

Full Paper

ZnO/MWCNTs/Au Based Nano Biosensor for Detection of Lactate in Food Samples

Pallavi Verma, Manvender Singh, and Vikas Dhull*

*Department of Biotechnology Engineering, University Institute of Engineering & Technology,
Maharshi Dayanand University, Rohtak-124001, Haryana, India*

*Corresponding Author, Tel.: +91-1262279083

E-Mail: v.nano87@gmail.com; vikasbtdhull.gf.uet@mdurohtak.ac.in

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Abstract- A lactate biosensor has been designed for the detection of lactate in a real sample. A combination of composite material comprising of multi-walled carbon nanotubes (MWCNTs) and zinc oxide nanoparticles has been used as working electrode, which were deposited on gold (Au) wire. This was treated as working electrode for the preparation of a LDH-based amperometric biosensor along with acetyl coenzyme, which acts as the mediator to enhance electron transportation. The biosensor showed excellent results in terms of stability, response time, and sensitivity. The sensitivity of the biosensor is 4.487 mA/ μ M and its linearity is between 10 μ M and 100 μ M. The biosensor has a 0.67 μ M limit of detection with a response time of 8 sec. The optimal temperature is 35°C, and the optimal pH is 8. All the results confirmed that the ZnO/MWCNTs/Au electrode, along with acetyl coenzyme, acts as a suitable matrix for the purpose of immobilizing LDH enzymes for the formation of lactate biosensors.

Keywords- Lactate; Biosensor; ZnO nanoparticles; MWCNTs; Cyclic voltammetry; UV Spectroscopy

1. INTRODUCTION

Lactate is a vital metabolite that acts as an indicator for various health-related problems, surgical surveillance, the quality of food products, etc [1]. Anaerobic respiration results in the production of lactate as the end product. An increase in the lactate concentration can lead to various health-related problems such as lactate acidosis, infections, heart failure, etc. Various fermented food items contain a good amount of lactate due to the bacterial actions during the

fermentation process. Moreover, lactate can also be used in various fields such as the cosmetic industry, pharmaceutical industry, chemical industry, etc. Lactate also plays a vital role in bioprocess engineering as the media of various processes consist of lactate; therefore, quantification of lactate is an important process. Several analytical techniques, including electrophoresis, spectroscopy [2-4], fluorometric methods, high-pressure liquid chromatography (HPLC), etc., can be used for lactate measurement in the given sample [5-8]. But these techniques are highly selective, specific, and expensive in nature .

Biosensors have emerged as a cutting-edge method in recent years, with applications ranging from the environment to biology. Contemporary biosensors are readily transportable, mass-produced, and miniaturised. Moreover, in order to detect abrupt changes in biological fluids, biosensors' capacity to test analytes in real-time is particularly useful. A biosensor is an analytical instrument that integrates a biological catalyst for the detection of analytes within the sample as a result of a physiochemical reaction. It comprises a transducer that converts the physiochemical reactions into quantifiable signals (electrical signals). Typically, classification of biosensors is based on the type of transduction technique employed. Biosensors transducers can convert a wide range of physical, biological, or chemical interactions into electrical signals [9-12].

Enzyme-based amperometric Lactate biosensors are basically made up of LOD (lactate oxidase), LDH (lactate dehydrogenase), or a combination of the enzymes. LOD biosensors are based on the principle of the conversion of lactate into pyruvate by reducing oxygen into hydrogen peroxide. The level of lactate in the sample is determined by detecting hydrogen peroxide. LDH biosensors are based on the principle of the conversion of lactate into pyruvate along with the oxidation of NADH. By quantifying NADH, it is possible to determine the amount of lactate present in the sample. To increase the sensitivity of the biosensor, fabrication can be done using nanomaterials such as nanoparticles, multi-walled carbon nanotubes, and a hybrid of both because of their good electrical, thermal, and mechanical properties. Among variety of nanoparticles zinc oxide nanoparticles can be used as they are highly stable, show good electrical properties, and compatibility [13-15].

The present work emphasis on the formation of LDH biosensor using zinc oxide nanoparticles (ZnO NPs), carboxylated multi-walled carbon nanotubes (c-MWCNTs), lactate dehydrogenase enzyme (LDH) (the main enzyme), and acetyl coenzyme as a mediator for the purpose of detecting lactate in the food samples, and hence the quality of the food samples can be detected.

