Biosensors and Automation for Bioprocess Monitoring and Control

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Biosensors and Automation for Bioprocess Monitoring and Control

Kumar M.A.
Department of Biotechnology
Doctoral Thesis
January 2011

Academic thesis which, by due permission of the Faculty of Engineering of Lund University will be publicly defended on Friday, January 14 at 10:30 a.m. in Lecture Hall C, at the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent: Docent Bengt Danielsson, Acromed Invest AB,
Magistratsvägen 10, 226 43 Lund

Bioprocess monitoring and control is a complex task that needs rapid and reliable methods which are adaptable to continuous analysis. Process monitoring during fermentation is widely applicable in the field of pharmaceutical, food and beverages and wastewater treatment. The ability to monitor has direct relevance in improving performance, quality, productivity, and yield of the process. In fact, the complexity of the bioprocesses requires almost real time insight into the dynamic process for efficient and effective control.

Recently, much attention has been focused on improving process monitoring tools, especially since the launch of the Process Analytical Technology (PAT) initiative by the US Food and Drug Administration (FDA) in 2003. PAT was introduced as a concept in GMP-based bioproduction in order to improve process knowledge via continuous monitoring without a compromised product quality. In this context, many tools based on advanced sensing techniques, novel biosensors and model based software sensors are being pursued to provide advanced insight into bioprocesses. Efforts are being pursued to integrate different approaches for enhanced real time view of the bioprocess. To achieve this objective, modular systems are necessary to improve the robustness of biosensors and integrate them with other complementary techniques.

This thesis work focuses on the development and automation of biosensors for process monitoring and control, employing flow-systems. Automation of biosensors in flow configurations like FIA, SIA or multi-commutation have been studied and interfaced with bioprocesses, choosing model analytes based on various biorecognition elements like enzymes, antibodies and microbial cells. Design and automation of biosensors adapting to online continuous monitoring and control of the fermentation of low molecular weight compounds like glucose, lactate, acetonitrile and higher molecular weight substances like proteins have been reported based on electrochemical and optical detections.

In this work, a general and versatile automation system is described having high stability for continuous monitoring of bioprocesses. A system for flow based immunoassay useful for monitoring of protein production in a bioprocess is described. The design of such sensing systems, and their successful integration with bioprocesses provide an important step in adapting such systems for process monitoring and industrial production.

An important issue is the stability of the biorecognition element. This has specifically been studied for enzymes and a method for improving the stability by a protein-based stabilizing agent is reported. In addition, a study aiming at improving the thermal stability of enzyme-based biosensors using the model glucose oxidase is described.

Biosensors are becoming increasingly attractive for environment monitoring and food safety and quality. Integration of a microbial biosensor for monitoring and control of a degradation process of the organic pollutant acetonitrile is discussed. In fact, environmental applications certainly need rapid and sensitive detection techniques and here a novel immunosensor based on IgY antibody for detection of methyl parathion using chemiluminescence is reported. The sensitivity obtained in this application was close to 10 ppt of the target pesticide methyl parathion. Furthermore, an automated analyzer for precise control and stable analysis of methyl parathion is described.

Finally, sensing systems utilizing both enzymes and microbial cells for the quality evaluation of food stuff and pharmaceutical applications have been developed. A highly specific microbial sensor for the detection of caffeine with rapid response time as well as a prototype for detection of polyphenols in tea has been developed and its applications illustrated.

Key words
Biosensor, bioprocess, flow injection, immuno sensor, ELISA, online monitoring, control, environmental

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I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature ___________________________ Date 2011-01-14
To my dearest ones..

Wife Srimathi, daughters Meghana and Sanjana
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1. List of Publications


VIII. K. Håkansson, **M.A. Kumar**, and B. Mattiasson, A Biosensor for on-line analysis and control of an acetonitrile degrading system (Manuscript)

IX. **M.A. Kumar**, M. Mazlomi, M. Hedström, B. Mattiasson, Versatile continuous flow system for Bioanalysis and Bioprocess control (Manuscript)
2. My Contributions to Papers

**Paper I** I contributed to the design of the glucose and L-lactate sensors, performed the experimental work, developed the flow injection analysis system interfacing it to the bioreactor, developed the amperometric electronic detection systems and did the programming for automated data acquisition and analysis. I also contributed to the writing of the paper.

**Paper II** is about enhancing operational stability of an amperometric glucose biosensor. I contributed to the design of the sensor and the experimental work. I also contributed in writing of the paper.

**Paper III** I developed the flow injection analysis system, contributed to the experimental work towards thermal stability of the enzyme and writing of the paper.

**Paper IV** I designed and developed the automated flow ELISA system with flow injection and electronic control unit and contributed to the experimental work for analysis. I wrote the paper along with my co-authors.

**Paper V** I devised and developed the imaging system for chemiluminescence detection of methyl parathion and supervised the work of Chouhan for immuno-detection of methyl parathion. I contributed to the writing of the paper.

**Paper VI** I contributed to the design of the sensor and developed the prototype of the tea polyphenol biosensing system. I also supervised the experimental work of Abhijith and contributed to the writing of the paper.

**Paper VII** I contributed to the design of the sensor, its optimization and writing of the paper.

**Paper VIII** I developed the flow injection analysis system for analysis of acetonitrile along with Kristina and contributed to the experimental work. I developed the software for automated data acquisition, control and analysis of the data from the amperometric sensor. I contributed to the manuscript.

**Paper IX** I devised and developed the versatile automatic flow injection system and the software for flow scheduling, pump and valve control, data visualization and analysis. I carried out the experimental work along with Mohammad. I drafted the manuscript.
3. Acknowledgements

This work was done primarily at two institutes, Department of Biotechnology, Kemicentrum, Lund university, Sweden and Central Food Technological Research Institute (CFTRI), Mysore, India and have interacted with many interesting people to whom I owe a lot for the completion of this work. It has immensely contributed to my learning and made this journey a truly memorable one.

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Thanks to Dr. V. Prakash, Director, CFTRI and staff of Central Instruments Facility and Services (CIFS) – my department at CFTRI, for all their help and stimulation given to pursue my PhD, made me recognize and respond to challenges in life. Thanks to Umamathi H., for his help in fabrication of some of the hardware and Asha M for all her help in CIFS department.

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I owe a lot to my lovely wife **Srimathi** and charming daughters **Meghana** and **Sanjana** for always being there for me. They have brought lot of light and cheer in my life and have endured quite admirably my staying away from home and made this work possible. I am proud of all of you for staying focused and continuing to excel in your respective work and studies. I eagerly look forward to be together soon.

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4. Introduction and Motivation

4.1. Bioprocess Monitoring and Control

Bioprocess monitoring and control is important in order to accomplish the broad objectives of maintaining a desired environment for growth of microorganisms. In fact, in many aspects the monitoring and control situation is necessary to reach an economically feasible production with acceptable product quality. An optimized process leads to streamlined performance, reduction in running and material costs and improvements in quality control [1]. The effective monitoring of bioprocess is necessary to develop, optimize and maintain biological reactors at maximum efficiency. Furthermore, due to the nature, type and volume of products produced in bioprocesses, there is a strong economic incentive for process monitoring for increasing yield and productivity.

Biotechnological processes are dynamic and involve continuous changes to the physico-chemical conditions of the medium. This in turn influences the functioning of the biocatalyst. The response to changes in the process environment is less reproducible making the tasks of process development, optimization and scale up difficult. Furthermore, bioprocesses inherently are batch oriented. Individual process steps need to be optimized and improved upon to adapt to the progress in process technology. This needs factual insight of the process state and understanding of the biochemical and metabolic control mechanisms. Production of recombinant protein is a fast growing area and the requirements of bioprocess monitoring and control in such processes is crucial for selecting optimal expression conditions. These emphasize the requirement of better tools and systems for online monitoring and control with insight into biochemical variables in the bioprocess.

Automation and process monitoring ensures adherence to standards and regulations and generates much related process documentation [2, 3]. The need for quality monitoring is higher for biotechnological applications which involve intense downstream processing steps. Furthermore, it is significant from the point of view of environmental control and food safety. This ensures better process engineering, stringent quality and optimized processes.

In addition, integration of individual processes is vital right from the field devices sensing the physical and biochemical parameters to higher layer controllers, supervisory and expert systems and application software. In fact, integrated control promotes optimization of bioprocesses and informed decision making in real time by employing Manufacturing Execution Systems (MES) [4]. Certainly, with the process analytical technology initiative (PAT) of FDA, this has gained more momentum.
4.2. Process Analytical Technology

Bioprocesses used in the pharmaceutical industry have the challenge to adhere to stringent regulations and quality control norms for assuring the customer/patient safety of the products produced. In fact, many of the analytical measurements are performed in offline mode and there is a clear need to minimize or eliminate delay times between sampling and analysis [5]. In fact, there is a general reluctance in the industry to adapt novel techniques for developing and improving production. This stems from the fact that it is cumbersome to validate new/novel process techniques. Recognizing this fact and to make the conditions more flexible for adopting newer technologies for quality control and management, US Food and Drug Administration (FDA) launched a new Process Analytical Technology (PAT) initiative entitled ‘Pharmaceutical cGMPs for the 21st century: A Risk based Approach’. With cGMP (current good manufacturing processes) initiative industry is encouraged to use newer and sophisticated pharmaceutical manufacturing techniques with emphasis of process monitoring and control. Timely measurements, critical quality and performance of both raw materials and the in-process materials are the key essence of PAT to ensure the final product quality [6]. The PAT framework itself is comprised of continuous improvement, risk assessment, knowledge management, and at/on-line sensors. These concepts assist in design, monitor, control, and prediction of the process performance. The PAT framework encourages exploiting the benefits of integrated process analytical control for obtaining real-time information of Critical Quality Attributes (CQA) of the product throughout the production line. It identifies critical sources of variability, beyond what may have been identified based on results from process validation studies. In order to gain the full benefits of PAT, there must be a solid understanding of the product’s CQAs and what parts of the manufacturing process, control them. In fact, it is desirable to understand the impact of measurable and controllable process parameters (e.g., pH, DO, nutrients) on product characteristics which are not directly being measured in real time. This leads to measurement of the biochemical variables in the process. To encourage PAT adoption, the FDA is permitting individual manufacturers themselves to determine which real time control or monitoring strategies constitute PAT [7]. Many aspects of biotechnological manufacturing are readily amenable to PAT for monitoring and control ensuring improvement in productivity, consistency, and quality. Detailed monitoring of biotechnological processes is also necessary in order to optimize the recovery process with regard to quantity as well as quality (e.g. biological activity) of the products.

4.3. Process automation and Biosensors

Process control aims at repeatable process conditions for consistent production of the desired product. Sound process design with excellent automation ensures to have
reliable processes. This is possible by exploiting advances in electronics and sensor development for biochemical parameters in combination with intelligent analytical systems for use under aseptic conditions. These analytical data should be best produced in situ, or at least on-line for continuously analyzing the desired parameter in real time. Further the analysis needs to cover a wide dynamic range (of activities and/or concentrations) for process control [2]. Currently such information is done by offline laboratory measurements, process operator supervision and efforts are there to adapt inferential measurements or software sensors in a feedback mode. Offline measurement though useful, cause delay in process control and hence are generally used for development of mathematical models and process development. In fact, the state estimation of software sensors can be improved by augmenting the model with online measurements. Inputs like concentration of substrate, products, and intermediates in real time facilitate a more accurate state estimation [8].

Bioprocess optimization is significantly influenced by the sensitivity of micro-organisms to their environment. Traditionally variables like pH, dissolved oxygen, temperature, pressure, level and flow are employed for monitoring and control of bioprocesses. The use of software sensors requires profound insight into the process and the performance depends on the initial model conditions applied. The initial conditions may vary from batch to batch. Furthermore, the reliability of these virtual sensors during product synthesis is quite low, which mainly comes from the poor understanding of the start-up procedure. This may result in substantial cell death and product inhibition with a severely compromised process economy as a consequence [9].

Process variability is another problematic issue and reasons causing it are poorly understood. However, it is concluded that process monitoring combined with process analytics significantly facilitates reduction in the variability. Advanced analytics by way of online measurement and analysis of key substrates, products like sugars, amino acids and vitamins, activity of key enzymes, nutrients, metabolites and cell biomass, aid to provide a better insight of the process and derive process states. Direct tools to monitor these analytes are possible through techniques like biosensing and IR spectroscopy. Integration of these measurement techniques with bioprocesses helps in generating database for elucidating origin-effect mechanisms of the underlying molecular mechanisms. This facilitates in developing better process models for state estimation leading to enhanced process information and control.

Though there are many biosensors available for measurement for substrates, products and intermediate metabolites, their use in bioreactors is scarce owing to drift or fouling. Hence they operate under conditions not adapted for continuous measurements. In fact, automation provides a favorable environment for reliably employing such sensors for online monitoring can be achieved. Furthermore, automation using biosensors also
provides flexibility for on-time improvement of process behavior e.g. process reproducibility and increased safety [3, 10-12]. Automation per-se reduces the number of repetitive tasks, both regard to a decreased number of human errors and increased operator motivation [2].

Process analytical technology plays a key role in process understanding, optimization and quality control and tremendous efforts will be necessary to meet future ecological and legislative demands. Process analytical approaches including deploying multivariate techniques along with on-line/at-line/in-line sensors and analyzers and the use of chemometrics in biotechnology have received significant attention in recent years. Applications include monitoring and control of microbial fermentations, cell culture and purification processes. For this, development of new measurement technologies for real-time bioprocess monitoring and control is also crucial [8, 10].

4.4. Environmental monitoring

Toxic chemicals in the environment are a major problem. Environmental pollutants like phenols, organic solvents are used extensively in industries. Genotoxins, and pesticides such as organophosphates, 2,4-D, carbamates are used for safe guarding the crops and improving the yield. However, with uncontrolled use, these substances have serious effects on human health and safety and have widespread environmental concerns [13]. Among the pesticides, organophosphate and carbamate species are the most used due to their high insecticidal activity and relative low persistence however they have high toxicity. Their toxicity is mainly due to their inhibitory effect on acetylcholinesterase, a key enzyme for the signal transmission between nerves [14]. Due to their toxicity, regulation agencies like EPA, EEC, FDA have laid out stringent regulations for their use and monitoring in e.g. industrial effluents, water treatment and agriculture. Sensitive, specific and yet rapid measurement systems for their analysis are required. Immunoassays provide possibilities of specifically and sensitively detect these pesticides however with the down side of being time consuming. Automated immunosensors provide rapid and sensitive means for detection.

Monitoring of environmental processes for wastewater treatment is vital in order to understand and ultimately control the microbial activities of a certain system. These processes are governed by stringent requirements in terms of toxic residues in treated effluent. Further, there is huge economic incentive for better utilization of capacity and devising early warning systems for disturbances in the process [15]. In this context, microbial biosensors are important tools in rapidly determining toxicity, chemical and biological pollutants. It also provides means to determine process over- and underload conditions and take decisions to control and safeguard the process [16, 17]. Improved concepts of microbial biosensors offer reliable methods for sensing operating conditions.
and thereby provide opportunities for better control of degradation bioreactors using these pollutants and toxic compounds as substrates.

5. Objectives and Scope of thesis

This thesis deals with devising approaches for automated online monitoring and control of bioprocesses employing flow injection assays and biosensors. Real time information being vital for bioprocesses, the emphasis is on automation and integration of biosensors for reliable analysis with good operational stability. Different biorecognition elements have been studied which include enzymes, antibodies and microbial cells for monitoring. Studies are focused on monitoring of model analytes which are of importance in bioprocesses. Optimization of sensor for online monitoring employing flow injection techniques and development of automatic technique for immunosensors are studied leading to their integration for online monitoring and control of bioprocesses. Further, design of new biosensors for monitoring pollutants with high sensitivity and rapid detection techniques and their optimization is addressed for use in food and pharmaceutical applications. Amperometric and immunoassay techniques are studied for high sensitivity analysis of trace impurities and pollutants.

The work comprises of automated amperometric biosensors (Paper I, II, III and VI: enzymatic; Paper VII: microbial) for analytes that are important in food and fermentation processes and detection of quality parameters in beverages, online monitoring and estimation in bioprocess (Papers I, VIII, IX), immunosensors and automation combined with optical detection (Papers IV, V and IX) and pesticide detection by applying image processing (Paper V).

Paper I deals with continuous online analysis of substrate and product in a recycle bioreactor for L-lactic acid with good dynamic range suitable for fermentation conditions. Online monitoring was realized through custom software developed in Visual C++ and sampling from the bioreactor was achieved through a dialyzer. Paper II describes use of protein based stabilizing agents for increasing the stability of biosensors even at high concentration of substrate. The operational performance of both single and multienzyme sensor was improved by incorporating inert proteins into the immobilization system. Paper III introduces technique for enhancing the thermal stability of immobilized enzyme by silanization. Paper VI and VII addresses development of stable enzyme sensors for quality analysis of beverages - estimation of polyphenols in tea and analysis of caffeine respectively, employing the improved immobilization procedure developed in Paper II. Paper IV and V describes new methods for detection of organophosphate pesticides using immunosensors with IgY antibody and HRP labeling. In Paper IV, a microcontroller based multi-commuted system was developed for flow-immunoassay for
high sensitivity detection of methyl parathion and comparative study with offline enzyme-linked immunosorbent assay (ELISA) was demonstrated. Paper V presents a novel method based on immuno-chemiluminescence and image analysis using charge coupled device. Using light enhancers a sensitive analysis of methyl parathion was possible and good comparison with competitive ELISA is demonstrated. Paper VIII exemplifies the use of microbial biosensor for control of an acetonitrile degradation bioreactor. The feed rate of acetonitrile was controlled based on the online biosensor measurements, for optimum utilization of the bioreactor capacity. Paper IX describes development of a comprehensive and versatile platform for bioanalysis and bioprocess monitoring and demonstrates its use for highly repeatable continuous flow ELISA for monitoring of IgG in a competitive flow ELISA mode. The system supports the various versions of flow assay – flow injection analysis (FIA), sequential injection, bead injection and multi-commuted flow.

4. Bioprocess Monitoring and Automation

Fermentation processes are considered complex for monitoring due to the intricate nature of biological system and its interaction with the surrounding physical and chemical environment [10]. The fermentation medium is a mixture of simple and complex ingredients wherein some are easy to characterize while others are not. Moreover it is very dynamic with products, byproducts being formed in different phases of fermentation. The catalysts are organisms (such as bacteria, yeasts, fungi, and animal/insect/plant cells) that vary considerably in their metabolic characteristics. In a bioprocess, substrates are consumed; products and metabolites are formed altering the physical conditions of the bioprocess. Controlling only the culture parameters like dissolved oxygen (DO), pH, pressure, temperature are not enough to reduce the variability in the process. In fact factors like concentration of nutrients, growth balance; intracellular metabolic products, energy charge are all relevant for understanding the process state. An essential requirement is that the sensing techniques employed needs to be rapid not only to facilitate this deeper understanding of the process but also to utilize it for process control [2, 15, 16].

Application of the biosensor technique provides additional insight into some of these parameters and facilitates decision making e.g. online monitoring of key primary and secondary metabolites provides a basis for determining the feed strategy to be applied. Further, biosensors offer advantages as alternatives to conventional methods due to their inherent specificity, simplicity and quick response. Combining online process monitoring with automation, it is possible to initiate process corrections with feedback control. However, with offline analysis, these corrections can be done only
retrospectively. Further online monitoring enables generating early warning signals based on observed process deviations and devise appropriate control strategies. In fact, with these insights, it is possible to achieve higher productivity e.g. by monitoring glucose and glutamine and maintaining the balance of these carbon and nitrogen sources animal cell cultures can be run at optimal conditions. This helps in reducing production of undesirable by-products like ammonia and lactate. Moreover, effective monitoring coupled with appropriate control strategy will facilitate increase of production of desired product with high quality. As a consequence, savings can be accrued by reducing costs on downstream purification requirements and use of reagents thereof [18, 19].

Bioprocesses rely upon controlling the environment of cultivated cells to influence their internal environment and economically produce useful products. In a typical bioprocess, multiple measurement and control systems/sub systems are employed. In such a context, automation provides a perfect platform to integrate these disparate systems. Reliability is enhanced due to reproducibility of sampling instants, sampling volumes, and interaction times with sensors and controllers. Other benefits include integrated online monitoring, process data visualization, intelligent operator interface. Further process documentation is enhanced by storing all measurements ensuring traceability.

