measuring oxygen consumption at a positive potential of 20 mV or by measuring the hydrogen peroxide generated at a potential of 600–650 mV. To prevent the oxidation of other electroactive compounds, hydrogen peroxide requires a high potential. However, measuring the generated hydrogen peroxide could be inconvenient due to the presence of potentially interfering species in the sample and the possibility of oxidation caused by the high potential required by hydrogen peroxide (Casero et al., 2014). Therefore, the main objective of this study was to develop a rapid technique for measuring lactic acid levels in craft beers using a LacOx sensor in conjunction with a Pt/Ag electrode.

2. Materials and methods

2.1. Chemical and reagents

LacOx E.C. 232–841-6 was sourced from Pediococcus sp., with 2.9 mg of protein per vial. The following chemicals were procured from Sigma (Sigma-Aldrich, St. Louis, MO, USA): L-(+) lactic acid, sodium L-lactate, glutaraldehyde (50 %), and Flavin adenine dinucleotide of HPLC-grade. Sodium phosphate and anhydrous sodium acetate were supplied by Panreac Química (Barcelona, Spain).

The pre-activated Immunodyne ABC membrane (Nylon 6,6, pore size 0.45 μm) was provided by Pall Europe (Portsmouth, UK).

2.2. Preparation of the LacOx solution

The commercial enzyme was dissolved in 10 mL of 10 mM buffer solution at pH 7, with an activity of 12.1 U/mL. It was stored in 0.5 mL Eppendorf tubes and frozen at −80 ℃ until use.

2.3. Equipment

The enzymatic sensor used in the study consisted of a platinum electrode, specifically the Dual Digital Model 20 (Rank Brothers, Bottisham, Cambridge, UK), with an immobilized enzyme attached.

The electrode was constructed with two internal sections: 1) a working electrode with a 2 mm diameter platinum disk, and 2) a reference electrode with a surrounding silver ring (Ag/AgCl). The thermostatic reaction cell and 12.7 μm oxygen-permeable cassava biopolymer film was utilized in the construction of the enzymatic sensor.

Chromatographic analysis was carried out using a YL9100 system produced by Young Lin Instrument CO., LTD (Korea).

2.4. Sample preparation

The beer samples were acquired from a local brewery. t degassing by means of agitation with a horizontal shaker (Advanced digital shaker, VWR, 3500, USA). The shaker was set to operate at 200 rpm for 60 min. Afterward, the beverages were immersed in an ultrasonic bath at

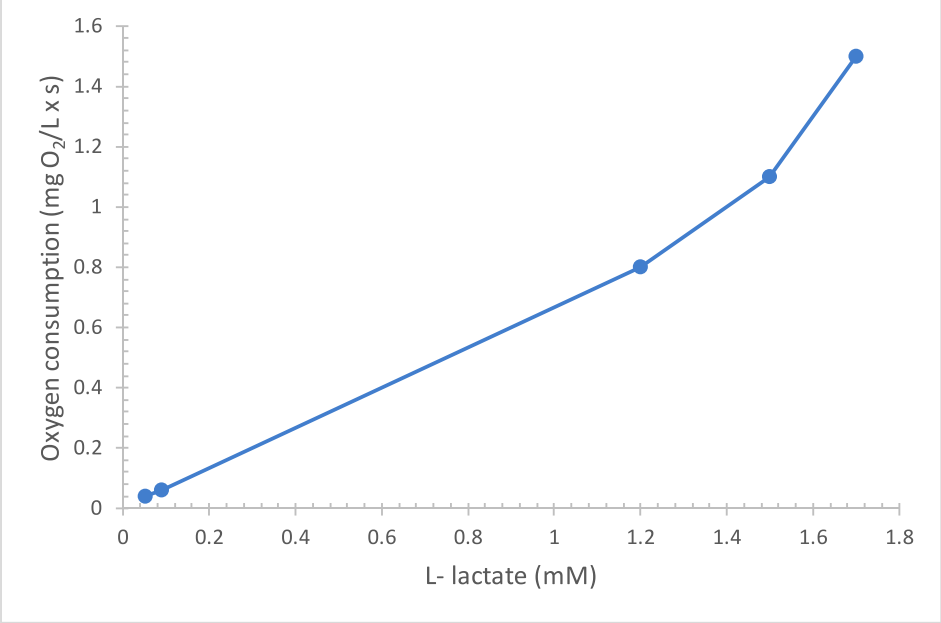

Fig. 2. Response of the equipment during substrate oxidation at 45 °C in potassium phosphate buffer 0.01 M, pH 7.

ambient temperature for an additional 30 min. Subsequently, the resulting solution was utilized for the analysis of lactic acid utilizing HPLC and the enzymatic sensor.

