ABSTRACT
Compatible solutes from hyperthermophilic bacteria, called hypersolutes, are very efficient for the preservation of the performance of a wide variety of biomaterials; ranging from proteins to whole cells and artificial tissues. The overall objectives of this work have been to investigate the application of hypersolutes to enhance the performance of enzyme based biosensors based on the stabilization properties offered by hypersolute compounds, particularly with respect to storage and operational lifetime. Based on a simple, rapid and low-cost measurement system, the optimum working conditions for the hypersolutes were determined for the following enzymes, selected due to their commercial importance: Glucose Oxidase (GOx), Alcohol Oxidase (AOx), Acetylcholinesterase (AchE) and Lactate Dehydrogenase (LDH). The sensor range, operational stability, storage stability, pH tolerance and thermal stability have been investigated. Ectoine and Firoin A presented a small stabilizing effect (+80%) against ionic strength variations. DGP gave the best stabilization against storage (+237%) with Firoin A and Hydroxyectoin (+218% and +176% respectively).

KEY WORDS
Enzyme biosensor, stabilization, hypersolutes.

1. Introduction
A biosensor is an analytical device incorporating a biological or biologically-derived sensing element, either integrated within or intimately associated with a physicochemical transducer. The biorecognition of the analyte by the receptor creates a response that is converted into a measurable signal by the transducer. A biosensor produces either a discrete or a continuous digital electronic signal related to a single analyte or analyte group concentration of. It is estimated that the market size for worldwide biosensors at year end 2003 was $7.3 billion. Even with unfavourable geopolitical events occurring and a weak global economy, the market is projected to improve and grow to $10.8 billion in 2007, a growth rate of ~14% per annum. [1,2]

Lack of stability of the biological component, in this case enzyme, is one of the most important drawbacks for biosensor applications. An ideal diagnostic enzyme should fulfill 2 important characteristics: to be stable and to be functional. Thus enzymes have to maintain a stable, essentially hydrophobic core, anchored by intra-molecular H-bonds. In order to be functional, the enzyme must maintain a stable active site. Various attempts have been made to increase the stability of the enzyme without hindering its activity. Both shelf life and operational stability may be enhanced by several approaches.

Immobilization to a supporting structure: While immobilization can give notable stability gains, it is generally undertaken to prevent loss of bioligand activity. However, such a practice may affect the protein conformation or be responsible for non-uniform distribution, parasite reaction or diffusion.

Chemical modification: This remains a useful solution, despite having been overshadowed by genetic strategies. Several methods have been used, including: cross-linked enzymes; covalent attachment to polymers; surface modification (by chemical modification of charged groups at the molecule surface).

Protein engineering: This involves the manipulation of the protein at the genetic level. Genetically modified proteins offer enhanced capacities compared to their native forms.

Additives: They involve the use of salts, polyols, divalent metal ions and sugars. These additives are believed to influence the microenvironment of the enzyme and modify the hydrophobic or hydrophilic interactions by disrupting the enzyme/water interactions. A range of low molecular weight additives, sugar derivatives, exert stabilizing effect, such as trehalose. The study of protein and enzymes from extremophilic organisms can give insight to protein stability as well as providing ready-made stable proteins and stabilizing agents for biotechnological applications. [3,4]
2. Materials and Methods

2.1 Hypersolutes

2.1.1 Stabilisation agents examined in this study

The stabilizing agents considered for this study were Firoin, Ectoine®, Hydroxyectoine, Diglycerol Phosphate (DGP) and Potassium Mannosyl-Lactate (PML), provided either by Bitop AG (Witten, D) or StabVida (Oeiras, P).

Firoin (αD-mannosglycerate (MG) has a MW of 306.2g and solubility in water of 537g/L (1.75M) at ambient temperature. Ectoine and Hydroxyectoine are compounds from the same family, Ectoines. They are highly compatible with cell metabolism and tolerated up to 1M concentrations. Ectoine, or (S)-2-Methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid has a MW of 142.2g and solubility in water of 569g/L at ambient temperature, e.g. 4M, whereas Hydroxyectoine, or (S)-2-Methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid, has a MW of 158.2g and a slightly higher solubility in water of 664g/L (4.2M) at ambient temperature. Diglycerol Phosphate (DGP) and Potassium Mannosyl Lactate (PML) are novel compounds. Due to their similarities with mannosyl glycerate (MG), they may function as stabilizers. PML is less well known, with a MW of 290g/mL and was provided in solution at pH=4.6. DGP has a molecular weight of 284.2g and its solubility in water is of 12.9M. All structures are shown in Figure 1.