Communication of data and process information is enhanced with other measurement and control systems, an essential attribute for integrated process monitoring and control. The amount of data collected in a bioprocess batch operation is huge owing to the nature of the process which can run from a few hours to days. Continuous data collection and increased productivity are characteristics of automated systems. Further, with so much data collected in a bioprocess, there is a pertinent need for data analysis and tools to translate them into useful operational information. This is handled by data analysis tools like partial least squares, artificial neural network to derive useful process information. In addition, automation at different levels such as sensor systems, controls provides means to collect, translate these data into meaningful process information for communication with other systems. Overall, automation provides a paradigm for integrated control of bioprocess by usefully combining information across the control hierarchy - field devices, individual controllers, supervisory and expert systems to manufacturing execution systems (MES) [9]. As a consequence of this integration, enhanced real time decision making can be achieved at various levels of automation.

In the food industry an attractive area for the biosensor application is the detection of pathogens, pesticides and toxins. Traditional methods to identify contaminants include physicochemical, biological and serological tests. However, many of these are laboratory based; require time-consuming sample preparation, have extended analysis time and lack sufficient sensitivity and selectivity. Some of the analyses may even take days. Biosensing methods based on immunosensing are good alternatives because antibodies
can be developed not only for recognizing proteins, but also for surface antigens of microorganisms and low-molecular weight compounds [20].

Biosensors with their bio-recognition element provide the required specificity for analysis and offer convenience in transforming it into portable devices. However, challenges for *in-situ* analysis in bioreactors still remains as they are not amenable for sterilization. *Ex-situ* analysis using biosensors with flow-injection analysis is an important tool for investigation of bioprocesses.

Table 1 provides some of the examples of Biosensors for online monitoring of Bioprocesses with different sensing principles.

<table>
<thead>
<tr>
<th>Bioprocess</th>
<th>Analyte</th>
<th>Biosensing Principle</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> and <em>Saccharomyces cerevisiae</em> fermentation monitoring</td>
<td>Glucose, in an animal cell culture</td>
<td>Amperometric</td>
<td>[21]</td>
</tr>
<tr>
<td>Lactic acid, <em>L. delbrueckii.</em></td>
<td>D-Lactic acid</td>
<td>Enzyme based, optical</td>
<td>[22]</td>
</tr>
<tr>
<td>On-line monitoring of glucose during the cultivation of <em>S. cerevisiae</em></td>
<td>Glucose</td>
<td>Enzyme based, Amperometric</td>
<td>[23]</td>
</tr>
<tr>
<td>Bioprocess monitoring, <em>Penicillium chrysogenum</em></td>
<td>Pencillin, Urea, Ethanol, Mannitol</td>
<td>Enzyme optrode Optical</td>
<td>[24]</td>
</tr>
<tr>
<td>Monitoring malolactic fermentation in wines</td>
<td>L-malic and L-lactic acids</td>
<td>Multi Enzyme, Amperometric</td>
<td>[25]</td>
</tr>
<tr>
<td>Apple juice fermentation <em>Lactobacillus plantarum</em> and <em>Lactobacillus brevis</em></td>
<td>L-Lactic acid</td>
<td>Enzyme based, Amperometric</td>
<td>[26]</td>
</tr>
<tr>
<td>Probiotic yogurts, <em>Lactobacillus Bulgaricus</em></td>
<td>L-lactate</td>
<td>Enzyme based, Amperometric</td>
<td>[27]</td>
</tr>
<tr>
<td>Ethanol fermentation, <em>Saccharomyces cerevisiae</em></td>
<td>Ethanol</td>
<td>Microbial, Amperometric</td>
<td>[28]</td>
</tr>
<tr>
<td>Beer Fermentation</td>
<td>Glucose and Ethanol</td>
<td>Enzyme, Amperometric</td>
<td>[29]</td>
</tr>
<tr>
<td>Recombinant protein production <em>Escherichia coli</em></td>
<td>D1.3 anti-hen egg lysozyme Fv antibody fragments</td>
<td>Immunoassay, Optical,</td>
<td>[31]</td>
</tr>
</tbody>
</table>
Table 1 (Cont’d.)

<table>
<thead>
<tr>
<th>Bioprocess</th>
<th>Analyte</th>
<th>Biosensing Principle</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol fermentation <em>Gluconobacter oxydans</em></td>
<td>1-3 Propanediol in the presence of Glycerol</td>
<td>Amperometric</td>
<td>[32]</td>
</tr>
<tr>
<td>Selective determination of 1,3-propanediol in the presence of glycerol</td>
<td>1,3-propanediol</td>
<td>Microbial-Amperometric</td>
<td>[33]</td>
</tr>
<tr>
<td>Wastewater Treatment</td>
<td>Phenol</td>
<td>Enzyme based, Amperometric</td>
<td>[34]</td>
</tr>
<tr>
<td>Wastewater treatment</td>
<td>Hydrogen peroxide and peracetic acid</td>
<td>Enzyme based, Amperometric</td>
<td>[35]</td>
</tr>
<tr>
<td>Water</td>
<td>Herbicide</td>
<td>Immuno assay, Electrochemical detection</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>2,4-dichlorophenoxyacetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wastewater</td>
<td>BOD</td>
<td>Microbial, Amperometric</td>
<td>[37]</td>
</tr>
<tr>
<td>Water</td>
<td>Methyl parathion</td>
<td>Enzyme based, Conductimetry</td>
<td>[38]</td>
</tr>
</tbody>
</table>

6. Bioprocess Monitoring and Control Requirements

6.1. Process Monitoring

Well defined process characteristics based on elaborate studies of major contributing factors and their interactions are available. However, even while following these established processes, batch to batch variability in product is quite common. Process monitoring helps to track the process and reduce this variability. Further, in order to achieve process optimization and control, it is desirable to maximize product production for a given substrate and other resources consumed. For any bioprocess the real-time view of key process variables that affect the critical quality attributes of the product being produced is crucial. Monitoring helps increase productivity and yield saving costs while improving efficiency. Further, process monitoring provides tools for risk management, diagnostics and continuous improvement of processes and addresses key requirement of PAT framework. Classical methods of analysis of samples from bioprocesses include chromatography and mass spectrometry. However, efforts are progressing to integrate and have rapid online analysis of biochemical parameters and in this context biosensors are important [3, 18, 39].
The sensors employed for analysis of biochemical parameters need to have high specificity and selectivity for the parameter being measured with least interference. This is especially important with complex culture media and possibility of it invalidating the sensor. With the requirement for sterility in bioprocesses, the sensors employed need to be steam sterilizable which essentially restricts the use of biosensors in-situ unless with elaborate arrangements [40]. Ideally, sensors are to be robust, need to perform accurately under all applicable operating conditions and be devoid of any matrix effects. This will ultimately enable accurate measurements to devise effective control strategies. Certainly, minimal invasive or non-invasive sensors are the best option and the installation of such sensor should not compromise the sterility barrier of the bioprocess. Further, the measurements need to be rapid, have low drift, with high sensitivity and precision. Additionally, the sensing systems need to cater for continuous measurements over the entire length of the process [11, 41]. Another important factor is that the sensors and systems employed should have minimal maintenance requirements for easy adaptation to the real world. As validation of measurements is vital for deriving meaningful process information, the sensors require means to check for any drift and adopt compensation techniques.

Owing to the dynamic and complex nature of bioprocess, different measurement systems are employed for physical, chemical and biochemical variables and a common platform is essential for interlinking and deriving process information. Further, industry standard data communication protocols like Fieldbus, Profibus, Highway Addressable Remote Transducer (HART) can then be employed [9].

With the process monitoring having a daunting task of deeper insight into the process, the measurement systems employed needs to fulfil certain criteria for satisfactory performance.

Accuracy and sensitivity are important parameters which are of significance in the measurement system. Accuracy is the measure of closeness of the measured value to the true value. Further, the measurement system needs to be precise as well which is a measure of consistency under the same operating conditions.

Sensitivity describes the output of the sensor per unit input; higher the sensitivity the lower the detection levels possible. Careful design of sensor, underlying principle of sensing, operating conditions and signal conditioning dictate the sensitivity of the sensor.

Usually a biosensor consists of subsystems in a sampling system, a biocomponent, a flow systems and detection and data processing units. Good instrumentation and automation ensures reliability at all levels with consistent measurement conditions and online diagnostics.
The minimum response time required for employing a sensor depends on the dynamics of the process which would in turn define the sampling frequency for the analyte. For mammalian cell cultures, sampling frequency of an hour could be admissible whereas in downstream processing, due to high throughput flow, the time could be in the order of few seconds or minutes [9]. Typically enzyme based amperometric sensors have lower response times ranging from few seconds to minutes. The immunosensors in the flow configuration have many defined process steps like equilibration, rinse etc which adds to their overall response times.

The range of analysis of the sensor is determined by the minimum and maximum concentrations that a biosensor can accurately detect. For process monitoring the sensor should have a useful dynamic range covering the expected lowest to the highest value of the analyte being measured.

The limit of detection is the least value of the analyte that the sensor can reliably measure. For measurement of trace impurities, biohazards and pollutants, lower detection limits are essential. For instance for impurities in bioproduction, capacitance measurements offer better detection limits (fg/ml) as compared to surface plasmon resonance (SPR) or ELISA technique [42]. In a sensor, the activity of the biorecognition layer, type of detection influences the signal level, its sensitivity, range and the limit of detection. The range of detection is optimized by varying the amount and characteristics of the bio-recognition element e.g. in an enzyme sensor, polymeric membranes surrounding the sensing unit induce mass limitation thereby affecting the analytical range. Fluorescence and chemiluminescence based sensors exhibit high sensitivity and hence lower detection limits are possible as compared to when using amperometric or potentiometric methods.

### 6.2. Process Control

Process control aims at achieving the objective of producing the desired product efficiently and effectively in a safe and profitable manner. Controllers operate on the error signal of the measured variable and generate a control signal which acts on a final control element or the actuator. This realizes a material change in the process to ensure the controlled variable is at desired set point, irrespective of process disturbances. For example, the controlled variable e.g. substrate concentration or dissolved oxygen is measured and influenced by a manipulated variable e.g. feed rate, agitation, nitrogen source, to ensure it is always at the desired value. Controllers employed range from simple on-off type to Proportional Integral (PI), Proportional Integral Derivative (PID) controls and Expert systems. A scheme of typical closed loop control is shown in Figure 1. In expert systems a knowledge base of the process is built into the control based on heuristics, artificial neural networks, fuzzy logic, multivariate statistical analysis etc. In
fact, with bioprocesses being dynamic and non-linear, building specific knowledgebase of each bioprocess is of high significance.

Figure 1 Closed Loop Feedback control in a bioprocess

A typical output of the PID controller with feedback is defined as below:

\[
u(t) = K_p e(t) + K_i \int e(t)dt + K_d \frac{de(t)}{dt}\]

The \(K_p\), \(K_i\), and \(K_d\) are the tuning parameters of the controller which can be adjusted by varying the dynamics of the control loop. Feed rates can also be adjusted based on an optimal objective function derived online or offline. The objective function targets to increase productivity or maximise operating profits. Sometimes combination of optimal and feedback controls are used [43].

Measurement of primary biochemical variables e.g. cell mass composition, concentrations of ATP, NAD\(^+\), NADH, nucleic acids, total protein, enzyme activity, etc. are essential to adopt control strategy. Indeed many of these key process variables may be measured online by biosensors by making use of their selectivity and automation integrates them to the bioprocess.

The type of control also depends upon the dynamics of the process, sensor, actuator and the loop dynamics. E.g. the dissolved oxygen (DO) control process is fast whereas the concentration or the biomass control loops are sluggish owing to slow dynamics and large mass volumes. PI and cascade control strategies are usually adopted for process variables like DO and temperature in a bioreactor. However, PID controls is most suited for loops involving concentration and biomass variables.

Certain process variables need better strategies for control wherein measurements by biosensors can complement other measurements and control. For example, it is evident that close control of DO to the set point helps to reduce process variability. But, there are challenges in maintaining a close control with DO with a native PID control. Due to non-linearity in the bioprocess, the tuning of DO done in one phase of fermentation may not be optimum in another phase. In such a scenario, real time information of substrate concentrations can be used in a feed forward loop to devise adaptive gain-schedule
control strategy for effective control of DO. Such an adaptive control takes into consideration the process dynamics, in effect providing smoother control [44].

Process dynamics influences the control strategy. The volumes of bioreactors vary from few liters to several cubic meters and they are characterized by inherent process lag. The growth of microorganisms results in production of metabolites with changes in biomass, pH and temperature. In such a time variant process there is also a requirement for intelligent predictive control. Supervisory systems are deployed wherein controller, controlled variables together with the available knowledge base are used to dynamically change the tuning parameters of control loops.

Bioreactors operate in either batch, fed-batch or continuous modes. In fed-batch reactor, feed is added as the process progresses. In the continuous type, feed is added and at the same time the biomass is removed. Numerous control strategies based on optimal feeding of nutrients are used with closed loop control of variables. In the event of a variable being not measured, estimates are computed based on process models. Process models can be data driven or mechanistic. In a data driven model, estimates of state variables are arrived using real time measurement of target variables and combining them with data reduction techniques like ANN, multivariate analysis like PCA, PLS etc. Mechanistic models estimate target variables based on available measurements e.g. physical variables like DO intake, temperature etc. In some cases combination of mechanistic and data driven models called hybrid models are employed. There seems to be a general consensus on shifting from open loop optimal feeding to adopt closed loop control with actual measurements or with estimation. Software state estimation techniques based on Kalman filters, particle filters, asymptotic observer etc. combined with computational tools like artificial neural networks and other chemometrics are increasingly being used for estimating some of the unmeasured values such as biomass, substrate, product concentrations etc. and derive process states in the bioprocess [3, 8, 45-48]. However, it is to be recognized that regulatory mechanisms in a bioprocess have their roots in molecular mechanisms. The function of biologically active molecules is determined by their molecular structure. The regulatory mechanisms on the level of populations are in fact a superposition of many individual but interdependent molecular mechanisms. There are challenges in studying exhaustively and exclusively at the level of isolated biological subsystems because important components are removed by isolation [49]. These considerations make defining accurate process models difficult and hence estimation using such models or software sensors has their own limitations. In such a situation monitoring of key process variables facilitates to compensate for lack of such information and ease control. A real time in-situ impedimetric biosensor has been reported for measuring microbial growth [50]. Combining such measurements the effectiveness of closed loop control can be further enhanced.
Control of feeding strategy depends on factors such as whether the product being produced is growth associated or substrate inhibited. It may require an inducer or enhancer for expressing a condition in the culture cells. In such an event, monitoring biomass or substrates or the inducer can provide insight into the feeding strategies for realizing better efficiency from the reactors [51]. For instance, in fed batch fermentation for production of L-lactic acid, L-lactate sensor was used to monitor the process. Employing exponential feeding strategy for glucose substrate, 56% increase in production of L-lactic acid (180.0 g/l) could be achieved as compared to pulsed fed batch feeding [53]. Likewise strategies on feeding depending upon the process states and key process variables can be adopted by online monitoring.

7. Analytical methods for bioprocesses

Bioprocesses require information from physical, chemical and biochemical constituents inside the reactor and hence many analytical techniques have been employed [10]. Established analytical tools are available to monitor process variables such as temperature, pressure, agitation, aeration and power input rates, liquid volume, foam level and broth turbidity. Apart from the conventional sensors for these parameters, other optical methods for direct sensing are employed for bioprocess monitoring.

NIR and mid infrared spectroscopy are employed for obtaining signature patterns for analytes such as sugars, glutamate, ammonium, carbon dioxide, biomass etc. However, as many overlaps exist in measured spectral information for different variables, statistical techniques are employed for calibration and data analysis. Multivariate analysis, neural network tools are used for the measurement model. Once the model is accurately setup, this method provides a means for non-invasive online monitoring of bioprocess. However, these spectroscopic measurements have to account for factor like cell debris during death phase, agitation, high cell densities, viscosity of broth, bubbles etc [54]. These factors vary considerably with time and operating conditions employed. Additionally, though mid infrared spectroscopy offers fingerprint spectra for many constituents, it has to account for the strong absorption of water in the band. It is also an expensive technique to adopt for all processes. Further for NIR spectroscopy, generally the target analyte needs to be in high concentrations [41, 54-58].

With the advance in laser technology, Raman spectroscopy and scattering of monochromatic light offers another non-invasive method for detection of concentrations of many analytes like sugars, lactate, formate and biomass. It has the advantage of less interference due to water absorption. On the contrary, interference due to fluorescence of biomolecules is a hindrance and needs compensation by employing expensive techniques like shifted subtracted Raman spectroscopy [41]. Moreover, this method also
relies on the process model thereby affecting the measurement accuracy. Inconsistencies due to the influence of characteristics of culture need compensation e.g. microbial cultivations involve more viscous bulks affecting the quality of spectra. Hence such effects are to be compensated and more so when conditions like aeration and agitation are varying during different phases of the process [54]. Overall, NIR and Raman spectroscopy techniques provide information about intracellular components, are non-invasive and do not require any sampling but require multivariate analysis and have their own challenges.

Electronic nose using metal oxide field effect transistor based (MOSFET) gas sensors are employed for off-gas analysis during biopharmaceutical productions. To reduce the response time of these sensors, they need to be operated at elevated temperatures typically around 400°C. Further, these techniques usually require tools like artificial neural network of principal component analysis [52]. With the analysis of off-odors, metabolic activity and physiological state of the process can be ascertained. Microbial and viral contaminations have also been possible to detect at an early stage [54]. Recently, techniques involving 2D fluorescence, dielectric capacitance spectroscopy have also been used for online bioprocess monitoring. Both fluorescence and dielectric capacitance methods are relevant especially for biomass monitoring. Dielectric capacitance provides information on viable cell density in the bioreactor but cannot provide information on intracellular and extracellular compounds. In 2D fluorescence emission from multiple wavelengths can be considered by wavelength scanning. Further, biologically relevant molecules like amino acids, enzymes, vitamins exhibit fluorescence. In fact, tryptophan fluorescence shows correlation to biomass. On the contrary, interpretation of fluorescence is not trivial. Fluorescence measurements have to be compensated for interfering phenomenon like filter effects (excitation being absorbed by non-fluorescent compounds) and cascade effects (additional excitation from another fluorophore). As in these methods provide series of spectra, meaningful information must be extracted for the variable being measured. To achieve this, data mining techniques for data reduction, noise elimination and analysis are applied e.g. principal components regression (PCR), partial least squares (PLS) or artificial neural networks (ANN) can handle dense flow of data generated from the real-time process analyzers. The accuracy of the extraction technique depends upon factors like assumptions made, initial conditions applied and the training set employed [54].

In order to derive information about the chemical environment and the biological state of the cells, electrochemical and optical sensors, biosensors or chromatographic procedures (e.g. HPLC, GC) are used [12]. Chromatography technique involves separation of compounds in a packed column based on partition, ion exchange or adsorption principles. Low volatile mixtures can be separated and detected by gas chromatography
whereas liquid chromatography is generally used for separation and detection of mono and oligosaccharides, organic acids, aldehydes, ketones, alcohols etc. The separation is achieved due to differential portioning between the packed stationary phase and the sample in a mobile phase. The separated compounds in liquid chromatography are detected by refractive index, UV-Visible absorption or by amperometric methods. Though chromatography provides sensitive and accurate analysis, it has longer analysis times and requires high maintenance. Further the technique is not specific and has to be compared with response of standards of expected compounds analysed in similar operating conditions. Additionally, coupled techniques like GC-MS, LC-MS provide mass information but are offline, sophisticated and require extensive sample preparation. Proton Transfer Mass Spectrometry analyzes off-gas from bioprocesses and is a promising tool for volatile organic and sulphur containing compounds [58].

Software sensors are another option employed for online measurement and analysis. A critical element in the synthesis of software sensors is the available knowledge of the process which is expressed as a mathematical model. It involves extensive data based on culture parameters, process employed and the operating environment. Existing knowledge base is used to estimate variables that are important and cannot be measured directly. Secondary variables of a bioprocess, e.g. temperature, gas-flow rates, oxygen and carbon dioxide concentration, dissolved oxygen and pressures, are measured online using hardware sensors. Process inputs like feed rates and some infrequent measurements of primary outputs, are supplied to software sensors. In the model, the relationship between a primary and secondary variables are described by use of first principles. In defining the model, careful treatment of process knowledge, assessment to consider all possible factors influencing the cultures during production is very important. Empirical models based on artificial neural network, fuzzy logic and multivariate analysis are employed [43, 46, 47]. Based on the accuracy of these measurements, the software outputs can be used for feedback control and optimization of the process.