2.5. Biosensor preparation and mode of operation

The sensor's operating method was previously outlined by Jadán (2019). The primary reaction takes place on the platinum electrode. To adjust the sensitivity of the instrument, the reaction cell is filled with stirred, oxygen-saturated distilled water. 100 % saturation is used as a baseline. The entire process takes less than 10 s.

The reaction cell is kept at the optimal enzyme temperature (45 °C) by recirculating water preheated with a thermostat. To separate the enzymatic solution (inside the reaction cell) from the electrode base, the immunodyne ABC membrane is used. This membrane isolates the electrode, allowing just the passage of oxygen.

2.6. Immobilized enzyme sensor

The immobilization procedure was based on Hernández-Cázares' work in 2010. The appropriate percentage of glutaraldehyde for the enzyme (LacOx) was studied in a range between 1 % and 8 %, and fixed at 2.5 %. Consequently, the total mixture (50 μL) contained 15 μL of LacOx solution (12.1U), 29 μL of 0.5 M sodium phosphate buffer, pH 7.0, and 6 μL of glutaraldehyde. This was then dropped onto the immunodyne ABC membrane (1 cm2) and left to dry for an hour before the immobilization process began. After drying, 5 μL of 2 % cellulose acetate solution in acetone were added to prevent any matrix interference (Qiong et al., 1998). Next, the two membranes (LacOx and teflon) were attached to the oxygen electrode by using a rubber ring and placed in the thermostatic reaction cell.

2.7. Effect of lactate oxidase on lactic acid

The depletion of lactate (substrate) in the presence of LacOx enzyme was studied using HPLC. To that end, 750 μL of enzyme solution (12.1U) and 750 μL of 10 mM lactate standard solution (1 mM in the reactor), or the beer sample (1/10 dilution), were added to 7.5 mL of phosphate buffer (pH 7.0 and concentration of 0.01 M).

The mixture was left to react at 31 °C with gentle agitation. Every 10 s, 900 μL aliquots were taken and immediately mixed with 100 μL of 2 % trichloroacetic acid to stop the reaction. These aliquots were filtered and centrifuged for analysis on HPLC. The concentration of protein present in 1 mL of solution corresponds to 21.1 U, which is the minimum concentration for enzyme reaction with the substrate.

2.8. Description of the chromatographic method

To determine the presence of lactic acid in beer or standards, we utilized a Rezex ROA-Organic Acid H+ (8 μm, x300 x7.8 mm) thermostatic column at 50 °C, along with sulfuric acid with a concentration of 0.009 N. We used 20 μL of the sample diluted with sulfuric acid and a flow rate of 0.7 mL/min in the mobile phase. Fluorescence detection at excitation and emission wavelengths of 230 and 450 nm respectively, were used. The gradient was linear, two solvents were used: 20 mM sodium acetate buffer, pH 7.2 (solvent A), and 100 mM sodium acetate pH 7.2 /acetonitrile/methanol (30:35:35) v/v as solvent B. The mobile phase passed through a 0.45 μm nylon filter and was subsequently degassed. The quantification was performed by means of a calibration curve between 10 μM and 2700 μM acid lactic.

2.9. Application of the enzymatic sensor to analyze samples of beer

The enzymatic sensor was used to quantify lactic acid in blonde (4–12 EBC), toasted (12–47 EBC) and black (>47EBC) beers. The method was validated and its results were compared with those obtained using HPLC.

2.10. Storage stability of the LacOx electrode

To assess the stability of lactate oxidase (LacOx) during storage at 4 °C, a series of amperometric tests were conducted over a 95-day period at 5-day intervals. Measurements were taken in triplicate to determine the mean value and standard desviation.