![Figure 1: Stabilising agent structures](image)

2.1.2 Stabilisation mechanism

The stabilising agents used may act in a similar manner to the well known stabilising agent Trehalose. Trehalose has already been thoroughly studied and two mechanisms have been proven to play a part in protein stabilization. The first is the formation of a glassy matrix and the second, the formation of H-bonds to replace water molecules. However, neither mechanism fully explains the stabilization process.

Sugar glasses have been used to stabilize protein during drying. They require a high Tg (glassy transition temperature), a poor hygroscopicity, a low crystallization rate and must contain no reducing group to be efficient stabilizing agents. The formation of a glassy matrix leads to the restriction of the molecule motion, thereby limiting degradation, as this process is thought to be directly linked to protein mobility i.e. its ability to have its conformation modified. According to this model, the glassy transition temperature (Tg) greatly influences stabilization. The higher a Tg the solute has the more stable the protein will be. However, the hydration levels of both protein and sugar influences their respective Tg. In addition, proteins in a dry state do have higher Tg than sugar. This system alone therefore can’t explain stabilization.

An additional explanation that completes the model is the formation of H-bonds to replace water molecules. In the absence of either water or sugar, H-bonding may occur between sites inside the protein, thereby eventually compromising its activity through a change of conformation. The remaining native protein conformation is the key to the remaining activity, the active site should remain untouched losing none of its activity: the sugar-protein interaction being less labile than the water-protein one, leading to an increased stability for the conformation. Obviously, the greater the levels of interaction are between the sugar and the protein, the more effective the preservation will be. Sugars have been proven to preserve the native α-helix. They also inhibit the dissociation of the protein into subunits, preserving its quaternary structure.

2.2 Enzymes studied

2.2.1 Glucose oxidase

Glucose oxidase is perhaps the most commercially important biocatalyst for diagnostic applications (blood glucose measurement). It is also commercially used in the production of gluconic acid and food preservation. The reaction pathway, leading to the electrochemical determination of glucose is detailed in Equ.1.

\[ C_6H_{12}O_6 + O_2 \xrightarrow{GOx} C_6H_{10}O_6 + H_2O_2 \]  \hspace{1cm} (1)

\[ H_2O_2 \xrightarrow{electrode, \text{surface}} O_2 + 2H^+ + 2e^- \]

The substrate, glucose, is oxidized to gluconolactone, thereby reducing the prosthetic group within the GOx active site. Free oxygen dissolved in the test medium is able to diffuse to the enzyme active site and act as an electron acceptor, oxidizing the prosthetic group within the enzyme structure and being reduced to hydrogen peroxide. The latter, on diffusing from the active site of the enzyme to the electrochemical transducer, is oxidized, liberating electrons to the electrode, thereby generating a measurable current and releasing free dioxygen back into solution. The magnitude of the current generated is directly related to the number of glucose molecules consumed, hence to the glucose concentration. [6,7]
2.2.2 Alcohol oxidase
Alcohol oxidase is widely used in the wine and beer industry, but also in biosensors for alcohol testing on breath. As with GOx, alcohol oxidase uses oxygen as co-factor (Equ.2), however this enzyme is inherently unstable and displays different characteristics, depending upon its origin. The reaction end product is hydrogen peroxide; hence electrode-mediated detection is similar to GOx. [8]

\[ R - CH_2OH + O_2 \rightarrow R - CHO + H_2O_2 \]  (2)

2.2.3 Lactate dehydrogenase
Lactate dehydrogenase is used in the food industry and in the monitoring of sports performance. The enzyme requires a co-enzyme (Equ.3) and has proven itself rather difficult to detect electrochemically. Lactate dehydrogenase is a relatively stable enzyme, but not as stable as glucose oxidase.

\[ \text{Lactate} + NAD^+ \rightarrow \text{Pyruvate} + NADH + H^+ \]  (3)