Biosensors offer methods for online determination of low as well as high molecular weight compounds in bioprocesses because of a vast number of types of biorecognition elements available e.g. enzymes, antibodies, microbes, nucleic acids, aptamers. They have high specificity, selectivity with rapid detection. Many different variations of detections are possible ranging from enzymatic sensors to immunosensing and whole cell based sensors. Detection options include electrochemical, calorimetric, optical and mass detection. Optical detection involving luminescence techniques offer higher sensitivity and lower detection limits. Amongst the electrochemical detections, amperometry has higher sensitivity as compared to potentiometry. Single target
analytes can be analyzed and used for monitoring even in complex media and requires no or minimal sample preparation (10, 16-18, 21, 22, 46, 47, 59, Paper I, VIII, IX).

Importance of biosensors in bioprocesses can be gauged by the fact that many compounds of interest such as amino acids, antibiotics (including isomers), proteins, sugars, alcohols, biomass, trace impurities etc. can quantified in quick time by biosensors. Amino acids are important both as a product of fermentation and as an important factor for production. It can be estimated by combining enzymes and electrochemical transduction. For instance determination of L-phenylalanine by an enzyme based sensor using L-phenylalanine ammonia lyase followed by potentiometric detection [59]. Another important amino acid glutamine which is of significance in mammalian cell culture can be quantified by using glutaminase and glutamate oxidase employing chemiluminescence detection. Monitoring of biosynthesis of antibiotic penicillin V based on enzyme thermistors has been reported [59].

Developments in DNA micro array provide improved insight into recombinant protein production. DNA biochips exploits hybridization of target DNA strands with fluorophore labelled cDNA probes which are optically detected. With high integration capacity of biochips, an array of immobilized DNAs can accommodate all the required gene expressions that need to be monitored during recombinant-protein production. It enables to provide a snap shot of the actual gene expression profile and carryout genome wide investigation. This makes it possible for qualitative and quantitative characterization of the metabolic stress on the host cell mechanism. Presently, it is an offline technique wherein the fluorescence of the expressed gene is analyzed by employing non-linear statistics. Advanced systemic information could be obtained by protein analysis in real time. Analysis of the expressed proteins at the cellular level, could provide more dynamic information of synthesis as well as degradation of proteins in the cell. This involves having arrays of microspots of immobilized capture probes of antibodies, enzymes, aptamers etc. The specific interaction between the sample and the target molecule can be detected by fluorescence and chemiluminescence techniques. These techniques are off-line, but developments in lab-on-chip concepts can provide promises for online DNA extraction, hybridization and analysis in the near future [41, 58].

8. Challenges in Bioprocess Monitoring and Automated Control

8.1. Process Dynamics

Bioprocesses are usually run in different batch modes for overproduction of economically important products. As this involves living microorganisms, this affects the intricate
cellular metabolism making the process complex. Though, it is ideal to have complete real time insight of the happenings inside the cell so as to maximize production, it poses a severe challenge in terms of methods and tools available for continuous monitoring. Some of the measurements are impractical and need to be estimated while others like substrate and product concentrations, ATP, ADP, NAD, NADH, total protein can be measured by techniques like biosensors with robust instrumentation [10, 11, 18]. Bioprocess being nonlinear and time variant has different possible outcomes for set point changes and process load disturbances. For instance, non-linearity observed in the metabolic activity of yeast – wherein at high glucose concentration switches from production of yeast to ethanol production. Likewise when glucose is limited, the order of the reaction changes from first order to zero order which has influences on the control strategy.

The culture parameters like specific and total oxygen uptake rates, carbon dioxide evolution rate are dependent variables. The enzyme and genetic make-up of innoculum are usually ill defined causing process variability. Further, bioprocesses have hysteresis effects making the control complex. So it is desirable to have online assessment of the state of the process. In this context, the need for reliable and accurate chemical and biochemical signals are enormous. In this PAT regime, emphasis is laid on quality-by-design (QbD) rather than for quality by testing. Variability of product actually arises due to complexity such as scaling-up effects, variations due to raw materials from batch to batch and operational factors [8, 54]. These effects being dynamic are difficult to measure and are best estimated by monitoring and virtual sensors. This necessitates real-time multi-variate process monitoring. Bioanalytical methods provide additional real time insight to complement the efforts for improved process understanding and control.

8.2. Sampling Systems

Sampling and sample handling are of utmost importance for online measurement systems. These systems have to address the essential requirement for a sterile barrier in the bioprocess to avoid contamination. Sampling systems obtain representative samples from the bioprocess at any given instance. Too frequent a sampling is also a hindrance owing to the sterile requirements. Bioprocesses involve both direct and indirect sampling methods and the type of sampling device chosen is based on many parameters like medium conditions, the susceptibility of the fermentation to contamination, and the sensitivity of the cultured cells to shear stress or oxygen limitation [4, 48].

Sampling devices are usually based either on filtration or on dialysis techniques which are by far the most frequently used techniques for online sampling and analysis [61]. There are essentially three main strategies for sampling — mechanical, chemical/physical, and heat barrier. For biosensors, cell free samples are desirable.
Sampling using a coaxial double lumen catheter is an interesting option for biosensors on two counts - it is extremely efficient with online sampling of small volumes with controlled dilution and it is approved by US, FDA. In fact, it is extensively used in medical applications and shown applicable for fermentation samples as well. The device has a coaxial inner and outer part. The sample is drawn in the inner lumen while simultaneously an inhibitory solution is pumped in the outer lumen. Further, the inhibitor arrests the metabolism of the sample making it highly representative. By controlling the flow rates in the coaxial catheter the required dilution can easily be achieved [60-62].

If the sample involves intracellular enzymes, then online sonification and subsequent filtration is essential. A particle-free solution can be sampled out by combining a sampling device with membranes as sterile barriers. Flow injection system provides different configurations for in situ sampling, at-line or online sampling from a bioreactor [61].

The sample drawn from the bioreactor can require some sample handling steps. It could be dilution, enrichment of sample, cell disintegration etc. Systems have to be integrated for effective sample handling so that reliable detection is possible. Care also needs to be taken so as to ensure real time representative samples are drawn from the bioreactor at all times.

In sampling with dialysis membrane, a concentration gradient of broth (donor) and buffer (acceptor) is generated resulting in a flux of solute molecules across the membrane. The solutes that pass through are determined by combination of factors such as composition, thickness and molecular cut-off of the membrane. By controlling the flow rates of buffer and the sampled broth, the concentration of sample can be controlled to cater to the sensitivity and linear range of the sensor employed. Dialysis membrane sampling is suitable for low molecular weight compounds and has been used during automated analysis of glucose and lactic acid. It provides reliable sampling for online monitoring in a bioreactor (Paper I). Filters were used for sampling from an acetonitrile degradation reactor for its monitoring and control (Paper VIII).
9. Biosensors for Bioprocesses

The general scheme of employing biosensors for process monitoring and control is given in Figure 2.

![Figure 2 Schematic presentation of a biosensor system for monitoring and control]

Bioprocess involves variety of analytical procedures and measurements are required at several stages – the production bioreactor, downstream processes and subsystems. The relevant parameters include pH, temperature, pressure, dissolved oxygen, nutrients, metabolites, products, biomass etc. Continuous monitoring systems provide online information of these variables to devise and adopt control strategies for efficient and realization of the bioprocess. It also builds up the knowledge base to arrive at new approaches of model based process control.

Biosensors offers a means to provide this information combining the biospecificity of biorecognition elements e.g. enzymes, antibodies, microbial cells, carbohydrates, aptamers, peptides, with a transducer such as electrochemical, optical, calorimetric or piezoelectric to generate a measurable signal. The biological sensing element produces a response which is translated into a physical signal by means of a transducer.

![Figure 3 Schematic representation of the operational principle of a biosensor]
A schematic representation of the operational principle of a biosensor is shown in Figure 3. Commonly employed biosensors are based on enzymes, antibodies or whole cells. Microbial sensors provide convenience for certain analytes which cannot be detected by commercially available enzymes or when requiring multiple enzymes or cofactor/coenzyme to generate a measurable product.

Biosensors in bioprocesses are mainly used for analysis of carbon sources like sugars, amino acids like glutamine, phenylalanine, antibiotics penicillin G and V, macromolecules like antibodies, therapeutic proteins, enzymes, antigen; glycerol, alcohol etc. [11, 42, 64-66]; biological oxygen demand and analysis of toxic chemicals and pollutants for environmental monitoring [37, 66, 67]. Recently aptamer or “chemical antibody” based sensors have received more attention. Aptamers have high specificity and in principle can be selected for detection of any target - small molecule to large proteins and even cells. Additionally, they have high sensitivity due to their significant conformational changes and possibilities to employ different sensing configurations [68-71]. In this work, biosensors based on enzymes, antibodies and whole cells have been used along with flow systems and are discussed further.

9.1. Flow Systems and bioprocess monitoring and control

Flow injection analysis (FIA) is a versatile tool for monitoring of bioprocesses, and by combining different bioelements and detector choices many options for sensing biochemical variables are available for process monitoring. FIA can easily be adapted to various analytical problems of bioprocess by aseptically drawing samples [1, 60, 62]. FIA introduced in 1987 [72] is a flexible technique which is utilized as an important sample handling tool for online monitoring. Its integration to bioprocesses through an aseptic sampling system is simpler and is an attractive means for at-line/ on-line analysis [1, 63]. Biosensors based on FIA have been used for bioprocess-monitoring of many analytes like sugars – glucose, maltose, sucrose, galactose and others products/substrates like lactate, glutamine, glycerol, methanol, antibodies, amino acids etc in allied fields like pharmaceutical, food, environmental medical etc. [18, 21, 48, 73-75].

Flow injection has gained popularity for biosensing applications in bioprocess monitoring because the sensing element cannot be steam sterilized and in situ biosensor has been limited to single analyte - glucose. The concept of autoclavable biosensor has been described only for glucose. The immobilized enzyme for recognition is housed in a reservoir with a semipermeable membrane sheath. The analyte diffuses through the membrane and the readout is potentiometric. It has advantages to limit interference but has limitations of a large response time(up to 10 mins) and range of analysis [40, 48].
Flow systems provide flexibility to combine different types of biosensing elements with different transducers. Furthermore, the biorecognition component and transducer can be spatially separated or used in close conjunction. This provides means of easy maintenance for continuous operation. A brief description of these different flow systems used in conjunction with biosensors is provided here.

In basic flow injection, a sample is introduced into a flowing carrier stream. The dispersed sample is physically, chemically or biologically manipulated and detected by various techniques like optical, electrochemical, mass, thermal, etc. For example, in a FIA system with amperometric detection, the sample is subjected to electrode processes and the resulting current is detected electronically. Furthermore, the flow system is easily realized by a single peristaltic pump, an injection valve and a detector. In fact, FIA provides the distinct advantage of consistency of analysis, ease of sampling, automation and flexibility. Moreover, different modes of flow injection analysis are employed like sequential injection (SIA), bead injection (BIA) and Lab-on-Valve concepts for bioanalysis [39, 60, 64, 74]. Coupling of micro columns with different solid supports like beads, gels, etc., used for capturing the target analyte is convenient in FIA and hence preferred for on-line process monitoring [74].

However, their application in the industrial production is impaired by the lack of reliable supervisory systems for on-line fault detection and correction. Automation of such a system provides repeatable sample dispersion in a carrier stream and makes continuous analysis possible. With FIA configuration, a single sample can be analysed at a time but variations with sequential injection analysis (SIA) makes it possible for multiple analytes [74]. Figure 4, shows the SIA system which uses a bidirectional pump and a multi-position valve along with the reaction and the holding coils.

![Figure 4 Sequential Injection Analysis (SIA) System](image)

The holding coil serves the purpose of holding and mixing the reagents and sample. The reaction coil is for kinetic reactions of the sample. A piston pump assures steady flow in the system. SIA offers the advantage of less hardware, along with possibility for multiple
analytes, stopped flow and reduced consumption of reagents. Precise control of pump and valve actions would be necessary and requires automation.

In bead injection analysis, micro beads onto which sensing materials are immobilized are injected into a manifold and made to interact with the sample to be analyzed. For each sample analysis, fresh beads are used so as to eliminate any cross interference from earlier samples. However, it is preferable to inject beads prepared from the same batch to eliminate effects of batch to batch variability during coupling. With bead injection, an advantage is the possibility to address analysis of different analytes each time by injecting beads with appropriate sensing materials.

The Lab-on-valve essentially uses the same hardware as SIA/BIA with the valve having an integrated micro conduit on the valve. This enables the downscaling of consumption of samples and reagents. Further, it is suitable for micro scaling and to employ integrated optical detection [74]. In addition, it has all the advantages of SIA of online calibration, programmable flow rates, stopped flow and increased sensitivity for kinetic reactions.

Additionally, in FIA systems, cofactors for bioanalysis can be provided easily and the operating conditions optimized to allow the enzymatic reaction to operate at optimal efficiency. As the amount of sample required for analysis is small, multiple analytes can be analyzed with reactors either in series or in parallel. Nonetheless, the investigation of interference as a result of the other components of the sample and optimization of conditions to ensure the long-term stability of the system are of utmost importance. Only under optimum and stable conditions will it be possible to reliably use biosensors in actual processes [3, 10, 19, 62]. The biosensor techniques based on enzymes and antibodies employed in flow mode have yielded comparable results to other established methods based on enzymatic assay [39]. Hence, all these principles are applicable to bioprocess monitoring, but the analytical range of each system has to be adapted to the concentration range of the analyte in the cultivation, by choosing suitable dilution rates. These dilution rates can be easily accommodated by FIA system and automation.

Automated FIA systems along with enzyme biosensors or immuno sensors provide a valuable option to adapt to requirements for online analysis and control in bioprocesses and these have been studied and illustrated in Paper I, IV, V, VIII and IX.

Flow systems have been employed for online monitoring to provide information of nutrients, elicit process information and improve performance. Novel biosensor tools for real time process monitoring have been a focus area in bioprocess engineering not only for monitoring but also for diagnostics and control. FIA technique along with bi-enzyme immobilization has been found to provide accurate and stable response over a prolonged period of time for glucose measurements in E. coli and S. cerevisiae fermentation [21].
An analytical microreactor with the immobilized biocatalyst was used wherein more than 21000 sample analyses were possible with the reactor column. An optical biosensor monitoring a recombinant protein product during recovery and purification has been illustrated for application in large scale fermentation [76]. The utility of the biosensor as a tool for process evaluation and fault diagnosis during different stages of production from fermentation to chromatography strongly suggests real time view of processes even for fermentation at industrial scale. By online monitoring of the biosynthesis of proteins, decisions can be made on the instant at which harvest needs to be done during production. Further during extraction and purification, it is provides a monitor for assessing any quantitative or qualitative loss in the product. This leads to improved processes with better efficiency. The benefits of rapidity of analysis of biosensors were utilized for automated analysis of biofuels, gasohols and hydrated alcohols for quality control. Integrated biosensor systems in SIA flow configuration were which enabled rapid analysis with high sensitivity, and online dilution helped to achieve the quality objective [77]. The possibility of rapid measurement of compounds like glucose by biosensors was exploited for fermentation control. A SIRErö biosensor-based fermentation control by continuous monitoring using FIA and having a one step ahead predictor has been reported [78] wherein the feeding concentration and rate were computed based on the biosensor measurements. The results suggest adaptability of biosensor for continuous control with good accuracy. In Paper I and VIII, amperometric systems have been used in conjunction with FIA. Signal conditioning boards were devised for amperometric detection in Paper I. The electronic signals which were proportional to the concentrations of glucose and L-lactic acid, respectively, were acquired by a computer through a data acquisition system. The computer controls the flow system, automated injection module, data acquisition from the sensor. Custom developed software ensured control of frequency of sampling, flow rates, selection of sensor, calibration and data analysis. Calibration module provided options for introducing known standards and calculating coefficients for quantification of bioprocess sample and to compensate for drift in the sensors due to loss of activity over a period of time. Further visualization and data processing abilities of the software enabled process documentation and reports. The system showed good reproducibility and linearity with consistent performance for online monitoring of the bioreactor. FIA systems find good number of applications in environmental monitoring using microbial biosensors [79- 81]. Microbial sensors for toxicity control and management of influent load has been reported both in aerobic and anaerobic configurations [82]. Early decisions are vital in environmental bioprocesses to not only for efficient operation but also to safe guard the processes. The immobilized biomass enabled control of nitrification process, monitoring of toxicity and over/under-load. Early detection of toxic phenolic
compound was possible to take control decisions by temporarily diverting the influent. Further process was operated at a lower feed rate to help quicker recovery of the process. **Paper VIII** discusses closed loop control of a two stage degradation bioreactor employing monitoring of acetonitrile by an amperometric whole cell biosensor (Figure 5). The feed of substrate acetonitrile was modulated to good effect to use the capacity of the reactor.

**Figure 5 Bioprocess monitoring and control by a microbial sensor**

Different ranges of expected acetonitrile concentration were set and the system demonstrated the adjustment of feed rate to the required level. Online multipoint calibration based on peak height and peak area was utilized. The frequency of sampling was selectable and the analysis time was around 3 mins. With automation the capacity of the reactor was better utilized. The automated flow system could recognize changes in sensor response due to membrane fouling and facilitated to initiate remedies. With the system of checks based on the sensor response, it was evident for a requirement of a more elaborate sampling technique with dilution.

Flexibility in operation is proven as flow injection can be used to intelligently switch between different sensing systems in a multiplexed configuration (**Paper I**, 83). Optical biosensing was combined with automated FIA for analysis of target protein in a differential arrangement with different ligands immobilized on separate channels. Two different affinity chromatography columns were used to observe any nonspecific binding and eliminate inferences due to non-specific interactions [83].

Tracking of process variables during production is vital. An automated at-line monitoring of a secreted protein and bioactivity in a mammalian cell bioreactor has been demonstrated based on Surface Plasmon Resonance technique (SPR) [83]. The secreted protein was detected with a peptide immobilized over the sensor surface. It was demonstrated that the protein concentration could be very well followed during...
production and also determine the bioactivity levels. In Paper IX another modular flow system for analysis of human IgG is described. The system employs immune-reactor for analysis and the conditions of operation are flexible to cater to different concentration ranges of the protein. As biosensing involves various types of flow systems such as FIA, SIA and BIA, Lab on Valve or multi-commuted flow, the system is versatile to easily adapt to any of these flow configurations. The software handles the flow control, continuous data acquisition and analysis. Different steps in flow analysis like holding, kinetic reactions, washing, dilution can be controlled and can program sequence of events. Additionally, the flow system can be setup in for continuous process monitoring and analysis. Further, the system has both analog (current and voltage) and digital input, and USB interface to conveniently integrate different type of detectors like electrochemical, optical, thermal, mass etc. Further, the flow system being generic and yet fully controllable different biorecognition elements can be employed e.g. membrane units in flow cell, packed bed, expanded bed reactors, microreactors etc.

9.2. Electrochemical biosensors

Development of biosensors is an interdisciplinary process involving biology, chemistry, physics, microcomputer and electronics. Electrochemical biosensors involve biocatalyst in close conjunction with an electro-chemical cell. A range of biocatalysts can be employed from enzymes, antibodies, microbial whole cells, DNA to aptamers.

Electrochemical sensors incorporate a pair of electrodes to produce a measurable signal. Based on its interaction with the electroactive species and the electrode an electrical signal is produced that is proportional to the concentration of the analyte. Electrochemical sensors combine the unique advantage of being rapid, quite simple and can easily be miniaturized. Biocatalysts provide them further merits of high specificity and sensitivity [85]. Noble and inert materials have been used as electrodes. Usually electrodes are metals such as platinum, gold, silver or carbon based such as graphite, carbon black, carbon nano tubes etc. Modified electrodes with polymeric membrane, self assembled monolayers are useful techniques to avoid interference, improve stability and ensure better performance from biosensors [86].