2.11. Validation tests

Linearity was achieved through triplicate analysis of standards ranging from 0.01 to 2.7 mM for the immobilized enzyme system using the Ordinary Least Squares (OLS) regression method. The dilution of the

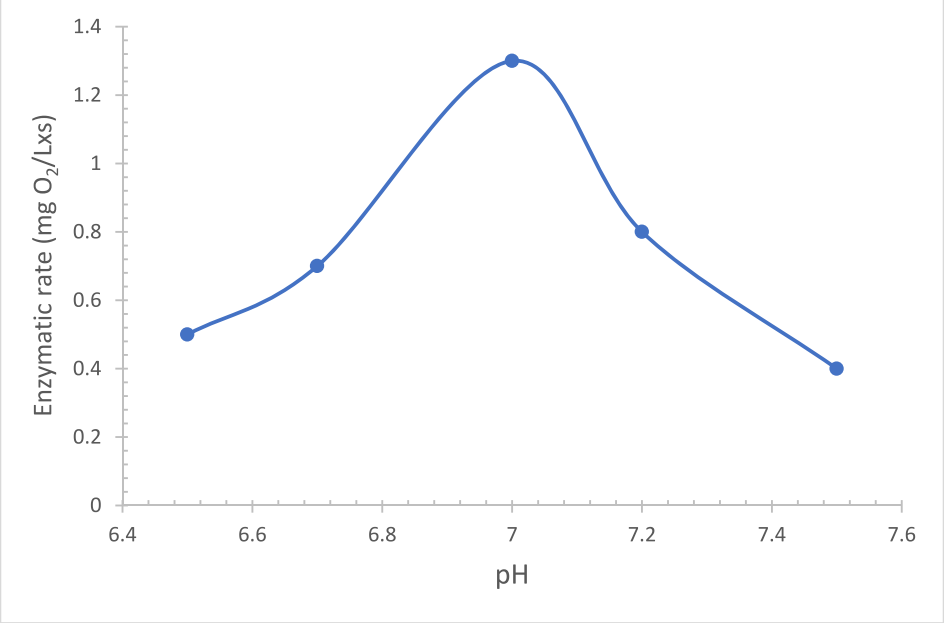

Fig. 3. Effect of pH on the rate of reaction of the enzyme lactate oxidase immobilised determined by the enzyme sensor. n = 3.

sample was 1/10 to obtain a strong signal, avoiding interference.

Reproducibility was established by injecting 0.5 mM lactic acid standards or beer samples consecutively 30 times in the immobilized enzyme system on the same day, with the same equipment, and under the same conditions. The stability of the membrane stored at different temperatures was assessed. The repeatability of the analysis was evaluated by performing 25 consecutive injections using standards of 500 μM (lactic acid) and beer samples.

To determine the membrane's stability under operational conditions, successive injections of 0.1 mM lactic acid were applied to a single membrane, and the response was recorded until 50 % of its initial activity was observed. The results were expressed as function of time. The correlation, using different concentrations, between the sensor and HPLC were compared.

2.12. Statistical methods

The increase in standards concentrations is analyzed using Statgraphics Plus software (v5.1). The data's linearity between the sensor and the HPLC is determined by the coefficient of determination (R^2) and the standard error (SE). A two-sample *t*-test (two-tailed) is used to determine if the HPLC and biosensor are statistically equivalent. The null hypothesis states that the population means are the same (H0: μ sensor = μ HPLC) while the alternative hypothesis states that the means are not equal (H0: H0: μ sensor ≠ μ HPLC).