NAD (Nicotinamide Adenosine Dinucleotide) is an essential element in the Lactate Dehydrogenase (LDH) mechanism. Lactate is converted to pyruvate while NAD is reduced by LDH to NADH which is then oxidised at the electrode surface, generating a signal in proportion to the amount of lactate present in the sample. However, NAD is a rather difficult molecule to detect electrochemically and requires a high overvoltage. This may result in a number of undesirable side-effects, including the strong adsorption of the oxidised form of NAD to electrode surfaces. It also generates electrode fouling and interfering background current problems. Finally, the process may also result in the electrochemical formation of enzymatically inactive forms of NAD. All these factors can contribute to the generation of unreliable responses, and in some cases the signal may be entirely removed. Systems based on the amperometric detection of NAD therefore lack stability and sensibility. Correspondingly, the use of a redox mediator (Meldola’s blue) has been considered. Meldola’s Blue (8-Dimethylamino-2,3-benzophenoxazine Hemizinc chloride) salt may be oxidized at suitably poised working electrodes at a considerably lower potential than NAD/NADH. The mechanism is as follows (Equ.4): [9]

\[ \text{Lactate} + NAD^+ \rightarrow \text{Pyruvate} + NADH + H^+ \]  (4)

2.2.4 Acetylcholinesterase
Acetylcholinesterase is used for organophosphate pesticide monitoring, in the agro- and food industries and plays an important role in some inherited diseases such as Alzheimer’s. Acetylcholinesterase requires a co-enzyme (TCNQ, Equ.5) and is unstable.

\[ ATch + H_2O \rightarrow \text{Acetic Acid} \]  (5)

2.3 Methods
2.3.1 Enzyme immobilisation
All experiments were performed at pH=7.5, close to the optimum working pH for all the enzymes studied here. The buffer used was 0.1M phosphate buffered saline (PBS) containing 0.1M KCl unless stated otherwise. Enzymes and stabilizing agents were immobilized by simple physical adsorption at the working electrode surface. Enzyme activity has been tested both in the presence and absence of stabilizing agent in order to determine any effects due to stabilizer addition. [7]

2.3.2 Transduction mechanism
The products of the enzyme reactions were determined amperometrically. Amperometry is a dynamic process in which electron flow at a working electrode is measured, whilst maintaining a constant applied potential to allow the oxidation/reduction of analytes. This redox process results in a flow of electrons through the working electrode, than can be quantified by an in-circuit ammeter. Tests were performed on a field-portable electrochemical analyzer (PalmSens, Palm Instruments BV, Utrecht, NL). Raw data were transferred into Matlab for further treatment.

2.3.3 Transducer design
A 3-electrode configuration has been designed, incorporating a working electrode consisting of a rhodined carbon film overlaying a basal carbon track, a carbon counter electrode and silver/silver chloride reference electrode (Fig. 2). The test solution was either applied as a droplet to the circular aperture within the blue insulation layer, or the 3-electrode assembly was dipped into the test solution. Full details of the screen-printing process are detailed elsewhere. The working ink is composed of up to 5% rhodium in a carbon paste, with added promoters for the favorable oxidation of certain electroactive species, notably peroxides, at a reduced potential. The open porous structure and physico-chemical properties of the electrocatalyst have proven to be an excellent medium for the simple physical immobilization of proteins. The matrix lowers the potential for amperometric detection rendering the use of mediators unnecessary in certain situations. A lower potential decreases the possible interferences, increasing biosensor specificity.

Screen-printed enzyme-electrode biosensors have been prepared in-house and offer certain benefits:

- Mass-manufacturing at low cost.
• Sensors can be used once and then discarded, negating electrode cleaning and regeneration issues.
• The approach mirrors that of the most commercially successful branch of the enzyme biosensor market.
• Multiple biosensor elements may be produced in single batches for testing purposes. [2,10]

3. Results

3.1 Enzymes alone

3.1.1 Glucose oxidase

A series of experiments were performed to determine the optimum loading of glucose oxidase on the working electrode surface. The optimum loading represents a balance between the maximum S/N (signal to noise) ratio and insulation of the working electrode by excess biological material. The optimum GOx loading was determined to be 2U per electrode. Calibration curves were constructed, with respect to the GOx-electrode responses to glucose in solution.