In electrochemical biosensors, the transduction element is the electrode. Depending upon the type of changes measured in the electrochemical cell, the sensors are classified as amperometric (current), potentiometric (voltage) and conductimetric (impedance) sensors. All these type of sensors are significant for bioprocesses with amperometric and conductimetric biosensors having more utility in bioprocesses. In the present work amperometric biosensors for bioprocess monitoring and control have been investigated.
9.2.1. **Enzyme based Biosensors**

Enzyme based biosensors employ different classes of enzyme like oxido-reductases, hydrolases and lyases as biocatalysts. Enzymes are proteins which catalyze specific reactions by converting a substrate to a product without being consumed. Detection is achieved by monitoring changes in substrate, cofactor or product due to the enzymatic reactions (Figure 6). For example oxidase and dehydrogenase enzymes generate electro-oxidizable peroxide or NADH, hydrolases are involved in hydrolysis reactions and lyases like citrate form carbonate ions for detection. Many enzymes requires one or several inorganic ions e.g. Fe^{2+}, Fe^{3+}, Mg^{2+}, Cu^{2+}, Zn^{2+} or nucleotide cofactors NAD^+, NADP^+, FAD and FMN, for activity [87, 88]. Enzyme based sensors exploit the selectivity, specificity along with the catalytic action of the enzyme. Each enzyme has a specific substrate or group of substrates. Biosensors can be developed with multiple enzymes to generate a sequence of enzymatic reactions so as to generate a product that is measurable. It can also be used as a strategy to eliminate certain interference compounds which affects the measurement, [48, 77, 89, Paper III]. Combined with the dual benefits of enzymes and the electrochemical detection technique, these sensors have become a potent force as sensing elements in the field of food, fermentation, pharmaceuticals and environment applications. The enzyme derives its specificity by its 3-dimensional active site to which the substrate binds.

![Figure 6 Oxidoreductase enzyme and amperometry](image)

**Figure 6 Oxidoreductase enzyme and amperometry**

The enzyme most widely used for glucose detection is glucose oxidase (GOD). It catalyses the oxidation of glucose to gluconic acid. The prosthetic group FAD at the active site of the enzyme exists in one of the two forms - oxidized (FAD) and reduced (FADH_2). FAD oxidizes glucose to gluconic acid, and the FADH_2 generated is oxidized to FAD by oxygen. In this reaction oxygen is consumed and hydrogen peroxide is released which can be measured to relate the concentration of glucose.

The sensor response is dependent on many factors like the enzyme activity, concentration of the substrate, its diffusion into the enzymatic layer, transformation into a product and finally its conversion into an electrical signal by the transducer. Common transducers are based on electrochemical, optical (absorbance, fluorescence,
chemiluminescence), and thermal detection [10, 59, 63, 90]. The microenvironmental conditions like pH, temperature, ionic strength etc. also affect the sensor response. Stopped flow technique can enhance the sensitivity of enzyme biosensor employed in a flow system. This will enable increased reaction time of the analyte with the sensing element [89].

For reuse, good sensitivity and faster response, enzymes are immobilized on to the sensor surface or to a support material. Binding of the enzymes to the sensors is done in several ways like adsorption, covalent attachment, cross-linking, entrapment and encapsulation. The immobilization method adopted should not only increase the stability of the bioreceptor but also retain its bio-recognition property to extend the useful life of the sensor.

Enzyme biosensors are relevant for bioprocesses as they provide possibility to detect several compounds relevant to fermentation e.g. sugars, amino acids, antibiotics, alcohol, glycerols and macromolecules.

9.2.2.  Microbial Biosensor

Microbes are ubiquitous and have excellent capability to metabolize many substances. Microbial biosensor couples microorganisms as biorecognition elements with a transducer. Consumption or degradation of a substrate results in an electronic response. Microbial sensors offer advantage of broad specificity, elimination of requirement of pure enzymes. In fact, they are best suited when multiple enzymes and/or cofactors are required for producing the desired product for detection. Further they provide a suitable alternative for detection of different target compounds as they can be easily manipulated to consume or degrade new substrate under certain cultivation conditions [17, Paper VII]. Further the activity of organisms can be modified by genetic manipulation to suit custom requirements. This is achieved by improving enzyme activity or expressing different enzyme or protein in host cell which can then be used as a marker for detection [91]. Microbial biosensors based on modification of pH electrode with genetically engineered E. coli intercellular expression of organophosphorus hydrolase and on the outer surface of cells of wild-type OP degrading bacteria Flavobacteium sp. have been reported [92]. Additionally, microbial sensors can be operated under broad pH and temperature range with several detection possibilities e.g. amperometric, potentiometric, conductimetric or luminescence techniques [16, 17].

The cells are immobilized either by adsorption, entrapment or crosslinking. For continuous online analysis, adsorption technique is not stable enough as the cells would be washed off and hence entrapment within polymeric membranes is preferred.
The major areas of application of microbial sensors is in environmental monitoring of BOD and pollutants, process monitoring and for analysis of food additives, contaminants, nutritional factors [69, 71, Paper VII, Paper VIII].

9.2.3. Amperometric Biosensors and Detection

The amperometric biosensors are the most commonly used electrochemical sensors for analysis of food and fermentation processes. Amperometric biosensors utilize the analytical capability of amperometry together with the specificity of enzymes or other bioelements resulting in sensors with high sensitivity and selectivity. Another attractive feature is bioelements like aptamers can be employed with electrochemical detection. This opens up many opportunities for detection of macromolecules in bioprocesses e.g. detection of thrombin was achieved by intercalating an electroactive species like methylene blue with an aptamer and tracking its release due to conformational changes [93].

Amperometric sensors are rapid and have the possibility for miniaturization, thereby representing options to develop array of sensors for multiple analyses at low cost [94]. The sensor comprises of either a two- or a three-electrode system - a reference, a working and a counter/auxiliary electrode (Figure 7).

![Figure 7 Amperometric sensing electrode and detection scheme](image)

In the two-electrode system, the potential between reference and working electrode is kept constant during measurement e.g. for detection of dissolved oxygen, a potential of -0.65 V is applied and +0.600 for hydrogen peroxide. The biocatalyst is immobilized on to a membrane and closely coupled with the electrode. The resulting current is directly proportional to the oxidation or reduction of the electroactive species at the working electrode and is amplified using a signal conditioning circuitry for final readout.

In a reversible electrochemical system,

$$Oxd + ne^- \Leftrightarrow Red$$
where Oxd and Red are the oxidized and reduced form of the electroactive species and there is no net charge transfer. The concentration of the oxidized and reduced forms can be controlled by controlling the applied electrode potential (E) according to the Nernst equation.

\[ E = E^0 + \frac{2.3RT}{nF} \log \frac{C_{\text{oxid}}}{C_{\text{red}}} \]

where \( E^0 \) is the standard potential, \( R \) is the universal gas constant, \( T \) is the Temperature in Kelvin, \( F \) the Faraday constant, \( C_{\text{oxid}} \) and \( C_{\text{red}} \) are the concentration of the oxidized and the reduced species respectively and ‘n’ is the number of electrons transferred in the reaction.

The resulting Faradic current ‘i’ is diffusion controlled and is directly proportional to the concentration \( C \) of the reduced species. It decreases with time and is given by Cottrell equation:

\[ i = \frac{nFAD^2C}{\pi \delta^2} \]

where \( D \) is the diffusion coefficient and \( A \) is the area of the electrode surface [95]. The reduced species is depleted at the electrode. In a flow system with amperometric detection, due to the forced convection, the current becomes dependent on the mass transfer of the reduced species at the electrode and is given by

\[ i = \frac{nFADC}{\delta} \]

where \( \delta \) is the thickness of the diffusion layer and is constant for a given configuration.

Convection can be induced by stirring the bulk solution or rotating an electrode or flow system. In Paper I, III, VIII flow systems were employed and in Paper II, VI and VII batch systems with stirring were employed for amperometric detection. Normally with the biocatalysts employed for analysis of compounds like glucose, lactate, ethanol or glycerol, the electron acceptor is oxygen and hydrogen peroxide (\( H_2O_2 \)) is produced. Either the consumption of oxygen or production of \( H_2O_2 \) can be monitored electrochemically.

Amperometric biosensors have good linearity and response time as compared to potentiometric sensors. At high electrode potentials, there could be interference from other electroactive species and care needs to be taken to eliminate them. Usually these interferences are minimized by careful selection of membranes with different molecular cutoff and surface charges. Alternatively, chemically modified electrodes with polymer films, inorganic films like metal ferrocyanide, clay etc. can also be used [96]. Further, by employing mediators like ferrocene, methylene blue, cytochrome b etc., the applied
potential can be lowered. Mediators transfer electrons from redox centers of enzyme to the working electrode. Another option is to have direct electron transfer between the enzyme and the electrode to achieve bioelectrocatalysis [97]. To improve stability generally a three electrode system is used wherein a counter electrode is used for measurements. The counter electrode completes the electrical path for the current avoiding the flow of current through the reference electrode.

9.2.4. Stability of Bioreceptors

Stability of the biomolecules used for recognition is essential for robust operation of the biosensor. While the biosensor is employed for bioprocess monitoring, the sensor is continuously used for sampling the complex matrix and experiences shear forces due the flow system. Different immobilization techniques are used to retain a stable activity of the enzyme and extend the useful life of the sensor. Suitable support materials like glass beads, noble metals, polymeric films, gels, carbon based materials, conducting polymers are employed for enzyme immobilization.

Adsorption tends to be less disruptive for enzymes as the binding involves hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions. However, it suffers from desorption effects. In the covalent bond method, some functional groups of the enzyme like carboxylic acid, thiol, imidazole, or phenolic groups which are not used for recognition are utilized to covalently attach them to activated supports like glass, polymers etc. Cross-linking involves multifunctional agents like glutaraldehyde (Paper I, II, III, VI, VII). Spacers like gelatin are used in such immobilizations.

Various methods have been used for increasing the operational stability of the electrodes e.g. formation of protein-polyelectrolyte complexes, use of sugar alcohols etc. [98 and 99]. A novel enzyme stabilizing techniques based on polyelectrolyte diethylaminoethyl-dextran has been reported. Glucose oxidase and lactate oxidase were stabilized with the cationic polyelectrolyte and physically adsorbed into a porous carbon electrode for biosensor application. The method yielded storage and operation stability up to 6 months. It was implied that the increase in response current from the sensors was due to improved conducting matrices [98]. Another sensing configuration involving chemically linked GOD with redox sensitive prussian blue on polyester foil has been reported with operational stability of 30 days. The concentration range for monitoring glucose was also in the sub millimolar range [100]. It is reported that inert proteins like gelatin and BSA have stabilizing effects when immobilizing enzymes with bifunctional agents like glutaraldehyde. This minimizes the excessive intramolecular cross-linkages within the enzyme [101]. In Paper II it was shown that lysozyme has a very good stabilizing effect for glucose oxidase for glucose sensor and multienzyme - invertase, mutarotase and glucose oxidase system in a sucrose biosensor. Operational stability was
found to be 230 days (750 samples) for glucose (555 mM) and about 40 days (400 samples) for sucrose (290 mM) as shown in Figure 8.

![Figure 8](image_url)

**Figure 8** Operational stability of immobilized GOD enzyme - Residual activity after repeated glucose (10%) analysis with biosensor using GOD immobilized with different protein based stabilizing agents.

The improved stability with lysozyme could be due to complementarity of surface properties with the enzymes involved and also due to ionic interactions. This method was further used in Paper VI for tyrosinase based biosensor for detection of tea polyphenols.

Thermal stability is another important factor for operation of enzyme-based sensors. Changes in conformation of enzymes in biosensors are influenced by factors like thermal shock, pH, ionic strength, proteolytic degradation etc. Improved thermal stability for glucose oxidase has been reported earlier by using polyelectrolyte like diethylaminoethyl dextran with buffered sorbose. The thermal stability could be improved by the enzyme-protein complex formation up to 100 °C for 15 min [102]. When immobilizing β-galactosidase in agarose up to 53 °C stabilization has been reported when measuring the transition temperature for denaturation [103]. Hydrophobicity is an important factor for stability of the enzyme. Taking advantage of this, use of silane was explored to increase the hydrophobicity of the surface for glucose sensor (Paper III). Studies showed that enzyme immobilized on glass beads with higher silane content was stable at 75 °C for 3 h with 180% activity (Figure 9.).
enzyme activity was maximum after incubation at 45 °C for 3 h only (Fig. 1). The enzyme immobilized on CPG retained 50% of its initial activity and was determined to be 250% of the initial value (Fig. 2) and the transition temperature increased to 75 °C. Among all the matrices used for the studies, glass beads and at a transition temperature (Tm) of 63 °C for 3 h only (Fig. 1). The enzyme immobilized on CPG showed an enhancement in the thermal stability of the enzyme and the m value of 63 °C temperature. The relative standard deviation in the experiments was in the range of 0.5–1.5%. Therefore, the error bars are not clearly visible in the figure. In case of 4% silane the activity remained constant (Fig. 2). The transi-

**Figure 9** Thermal stability study of GOD on various solid supports

This indicates that the system can be used in applications at higher temperatures for better stability in biosensors e.g. for online monitoring of processes with packed bed bioreactors.

### 9.2.5. Amperometric sensors and Automation

In the food industry, the quality of a product is evaluated through periodic chemical and microbiological analysis. In this context, rapid and affordable assay methods are required. Moreover, the food and drink industries need rapid and affordable methods to determine compounds that have not previously been monitored and to replace existing analytical methods [104]. This indicates a clear need for quick analysis tools which reduce expensive sample pretreatment steps typical of the conventional techniques [20].

Continuous production of lactic acid by fermentation is important as it has wide applications in food processing and pharmaceutical industries. Production of L-lactic acid is now constantly increasing because of its potential use as feedstock for biodegradable polymers [105]. Further, rapid detection of sugars like glucose is of importance in fermentation for e.g. for determining feed strategy. Analysis of such analytes are possible by enzyme based biosensors e.g. sensors for glucose, lactic acid, ethanol and glycerol in fermentation processes [64]. Further, glucose biosensors are widely employed in the fermentation of dairy products, wine, fruit juices, drinks, ethanol etc. [20, 64, Paper I].

For fermentation monitoring, in-situ enzyme sensors could be preferred but due to the fragile nature of the sensor it is employed along with flow systems. Attempts have been reported on an in situ electrode, which is inserted directly in the reactor resulting in response times of about 1 - 3 min. But this electrode is optimised to date only for glucose. The major disadvantages of such device are its complicated setup, limited linear range (1 to 10 g/l). Additional problems may appear when there are major differences in pressure between the internal buffer in the electrode and the cultivation medium,
because this will lead to a permeation of liquid through the membrane at the electrode face [39]. Apart from monitoring concentrations of analytes, biosensor measurements can also follow substrate uptake kinetics of microorganisms which was demonstrated by carrying out studies using *Saccharomyces cerevisiae* [106]. Such measurements provide indications of the dynamics of metabolic pathways of microorganisms. L-lactic acid measurement during wine making is important as it affects the malo-lactic fermentation. On-line lactic acid determination provides a profound insight during the wine making process to manage fermentation and eliminate off-odors [107]. Additionally, the malo-lactic fermentation has to be triggered at the end of ethanol fermentation for better efficiency and quality of the product. Such phase changes in fermentation can be best monitored by following key process variables like concentrations of ethanol and lactic acid.

Different techniques have been employed for analysis of important constituents during fermentation. Possibilities of employing enzyme thermistor-based automated sensor for online analysis of penicillin V, L-Lactate, glucose and ethanol in industrial fermentations have been reported, with the detection limit of 0.15 mM for L-lactate [108]. The response time was 5 min. The technique required pure and substrate specific enzyme. Another example of lactate biosensor is based on integrated enzyme field-effect transistor with potentiometric measurement. It had a detection range from 0.1 mM to 10 mM and a quick response time of 15 s [109].

Fourier transform IR (FTIR), FT-Raman and NIR techniques for monitoring of multianalytes during lactic acid fermentation has also been reported for analysis of glucose, lactic acid and biomass [57]. FT-MIR technique was found more reliable as compared to FT-Raman and NIR. IR spectroscopy involves expensive instrumentation, statistical data processing and was reported for offline analysis.

Combining biosensing with automated flow analysis, on-line monitoring of lactic acid fermentation for measurement of both glucose and lactate was illustrated by amperometric enzyme sensors (*Paper I*). The use of immobilized enzyme with good stability meant a useful life of 45 days for the biosensor with good linearity and the range of detection being 1-60 g/l for L-Lactate and 2-100 g/l for glucose. The biosensor results agreed well with the results from the reference method (Figure 10).

During the automated analysis of glucose and lactic acid, dialysis membrane was used for sampling. It was demonstrated to provide consistent and representative sampling from the bioreactor. A potentiostat for interfacing the biosensor with the computer along with software in Visual C++ was developed.
Figure 10 Online monitoring of L-lactate and glucose in a recycle bioreactor during lactic acid fermentation

For L-lactate measurement, reduction of oxygen at the cathode is monitored. High gain amplifiers with high input resistance were used to measure the resulting current in a differential configuration to achieve high signal to noise ratio for the measurements. Software employed had options for running average calculation for signal filtering and noise reduction.

In a flow system for continuous analysis adherence to certain operating norms is very much essential. The sample and carrier flows and online dilution was controlled for accurate and precise measurements. Further, bubble-free flow was ensured in the FIA system by automated valve operations for sampling and injection and flow control. Automation ensured quick and continuous measurements and hence aided to achieve good optimized conditions for online analysis and provided a basis for changes to optimize the sensor for process monitoring. With control of flow and on-line calibration, the time the sample is exposed to the sensor could be varied. Higher flow rates meant shorter interaction times and less diffusion of sample through the membranes. In combination with the repeatability achieved by automation, the required concentration range of analysis could be accommodated. The current signal was converted to a voltage signal for easy interface with data acquisition system. The software provided features for online flexible data acquisition, data visualization, multi-point calibration, data analysis and storage.

Microbes are useful choice as sensing elements for degradation and detection of toxic chemical compounds as they have the natural ability to consume these products. These can be usefully combined for detection by amperometric methods. Microbial biosensors have been reported for toxic compounds like phenols, naphthalene, acetonitrile etc. [110-112]. Monitoring and control of a two-stage degradation bioreactor was studied using an amperometric microbial sensor (Paper IX). An immobilized whole cell biosensor based on amperometric detection was employed for controlling the acetonitrile
feed into the degradation reactor. Continuous monitoring of acetonitrile contributed to closed loop control of the reactor with good success. The feed rate to the reactor was modulated by sampling and measuring acetonitrile at a low sample frequency (1 hour). Calibration of the sensor was possible based on both peak area as well as height. With higher frequency of sampling and higher concentrations of acetonitrile, the system required robust sample handling as the sensor was found to be inhibited.

Another valuable use of amperometric biosensors are for purposes of quality assessment. Caffeine is an important quality marker and is found in beverages, pharmaceutical products, cocoa and cola nuts. Caffeine is used as a therapeutic agent but in significant doses can have adverse effects. Caffeine can be analyzed by several methods like chromatographic separation, UV-visible spectrophotometry, mass spectrometry and immunological methods. It is analyzed usually by chromatographic techniques like HPLC, electrokinetic capillary chromatography etc. which involve considerable steps of sample preparation, extraction, separation and detection. The detection limits were in the order of 30 mg/g. [113, 114]. Moreover, these techniques are not portable. Biosensing methods provide rapid, reliable detection and possibility for online monitoring as well as portable devices. A caffeine biosensor based on piezoelectric detection in a flow injection mode has been described [115] and is based on molecular imprinted polymer and quartz crystal microbalance detection. In this sensor a supported liquid membrane was used for elimination of interference which requires additional steps in fabrication. The response time was found to be 10 mins with regeneration time of 5 mins between analyses. The detection range was found to be 10-1000 ng/ml. A simple, yet rapid and accurate method of detection of caffeine based on biosensing is described in Paper VIII. The microbial amperometric sensor uses whole cells of Pseudomonas alcaligenes for detection of caffeine. A novel selection strategy that incorporated isolation of caffeine degrading bacteria capable of utilizing caffeine as the sole source of carbon and nitrogen from soils and induction of caffeine degrading capacity in the microbe led to the development of the biosensor. The tri-methylxanthine caffeine is sequentially demethylated into theobromine and paraxanthine. It had good linearity in the range 0.1 to 1 mg/ml and was tested with commercial coffee and soft drink as well and had least interference. The optimum pH was found to be 6.8 and temperature was 32 °C. The response time was 3 mins with about 3 mins time to regenerate the sensor in-between analysis.

Another quality assessment tool based on biosensing is the concept of electronic nose. Tea-quality detection has been described based on this principle using principal component and linear discriminant analysis techniques [116]. This method involves multivariate analysis and as it is based on non-specific sensing technique the response depends upon other factors like epigallocatechins, total catechins and sugar content.
Moreover, employing this technique for product differentiation requires different process models depending on tea products selected and has to account for their variability. A tyrosinase based amperometric biosensor system was developed and tested with commercial samples to grade tea (Paper VII). The sensing technique compared well with established UV-visible spectrophotometric method.

\[
\text{QH}_2 + \frac{1}{2} \text{O}_2 \rightarrow \text{Q} + \text{H}_2\text{O} \\
\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}
\]

where QH2 and Q are the reduced and oxidized form of polyphenols.