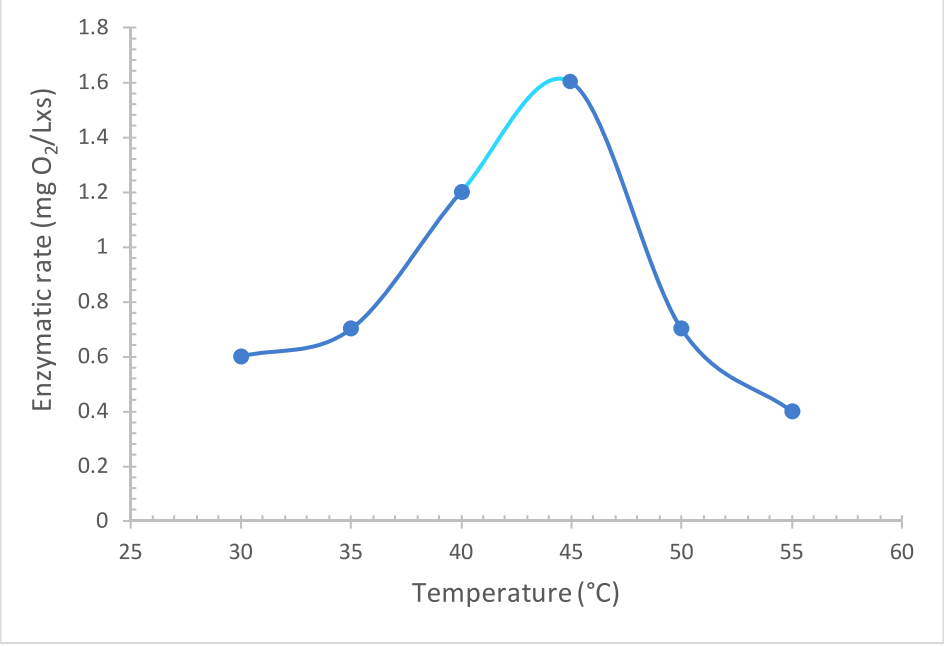

Fig. 4. Effect of temperature on the rate of reaction of the enzyme lactate oxidase Immobilised determined by the enzyme sensor. n = 3.

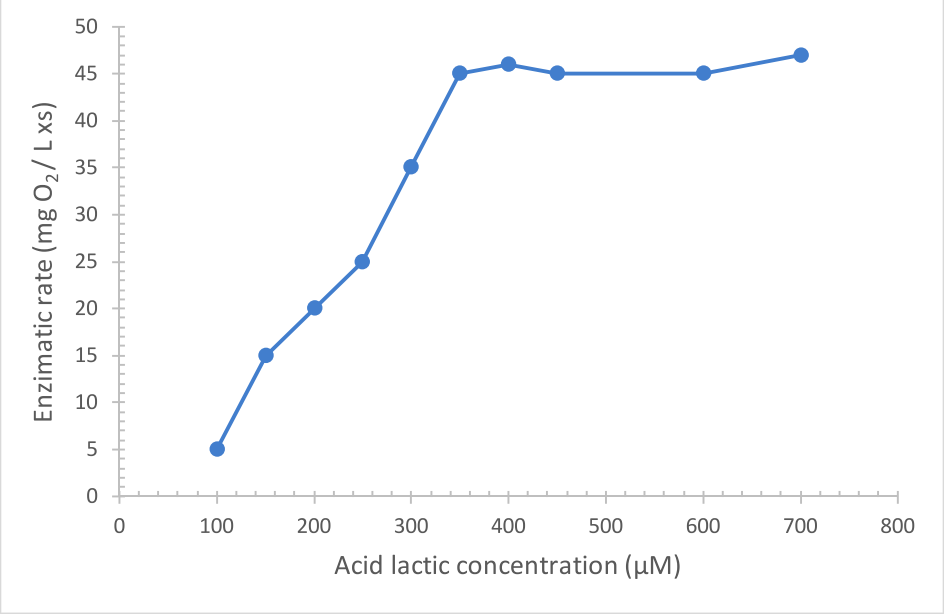

Fig. 5. Enzyme sensor response represented as the enzyme reaction rate as a function acid lactic concentration (n = 3).

3. Results and discussion

3.1. Biosensor optimization

The sensor was optimized in all its parameters, in particular the LacOxAg/AgCl electrode was evaluated at different working potentials within a range of −0.1 to −0.8 mV. The best interference-free response was observed at 3 s with an optimal potential of −0.6 mV.

This was due to the fact that the emitted noise remained below the baseline, resulting in an optimal signal in this sensor. Signal recognition occurs on a platinum base (2 mm diameter) utilizing a surrounding silver ring as the Ag/AgCl reference electrode.

It was established that during the oxidation of L-Lactate, oxygen consumption was proportional to the concentration of lactic acid (Fig. 2).

The repeatability expressed as the coefficient of variation (CV) of the sensor was determined by analyzing 30 consecutive injections of either a 1 mM standard pattern or beer samples on the same day, with the same equipment, and under the same conditions, while using a new membrane on the sensor with immobilized enzyme each time. The CV values for beer and standard pattern injections were 3.15 % and 4.9 %, respectively, indicating acceptable sensor performance. The variability was higher in the diluted samples compared to the standard solutions, which may be attributed to potential membrane fouling issues (Hernández-Cazares et al., 2010).

This allowed for good specificity, selectivity, and accuracy in the quantification of lactic acid. The pH range was between 6.5 and 7.5 using potassium phosphate (0.01 M). The optimal pH was 7 (Fig. 3). In parallel, the optimal reaction temperature was studied and was established at 45 °C (Fig. 4).