GOx follows a classical enzyme kinetic profile over the 0-10mM range. Fig.3 illustrates a typical Michaelis–Menten substrate reaction rate response, with a first order relationship evident at low glucose concentration (< 2mM), then tending to a zero order reaction at higher concentration. A LineWeaver-Burke plot of the response was then produced and the kinetic values of the enzyme calculated. These kinetic values will be used to aid the assessment of the stabilizing agents, when used for enzyme-electrode stabilization purposes.

The \( K_m \) was lower for the immobilized GOx than for GOx in solution, which confirm that immobilization affects the enzyme configuration, thereby lessening its activity. The \( i_{\text{max}} \) value is higher for the immobilized enzyme as the enzyme and the reaction products are closer to the electrode and therefore more \( \text{H}_2\text{O}_2 \) is likely to be detected for the same period of time. As the \( K_m \) value was 4mM, the studies involving investigation of stabilizing agents were performed with a glucose concentration of 20mM, e.g. \( 5 \times K_m \), this value being empirically proven to be located in the plateau of the Michaelis-Menten kinetic profile for GOx.

### 3.1.2 Enzyme kinetic data

The results obtained for GOx, AOx and AchE are summarized Table 1. All enzymes have been found to display a classical Michaelis-Menten curve. The AOx has been confirmed to be the less stable and satisfactory enzyme with a RSD of 11.6%. The remarks concerning the kinetic values of free vs. immobilized enzyme apply for GOx as well as for AOx and AchE.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Activity deposited [Substrate]</th>
<th>Km</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOx</td>
<td>glucose</td>
<td>2U</td>
<td>6.2mM</td>
<td>9.2µA</td>
</tr>
<tr>
<td>AOx</td>
<td>ethanol</td>
<td>500mU</td>
<td>4.60%</td>
<td>2.76µA</td>
</tr>
<tr>
<td>AchE</td>
<td>acetylthiocholine</td>
<td>1U</td>
<td>14.7mM</td>
<td>76.9µA</td>
</tr>
</tbody>
</table>

### 3.1.3 Lactate Dehydrogenase

The amperometric conditions for LDH on SPE (screen-printed electrodes) were found in literature to be, at pH=7.5, \( E=+350\text{mV} \) on Rh-C vs. Ag/AgCl over the range 5-4000mU, with a cofactor concentration of 1mM. The cofactor, NADH, is difficult to determine electrochemically. Volumes (10µL) of enzyme solution were deposited at the SPE surface. Poor performance of the system indicated that a mediator (in this case Meldola’s Blue) was required in order to reduce the amperometric detection potential.

Optimum deposition condition and calibration curve characteristics

NAD was first studied in presence of Meldola’s Blue (MB). Different concentrations of NAD were tested, in the range 0.5-50mM NAD with 6.6mM MB. The detection conditions were optimized with an assay duration of 150s and a substrate volume of 20µL. The optimum signal:noise ratio was obtained at an NAD concentration of 25mM. Cyclic voltammetric studies of 25mM NAD, 6.6mM MB were then realized in order to determine the optimum working potential (+100mV).

### Table 1: Optimized parameters and results for GOx, AOx & AchE

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Km</th>
<th>i max</th>
<th>RSD</th>
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<tr>
<td>GOx</td>
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<td>9.2µA</td>
<td></td>
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<td>acetylthiocholine</td>
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<td>76.9µA</td>
<td></td>
</tr>
</tbody>
</table>

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Lactate deposition was then optimized at the electrode surface. Several deposition method were first studied: 10µL of a 100U/mL LDH solution were deposited at the electrode surface and left to dry overnight, either alone, or with 10µL of NAD 25mM, or with 10µL of NAD 25mM and 10µL of MB 6.6mM. They were tested with 30µL of a solution of lactate 20mM and 10µL of a solution of NAD 25mM and MB 3.3mM. Very similar results were obtained, but the electrodes where lactate, NAD and MB were deposited simultaneously presented the lowest RSD (12%). A cyclic voltammetry study was then realized on these electrodes, in order to confirm the validity of the electrochemical parameters used. Meldola’s blue exhibited an oxidation peak at +80 mV, thus an amperometric detection potential of +100 mV was selected on the basis of a more positive oxidation potential, whilst minimising the possible oxidation of any electroactive interferents that may be found in ‘real world’ samples. The relation between the MB oxidation at the electrode surface and lactate degradation into pyruvate is proportional, the electrochemical signal measured hence being directly related to the lactate concentration deposited at the electrode surface. The calibration curve for LDH was then determined over the range 0-100mM. The curve was found to be linear over the range 0-5mM. The RSD was confirmed to be 12% for lactate at 20mM.