**Figure 11 Amperometric biosensor system for polyphenol detection in tea**

A prototype of the tea biosensor was also realized based on a PSoC microcontroller. With the system on chip architecture of the microprocessor employed, the device could be made small and portable (Figure 11 and 12). The biosensor had options for two or multipoint calibration. The sensor had a linear range of 10-80 mmol/L and operational stability of 30 days. Each enzyme membrane could be used for about 80 samples. Different commercial beverage samples were tested and the results compared well with established method.

**Figure 12 Biosensing system for detection of polyphenols in tea**

9.3. **Chemiluminescence based Biosensors**

Chemiluminescence - a category of photoluminescence sensors are based on light produced due to chemical reactions. They have high sensitivity and do not have any background light as the reaction is carried out in a dark cell. All the light or photons detected are due to the phenomenon being studied. Their high sensitivity makes them ideal candidates for biosensing based on immunoassays and DNA hybridization.
The chemiluminescence reaction involves oxidation of certain compounds producing one or more intermediates in an excited state which emits light at a particular wavelength. Example is the chemiluminescence generated by oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) which when reacted with hydrogen peroxide (H₂O₂) and in the presence of horseradish peroxidase (HRP) enzyme produce an intense blue light.

\[
\text{Luminol} + H_2O_2 \rightarrow 3 - \text{Aminophthalate} + N_2 + 3H_2O + \text{Light}^* \n\]

The amount of light produced is proportional to the concentration of hydrogen peroxide. The light produced can be detected by variety of detectors – photomultiplier tube, charge coupled device camera, avalanche photo diode etc. Combinations of different biomaterials like enzyme HRP, DNA, antibodies with photoluminescence materials are attractive options for detection of macromolecules, toxic compounds, DNA, trace impurities etc [117]. These devices can be miniaturized and combined with their advantage of minimal sample preparation steps, result in field applicable optical biosensors for food and environment applications [118, 119, Paper VI]. Further, these optical sensors have benefits of non-invasive measurements and possibly less interference due to wavelength selectivity. The downside is requirement of dark reaction cell, to eliminate ambient light effects and the need for additional reagents for luminescence.

Automated immunoassays with optical detection are promising options for quantification of proteins as well for monitoring pesticide residues [119, Paper IV, V, IX]. In a related development an array of a chip based biosensor for analysis of different target proteins with fluorescence labels have been reported [71] indicating the possibility of microfabrication of such sensors for rapid detection.

### 9.4. Affinity Based sensing elements

Immunosensors exploit the intrinsic affinity between the antibody (Ab) and the antigen (Ag) to generate a measurable signal. The binding of antigen happens in Fab portion of the antibody to produce immuno-complexes. An antibody has high specificity and affinity to bind with a particular antigen. Further, antibody-antigen kinetics is reversible. The binding forces responsible for the Ab-Ag interaction are non-covalent in nature and include non-polar hydrophobic interactions, electrostatic forces, hydrogen bonding and van der Waals interactions. Their interaction is characterized with an association and a dissociation reaction rate constant, kₐ and kₐ, respectively.

\[
\begin{align*}
\text{Ab} + \text{Ag} & \rightleftharpoons \text{Ab} - \text{Ag} \\
\end{align*}
\]

48
The dissociation constant is defined as

$$K_D = \frac{k_d}{k_a}$$

$$K_D = \frac{[Ab][Ag]}{[Ab - Ag]}$$

where \([Ab]\), \([Ag]\) and \([Ab-Ag]\) are the concentrations of the antibody, antigen and complex in the solution, respectively. Smaller dissociation constants imply higher affinity. The reversibility of the reaction makes it possible to reuse the sensing element by dissociation. The association rate is a factor of diffusion of antibody and antigen whereas the dissociation rate is determined by the strength of the antibody-antigen complex. Dissociation is achieved by harsh conditions like extreme pH (low or high), denaturants etc.

The specificity of antibody-antigen reaction is like enzyme-substrate but unlike the latter, no new species of molecules are produced. Therefore the detection of the Ab-Ag association is done normally through labelling. Detection could be by fluorescence, chemiluminescence, or enzyme labelling. Enzyme linked immunoassay utilizes labels like alkaline phosphatase, horseradish peroxidase for optical detection [121]. Direct detection of Ab-Ag interaction without any labels is also possible by surface plasmon resonance (SPR), quartz crystal microbalance (QCM), electrochemical and micro mechanical measurements [122]. Label free immunoassay is less complicated and makes it possible to reduce the time for analysis by eliminating certain incubation steps as the number of reagents are less. Flow based enzyme linked immunoassays have been studied in the present work.

Trends indicate immunoassay is moving towards miniaturization for rapid analysis of multiple analytes and regeneratable devices [123]. Further, miniaturized sensors provide advantages of multiple analytes, higher sensitivity, lower detection limits, short response times, and high signal to noise ratio.

Apart from their specificity and affinity, antibodies can be produced both for macromolecules and low molecular weight compounds. Affinity sensors generally have higher sensitivity as compared to enzyme based sensors. These characteristics of immunosensors make them notable candidates for bioprocess monitoring and environmental applications.

Usually, immunosensors developed for process monitoring and environmental applications are heterogeneous, with either the antibody or the antigen immobilized on to a solid support. Upon introduction of the sample containing the other immunoagent, an immunocomplex is formed. The unbound molecules (usually proteins in this context) are removed by washing steps and the response obtained from the labels is proportional.
to the amount of protein bound. There are mainly two heterogenous immunoassay formats competitive and sandwich. In a direct competitive assay format antibody (or antigen) is coated onto a solid support. The sample antigen (or antibody) is introduced along with a labelled antigen (or labelled antibody). Enzymatic labelling is used to amplify the signal due to the enzyme – substrate turnover so that it is detected by an optical or electrochemical transducer [123- 125, Paper IV, V, IX].

Electrochemical detection of immune-interaction can be performed both with and without labeling. An amperometric immunosensor can be devised wherein proteins are labeled with enzymes. Biotinylated aptamers are also used to increase the selectivity [125]. Direct detection without labeling is performed by cyclic voltammetry, chronoamperometry, impedimetry, and by measuring the current during potential pulses (pulsed amperometric detection). These methods are able to detect a change in capacitance and/or resistance of the electrode induced by binding of protein [69]. Another possibility of unlabeled detection is by amperometry wherein an electroactive product produced due to an added substrate is monitored [70, 95, 96].

In the competitive format, the unlabeled (sample) and the labelled species compete to bind to the antibody (or antigen). There is an inverse relationship wherein an increase in analyte concentration results in decrease of signal for the enzyme substrate. Figure 13 shows a scheme of the competitive assay with antibody bound to a solid support like Sepharose.

Figure 13 Scheme for competitive flow ELISA

In an indirect competitive immunoassay, the antigen could be immobilized and a second labelled antibody (covalently coupled with enzyme or other tracer) is used which binds to
the bound antibody. The competitive assay format can be a single or a two-step. In the single step format both the competing species and the labels are introduced together whereas in the two-step format they are introduced sequentially. The immobilized antibody is limited to induce competition, whereas the antigen and the labelled antigen are in excess. In a two step assay, the interaction time for antigen and the labelled antigen can be separately varied providing control to modulate sensor response. For higher time of the antigen with the immunosorbent, the labelled species would have fewer binding sites to occupy and vice versa. Automation provides precise control of assay protocol, the sample and reagent volumes. It provides levers for altering the sensitivity based on analytical requirements. Furthermore it enhances repeatability and above all it provides a means for coupling this powerful technique for continuous process monitoring. In Paper IV, V and IX competitive ELISA formats have been utilized to detect low molecular weight compound (methyl parathion) as well for a protein with good linearity.

In a non-competitive immunoassay, a sandwich assay format is usually configured. The antibody is immobilized onto a support. The analyte is introduced in the next step. A second antibody with a labelled marker is introduced to sandwich the bound analyte. For this the analyte needs to have multiple antibody binding sites. The sandwich mode is useful for trace analysis of impurities or other biological markers for diagnostics.

Usually optical or electrochemical detection is combined with immunoassays. Options for detection include absorbance, chemiluminescence, fluorescence, surface plasmon resonance and amperometric measurements. For direct immunoassays without any labels, piezoelectric principle employing quartz crystal microbalance and capacitance are employed.

The immobilization of antibodies onto solid support is critical aspect so that the binding capability is intact and orientation is proper without any sterical hindrances. Common methods involve that the capturing antibody is physically adsorbed, covalently attached or entrapped in a polymer matrix. Antibody binding protein like protein A or protein G can be used as these proteins bind antibodies through their non-antigenic Fc region [126, Paper IX]. Biotinlyated bioreceptor can be employed with electrodes coated with avidin or streptavidin [124, 125].

9.4.1. Affinity based sensors and Automation

Flow Immunoassays provide a convenient means for online process monitoring for a range of compounds with high precision and accuracy. With advancement in technology in the field of miniaturization, microfluidic devices there have been efforts to integrate
immunoassay and provide it as a platform for assay for biological, medical and environmental applications [127-129].

In flow immunoassays, different techniques like BIA, SIA, LOV can be used and these systems detect signal changes at a non-equilibrium state. An affinity reactor is employed through which sample and reagents are sequentially passed to realize the competitive or the sandwich assays described earlier. It typically involves the affinity interaction, equilibration, incubation of the enzyme-label with proper substrate, rinse and regeneration steps. The sensitivity of detection is controlled by controlling reaction times and amounts of reagents introduced during each step. Even though the response for given conditions is not maximum due to non-equilibrium conditions, automation ensures repeatable scheme of operations. Apart from the sensitivity of the affinity column and the sensing chemistry, the control of each step and stability of flow through the column determines the accuracy and reproducibility of assay. Very sensitive assays are possible using the flow ELISA setup as is evident in Paper IV, V and IX. Consistent reaction and washing times are achieved. At the end a dissociation buffer is passed through the reactor column, to regenerate the immunoreactor for subsequent analysis. Automation and integration also ensured ease of operation without needing any special skills and reduced the analytical time.

Requirements of online analysis for bioprocess vary from small molecules to large proteins with different demands for sampling frequency depending on the process dynamics. Mammalian cell cultures could require a sampling frequency of hours whereas in downstream processing for recovery of materials, it could be in terms of few minutes.

In the recent past many approaches for measurement of proteins during production is reported. Production rate of inclusion bodies during fermentation has been monitored by ELISA using cryogel minicolumn plates. This technique is off-line but shows the possibility of monitoring proteins using labelled technique coupled with ELISA [130]. Another promising technique published is the use of green fluorescence protein (GFP) reporter in production of economically important recombinant proteins in *Escherichia coli* based host/vectors [131]. The fluorescence of the cell was measured off-line and showed good correlation to the protein production and needs requirement of reporter gene in the production of target protein. Multi-wavelength fluorescence spectroscopy has been used in conjunction with state variable techniques in heterologous protein production by *Pichia pastoris* [132]. Here the correlation of production had significant bearing on the chemometric modeling used especially in the stationary phase as error up to 20% was reported. Apart from biosensing, software sensors for state estimation are other approaches being pursued for measuring protein production. Software sensors were evaluated for estimation of product and substrate rates in an *E. coli* cultivation expressing a recombinant protein [133]. Biomass inputs and HPLC data for determining
substrate and product rates were used in the estimation. The challenges in estimating in real time or near real time persists as there was delay in HPLC and requirements of maintenance. It is notable that integration of hardware sensors into the architecture resulted in better estimation and control [134]. Flow ELISA is another tool which has potential for online determination of protein concentration. **Paper IX** describes a versatile automated system and its application for determination of IgG in a continuous set up is discussed. The architecture of a versatile flow system is shown in Figure 14. The flow immunosensor showed very good linearity from 5 μg/ml to 400 μg/ml with high repeatability and S.D values of less than 2.5% and good stability (Figure 15-19).

![User Interface and Visualization](image)

**Figure 14 Software architecture of versatile flow system**

**Figure 15 Automated Flow ELISA configuration**

The protein being quantified could be intracellular requiring online lysis steps. Further, for monitoring protein production the concentration range is entirely different from that when analyzing trace impurities. Different sensitivities and dynamic range along with different transducers need to be employed. Considering these aspects, a versatile
platform for bioanalysis involving different flow configuration has been described in Paper IX wherein it is possible to support all the common schemes of flow analysis employed. Its usefulness was illustrated with an immunoassay of human IgG as a model analyte (Figure 15). The immunoreactor column could be used for over 150 IgG samples with good stability. Complete control of amount of sampling, flow rates, dilution and the frequency of sampling meant that flexible assay protocol could be employed to suit the dynamic range of analysis with no changes to the immunoreactor column. Automation also enabled dynamic calibration of sensor at desired frequency. Further data analysis plays vital role in terms of handling measurements and monitoring the regeneration of the immunoreactor column to evaluate its performance. It was noted that the time taken for ELISA is reduced from hours to few minutes and the reproducibility is enhanced as the interaction times as well as washing steps are very reproducible.

Figure 16 Schematic of versatile automated flow system for bioanalysis

The software architecture is modular in nature to allow plug and play and to add or modify different features required for monitoring and control. The different modules for the flow system include device drivers which translates the control signals from the computer to a form recognizable by the pump, valve and other hardware, graphical visualization, flow scheduler, hardware configuration, data analysis, calibration and report generation (Figure 16).

To integrate the data into other applications or software, the analysis and the configuration data are stored in compatible formats and use of technologies such as ActiveX controls for exploiting features of other applications for data processing and analysis. For example, invoking a spreadsheet application from measurement/control software and using the data analysis capabilities in MS Excel. The calibration curve and the repeatability of analysis are depicted in Figure 17 and 18.
The sensor was also subjected to a model first order kinetics of target protein concentration and the results from the sensor agreed well with the estimated concentration. The system being very flexible, different flow configurations are easily setup and the dynamic range of analysis can be adapted by varying different flow conditions for measurement. The system is useful for online analysis and control of bioprocesses employing biosensor. It has comprehensive control over the operating conditions and diagnostics is built in for monitoring the assay. The capture column can
be easily changed to analyze different target protein and different detection schemes can be employed. It is also flexible in terms of choosing the number and type of pumps, valves or detectors to be employed. This provides options in choosing flow configurations for any process monitoring to choose any of the popular detection schemes in combination with an enzyme or affinity bioreactor column. Moreover there could be a need for enzymatic pretreatment and ultrasound to release intracellular enzymes from cells for analysis. The control of the operation of a sonicator could be useful for online sample handling and FIA analysis. Furthermore, with this control over these devices, precise events can be set up for their management providing operational flexibility and approach for diagnostics. Additionally, the system can interface with other measurements and control which is vital for integrated control architecture for a bioprocess.

Immunosensing is a preferred technique for detection of toxic compounds and contaminants alike. Mainly organophosphates, carbamates are used as pesticides to control infestation in crops and increase productivity. Organochlorides have been replaced by organophosphates and carbamates in recent years. These pesticides are acutely toxic and need to be monitored at sensitive levels for reasons of food safety and environmental regulations. There are several methods for sensitive detection of pesticides in food and water samples. Many of them are based on chromatographic techniques involving gas or liquid chromatography, GC-Mass spectrometry, ELISA etc. They are accurate and sensitive. However, they are laboratory based and need expensive instrumentation. Moreover it involves considerable sample preparation, skill and quite laborious.

Rapid, sensitive and selective detection of organophosphates are possible using biosensing principles [133, 134]. Enzyme inhibition sensors are used for analyzing organophosphates. For example acetyl cholinesterase (AChE) enzyme is inhibited by many of the organophosphates and can be used as a basis for detection. However, the inhibition based techniques involve multiple steps of measuring the activity of enzyme - without inhibition, with inhibition and regeneration of enzyme. It is a time consuming process and the regeneration may not be complete requiring careful standardization [11]. Other options include combination of enzymes like choline oxidase with AChE to lower the operating potential of the electrode. Hydrolysis using organophosphorous hydrolase (OPH) have also been employed for OP pesticides. But, OPH has broad specificity for all OP pesticides [13 and 136]. Acid phosphatase sensors are used in conjunction with glucose oxidase enzyme and standardization of these sensors for having optimum conditions for both enzymes is challenging [137-139]. Optical detection provides good sensitivity e.g. high sensitivity sensor for detection up to 50 ppb of paraxon has been reported with a chemiluminescent biosensor [140]. Alkaline
phosphatase with biotinylated conjugated copolymer, poly(3-undecylthiophene-co-3-thiophencarboxyldehyde-biotin-LC-hydrozone) on a glass surface is used in a chemiluminescent strategy for OP detection. The OP pesticide inhibits the enzyme activity which is detected by the decrease in chemiluminescence [140].

Conventional enzyme linked immunosorbent method (ELISA) is time consuming and laborious as it has lot of incubation and washing steps and automation is important [30, 123, 141, Paper IV, IX]. There is interest in flow immunosensors and reliable detection of analytes in the field of food analysis, environment and bioprocess monitoring [29, 121, 141-143, Paper IV, IX]. An automated regenerable microarray based on immunoassay has been demonstrated for analysis of antibiotics in milk and aflatoxins in food and beverage samples using sensitive CCD imaging of chemiluminescence and enzyme labelling [144].

In Paper IV and V flow immunoassays with high sensitivity were setup with the biorecognition element in a packed reactor column for continuous analysis with high sensitivity and optical detection. A microcontroller controls the different flows, sample, reagents and the time between the equilibration, wash and regenerate steps. Additionally, the sampling amount, reaction times and flow configuration can be flexibly varied to account for the different range of concentrations expected and to optimize the sensor performance. Further with the data processing ability, baseline compensation and online calibration, concentration of analyte can be quantified in real time. The concept of flow ELISA helps in continuous monitoring and reuse of the immobilized antibody column. Moreover, with the developed system, different formats of immunoassay can be easily accommodated without any changes to the flow setup. Another advantage is the configuration uses only solenoid valves without the expensive injection valves.

In Paper V immuno-chemical reaction was exploited for detection of methyl parathion down to 10 ppt based on CCD imaging. Image enhancement technique combined with promotional influence of K₃Fe(CN)₆ was used for detection of the pesticide. The different conditions like amount of antibody, luminol, urea H₂O₂, K₃Fe(CN)₆, reaction time were optimized to achieve the detection (Figure 20). The results showed good linearity for detection of methyl parathion (Figure 21) and compared well with ELISA method used as a reference. However, with ELISA the lower limit of detection was only up to 2.2 ppb.

Automation based systems described in Paper IV, and IX offer convenient platforms for development of integrated biosensing systems for bioreactors. These devices can be integrated to industrial bioreactors as well as laboratory fermentors with different biosensing configurations. In effect it is useful for process monitoring as well as process development studies. Miniaturized versions are useful for environmental applications.
Luminol + urea + H$_2$O$_2$ + K$_3$Fe(CN)$_6$ + matrix. Distinguishing between sample and control is very poor because the entire reaction takes place before addition of matrix.

Table 3

<table>
<thead>
<tr>
<th>Concentration (ppb)</th>
<th>Absorbance (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.23</td>
</tr>
<tr>
<td>100</td>
<td>0.45</td>
</tr>
<tr>
<td>500</td>
<td>0.67</td>
</tr>
<tr>
<td>1000</td>
<td>0.89</td>
</tr>
<tr>
<td>5000</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Fig. 3.

Selective detection of pesticides, particularly methyl parathion, in water. With the significant advantage of higher sensitivity compared to the plate ELISA method, which could detect only up to 3.3 ppb, the FIA has been successfully carried out and it was possible to achieve 13 use cycles with very good dissociation. With FIA automation, the analyst is not faced with the problem of accuracy or absorbance. A further significant advantage is the absorbance of the eluent after dissociation. With either increase or decrease in incubation time, the gradient was observed in the range 50–500 ppb, with an absorbance of 0.045 in the range 50–500 ppb. A good linearity was obtained as the optimum antibody concentration for the different dilutions of MP–HRP conjugate (1/1K, 1/2K and 1/5K) was found to be optimum and was employed for further experimentation.

Table 4

<table>
<thead>
<tr>
<th>Antibody Concentration</th>
<th>Absorbance (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.045</td>
<td>0.01</td>
</tr>
<tr>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td>0.15</td>
<td>0.03</td>
</tr>
<tr>
<td>0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>0.25</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Fig. 5. Calibration graph for the analysis of methyl parathion using flow ELISA. A good linearity and high repeatability with a standard deviation ranging from 0.01 to 0.045 in the range 50–500 ppb were observed. The experiment was repeated several times.