3.2. Biosensor features

The study of enzyme specificity for the substrate was analyzed (Fig. 5). The Michaelis-Menten constant (K_M) was found to be 450 µM, indicating good enzyme's affinity towards the substrate.

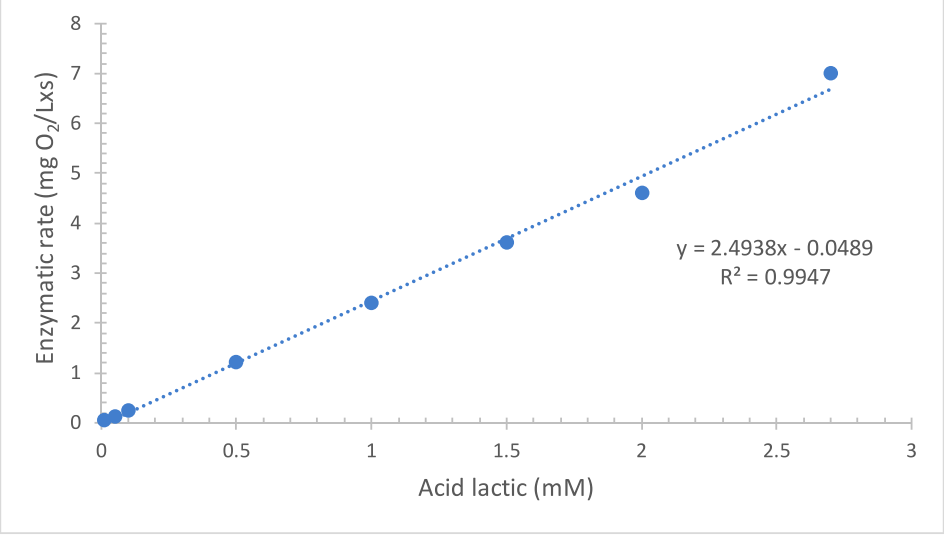

Fig. 6. Calibration curve obtained by the enzyme sensor with the Immobilised enzyme.n = 3.

Table 1
Measurements lactic acid levels by the enzime sensor and HPLC. Results are expressed in μM ± CV (n = 3).

Sample	EnzymeSensor	HPLC	Sample	EnzymeSensor	HPLC (mM)
1	500 ± 2.2	505 ± 0.2	16	490 ± 4.2	495 ± 0.7
2	503 ± 2.5	501 ± 1.2	17	495 ± 3.5	490 ± 1.2
3	507 ± 2.3	505 ± 0.6	18	498 ± 3.1	495 ± 0.3
4	504 ± 3.6	504 ± 0.5	19	420 ± 2.6	422 ± 0.1
5	510 ± 3.1	505 ± 0.2	20	410 ± 1.8	415 ± 1.3
6	502 ± 1.5	503 ± 1.3	21	430 ± 2.7	425 ± 0.9
7	510 ± 2.2	505 ± 0.9	22	460 ± 1.4	455 ± 1.6
8	500 ± 4.3	502 ± 0.3	23	410 ± 2.6	412 ± 1.3
9	507 ± 1.5	510 ± 0.7	24	450 ± 1.6	455 ± 0.4
10	501 ± 2.4	505 ± 0.3	25	420 ± 3.5	421 ± 0.7
11	505 ± 1.6	501 ± 0.5	26	430 ± 3.4	426 ± 0.2
12	504 ± 4.2	505 ± 0.4	27	445 ± 3.2	443 ± 0.8
13	501 ± 3.4	498 ± 0.6	28	460 ± 3.9	462 ± 1.7
14	505 ± 1.6	504 ± 1.7	29	480 ± 1.8	479 ± 0.3
15	502 ± 2.4	505 ± 1.9	30	415 ± 3.4	418 ± 1.3

The enzyme's activity was also studied, and it was affected when immobilized, approximately by 19 %. This could be due to the final position it acquires on the membrane; not all enzymes are correctly positioned for coupling with the substrate. These results were observed by Avramescu et al., 2001; Gros et al., 2004.

3.3. Calibration curves

To determine the linear range, in which the concentration of acid lactic can be accurately quantified using the biosensor, a linear response was constructed in the range of 0.01 to 2.7 mM for lactic acid (R^2 = 0.9947) (Fig. 6) when the substrate concentrations were increased. The sensor's lower limit of detection is low, demonstrating good sensitivity.