3.2 Addition of stabilizing agents
3.2.1 Glucose oxidase
Several tests were performed to assess the optimum method for addition of the stabilising agents to GOx. Four possibilities were compared: deposition of the Hotsolutes, the electrode being then left to dry about 16h before GOx was deposited, deposition of GOx, left to dry about 16h before the Hotsolutes was added, preparation of a solution containing both enzyme and stabilizing agent, a droplet of this solution being afterwards deposited onto the electrode and let to dry, simultaneous deposit of GOx and stabilizing agent at the electrode surface.

The latter method was proven to be the most effective and simplest method. The first two solutions required a longer preparation time and give lower current responses. This was attributed to the difficulty for either H₂O₂ or glucose to reach the electrode surface or GOx to blockage/masking of enzyme active sites. The efficiency of the stabilization by the Hotsolutes is also thought to be lessened in those conditions.

The preparation of a solution requires more liquid handling step and was found to yield slightly lower results, probably because of a stronger interaction between the stabilizing agent and GOx, due to a longer time in the presence of both before physical immobilization. It might also lead to better stabilization results. As the liquid handling required in this case would be more important, this solution was not selected, event though it would surely be considered for industrial applications.

The deposition of both enzyme and stabilizing agent was thus done following the steps described here: 10µL of GOx was deposited at the electrode surface,
This was followed immediately by the application of 10µL of STAB.
The resulting droplet was carefully stirred and homogenized at the electrode surface.
The electrode was then left to dry at ambient temperature (20°C) for 16h.
The electrode was then either used immediately or put in storage.

3.2.2 Optimisation of stabilising agent concentration
The concentration of stabilizing agents was studied over the range 50-1000mM. The deposition was performed as previously detailed. The electrodes were stored at room temperature and tested at regular intervals. A significant difference of response for the electrodes with different Hotsolutes concentration was noticeable after 1 month of storage for alcohol oxidase and acetylcholinesterase, and after two months storage for glucose oxidase. The curve obtained for all the associations of enzyme and stabilizing agent presented a similar profile, with an optimum concentration between 50-100mM. The optimum concentration was considered to be that giving the highest S/N ratio. The optimized concentration determined for the enzymes with the different stabilizing agents are displayed in Table 2 & 3. A typical curve is showed Fig.4.

![Figure 4: Influence of hydroxyectoine on GOx activity upon 2 month-storage](image)

3.3 Stabilisation agent performance
The influence of stabilizing agent on enzyme-based sensor was tested for several parameters: enzyme desorption, pH, salt influence, and storage.

| Table 2: Optimum loading of stabilizing agents on enzyme-based biosensors |
|-----------------------------|----------------|----------------|----------------|----------------|----------|
|                             | FOx            | AOX            | AchE           | LDH            | GOx      |
|                             | 50mM           | 100mM          | 50mM           | 100mM          | 55mM     |
|                             | 55mM           | 50mM           | 80mM           | 60mM           | 55mM     |
|                             | 60mM           | 100mM          | 100mM          | 50mM           | 50mM     |

<table>
<thead>
<tr>
<th>Table 3: Stabilizing agent addition - LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyectoine 80mM</td>
</tr>
</tbody>
</table>

![Figure 4: Influence of hydroxyectoine on GOx activity upon 2 month-storage](image)
3.3.1 Enzyme desorption
The stabilizing agents have been found to have a stabilizing effect on enzyme desorption using PBS-T (phosphate buffer saline-Tween). A variation in the loss of enzyme material deposited at the electrode surface was investigated. The electrode with immobilized enzyme with or without stabilizing agent was stirred for 5 seconds in a solution of PBS-T, then was interrogated amperometrically. Without stabilizing agent, a loss of signal of 33% was observed, this loss being reduced to 18% in presence of a stabilizing agent. The RSD of the electrode with physically immobilized enzyme was found to vary between 8 and 15%. For GOx, whose results are presented Fig.5, the RSD was 8%. The stability of the enzyme preparation at the electrode surface was significantly increased by the presence of a stabilizing agent.