Fig. 6. Image intensity as a function of methyl parathion.

y = 0.0269x + 0.2277

RF = 0.9780

Fig. 20 Auto analyzer and chemiluminescence system for methyl parathion detection.

Fig. 21 Linearity of detection methyl parathion by absorbance and chemiluminescence detection.

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10. Conclusions and Future Perspectives

This thesis summarizes my research work and gives an overview over the ongoing development and trends in the area of automated bioprocess monitoring and control using flow injection assay and biosensors. The complex dynamics of bioprocesses require automated tools for having a greater insight into the process in real time. In fact, with the PAT initiative, new directions are being sought for integrated monitoring and control. Identification of phase states in bioprocesses and predicting the direction of biosynthesis requires knowledge of biochemical as well as physical and chemical variables. Biosensors offer a rapid and sensitive method for process monitoring and control of both low and high molecular weight compounds in bioprocesses. With robust and yet flexible automation they provide a powerful tool to complement the other existing methods. No single analytical tool satisfies all the challenging requirements of bioprocess monitoring and control that ranges from microbial cell cultivation to mammalian cell processes. Advances in other techniques like software sensors, FTIR spectroscopy, 2D fluorescence, and di-electric spectroscopy are promising and have other pros and cons for analyte measurements. With the advent of PAT regime, focus on improvements in bioprocess monitoring methods is evident with the increased number of publications in this area [8, 9, 41, 42, 48, 64, 58, 70, 133, and 145-148]. Biosensors provide a proven basis for building knowledgebase of bioprocesses. Software sensors are restricted to specific applications for which they are employed and need significant modifications for other applications. Model based predictive control is increasingly being sharpened and research indicates that hardware sensors for online information on biochemical parameters is indispensable to complement the model based approach. Biosensors provide specific and more authentic information to build robust process models and approaches towards integrated control. Furthermore, work needs to be focused for integration of such sensing systems with other measurements at production level to quantify benefits on process improvement, productivity, and quality. This would provide new impetus to routinely adapt such technologies in bioprocesses and promote a truly integrated control environment.

For biosensor based systems electrochemical and optical biosensors have a distinct advantage for process monitoring. It has been demonstrated that automated flow system, with intelligent data processing provide means for continuous online measurement while accommodating entire range of analysis and compensating inherent loss of activity of biosensor-based detection systems. The automation system is flexible to accommodate different types of biosensors and their applicability with enzyme- and immuno-based sensing with electrochemical, optical detection have been illustrated.
Automation of laboratory based methods like ELISA extends its application for process monitoring and sensitive detection of toxic compounds with reliable operation. Novel detection methodology based on immunosensing with chemiluminescence provides very sensitive method for detection of pesticide residues. Use of IgY antibody for such sensitive detection provides alternative choices for materials for sensor design. Enzyme based biosensors are potential candidates for quality estimation in beverages like tea and coffee and other caffeine products in pharmaceutical production. In addition these techniques are convenient for miniaturization and hence suitable for field applications. Operational stability of such sensor systems can be significantly increased by utilizing protein based stabilizing agents.

Novel sensing elements like aptamers provide new and better approaches for detecting macromolecules due to their relative ease of production and possibility of detection by established electrochemical and optical methods. Advances in microfluidics, nanomaterials and supporting fabrication facilities make it possible to miniaturize biosensing devices to develop DNA microarrays and protein biochips with electrochemical and optical detection. Much research is essential to integrate such devices for real time process information. Presently, work on biochips for analysis of genome and proteome using DNA- and protein-arrays is mainly restricted to strain optimization and process development. Online application of these techniques would have immense significance for bioprocess monitoring and control. An equally important area where biosensors would play an increasingly important role is the analysis of toxic compounds and quality assurance programs during production. Miniaturization of these sensors would make it possible for simultaneous detection of multiple analytes and development of hand held tools for field applications in food and environmental areas.
11. References


An automated flow injection analysis system for on-line monitoring of glucose and L-lactate during lactic acid fermentation in a recycle bioreactor

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Summary

The study concerns on-line sequential analysis of glucose and L-lactate during lactic acid fermentation using a flow injection analysis (FIA) system. Enzyme electrodes containing immobilized glucose oxidase and L-lactate oxidase were used with an amperometric detection system. A 12-bit data acquisition card with 16 analog input channels and 8 digital output channels was used. The software for data acquisition was developed using Visual C++, and was devised for sampling every hour for sequential analyses of lactate and glucose. The detection range was found to be 2–100 g l$^{-1}$ for glucose and 1–60 g l$^{-1}$ for L-lactate using the biosensors. This FIA system was used for monitoring glucose utilization and L-lactate production by immobilized cells of Lactobacillus casei subsp. rhamnosus during a lactic acid fermentation process in a recycle batch reactor. After 13 h of fermentation, complete sugar utilization and maximal L-lactate production was observed. A good agreement was observed between analysis data obtained using the biosensors and data from standard analyses of reducing sugar and L-lactate. The biosensors exhibited excellent stability during continuous operation for at least 45 days.

Introduction

Biotechnological processes are dynamic, involving continuous changes in the physico-chemical conditions of the medium, which in turn influence the functioning of the biological catalyst and hence the productivity. On-line monitoring of the critical metabolites including substrate and product is desirable in order to facilitate rapid optimization and also to control a bioprocess. This has led to a tremendous interest and development in the field of biosensors. Although the literature on various biosensors is extensive, examples of their use for on-line monitoring are rather limited. To make the use of biosensors realistic for on-line monitoring, their development should be studied as an integral part of the bioprocess as a whole. The critical issues that need to be considered while setting up a system are the sensitivity of the biological component in a biosensor to contamination and operational stability. Hence, the use of in situ biosensors for on-line bioprocess monitoring is restricted, even though in situ analysis by an enzyme electrode, placed in an autoclavable housing, has been demonstrated (Cleland & Enfors 1984a).

The analytical technique that has shown the most promise for monitoring of bioprocesses using the biosensors is flow injection analysis (FIA) (Schepers et al. 1996; Raricka & Hansen 1985). The technique has the advantages of quick analysis and low incidence of infection to the bioreactor. The technique is also easily adapted to measurement of two or more components co-existing in the bioreactors (Scheller & Karsten 1983; Yao & Sato 1985; Brand et al. 1991; Renneberg et al. 1991; Dremel et al. 1992; Chen & Matsumoto 1995; Kyrolainen et al. 1995). Glucose and lactate have been by far the most popular analytes subjected to determination by biosensors, which have been based on chemiluminescence (Nielsen et al. 1990), NADH fluorescence (Vanderpol et al. 1994), calorimetric (Mandeni et al. 1985; Chen & Matsumoto 1995), amperometric (Yao & Sato 1985; Wentz & Schügerl 1992; Kyrolinen et al. 1995), potentiometric (Brand et al. 1991; Renneberg et al. 1991), and optical (Dremel...
et al. 1992) detection modes. Nielsen et al. (1990) have applied multi-channel FIA to analyse even protein and biomass besides sugars and lactic acid during lactic acid fermentation.

In this report, we present a study on integration of FIA using amperometric biosensors to a lactic acid fermentation process for on-line analysis of glucose and lactate. Lactic acid has wide applications in the food, chemical and pharmaceutical industries. The microbial production of lactic acid is gaining much interest in recent years because of the need for optically pure lactic acid for certain applications like production of poly(lactic acid) (Datta et al. 1995). Over the years, there have been attempts to improve the fermentation process, which is limited in productivity because of the inhibiting influence of the lactate on cell growth and metabolism. We have earlier developed a process based on immobilized lactic acid bacteria in a recycle batch reactor (Senthuran et al. 1997). The immobilized cells are used repeatedly for sugar conversion to lactate. In order to facilitate the progress of fermentation and to enable repeated fermentations without unnecessary time lag, on-line monitoring of the process was desirable.

Materials and Methods

Materials

The following materials were purchased from Sigma Chemical Co., St. Louis, MO: Lactate oxidase (LOD, from *Pedicoccus* species, 20–40 units/mg (EC number not available), D-glucose oxidase (GOD, type X-S from *Aspergillus niger*, 250 units/mg, EC 1.1.3.4), L-lactate (lithium salt), enzymatic kit for L-lactate measurement, polyethylenimine (PEI, 50% aqueous solution), bovine serum albumin (BSA), and 3,5-dinitrosalicylic acid (DNS).

Cellophane membranes for construction of enzyme electrodes were procured from SpectraPor, Houston, Texas, USA and Gambro AB, Lund, Sweden. The Clark type oxygen electrode of 10 mm diameter was purchased from M/S Century Instrument, Chandigarh, India. Poraver® beads (prepared from recycled glass, 4–8 mm mean diameter) were a kind gift from Dennert Poraver (Postbauer-Heng, Germany). The remaining chemicals were obtained from standard sources and were of analytical grade.

Microorganism and culture medium

*Lactobacillus casei* subsp. *rhamnosus* (DSM 20021) was maintained on MRS-agar (Merck) medium and subcultured fortnightly. The medium used for lactic acid production contained (per liter) 10 g yeast extract, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 1.0 g sodium citrate, 0.005 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.0031 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.002 g FeSO<sub>4</sub>·H<sub>2</sub>O, 0.005 g ascorbic acid, and 50 g sugar, the latter comprising of 47.5 g glucose and 2.5 g lactose (Senthuran et al. 1997). Sterilization of the medium components was performed by autoclaving at 120 °C for 20 min. Sugar solution was autoclaved separately prior to mixing with rest of the medium.

For the preparation of *L. casei* inoculum and for immobilization of cells to Poraver beads, medium based on cheese whey, after protein hydrolysis and supplementation with 2.5 g yeast extract, 0.03 g MnSO<sub>4</sub>·H<sub>2</sub>O, and lactose to give a final sugar concentration of 40 g l<sup>−1</sup> (Senthuran et al. 1997), was used.

**Immobilization of *L. casei* and lactic acid production in recycle batch reactor**

The recycle batch reactor used for the present study has been described earlier (Senthuran et al. 1997). It consisted of a column connected via both ends through tubing to a stirred tank reactor (1 l). The column was fully packed with Poraver beads (350 ml) precoated with PEI, by suspending in 2% (w/v) polymer solution as described earlier (Senthuran et al. 1997). To start up the system, the stirred tank reactor was filled with the whey medium (700 ml), inoculated with 5% (v/v) of overnight-grown culture of *L. casei*, and the cells allowed to grow in the reactor for 8 h, using a stirrer speed of 250 rev min<sup>−1</sup>, until a cell density of 1.7 g l<sup>−1</sup> was reached. The pH value was controlled in the reactor at 6 by titration with 6 M NH<sub>4</sub>OH. Thereafter, the medium was continuously circulated through the column and then back to the reactor until the sugar was completely utilized. The reactor was then emptied, washed by circulating 700 ml of 0.9% NaCl solution, and later filled with 800 ml of fresh fermentation medium containing 50 g sugar l<sup>−1</sup>. The medium recirculation was initiated immediately and the fermentation followed by withdrawing samples at definite time intervals for analyses of reducing sugar and lactic acid.

**Construction of enzyme electrode**

Enzyme electrodes for analyses of lactate and glucose were based on lactate oxidase (LOD) and glucose oxidase (GOD), respectively, catalysing the following reactions:

L-Lactate + O<sub>2</sub> ⇄<sup>LOD</sup> Pyruvate + H<sub>2</sub>O<sub>2</sub>

Glucose + O<sub>2</sub> + H<sub>2</sub>O ⇄<sup>GOD</sup> Gluconate + H<sub>2</sub>O<sub>2</sub>.

The oxygen consumption, at an applied potential of (~) 650 mV is accompanied by acceptance of electrons resulting in the following cathodic amperometric reaction:

O<sub>2</sub> + 2H<sub>2</sub>O + 4e<sup>−</sup> → 4OH<sup>−</sup>

which results in a decrease in current proportional to the substrate concentration in the sample. The current was
converted into voltage and scaled to suit the requirements of the successive data acquisition system.

The enzymes were immobilized to the cellophane membrane by cross-linking with glutaraldehyde in the presence of gelatin. To the membrane (1 cm diameter) on nylon mesh of Monodur (No. 63, AB Derma, Sweden) as a supporting matrix was added glucose oxidase, 8 IU; or lactate oxidase, 20 U, and 60 μl of 10% (w/v) gelatin solution in phosphate buffer, pH 7.0. After keeping for 5 min at 4 °C, immobilization of enzyme was initiated by addition of 25 μl of 25% (v/v) glutaraldehyde solution, followed by incubation at room temperature for 1 h. The membrane was then washed three times with 1 ml phosphate buffer. This enzyme-loaded membrane was sandwiched between a SpectraPor membrane (MW cut-off of 12,000–14,000 and thickness of 25 μm) as the outer membrane and Teflon as the inner membrane for better oxygen permeability. The sandwich enzyme-membrane system was attached to the Clark electrode by means of an ‘O’ ring.

**Construction of FIA set-up for off-line analysis**

The FIA set-up for off-line analysis of lactate and glucose is shown in Figure 1. Two peristaltic pumps, P1 and P2 (Gilson Minipuls-3, France, and Alitea-XV, Sweden) were used to draw the sample and buffer, respectively. The valves used were of electric solenoid type (Neptune Research, USA, DC 24 V), and were operated by means of a switch connected to a 24 V DC power supply (Mascot, Norway). The ON time of the valve V1 determined the amount of sample taken for analysis, which was mixed with the buffer carrier stream. A dialyser with cellophane membrane (8000 MWCO, SpectraPor, USA) was used to dialyse the sample, against the same buffer used above, so as to ensure the elimination of cells and suspended particles before it reached the electrodes. The channel dimensions of the dialyser unit was 33.69 cm long with a cross-section of 1.8 × 0.8 mm, while dialysis unit was 4 cm in diameter. The tubing carrying the buffer and the sample to the sensors had an i.d. of 0.15 mm. Valve V2...
directed the dialysed sample either to the L-lactate sensor (V2 ON) or to the glucose sensor (V2 OFF).

Construction of FIA set-up for on-line analysis

Figure 2 shows the FIA set-up used for on-line analysis of the broth from the bioreactor. The configuration is basically similar to that of the off-line set-up, the distinguishing feature being the injection valve, V1 (24 V DC) having a loop of 30 cm length and 0.15 cm inner diameter (volume 530 µl), which in association with peristaltic pump, P1 provided precise sampling of the fermentation broth. The electrically operated injection valve consisted of 9 ports (Figure 3). In the LOAD position of the valve, the sample from the bioreactor passes through the loop, thereby filling it and then into the waste. In this position, the buffer bypasses the sample loop and the electrode will receive only the buffer. In the INJECT position, the known volume of fermentation sample in the loop will reach the electrode through the dialyser unit.

The sample loop of the injection valve was filled with the sample from the fermentor, after taking into consideration, the dead volume from point A to B in the flow injection system (Figure 2). The carrier buffer was pumped at a rate of 1.2 ml min\(^{-1}\) by pump P2. The pumps and the valves could be operated at precise intervals through the computer by means of driving circuitry. The sample stream after dialysis was passed through the three-way solenoid valve to reach enzyme electrodes for the analysis of L-lactate and glucose, sequentially.

Detector system

Clark type electrodes connected to a home-made potentiostat were employed for amperometric detection. A voltage of \(-0.650\) V was applied to the gold electrode with reference to the silver electrode. High gain FET OP27 amplifiers were used for amplification of signal coming from the electrode. The amplified signals were conditioned for on-line data acquisition and monitoring.

Data acquisition

A Pentium PC, 166 MHz with 64 MB RAM was used, along with a 12-bit data acquisition board having 16 analog input channel and 8 digital output channel (AT MIO-16E; National Instruments, USA) for data acquisition and control. The software for data acquisition and automation was developed using Visual C++. The data was read by direct memory access making the acquisition fast and reliable. The software was developed to operate in MS Windows environment, and was programmed such that the sampling is done every hour for the sequential analysis of L-lactate and glucose. For
data acquisition during sampling, the signals from the electrodes were scanned at regular intervals of 1 s. The valves were steered using the computer by means of a home-made driving circuitry consisting of optocouplers, PVA 3354.

Reference analytical methods

The concentration of total reducing sugar was measured by a slight modification of the procedure using DNS reagent (Miller 1954). One ml of sample was mixed with 1 ml of DNS reagent in a test-tube and heated for 5 min in a boiling water bath. After cooling, 4 ml of distilled water was added and the absorbance measured at 550 nm.

The concentration of L-lactate was estimated using an enzyme kit (Sigma), in which H₂O₂ liberated by the oxidation of lactate by lactate oxidase is used by peroxidase for oxidative condensation of chromogen precursors to produce a coloured dye with an absorption maximum at 540 nm.

Results and Discussion

Optimizing the enzyme electrode for glucose and lactate analyses

A pre-requisite for the biosensor system was to be able to cover measurement of a wide concentration range of the sugar and lactate present in the bioreactor, which vary between nil and several grams per litre during the course of lactic acid fermentation. A measuring range of 0–30 g glucose/l and 0–20 g lactate/l by enzyme (glucose oxidase and lactate monoxygenase) cartridge flow injection system was earlier reported by Jürgens et al. (1995). However, during in situ glucose monitoring by an enzyme sensor with a Clark electrode principle, the linear range of analysis was extended by varying the buffer flow in the dialyser unit (Cleland & Enfors 1984). Due to the high specificity of the Clark electrode combined with a lower response time and suitability for detection of a wider range of target analyte, it was chosen for constructing the enzyme electrode. The unstable baseline usually associated with Clark electrode was compensated by having differential measurements.

To adapt the analyses to the desired concentration range during the lactic acid fermentation, the flow injection analysis system was optimized by off-line measurements. Cellophane membranes of varying thickness, 10 μm (‘AKZO’, Gambro AB Sweden) and 25 μm (SpectraPor, Spectrum Medical, USA) were used for enzyme immobilization. The sampling time was kept at 30 s. With the 10 μm membrane, the response for glucose was linear between 1 and 30 g l⁻¹ (regression coefficient, $R^2 = 0.961$), whereas the 25 μm membrane showed a linear response range from 2 to 100 g l⁻¹ ($y = 0.0779x + 0.0062; R^2 = 0.999$). For further study the SpectraPor membrane was used because of the wider detection range (2–100 g l⁻¹) including higher concentrations of sugar normally used in the fermentation processes. Observations to a similar effect were made during L-lactate analysis. With 10 U lactate oxidase used for immobilization to the 10 μm membrane the linearity range was 0.5–10 g l⁻¹ ($0.2229x + 0.3254; R^2 = 0.998$) as compared to the linearity range of 0.5–25 g l⁻¹ (0.3256x + 0.3764; $R^2$ of 0.998) with the 25 μm membrane. Thickness of the membrane plays an important role in modulating the differential barrier for the analyte, the thinner membrane displaying higher permeability for glucose and lactate, as a result of which only a limited concentration range of the compounds was accommodated.

As the desired range of detection of L-lactate concentration (1–50 g l⁻¹) was not achieved by changing the thickness of the membranes alone, the possibility of increasing the range of L-lactate detection above 20 g l⁻¹ was investigated using a different concentration of L-lactate oxidase enzyme for immobilization to the membrane. Increase in L-lactate oxidase enzyme amount from 10 to 20 units for immobilization yielded good linearity in the measurement range of 1–60 g l⁻¹ of L-lactate (0.0567x + 0.0824; $R^2$ of 0.994).

Off-line analysis of glucose and lactate in fermentation broth

Samples were withdrawn aseptically every hour during fermentation for analysis using the FIA system. Glucose
and L-lactate concentrations were also determined by DNS and the enzyme kit method, respectively. We have been using the DNS method for sugar estimation to follow the total sugar utilization during the lactic acid fermentations which were often performed in a medium composed of a mixture of lactose and glucose (Senthuran et al. 1997, 1999). Lactose was incorporated as a minor fraction of the total sugar amount in the fermentation medium, as it was shown to be essential for achieving efficient lactate fermentation with complete sugar utilization (Senthuran et al. 1997, 1999). Figure 4a shows that results obtained by the different analytical methods showed a good correlation. It must be noted that due to the presence of lactose in the medium which is not read by the biosensor, the sugar concentrations measured in the initial phase of the fermentation are slightly lower than that provided by the DNS method. Towards the end of fermentation, both methods showed complete substrate utilization. The slight variation in the values obtained by the biosensor and the reference analytical method could be due to the differences in time elapsed from sampling up to analysing the sample. The reference analyses were performed after immediate removal of cells by centrifugation, however there was a time lag from the point of sampling to its analysis by biosensors.