2. EXPERIMENTAL SECTION

2.1. Materials and Instrumentation

LDH (L-Lactate dehydrogenase) from hydrophilized powder of rabbit muscle purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India, Acetyl coenzyme A, trilithium salt

extrapure acquired from Sisco Research Laboratories Pvt. Ltd., Maharashtra, India, zinc oxide nanoparticles (self-made), MWCNTs obtained from Sisco Research Laboratories Pvt. Ltd., India, glutaraldehyde, food sample (soy sauce, milk, yogurt; collected from Q Mart, Shivaji Colony, Rohtak, Haryana), and distilled water. The following instrumentation has been used: magnetic stirrer along with hot plate (Jain Scientific Glass Works), centrifuge (REMI, R-24), UV-VIS-NIR spectrophotometer (Shimadzu, UV-3600 Plus), particle size analyser (Malvern, ZEN3600), potentiostat (Metrohm, Autolab 302N potentiostat-galvanostat), scanning electron microscope (Tabletop microscope, TM3000), dry heat oven (Narang Scientific Works PVT. LTD.), digital pH meter (Contech, pH-103), refrigerator (Samsung).

2.2. ZnO nanoparticles synthesis

For the chemical synthesis of ZnO nanoparticles, one molar solution of zinc sulphate ($\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$) (100 ml) is prepared in distilled water as the zinc source and oxalic acid ($\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) as the precipitating agent. Zinc sulphate ($\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$) solution is stirred on a hot magnetic stirrer at 400 rpm for approximately 15 to 20 minutes at room temperature, and oxalic acid ($\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) is added dropwise until a cloudy appearance (white color) occurs. Following a brief period of cooling at room temperature, the solution underwent centrifugation (REMI R-24). Pellets are obtained and allowed to dry at room temperature [16-19].

2.3. Characterizing ZnO nanoparticles using UV and PSA

UV-visible characterization has been done to investigate absorbance of UV light by the ZnO nanoparticles. Performed in the Aryabhata Central Instrumentation Laboratory, MDU, Rohtak, Haryana, using a UV-VIS-NIR spectrophotometer (Shimadzu, UV-3600 Plus) [10-22]. A particle size analyser is a technique that helps us detect the average size of the ZnO nanoparticles. This was also performed at Aryabhata Central Instrumentation Laboratory, MDU, Rohtak, Haryana, using a particle size analyzer (Malvern, ZEN3600). Zeta potential helps us detect the potential (electron negativity) of the ZnO nanoparticles. The more negative value of zeta potential corresponds to more stability of nanoparticles. Otherwise, lower zeta potential may lead to formation of clumps affecting stability of nanoparticles. Performed at the Aryabhata Central Instrumentation Laboratory, MDU, Rohtak, Haryana, using a particle size analyzer (Malvern, ZEN3600).

2.4. Fabrication of Electrode

The Au/ZnO/MWCNTs electrode was prepared by applying a 2 μL suspension of ZnO nanoparticles and MWCNTs in GAL (glutaraldehyde, which acts as a cross-linker) on the surface of the gold electrode (22 carrot wire, purchased from a local jeweller shop, Rohtak) in a plastic tube and placing it in the dry heat oven for drying at 30°C. An acetyl coenzyme

mixture was prepared using 5 U of the acetyl coenzyme powder and phosphate buffer solution of 0.1 mol L^{-1} with pH approximately 7.4, which acts as a mediator. Pipette out 5 U of the acetyl coenzyme solution on the Au/ZnO/MWCNTs electrode surface along with GAL and allow for drying at room temperature. A lactate dehydrogenase mixture was prepared using 5 U of LDH powder and 0.1 mol L^{-1} phosphate buffer solution (pH approximately 7.4). Pipette out 5 U of the LDH solution on the surface of the Au/ZnO/MWCNT/AC electrode along with GAL and allow for drying at room temperature. After solidification, we removed the fabricated electrode from the tube, washed it with distilled water to remove excess unbind material, and later stored it in a refrigerator, which was later used as a working electrode. Figure 1 shows the schematic illustration of electrode fabrication.