The sensor presents like upper limit of detection (2700 μM) showing a good correlation with the results obtained by the HPLC method (coefficient of determination R^2 = 0.9941) with a detection limit of 10 μM. High precision was observed, with no loss of stability or sensitivity.

The linear range is similar to what has been reported in the literature (Zanini et al., 2017; Serra et al., 1999). As a result, it was necessary to dilute the beer samples to bring them within the linear detection range.

Table 1 shows the lactic acid content in the beer samples, quantified using HPLC, and the values obtained experimentally with the LacOx biosensor.

3.4. Enzyme storage stability

The stability of the lactate oxidase membrane was strongly associated with temperature, using 4 °C as a reference. Within 50 days of storage, 90 % of its initial activity was maintained, with a 50 % decrease of this initial activity observed at 70 days of storage (Fig. 7). This decrease may have been due to rapid denaturation, as the values of pH remained near neutrality (Kumar et al., 2008).

3.5. Acid lactic measurements using the biosensor compared with HPLC measurements

The sensor was developed for the evaluation of lactic acid in commercial beers as a quality marker. To accomplish this, it was necessary to evaluate the accuracy of the enzymatic sensor by using six diluted samples.

These values were then compared to those obtained by high-performance liquid chromatography (HPLC). A good correlation was observed with a coefficient of determination of R^2 = 0.9941 and a standard error of 0.01 (Fig. 8).

The coefficient of determination value is comparable to that of other analytical techniques, such as Enzymatic Colorimetric Determination (reported in Ran et. al, 2023). In addition, a two-sample *t*-test was conducted to statistically assess ethanol concentration (mM) using both the sensor approach and HPLC at a significance level of α = 0.05.

The difference in lactic acid concentrations measured by both the biosensor ($\bar{x}$=1.9 and s = 0.2244) and HPLC ($\bar{x}$=1.55 and s = 0.1335) were not significantly different, as evidenced by t-value = 0.0930 (df = 30) and p-value = 0.9126. These measurements support the reliability of the biosensor, as it was capable of detecting methanol within the measurement range.

3.6. Effect of lactate oxidase on lactic acid

The substrate consumption (lactic acid and its sodium lactate salt)

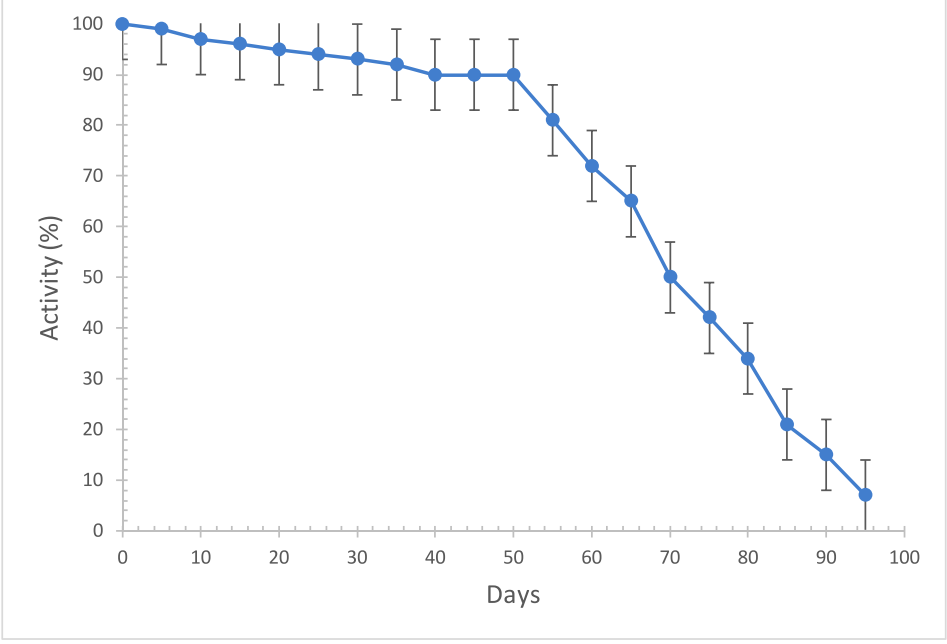

Fig. 7. Stability of lactate oxidase in solution stored in refrigeration (4 °C). n = 3.