![Figure 5: Influence of Hotsolutes on enzyme activity – Gox](image)

3.3.2 pH
No influence of the stabilizing agent on the immobilized enzyme preparations was observed with pH. The electrode were prepared using a PBS solution pH=7.5 for the enzymes and the stabilizing agents. The influence of pH was determined by introducing the relevant enzyme substrate under different pH conditions. The range studied was pH=3 to 11. The results for AchE are presented Fig. 6. The electrode was tested using acetylcholine (ATch) substrate solutions at various pH levels. No significant difference was observed in the presence or absence of the stabilizing agents. The study was repeated with all enzymes but LDH and the results are concordant with those presented here.

![Figure 6: Influence of Hotsolutes under pH variations – AchE](image)

3.3.3 Ionic strength
The influence of stabilizing agents on ionic strength variation was tested at various concentrations of PBS. Results for GOx are presented Fig.7. Ectoin and Firoin A showed a stabilizing effect of +80%, whereas the other stabilizing agents did not have any significant effect on GOx activity. Similar results were obtained for AOx and AchE. DGP did have a small stabilizing effect with AOx, even though it was less than Firoin A.

![Figure 7: Influence of Hotsolutes under salt variations – Gox](image)

3.3.4 Electrode storage
Electrodes coated with an enzyme (GOx, AOx, AchE) with and without stabilizing agent were stored at room temperature and at 4°C and tested between 2 and 8 months. The electrodes were tested after 2, 4 and 6 months when stored at room temperature, and at 4, 6 and 8 months when stored at 4°C. It is to be noted that DGP containing electrodes take longer to dry than the other electrodes. At 4months of storage, whether at RT or 4°C, it was observed that AOx was depredated. Also, a white depot had formed at the surface of the electrodes containing Firoin, and very little signal was preserved when using this stabilizing agent. The nature and origin of these depositions is still unknown and is currently being investigated.

The results obtained as to enzyme activity preservation are highly coherent over that time period and regardless of the enzyme considered or the storing temperature. Ectoine is the less stabilizing agent of all (+36% only), with the exception of Firoin. Firoin A (+218%) and Hydroxyectoine (+176%), and DGP (+237%) do provide efficient stabilization. The comparative enzyme activity for GOx stored 8 months at 4°C, tested against glucose 20mM, in PBS 10mM, is presented Fig.8 and is representative of the observations made.

![Figure 8: Enzyme activity preservation – GOx, 8months storing, 4°C](image)
4. Conclusion

4.1 Conclusion
Biosensors were fabricated by physical adsorption of the enzyme onto the working electrode surface. Tests were performed on screen-printed sensors, using a 3-electrode system and an electrocatalyst was added at the working electrode surface to lower the working potential; the reference electrode was Ag/AgCl.

Electrodes were first characterized with hydrogen peroxide. The optimum working conditions for the enzymes (glucose oxidase, alcohol oxidase, acetylcholine esterase) were determined.

Addition of stabilizing agent was optimized on the sensors. The influence of pH on the enzyme and immunosensor (without/with stabilizer) was determined, although first experiments indicate that the stabilizing agents have no significant stabilizing effect on the enzymes tested across the pH range 3-11.

The enzyme storing stability, temperature effect and shelf-life is currently being studied and the influence of the stabilizing agents assessed.

Preliminary results upon storage stability have bee obtained over a 2 months period: The best enzyme activity preservation was observed with DGP.

4.2 Future work
• The parameters for lactate dehydrogenase without, then with stabilizing agent, are to be determined.
• The influence of stabilizer on the enzymes and immunosensor is to be characterized.
• The enzyme shelf-life is to be studied and the influence of the stabilizing agents assessed.
• Testing of the Hotsolutes on other biosensors (immunosensors, DNA-based sensors) is under development.

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