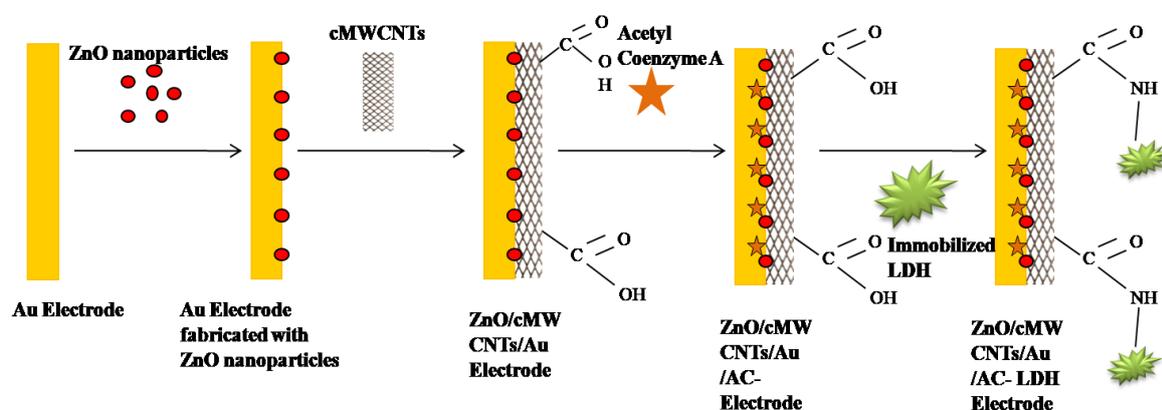


Figure 1. Step-by-step schematic representation of the fabrication of the gold electrode using zinc oxide nanoparticles, multiwalled carbon nanotubes, acetyl coenzyme A, and Immobilised LDH

2.5. Characterization of Fabricated Electrode

Morphological study of the fabricated electrode was done using SEM (Tabletop microscope, TM 3000). Performed at the Spectro Analytical Pvt. Ltd., Okala, New Delhi. ZnO/Au electrode, ZnO/MWCNT/Au electrode, and ZnO/MWCNT/Au/AC-enzyme immobilized electrode was all detected using SEM.

2.6. Electrochemical Experiment

Electrochemical experiments were performed using an electrochemical workstation (Metrohm, Autolab 302N potentiostat-galvanostat), which consists of a three-electrode system with an enzyme immobilized gold electrode fabricated with ZnO/MWCNT/AC, a counter electrode of platinum, and a reference electrode of Ag/AgCl as a reference electrode at room temperature. Performed in the Central Instrumentation Laboratory, Centre for Biotechnology,

MDU, Rohtak, Haryana [23]. Figure 2 shows the conceptualization of the electrochemical process necessary for lactate measurement.

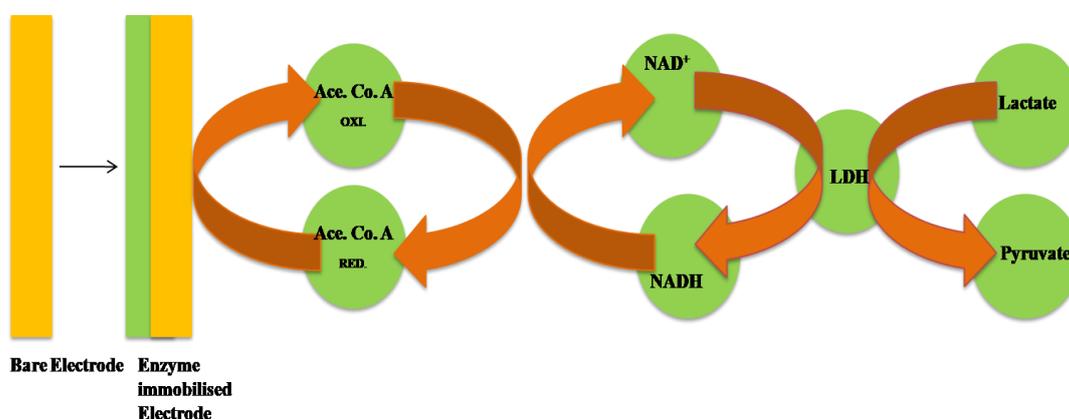


Figure 2. Schematic representation of the electrochemical reaction due to the addition of a mediator (Acetyl Coenzyme A) and an immobilised LDH enzyme

2.7. Study of Optimum Parameters for Biosensor

At first, we study the effect of analyte (lactate) concentration in the sample on the reaction current by plotting a graph of the current on the y axis versus the analyte (lactate) concentration on the x axis. Secondly, to detect the optimum temperature for the lactate biosensor, we study the effect of temperature (20°C–70°C) on the reaction current. We investigate the impact of various pH levels on the reaction current to determine the ideal pH for the biosensor's operation; for this, using sodium phosphate buffer, we created numerous samples of 0.1 M KCl solutions with varying pH levels. Next, we investigated the impact of incubation period on the biosensor's activity from 1 second to 12 seconds. At last, we use the Michaelis-Menton equation to plot the Lineweaver-Burk plot, which shows the relation between the reciprocal of current on the y axis and the reciprocal of analyte concentration on the x axis.