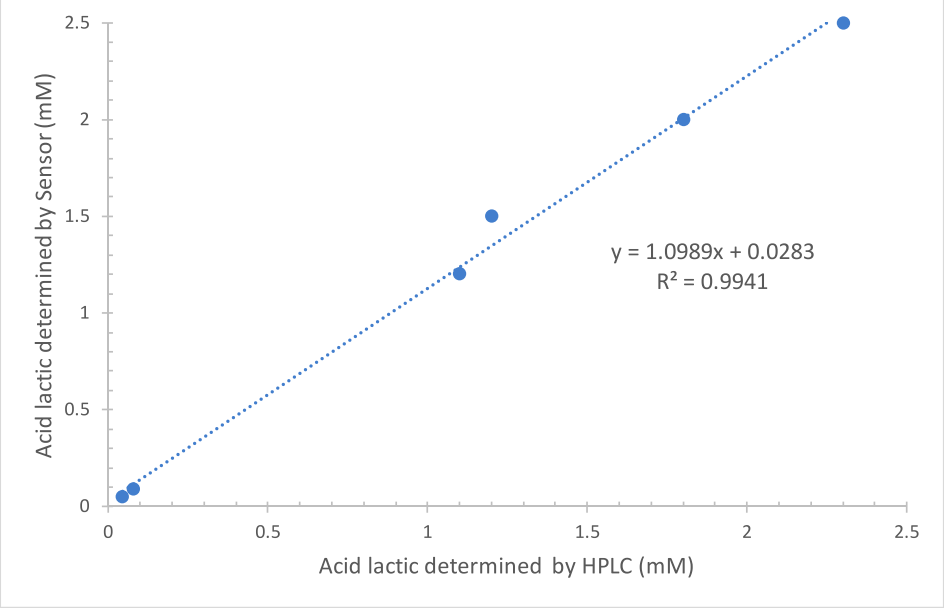

Fig. 8. Correlation between the contento f acid lactic in beer obtained with the enzyme sensor system and HPLC. n = 3.

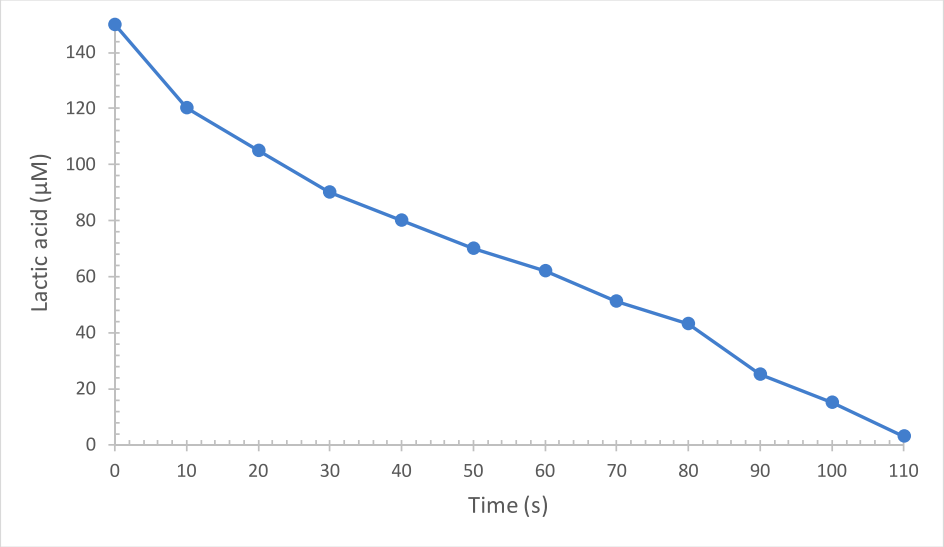

Fig. 9. Determination of acid lactic in the presence of lactate oxidase at pH 7.0 and 45 °C.

was analyzed in the presence of LacOx. The results are shown in Fig. 9, demonstrating a reduction completion time of 110 s, confirming LacOx's high affinity for lactic acid.

4. Conclusion

Based on the obtained results, this sensor is suitable for monitoring the concentration of lactic acid during the wort fermentation process and in the finished product. The method showed good sensitivity (10 µM), stability, and low interference despite the presence of other compounds in the sample. The immobilization of the enzyme provides an advantage as it allows for optimization of the enzymatic solution volume without sacrificing activity in each measurement, thereby reducing operational costs. The results were comparable to those of high-performance liquid chromatography. In conclusion, this method is a rapid test for monitoring the formation of lactic acid during alcoholic fermentation. It is a useful tool in the food industry due to its ease of use and short quantification times.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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