For on-line monitoring of the samples, modification of FIA was desired. Having an electrically operated injection valve, and controlling pump P1, precise volume intake of the sample, as and when required, into the FIA was ensured. During sampling, care was taken to discard the old sample retained in the tubing, due to the dead volume of flow line. This was done by switching on pump P1 for a definite period with valve V1 in LOAD position. The assay was operated in a sequential mode in the sense that first one enzyme electrode read its substrate followed by the second one. L-lactate was analysed prior to glucose. The phosphate buffer flow rate was maintained at 1.2 ml min⁻¹. The calibration curves for analysis of L-lactate \((0.0428x + 0.0594; R^2 = 0.9975)\) and glucose \((y = 0.0268x + 0.3413; R^2 = 0.9984)\) using the on-line approach showed good linearity. On-line monitoring of the fermentation also showed a reasonable agreement with the reference measurements although the latter measurements were slightly higher during the first 6 h. (Figure 4b) At zero time the sugar concentration in the bioreactor determined by the DNS method was 51 g l⁻¹, while the biosensor gave a value of 47.9 g l⁻¹ for glucose. According to both methods, there was no sugar left at the end of fermentation. L-lactate concentration increased from 4.8 g l⁻¹ at 1 h to 45.4 g l⁻¹ when the fermentation ended after 13 h.

One sample analysis took 3 min and washing time was kept at 2.0–2.5 min. More frequent analyses are fully possible, however when used in a stable process as the one studied, an interval of 1 h was considered sufficient. The enzyme electrodes exhibited very good operational stability. The response of the electrodes was examined in between on-line sample analyses by introducing the respective standard solutions. No change in response was noted. The electrodes maintained the same linear range even after continuous operation for analysis of about 300 samples over a period of 45 days at room temperature.

Concluding remarks

The FIA set-up for sequential determination of glucose and L-lactate using biosensors has given satisfactory results with good correlation with the results obtained using other standard methods of analysis. The biosensors could effectively cover the analyses of a wide range of concentration of the analytes. With some modification of the flow lines, simultaneous analysis of L-lactate and glucose is possible. The system has the flexibility to choose the target analyte, either glucose or L-lactate or both depending upon the need for analysis. Also, the system has the facility to store and process data for quantification. Since the flow injection system is automated for data acquisition and control, it is possible to...
incorporate calibration routines in the software. This will enable daily calibration of the biosensor and enhance the useful period of the sensor. On-line monitoring of any fermentation process using suitable transducers is also possible using this FIA set-up.

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References


Paper II
Enhancement of operational stability of an enzyme biosensor for glucose and sucrose using protein based stabilizing agents

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Abstract

With the incorporation of lysozyme during the immobilization step, considerable enhancement of the operational stability of a biosensor has been demonstrated in the case of an immobilized single enzyme (glucose oxidase) system for glucose and multienzyme (invertase, mutarotase and glucose oxidase) system for sucrose. Thus an increased number of repeated analyses of 750 samples during 230 days for glucose and 400 samples during 40 days of operation for sucrose have been achieved. The increased operational stability of immobilized single and multienzyme system, will improve the operating cost effectiveness of the biosensor.

Keywords: Biosensor; Immobilized enzymes; Operational stability; Protein based stabilizing agents; Detachable membrane unit

1. Introduction

Immobilized enzyme based biosensors have been widely used for analyses in food and fermentation industries (Coulet and Bertrand, 1979; Scheller and Karsten, 1983; Sheper, 1992), in environmental monitoring (Schmidt and Scheller, 1989) and in clinical diagnosis (Mason and Townshend, 1984). Biosensors for glucose and sucrose have been widely used for food and fermentation sample analysis (Danielsson, 1994; Hundech et al., 1992; Scheller and Renniberg, 1983; Xu et al., 1989; Matsumoto et al., 1988). An important consideration in the practical application of biosensors is the operational life of the sensing element particularly while monitoring the food and fermentation analysis where the substrate concentrations are high. When immobilized enzymes are used for this purpose, the activity loss due to denaturation and deactivation of the enzyme diminishes the life of the sensor. Therefore techniques to enhance the storage and operational stability of the enzyme electrode are important in the application of electrochemical biosensors. Attempts to stabilize enzymes reported in literature include the use of cationic polyelectrolytes (Gavalas and Channiotakis, 2000), polyelectrolytes and sugar alcohols (Gibson et al., 1992), immobilization of enzyme and polyelectrolyte complex on CPG (Appleton et al., 1997) and immobilization of enzyme on carbon paste electrodes in the presence of various additives (Lutz et al., 1995).

One of the suitable enzyme immobilization methods for biosensor applications is crosslinking by using glutaraldehyde. Glutaraldehyde being a strong bifunctional reagent, modifies the enzyme drastically, leads to conformational changes and loss of activity (Broun, 1976). This deleterious effect can be minimized by using inert proteins like BSA, gelatin, thrombin and lysine. These proteins avoid excess intramolecular crosslinkages within the enzyme and enhance the intermolecular linkages between the enzyme and inert proteins (Brount et al., 1973). While it is known that inert proteins can act as enzyme stabilizing agents, its application has been restricted to BSA and gelatin. In view of the reported observations that complimentary surface protein (Chang and Mahoney, 1995) and durability of carrier protein (Gabel et al., 1970; Gabel, 1973) play an important role in stabilization of enzymes, it is quite possible that stable proteins other than BSA and gelatin...
showing better complimentarity with the enzyme provide a better stability of free as well as immobilized enzyme preparations. Stabilization of desired enzyme can be achieved by using certain proteins, which may be catalytically active or inactive. In this paper we refer to them as protein based stabilizing agents (PBSA). However, one should keep in mind, that if the PBSA is an enzyme, its products should not interfere with the biochemical reaction of the desired enzyme electrode. In our laboratory we have constructed a batch biosensor for glucose using immobilized GOD and for sucrose using immobilized multienzyme system (invertase, mutarotase and glucose oxidase) and tested for repeated use. In an attempt to enhance the operating stability of the biosensor, we have found that incorporation of lysozyme as PBSA can achieve this objective substantially, and the results are reported in this paper.

2. Materials and methods

2.1. Materials

Glucose oxidase (E.C. 1.1.3.4.) from Aspergillus niger (specific activity—180 IU/mg), invertase (E.C. 3.2.1.26) from yeast (specific activity—400 IU/mg), mutarotase (E.C. 5.1.3.3.) from hog kidney (specific activity—2500 IU/mg), lysozyme, BSA, gelatin and glutaraldehyde were procured from M/s sigma, USA, the cellophane membrane molecular weight cut-off 6000 from Spectra/por, USA and oxygen permeable teflon membrane from WTW, Germany. For the biosensor, the Spectra/por membrane was chosen from a large variety of these factors, and the objective was to find PBSA that retains highest activity for a longer period of time. We have worked with several PBSAs and the results are reported in this paper.

2.2. Enzyme immobilization

Glucose oxidase as well as the multienzyme system was immobilized by crosslinking method reported by Wilson (1990), modified slightly. Ten milligrams of glucose oxidase (1800 IU), 1 mg of each invertase (852 IU) and mutarotase (2500 IU) were dissolved in 1 ml of 0.05 M phosphate buffer, pH 6.0. Glutaraldehyde solution (2.5% w/v) was prepared by appropriate dilution of 70% (w/v) glutaraldehyde. On a 2 x 2 cm cellophane membrane, predetermined concentration of the invertase, mutarotase and GOD and 30 µl (6mg) of the PBSA were placed and mixed thoroughly using a glass rod. Fifty microlitres of 2.5% glutaraldehyde was then added and mixed thoroughly again so that enzyme and the PBSA got distributed uniformly throughout the enzyme membrane. After 1 h, the enzyme membrane was washed three times with 0.05 M phosphate buffer, pH 7.0, to remove the excess glutaraldehyde. For the immobilization of glucose oxidase, instead of using multienzymes, single enzyme was used.

2.3. Enzyme electrode construction

The details of the construction of the enzyme electrode and operation have been reported earlier (Gouda et al., 1997). A detachable membrane unit (DMU) consisting of a teflon membrane and a cellophane membrane with the enzyme layer, held in a sandwich form and secured tightly with an ‘O’ ring fitted on to the electrode surface as reported by Gouda et al. (2001) has been used.

2.4. Operational stability studies

The operational stability studies on the single and multienzyme system immobilized with different PBSAs was carried out at 25 ± 1 °C. The enzyme activity was measured by immersing the enzyme sensing element in a 25 ml glass container having 5 ml buffer, kept agitated continuously with air bubbled through an aquarium pump. After bubbling of air for 2 min (for saturation) the dissolved oxygen meter was set by fixing the dissolved oxygen at 100%. Fifty microlitres of 10% glucose or sucrose solution was now injected and decrease in the % dissolved oxygen at the end of 3 min (time taken to reach steady state) was monitored, which represents the activity of the immobilized enzyme for that concentration of glucose or sucrose. If the first analysis response in % dissolved oxygen is ‘a’ and response at any time on the nth analysis is ‘b’, then % activity retained is calculated as \[ b/a \times 100 \]. In order to quantify the operational stability of several PBSAs incorporated into the immobilized enzyme preparations simultaneously, a DMU was constructed for each PBSA separately. The DMU containing the immobilized enzyme membrane system was then fixed to the electrode and the change in the response in % dissolved oxygen was measured by injecting 50 µl of 10% glucose and 100 µl of 10% sucrose, respectively, for the single and multienzyme membrane system. After analysing about six samples of the same concentration of the sugar, the DMU was now kept in 10 ml of buffer at 27 ± 1 °C. Another DMU containing a different PBSA now replaced the earlier one and the activity was measured. The same procedure was repeated for all the DMUs, every day until the activity fell down to less than 40% of the initial value.

3. Results and discussion

Fig. 1 demonstrates the operational stability of the enzyme electrode for glucose containing GOD immobilized with different PBSAs in 50 mM phosphate buffer,
pH 6.0. Lysozyme was found to be the best for the stabilization of GOD among the PBSAs tested, followed by BSA and gelatin. Immobilization without any additive gave virtually no enzyme activity. Repeated measurements with 30 μl of 10% glucose solution were carried out. For GOD immobilized with lysozyme, it was possible to analyse 750 samples during 230 days of operation at the end of which 50% of the initial activity of the immobilized GOD was retained. With BSA as PBSA it was possible to analyse slightly reduced number of 520 samples during 150 days of operation and with gelatin as PBSA it was possible to analyse only 245 samples during 60 days of operation. Another significant observation from Fig. 1 is that the reproducibility of the biosensor signal for GOD immobilized with BSA is poor. Corresponding behaviour of GOD immobilized with lysozyme as PBSA was very stable.

Fig. 1. Residual activity after repeated glucose (10%) analysis with biosensor using GOD immobilized with different PBSA.

Fig. 2. Residual activity after repeated sucrose (10%) analysis with biosensor using multienzyme preparations with different PBSA.

Fig. 2 demonstrates the operational stability of the sensing element for sucrose containing the multienzyme system immobilized with different PBSAs in 50 mM phosphate buffer, pH 7.0. Lysozyme was found again to be the best among the PBSAs tested, followed by gelatin and BSA. Repeated analysis of 10% sucrose showed that with the multienzyme system immobilized with lysozyme as PBSA it was possible to analyse 400 samples during 40 days of operation at the end of which 50% of the initial activity remained. Gelatin as PBSA enabled the analysis of 230 samples during 30 days of operation and with BSA as PBSA it was possible to analyse only 150 samples during 25 days of operation. Though the stability of multienzyme system increased by incorporating lysozyme during immobilization, when compared with the single enzyme system the stability of the multienzyme system was low. This may be probably...
due to the complexity of the multienzyme system, wherein the inactivation of any one of the enzymes may lead to loss of overall activity. The inactivation of the multienzyme membrane was probably owing to the inactivation of the invertase at pH 7.0. Though the higher thermal stability of soluble invertase at pH 5.0 has been reported (Wang et al., 1996), the significant decrease in thermal stability was observed as the pH increased from 5.0 to 7.0. Since the optimum pH for the multienzyme system immobilized with various PBSAs is in the range of 6.8–7.0, stability of invertase around this pH is crucial for the stabilization of multienzyme system for better usage.

Although, it has been claimed (Xu et al., 1989; Matsumoto et al., 1988; Guilbault and Montalvo, 1969 Mohammad et al., 1987; Peteu et al., 1996, 1998) that higher operational stability of the single and multienzyme systems for the estimation of glucose and sucrose (about 1000 and 500 analyses, respectively) has been obtained, it should be noted that concentrations of substrates employed in these reports for the quantification of the operational stability have been very low (1–5 mM). According to Carr and Bowers (1980) and recent IUPAC recommendations (Thevenot et al., 2001), the concentration of substrate employed is a decisive factor for operational stability of the biosensor, lower substrate concentrations of substrate giving higher operational stability and vice versa. In our studies, much higher substrate concentrations of 555 mM (10%) for glucose and 290 mM (10%) for sucrose have been employed to quantify the operational stability of single and multienzyme systems, respectively, since our investigations have been aimed towards application in food and fermentation samples, wherein higher concentrations of glucose and sucrose solutions are expected in actual use. In view of the considerably increased stability observed by us under these conditions, at the lower substrate concentrations reported in the literature, incorporation of lysozyme as PBSA is expected to give even much higher operational life. Furthermore, we have analysed a minimum of six samples per day to check the day-to-day performance of enzyme membrane.

In this work, we have found that the incorporation of lysozyme during the process of immobilization contributes to the long-term operational stability of the biosensor based on immobilized enzyme for the analysis of glucose and sucrose. Among the PBSAs studied for enhancing the stability of the biosensor for the analysis of glucose (based on GOD) and sucrose (based on invertase, mutarotase and GOD), lysozyme was found to be the best. It may be speculated that this may be due to a complimentarity of surface properties between the desired enzyme and lysozyme and also the ionic interactions involved.

The detachable membrane unit (DMU) used by us for these studies has enabled a convenient method to follow the activity of the immobilized enzyme over a relatively long duration using a number of composite enzyme membrane units, but using a single dissolved oxygen electrode. It has made possible a relatively large number of biosensor operational stability observations in a convenient and economical way. The biosensor technique has been useful here to track the activity of the immobilized enzymes used in this study. This may not be possible in the case of other enzymes. However, the results obtained by us may be useful to throw a better light on the action of inert proteins on enhancing the stability of immobilized enzymes.

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References


Paper III
Short communication

Stabilization of immobilized glucose oxidase against thermal inactivation by silanization for biosensor applications

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Abstract

An important requirement of immobilized enzyme based biosensors is the thermal stability of the enzyme. Studies were carried out to increase thermal stability of glucose oxidase (GOD) for biosensor applications. Immobilization of the enzyme was carried out using glass beads as support and the effect of silane concentration (in the range 1–10%) during the silanization step on the thermal stability of GOD has been investigated. Upon incubation at 70 °C for 3 h, the activity retention with 1% silane was only 23%, which increased with silane concentration to reach a maximum up to 250% of the initial activity with 4% silane. Above this concentration the activity decreased. The increased stability of the enzyme in the presence of high silane concentrations may be attributed to the increase in the surface hydrophobicity of the support. The decrease in the enzyme stability for silane concentrations above 4% was apparently due to the uneven deposition of the silane layer on the glass bead support. Further work on thermal stability above 70 °C was carried out by using 4% silane and it was found that the enzyme was stable up to 75 °C with an increased activity of 180% after 3-h incubation. Although silanization has been used for the modification of the supports for immobilization of enzymes, the use of higher concentrations to stabilize immobilized enzymes is being reported for the first time.

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Keywords: Glucose oxidase; Controlled pore glass; γ-Aminopropyltriethoxysilane; Hydrophobic interactions; Thermal stability; Flow injection analysis; Thermal denaturation; Transition temperature; Half-life

1. Introduction

Thermal stability of enzymes is important for use in immobilized enzyme based biosensors, which have applications in monitoring food and fermentation processes. Glucose oxidase (GOD), is the enzyme used in glucose biosensor and increasing its thermal stability is relevant to its practical application.

Thermal denaturation of GOD is mainly due to the destabilization of ionic and hydrophobic interactions and breakage of the hydrogen bonds, Van der Waal’s forces and ionic interactions which lead to a conformational change in the tertiary structure of the enzyme and thus render it inactive (Tsuge et al., 1975). In the case of GOD, it is known that dimers of GOD contain two disulfide bonds and two free thiol groups. At higher temperatures the disulfide bonds break and misfolding may occur, making the free sulfhydryl groups, which are very sensitive to thiol-disulfide exchange reaction, available to form inter- or intra-native disulfide bonded enzyme molecules that destabilize the enzyme and leads to thermal inactivation of GOD (Tsuge et al., 1975; Ye and Combes, 1989).

Many attempts have been made to stabilize GOD against thermal denaturation such as modification of the enzyme itself (Gouda et al., 2001), immobilization in carbon pastes (Liu and Wang, 1999; Chang et al., 1985), zeolites (Boahong et al., 1997) and modification of the microenvironment (O’Malley and Ulmer, 1973). Hydrophobic interactions are considered as the single most important factor in stabilization of the enzyme structure (Fersht, 1977). Therefore strengthening of these interactions should impart structural rigidity to the enzyme molecules and thus make them more resistant to thermal unfolding. Another popular theory is that thermostable proteins have more electrostatic and hydrogen bonding interactions and these are responsible for the high stability (Chen et al., 2000). Another method of enzyme
stabilization is immobilization on solid supports for repeated use. Previous studies in our laboratory have revealed that immobilization provides enhanced stability against thermal denaturation, variations in pH and other conditions (Gouda et al., 2001, 2002a,b). Available literature on the thermal denaturation of immobilized enzymes is still scanty. Attempts have been reported on increasing the thermal stability of GOD through immobilization on various supports like silica, CPG, chromatographic media (Robinson et al., 1979), activated charcoal, sol–gels, carbon paste (Popescu et al., 1995), cellulose, dextrins, etc. (Rosevear, 1987). Silane has been used for the surface modification of solid supports for the immobilization of enzymes, wherein the non-reactive groups in silane such as alkyls provide hydrophobicity and render the surface positively charged (Slobodianikova et al., 1979). The efficiency of this action is dependent on the surface roughness, concentration and type of silane used (Bhatia et al., 1993; Cras et al., 1999). In the present study, the enhancement of thermal stability of GOD immobilized on four solid supports viz., controlled pore glass (CPG), glass beads, permutit and brick dust using varying silane concentrations during immobilization has been investigated.

2. Materials and methods

2.1. Materials

Glucose oxidase (E.C.1.1.3.4), type II from Aspergillus niger (23.9 U/mg solid) and CPG (PC500-200), 120–200 mesh size, 500 Å were obtained from Sigma Chemical Company, USA; glass beads (0.5–0.75 mm diameter) were obtained from Anwendungstechnik, Basel, Switzerland; brick dust was prepared by grinding burnt bricks and sieving through standard sieves of 72 and 60 mesh size; Permutit was from Howard and William Fine Chemicals, England; γ-aminopropyl triethoxysilane (APTES) was purchased from Fluka Chemicals, USA; sodium cyanoborohydride was from Janssen Chimica, Belgium. All the other reagents of AR grade were purchased from Ranbaxy limited, India. The dissolved oxygen electrode was procured from M/S Century Electronics, Chandigarh, India.

2.2. Methods

2.2.1. Immobilization of GOD on various matrices

Immobilization of GOD was done on brick dust, CPG, Permutit and glass beads (0.5–0.75 mm diameter). The supports were first cleaned with distilled water, then boiled in concentrated nitric acid and activated as described by Weetal (1976). Activation was done by boiling the supports in 5% nitric acid at 80 °C for 3 h and subsequent washing with distilled water three–four times. Silanization of all the supports was done by evaporative deposition of 1% APTES in acetone. Only in the case of glass beads the silane concentration was varied in the range of 1–10%. The silanized supports were heated at 115 °C overnight in an oven and then treated with 2.5% glutaraldehyde in phosphate buffer for 1 h with and without vacuum. The supports were then thoroughly washed with distilled water and buffer. GOD (1000 unit) dissolved in buffer was mixed with 1 g of the support and kept at room temperature for 3 h with intermittent shaking. Sodium cyanoborohydride (50 mg) was then added to the above solution to reduce the Schiff’s bases formed, kept for 1 h at room temperature and then for 19 h at 4 °C. The immobilized enzyme support was washed with water and buffer four to five times each. Blocking the unoccupied regions of the support was done by adding 0.1 M glycine solution, keeping at room temperature for 1 h and then washing thrice with distilled water and buffer. Sodium azide (0.002%) was added to the buffer to prevent microbial contamination. The immobilized enzyme was stored at 4 °C until further use.