3. RESULTS AND DISCUSSION

3.1. Characterization of ZnO nanoparticles

UV spectroscopy results of zinc oxide nanoparticles are shown in Figure 3(a), which is used to detect and identify the nanoparticles. The obtained absorbance of ZnO nanoparticles is 378 nm at room temperature, which is close to the standard absorbance of ZnO nanoparticles and hence confirms the formation of zinc oxide nanoparticles. Figure 3(b) shows the particle size analyzer (PSA) of the ZnO nanoparticles. The size of the ZnO nanoparticles lies between 10 and 100 nm. The Z-average obtained is 37.74 d.nm., the pre-delivery intercept is 0.455, and the intercept is 0.825. Size, intensity, and standard deviation for peak 1 are 61.40 d.nm., 85.2%, and 26.25 d.nm., respectively. Size, intensity, and standard deviation for peak 3 are 4610 d.nm.,

2.8%, and 823.5 d.nm., respectively. Figure 3(c) shows the zeta potential of the zinc oxide nanoparticles. The ZnO nanoparticles' zeta potential ranges from -100 to 0. The zeta potential, zeta deviation, and conductivity obtained are -35.1 mV, 13.7 mV, and 3.65 mS/cm, respectively. Peak 1 has the following measurements: a mean value of -432 mV, an area of 62.8%, and a standard deviation of 7.97 mV. Peak 2 has the following characteristics: a mean value of -20.7 mV, an area of 37.2%, and a standard deviation of 6.29 mV. Peak 3 has the following metrics: 0.00 mV for the mean, 0.0% for the area, and 0.00 mV for the standard deviation.

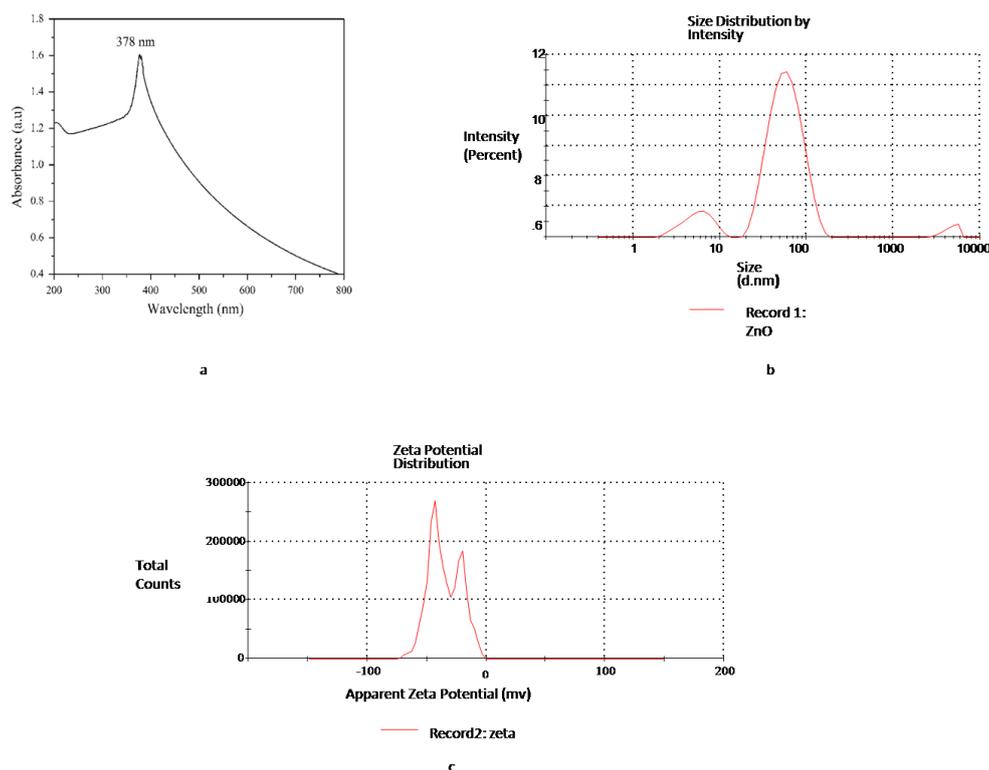


Figure 3. (a) UV-Visible graph of the ZnO Nanoparticles is obtained at 378 nm at 25°C, (b) Particle Size Analyzer of the ZnO Nanoparticles, (c) Zeta Potential of the ZnO Nanoparticles

3.2. Characterization of Fabricated electrodes

Figure 4 shows a comparison between scanning electron microscopy (SEM) images of a gold electrode fabricated with ZnO nanoparticles and SEM images of a gold electrode fabricated with ZnO nanoparticles, multiwalled carbon nanotubes, LDH (the main enzyme), and acetyl coenzyme (as a mediator) at $\times 30$ resolution. Characterisation of the gold electrode is done to check whether ZnO nanoparticles, multiwalled carbon nanotubes, LDH enzyme, and acetyl coenzyme stick to the surface of the gold electrode properly or not.

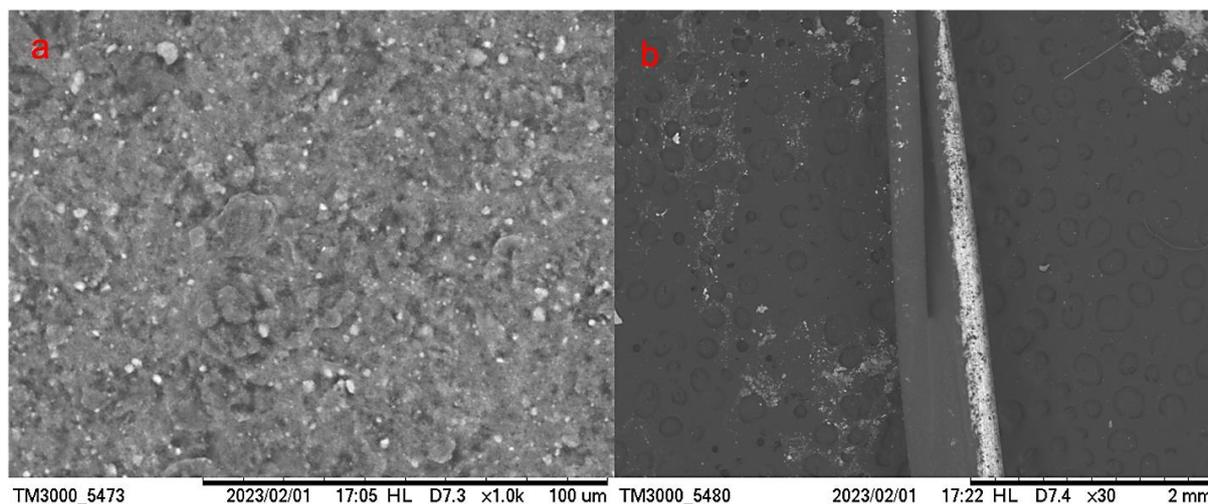


Figure 4. (a) SEM of the gold electrode fabricated with zinc oxide nanoparticles; (b) SEM of the gold electrode fabricated with zinc oxide nanoparticles, multiwalled carbon nanotubes, LDH enzyme, and acetyl coenzyme

3.3. Cyclic Voltammetry Analysis

Figure 5 shows the cyclic voltammetry of the LDH-based biosensor, which is performed between 0.0 and 0.3 V. In this cyclic voltammogram, positive current (i_{pa}) shows the anodic oxidation of NAD^+ , which is obtained at 0.00004 A, and negative current (i_{pc}) shows the cathodic reduction of NADH, which is obtained at -0.00014 A, which indirectly tells us about the lactate concentration in the sample. The continuous cycle shows the breakdown of lactate into pyruvate. The oxidation of NAD^+ takes place at 0.21 V.

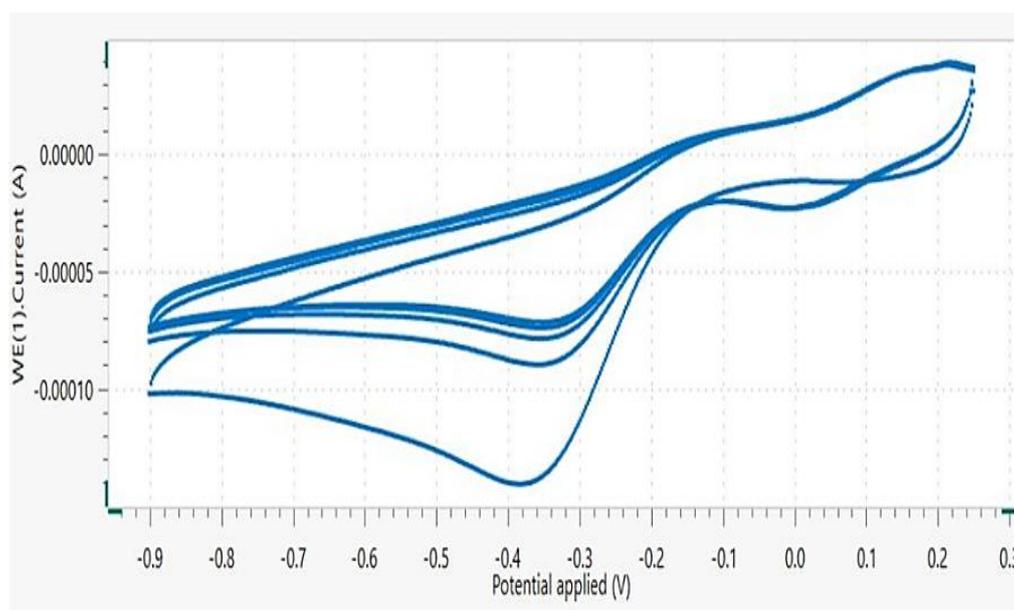


Figure 5. Cyclic Voltammetry between 0.0 and 0.3 V and at room temperature

3.4. Effect of Enzyme various parameters on Biosensor

At first, we assessed the effect of the amount of LDH enzyme used in the preparation of the lactate biosensor, and it was found that the biosensor is highly responsive to the presence of LDH enzyme. As we increased the amount of LDH enzyme, the responsiveness of the lactate biosensor increased due to the catalytic activity of LDH. The reaction current is affected by enzyme concentration, as seen in Figure 6. On the reaction current, we evaluated the impact of various temperatures. The obtained result is calibrated in the form of a graph. The reaction current's relationship to temperature is depicted in Figure S1 of supplementary file. The maximum output is obtained at 35°C.

We assessed the effect of different pH values from 6 to 10 on the reaction current. The obtained results are calibrated in the form of a graph, with maximum output at pH 8. The influence of pH on the reaction current is seen in Figure S2 in supplementary file. The response time is the period of time that the transducer needs to spend in order to detect lactate in the biosensor. Response time is also known as reaction time. Between 0 and 12 seconds, the reaction time was examined. Figure S3 of supplementary file shows the incubation time, with the best activity occurring at 8 sec. To study the enzyme kinetics and determine K_m and V_{max} we use Lineweaver Burk plot. Figure 7 shows a Lineweaver-bruk plot that is plotted by using the Michaelis-Menton equation:

$$\frac{1}{C} = \frac{K_m}{C_{max}} \frac{1}{S} + \frac{1}{C_{max}} \quad (1)$$

S is the concentration of the substrate, C is the reaction current, K_m is the Michaelis-Menton constant, and C_{max} is the maximal reaction current.

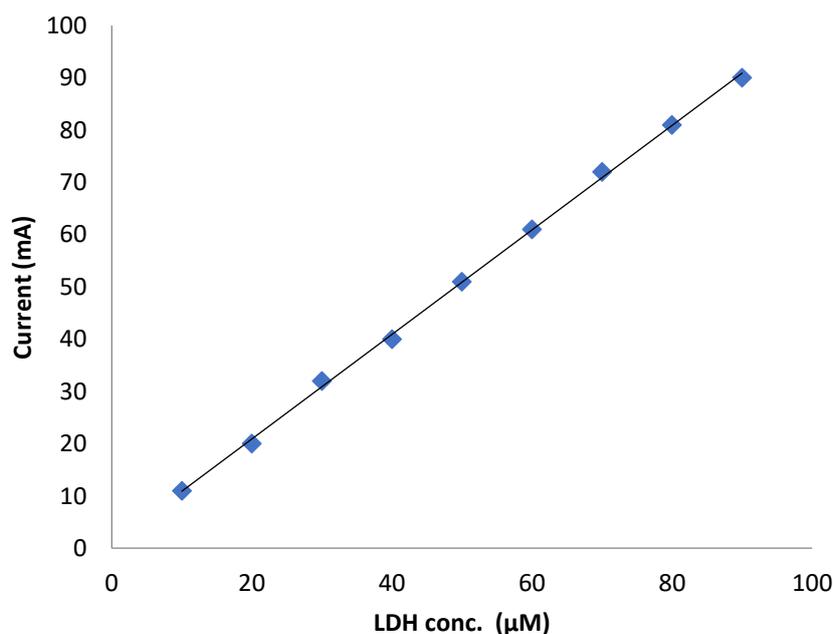


Figure 6. Effect of LDH concentration on the reaction current at room temperature

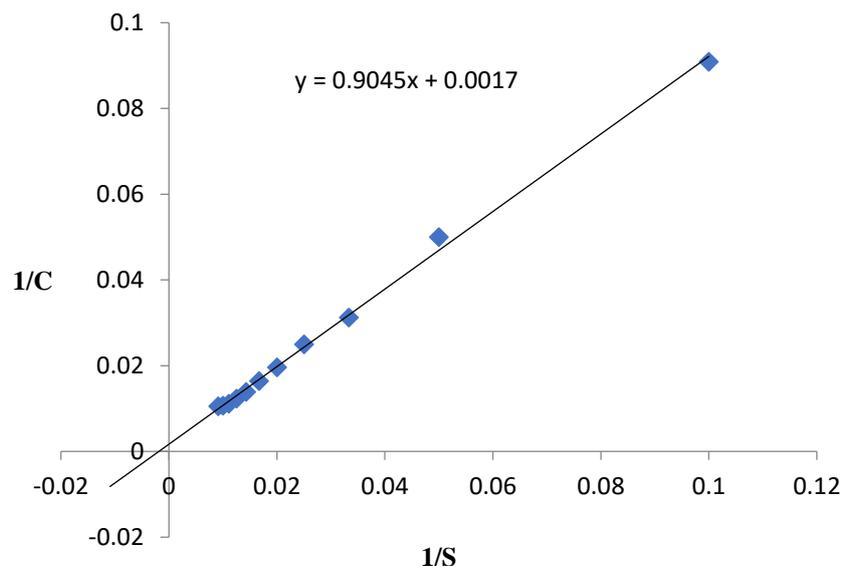


Figure 7. Lineweaver Bruk Plot of fabricated biosensor at room temperature

3.5. Effect of Interfering Substances and accuracy

To study the activity of the biosensor in the presence of other substances, we added various other substances to the sample, including glycine, ascorbic acid, glucose, and citric acid, which can affect the working of the self-made biosensor. Figure S4 of supplementary file shows the effect of various substances on the activity of the biosensor. To check the accuracy of the biosensor, we tested it on various soya sauce samples. The results obtained by a self-made biosensor are compared with the results obtained using a spectrophotometer. The spectrometry technique is used to measure the presence of lactate in the soya sauce sample which is used as a reference or control at room temperature [14].

Table 1. Detection of lactate in various soya sauce samples is done at room temperature using a newly fabricated biosensor

S No.	Sample used	Biosensor	Spectroscopy	Deviation	Relative Standard Deviation
1	Soya Sauce	0.26	0.28	-0.02	7.1
2	Soya Sauce	0.68	0.64	0.04	6.25
3	Soya Sauce	1.08	1.13	-0.05	4.4
4	Soya Sauce	1.67	1.59	0.06	3.7
5	Soya Sauce	1.96	1.92	0.04	2.0

It is performed at the Aryabhata Central Instrumentation Laboratory, MDU, Rohtak, Haryana. Table 1 contains a summary of the outcomes that were collected. The results obtained are quite similar, with less deviation. To detect the % recovery by the biosensor, we added lactate ranging from 0.5 mg/dl to 2.0 mg/dl to various food samples at room temperature. The outcomes attained tell us about the recovered lactate in various soya sauce samples by the newly fabricated biosensor, which are tabulated in Table 2.

Table 2. %Recovered by the newly fabricated biosensor after addition of known amount of the lactate to the sample at room temperature

S No.	Lactate added to sample (mg/dl)	Lactate recovered from (mg/dl)	%Recovery by Biosensor
1	0	1.68	-
2	0.5	2.10	96.3%
3	1.0	2.58	96.2%
4	1.5	3.00	94.3%
5	2.0	3.48	94.5%

3.6. Evaluating Biosensor

The biosensor was evaluated by determining storage stability, sensitivity and accuracy. It was found that the response current reduced only to 88% after 120 days, which shows good stability of the biosensor. The reason behind the decrease in stability is the un-immobilization of the enzyme after a few months. Figure S5 of supplementary file shows the stability of the biosensor in the form of a graph (% activity vs. days). The biosensor is stored in a cool, dry place when not in use. The sensitivity of the biosensor is 4.487 mA/ μ M, which we calculated by using the standard formula: sensitivity = slope (current vs. concentration of lactate) (0.920)/standard deviation of blank (0.205). For the purpose of determining the blank's standard deviation, we measure the cyclic voltammetry of the modified electrode without analyte (lactate) for 10 to 20 times and measure the current of the blank at the potential where our analyte gets oxidised. The limit of detection is 0.67 μ M, which is calculated using the standard formula: 3*standard deviation/slope (current vs. lactate concentration). The biosensor's linearity is attained between 10 and 100 μ M. Figure S6 of supplementary file shows the calibration curve of the biosensor. A comparison between various biosensors on the basis of their working electrode used, sensitivity, linearity, limit of detection, sample used, response time, and storage stability has been tabulated in Table 3. On the basis of the comparison, the present work is better in comparison to others on the basis of storage and stability [24-30].

Table 3. Comparison between Various Biosensors on the Basis of Various Parameters of the Biosensors

Working Electrode	Sensitivity	Linearity	Limit of Detection	Sample Used	Response Time	Storage Stability	Ref.
ZnO/MWCN T/AC/AU electrode with LDH enzyme	4.487 mA/ μ M	10 μ M to 100 μ M	0.67 μ M	Soy Sauce	8 sec.	4 months (88% accuracy)	Present Work
CeO ₂ , GCE,NADH	571.191 μ A/mM	0.2 to 2 mM	50 μ M	NA	<4 sec.	12 days (89% shelf life)	[24]
ZnO, MWCNTs, GCE, Nafion membrane/film	NA	0.01 to 10 μ mol/L, 10 to 200 μ mol /L	4 nmol/L	Blood Plasma	NA	1week (80% accuracy)	[25]
Fe ₃ O ₄ , MWCNTs, GCE	7.67 μ A/ mM	50 to 500 μ M	5 μ M	Human Serum	50 sec.	NA	[26]
Meldeola blue-reinecke salt, SPEs, cellulose acetate membrane	NA	0.55 to 10 mM	NA	Calf Serum (bovine) sample	10 sec.	17 days	[27]
SPEs, Sol-gel matrix(MB + NAD ⁺)	260 \pm 13 μ A/M	1.25 \times 10 ⁻⁴ to 2.48 \times 10 ⁻³ M	0.11 mM	NA	NA	NA	[28]
GCE, Alginate hydrogel	Good	5 to 30 μ M	2.52 μ M	Artificial Sweat	NA	NA	[29]
Poly-carbomyl-sulfonate, Hydrogel, Teflon membrane	3.05 to 276.35 mA mmol ⁻¹ L	10 to 400 μ M	4.3 μ M	NA	2 sec., total test time (4 min)	NA	[30]

*NA = Not Available

4. CONCLUSION

A highly responsive and stable lactate dehydrogenase-based biosensor is created using ZnO nanoparticles and MWCNTs. ZnO/MWCNTs/Au layers, along with acetyl coenzyme, which acts as a mediator, are responsible for the suitable environment for the MWCNTs strong catalytic performance and the LDH enzyme's biological activity regarding H₂O₂ with a sensitivity of 4.487 mA/ μ M, linearity between 10 μ M and 100 μ M, 0.67 μ M limit of detection, and 8 sec response time. The result obtained using this lactate biosensor is quite similar to that obtained using spectrophotometry, which shows the good sensitivity of the biosensor. The most

suitable pH and temperature for the working of the biosensor are 8 and 35°C respectively, with a stability of 120 days (accuracy of 80%), which is far better as compared to other biosensors. This method can be used to determine or identify lactate in various food items in addition to the soy sample.

Declarations of interest

The authors declare no conflict of interest in this reported work.

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