2.2.2. Activity of the immobilized enzyme

The setup used for the determination of the enzyme activity was a flow injection analysis system with an amperometry based biosensor unit reported earlier (Kumar et al., 2001). The system consists of two pumps P1 and P2 for the buffer and sample, respectively. The sample is injected through a valve, which can be controlled either manually or automatically. The sample is then dialyzed against the buffer, which flows at a rate of 0.8 ml/min. The dialyzed sample then passes through the immobilized enzyme column where the biochemical reaction takes place, causing a drop in the dissolved oxygen, which in turn is sensed by the dissolved oxygen electrode in terms of a drop in voltage. Freshly prepared glucose solution (of different concentrations in the range 1–10%) in phosphate buffer (with sodium azide 0.02%, to prevent microbial contamination) was kept for 4 h for mutarotation and was injected at the rate of 200 µl/min for 30 s. The enzyme activity resulted in a continuous drop in the electrode output voltage, to reach a minimum in about 2 min time before rising. The difference between the baseline and the minimum value in the voltage response was plotted against glucose concentration, which showed an excellent linearity.

2.2.3. Thermal stability studies

Thermal stability studies were carried out by heating the immobilized enzyme to the required temperature in a shaking water bath (Julabo, Elsenbahnstrabe, Germany) for different time intervals and loading it into the column. The residual activity was calculated by the formula:

\[ \text{Percentage residual activity (A)} = \frac{b - a}{a} \times 100 \]

where \( a \) is the activity before heat treatment and \( b \) the activity after heat treatment.

Transition temperature (\( T_{\text{m}} \)) is the maximum temperature at which the enzyme retains 50% of its initial activity after 3-h incubation.
Half-life of the enzyme ($t_{1/2}$) is the time at which the enzyme retains 50% of its initial activity and was determined at 70 °C temperature.

For all the enzyme stability experiments the glucose concentration in the sample used was 50 g/l and the effect of temperature (25–75 °C) on the stability of GOD with different concentrations of silane (APTES) was studied. All the experiments were carried out triplicate and the average of the results was reported in the figures.

3. Results and discussion

3.1. Thermal stability on various supports

Thermal stability studies on GOD immobilized on various supports were carried out. Immobilization itself showed an enhancement in the thermal stability of the enzyme and enzyme activity was maximum after incubation at 45 °C for 3 h. However, GOD immobilized on brick dust was inactivated rapidly above 55 °C with only 7.4% activity remaining at 60 °C (Fig. 1). The relative standard deviation in the experiments was in the range of 0.5–1.5%. Therefore, the error bars are not clearly visible in the figure. The transition temperature ($T_m$) was 52 °C, above which the enzyme showed a loss in the activity.

Studies on thermal stability of GOD showed that immobilization on CPG was found to stabilize the enzyme up to 60 °C for 3 h only (Fig. 1). The enzyme immobilized on CPG shows a $T_m$ value of 63 °C with a $t_{1/2}$ of 3.5 h. GOD immobilized on Permutit showed a higher stability up to 70 °C with 78% activity remaining after 3 h of heating (Fig. 1), and a transition temperature ($T_m$) of 71 °C with a half-life of 9 h. Among all the matrices used for the studies, glass beads with 5% silane showed the highest degree of protection to the enzyme with 114% activity remaining after incubation at 70 °C for 3 h (Fig. 1) with a $T_m$ of 72 °C and a $t_{1/2}$ of 16 h at 70 °C. Temperatures beyond an optimum value leads to the thermal denaturation of the enzyme. However, these studies on the effect of silane on stability had to be limited only to glass beads, as higher silane concentrations (above 1%) in case of the other matrices lead to impediment to flow, resulting in high pressure drop in the column and also formation of lumps of the matrix on treatment with glutaraldehyde.

3.2. Effect of silane concentration on the thermal stability of GOD immobilized on glass beads

The effect of silane on the stability of GOD immobilized on glass beads at higher temperatures was studied and it was found that at high silane concentrations the enzyme was stable above 70 °C without any loss of the original activity. GOD immobilized on glass beads with 2% silane was stable at 70 °C with full activity remaining after 3 h (Fig. 2). The relative standard deviation in the experiments was in the range of 0.5–1.5%. Therefore, the error bars are not clearly visible in the figure. In case of 4% silane the activity retained was 250% of the initial value (Fig. 2) and the transition temperature increased to 75 °C with a $t_{1/2}$ of 19 h. The corresponding activities retained after 3 h of heating at 70 °C, drastically reduced, giving values of 60.9 and 76.1% for 6 and 10% silane concentrations, respectively (Fig. 2). However, there was a decrease in the $T_m$ and $t_{1/2}$ values, which were 70, 64 °C and 13, 12 h, respectively. The maximum stability was shown in the presence of 4% silane. One reason for the increased stability of the enzyme at higher silane concentrations (upto 4%) is the presence of higher number of positive charges on the support surface enabled by higher silane levels. These charges will help in the protection of the ionic interactions in a manner similar to the addition of salts such as NaCl, a known ionic interaction stabilizer (Gavalas et al., 1998) in the buffer for the protection of the enzyme against thermal inactivation. A second reason for the increased stability of the enzyme could be the increased hydrophobicity of the support surface at increasing concentration of silane (upto 4%), which protects the enzyme against thermal denaturation (Cras et al., 1999). However, at silane concentrations above 4% the enzyme stability...
decreases, probably due to the inability to have a uniform silane layer on the support surface. This non-uniformity of silane layer on the support has been confirmed experimentally by microscopic measurements of the bead size before and after silanization using a binocular microscope (Leitz Laborlux S) with ocular micrometer attachment under 10× magnification. Glass beads silanized with 4% silane were also found to have the maximum bead size. Silane levels above 4% not only did not confer any advantage but in fact showed a considerably reduced level of residual activity of the immobilized enzyme preparation. Thus, we can conclude that a 4% silane concentration during immobilization is optimum for providing stability to the enzyme at higher temperatures.

An interesting feature of the work is that the glass beads used for immobilization of GOD were found to be superior to CPG in terms of thermal stability, ease of handling and cost.

4. Conclusions

The thermal stability of immobilized GOD on various supports has been studied. Glass beads as the immobilization carrier treated with 4% silane was found to confer maximum thermal stability. The immobilized enzyme was stable at 70°C for 3h and at 75°C for 90min without loss of activity. This indicates that the system can be used in applications at higher temperatures, involving protein immobilization studies and also in biosensors for online monitoring of processes. This can also be used in the preparation of heat sterilizable probes. Glass beads as an economical support for immobilization of enzymes will be suitable for application in packed bed reactors and columns for use in FIA systems. Work is in progress on further enhancement of the thermal and operational stability of enzymes by either modifying the enzyme sugar moieties or immobilizing the enzyme–polyelectrolyte complex on to the solid matrices.

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References


Paper IV
Automated flow enzyme-linked immunosorbent assay (ELISA) system for analysis of methyl parathion

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Abstract
Sensitive detection of pesticides is of utmost importance in environment and food analysis. Immunological methods are widely used to detect pesticides in agricultural and environmental samples wherein antibodies are employed against the target molecules. Accurate diagnosis depends on the affinity and specificity of the antibody preparation used, and high affinity antibodies are essential for the detection of very small amounts of pesticides. Enzyme linked immunosorbent assay (ELISA) coupled with flow injection analysis (FIA) technique provides a very high sensitivity with high throughput of analyses. Automation of this analysis scheme ensures precise detection with high accuracy. The present development aims at providing a user-friendly system for achieving this objective. It employs a 8952 microcontroller for precise flow of reagents, samples, substrate and conjugates used for analysis to be passed through an immobilized antibody column at predetermined time. With the sequence and flow control of buffers used, it also provides the option for reuse of the immobilized antibody column. The system is flexible to accommodate multiple sequences up to a maximum of 99 steps. It is customizable for different flow ELISA applications. It can control up to eight solenoid valves (dc 24 V) and two peristaltic pumps and has one 12 bit analog channel for data acquisition. With the serial interface port, the system provides convenient means for data acquisition into the computer. The system has been successfully tested for immuno analysis of organophosphorous pesticide methyl parathion. © 2005 Elsevier B.V. All rights reserved.

Keywords: ELISA; Flow ELISA; Immunosensor; Methyl parathion; Microcontroller

1. Introduction
The use of integrated methodological approaches may provide more reliable predictive data in the risk assessment of contaminants in future[1]. For the rapid detection of pesticides, which are important environmental pollutants, with minimal manual intervention, automated flow ELISA is an ideal choice. Advanced techniques like HPLC, GC, and spectrophotometry are time consuming and need competent technical support. Ability of methyl parathion with other OP pesticides, to inhibit choline esterases has found application in some analytical techniques such as flow injection [2] and biosensor [3–5] analysis. Flow injection analysis (FIA) system is user friendly and gives appropriate result within a short duration of time. Flow system also allows flexibility as to the type of detection system [6]. Immunoassay techniques for methyl parathion detection appear to have practical advantages of rapidity, accuracy and simplicity over existing conventional instrumental methods [7]. Automation makes routine tasks easier and less cumbersome. Immunoassay of methyl parathion has been reported by using ELISA and fluorescence polarization immunoassay (FPIA) [7,8]. In general, plate ELISA technique is laborious and requires specific skills. Though the FPIA technique reported is rapid, the sensitivity is low (15 ng/ml). Parathion detection by amperometric sensor has been reported [9] using multi-walled carbon nanotube/nafion electrode with HPLC technique. Recently [10] it has been reported that thin films of molecular imprinted sol–gels can also be used for recognition of parathion using quartz crystal microbalance. Chromatographic techniques for detection and quantification of organophospho-
rous pesticides has been reported [11] wherein capillary chromatography with nitrogen-phosphorous detector is used and the limit of quantification is 5 ppb. FIA technique has been reportedly employed for detection of chlorinated pesticides [12] wherein an immobilized antigen column is used wherein it takes 45 min in indirect ELISA and limit of detection is 1 nM. The present study reports the development of an automated analyzer for immunoassay based on flow ELISA, using IgY antibody immobilized on a solid surface matrix [13], that has been applied for the analysis of organophosphorous pesticide-methyl parathion (MP). The device is based on the principles of FIA and immunoassay wherein a glass capillary column filled with antibody against the pesticide being analyzed is employed. The use of solenoid valves incorporated into a flow system is a recent approach, which permits additional analytical features of flow analysis, namely, reproducibility, possibility of automation and reduction of sample and reagent consumption. However this strategy has been scarcely exploited to date [14,15]. Automated analysis using chemiluminescence has also been reported for the analysis of aldicarb [16]. Automated analysis using expanded bed immunoreactor has also been reported for analysis of nisin [17]. In the present work, FIA has been used to carry out immunoassay in an automated mode, for the detection of methyl parathion. Analyses time of less than 45 min could be achieved, using sample volumes of less than 100 μl. The assay was sensitive in the range of 500 ppb down to 50 ppt. This FIA-System could be useful for monitoring pesticides in industrial wastes, agriculture, water resources, food samples, etc. The automated assay showed very good correlation with the plate ELISA method. The study employs IgY antibodies for immunoassay of methyl parathion, which provides higher antibody yields and is more economical. Further, the sensitivity of detection was found to be excellent. The immobilized antibody column could be repeatedly used for 13 cycles with 95% dissociation of the antigen being achieved. Studies on the dissociation of the antigen from antibodies in the antibody column have been reported earlier [18].

2. Experimental

The automated system for flow ELISA was developed around an Atmel 8952 microcontroller and shown in Fig. 1. The Atmel 8952 is a high performance 8 bit CMOS microcontroller with 8kB of flash programmable, erasable programme memory. It has 256 bytes of RAM, 32 I/O line and three 16 bit timers/counters, which are used for configuring the autoanalyzer for valve control, sequencing and for precise timing of reagent flow. The system has 10 bit analog to digital converter, 2 digital to analog controllers for pump control, 8 digital I/O’s for solenoid valves. Peristaltic pumps (ISMA TEC, Switzerland) were employed. Solenoid valves, 24 V, dc (Neptune Research, USA) were employed. The microcontroller program was devised to program up to 99 sequence steps. At each sequence step, the ON/OFF switching times of valves, flow rate of pumps could be prescribed. This could facilitate flow of reagents in a precise manner to carry out flow ELISA. Fig. 2 shows the schematic diagram of the flow ELISA system. The system software controls the flow ELISA sequence of operations in a phased manner. The amounts of reagents and buffers that can flow through the valves were regulated by the flow rate of the two peristaltic pumps (P1 and P2).

Methyl parathion obtained from M/S Greyhound, England, association buffer (phosphate buffer) and pesticide conjugate with horseradish peroxidase (MP–HRP) were pumped through P1, whereas pump P2 was employed for the dissociation and wash buffers. Solenoid valves (V1–V6) were operated by the microcontroller along with the pumps in such a way as to allow the different reagents through the immobilized antibody column as described in Table 1.

One hundred and fifty microliters of MP antibody was immobilized on a Sepharose support as described in an earlier report.

![Fig. 1. Automated system for flow ELISA.](image1)

![Fig. 2. Schematic of flow ELISA system for pesticide analysis.](image2)

<table>
<thead>
<tr>
<th>Reagent through the column</th>
<th>P1</th>
<th>P2</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>V4</th>
<th>V5</th>
<th>V6</th>
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<tr>
<td>Association buffer</td>
<td>ON</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
</tr>
<tr>
<td>Sample</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
</tr>
<tr>
<td>MP–HRP</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
</tr>
<tr>
<td>Dissociation buffer</td>
<td>OFF</td>
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<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
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<tr>
<td>Substrate</td>
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<td>OFF</td>
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</tr>
<tr>
<td>Wash buffer</td>
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<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>ON</td>
</tr>
</tbody>
</table>

Table 1: ON-OFF position of valves and pumps for flow ELISA
parathion and MP–HRP conjugate were added (100 blocked by 1% BSA in PBS. Different concentrations of methyl plate may have some unbound sites for the antigen, which were remove unbound antibodies. The antibodies bound to the ELISA with PBS wash buffer having 0.05% Tween 20 (pH 7.4) to remove excess pesticide/MP–HRP complex. One hundred and fifty microliters of substrate solution TMB was added to initiate the reaction of 20 min. After incubation, the plate was washed three times with PBS wash buffer having 0.05% Tween 20 (pH 7.4) to remove unbound antibodies. The antibodies bound to the ELISA plate may have some unbound sites for the antigen, which were blocked by 1% BSA in PBS. Different concentrations of methyl parathion and MP–HRP conjugate were added (100 μl) to each well and allowed to bind with the antibody for 1 h at room temperature. The plate was washed with wash buffer three times to remove excess pesticide/MP–HRP complex. One hundred and fifty microliters of substrate solution TMB was added to initiate the development of color. The reaction was allowed to proceed for 30 min at room temperature and was stopped by adding stop solution, at which instant the solution turns yellow from blue. The plate was read at 450 nm (Versamax microplate reader).

### 2.2. Plate ELISA method

This was employed as a reference method for analysis of pesticides for comparison with the flow ELISA. From a 7.35 mg/ml stock, 1 μg/ml and 0.5 μg of MP antibody was taken in duplicate and coated inside ELISA wells using sodium bicarbonate buffer (pH 9.0) and incubated overnight at room temperature (27 °C). After incubation, the plate was washed three times with PBS wash buffer having 0.05% Tween 20 (pH 7.4) to remove unbound antibodies. The antibodies bound to the ELISA plate may have some unbound sites for the antigen, which were blocked by 1% BSA in PBS. Different concentrations of methyl parathion and MP–HRP conjugate were added (100 μl) to each well and allowed to bind with the antibody for 1 h at room temperature. The plate was washed with wash buffer three times to remove excess pesticide/MP–HRP complex. One hundred and fifty microliters of substrate solution TMB was added to initiate the development of color. The reaction was allowed to proceed for 30 min at room temperature and was stopped by adding stop solution, at which instant the solution turns yellow from blue. The plate was read at 450 nm (Versamax microplate reader).

### 3. Results and discussion

#### 3.1. MP analysis by plate ELISA

The calibration graph for the analysis of MP using ELISA is shown in Fig. 2. A good linearity was obtained in the range of 3.3–1000 ppb with a regression value $R^2 = 0.9841$.

#### 3.2. Optimization of parameters for automated flow-ELISA

Production of immunoreagents (immunogens, antibodies, etc.) as well as optimization and validation of an analytical sys-
were collected. After adding TMB it was observed that color was developed in samples from 2nd minute onwards, increased till 6th minute and declined to zero after the 10th minute. These samples (2nd–10th minute) were pooled and its absorbance at 450 nm was recorded. The experiment was repeated several times to check the repeatability of the automated assay. The column was stable and detection of unbound MP–HRP was repeatable in terms of the absorbance of the eluent (Fig. 4). Of the different dilutions of MP–HRP conjugate (1/1K, 1/2K and 1/5K) tried, (1/5K) was found to be optimum and was employed in the studies for MP measurement and dissociation. Having standardized the detection of unbound MP–HRP, pesticide measurements were tried. All analyses were carried out in duplicates using the optimized protocol. The automated assay showed very good repeatability with a standard deviation ranging from 0.01 to 0.045 in the range 50–500 ppb.

The results of analysis of methyl parathion using the automated flow ELISA, are shown in Fig. 5. A good linearity was observed in the range 50–500 ppb, with an $R^2$ value of 0.9783. These results have shown that FIA was a more sensitive method for the detection of MP going up to 50 ppt, whereas the plate ELISA method could detect only up to 3.3 ppb. The flow ELISA proved to be more sensitive possibly due to the higher signal amplification obtained because of higher concentration of MP antibodies used and larger surface area for MP-antibody interaction. Also, as the concentration of pesticide is directly proportional to the concentration of the unbound HRP-conjugate, at lower pesticide levels, higher absorbance readings are obtained resulting in a higher signal amplification and consequently higher sensitivity of detection. A further significant advantage of the automated flow ELISA method is that the use of dissociation buffer enables multiple uses of the immobilized antibody column. The multiple use of the immobilized antibodies was tested by repeatedly passing the pesticide (500 ppb) through the column. This was followed by passing the conjugate (1/5K) through the column, recording the absorbance of the eluent at 450 nm (Fig. 6) and thereby checking the dissociation of the antigen from the antibodies. The dissociation was computed as follows:

$$\% \text{Dissociation} = \frac{A - B}{A} \times 100$$

where $A$ is the absorbance of the eluent before dissociation and $B$ is the absorbance of the eluent after dissociation. The MP antibodies immobilized on Sepharose were stable up to 13 cycles using 1% methanol + gly-HCl (pH 2.4) dissociation buffer with more than 95% of initial binding capacity retained after the 13th cycle, which, steeply declined, thereafter.

4. Conclusion

Selective detection of pesticides, particularly methyl parathion at high sensitivity (parts per trillion) levels is the main attention of this paper. The flow injection immunosassay has been shown to be an efficient method for the detection of MP pesticide in water. With the significant advantage of higher sensitivity than the plate ELISA technique. The reusability of the immobilized antibody column, a very important practical requirement for the FIA has been successfully carried out and it was possible to achieve 13 use cycles with very good dissociation. With FIA technique the task was much simpler as compared with plate ELISA. Optimization of the flow ELISA requires less time with automation. On the other hand, plate ELISA technique requires more skill, precision and accuracy. The added advantage of automated flow ELISA is that the possible sources of human error due to the analyst are eliminated. For precise flow and timing control of reagents used, the use of microcontroller made the task easier and accurate determination of pesticide at ppt levels was possible. A further advantage is that the complete run time for a single analysis of methyl parathion was less than 44 min.
Acknowledgements

Authors are thankful to the Director, CFTRI, Mysore for providing facilities and encouragement. Mr. Akmal Pasha of FPIC Department, CFTRI is thanked for providing the antibody conjugate. Department of Biotechnology (DBT), India and Swiss Development Corporation (SDC), Switzerland are acknowledged for financial support under the Indo-Swiss project on Immuno-biosensors for pesticides.

References

Detection of methyl parathion using immuno-chemiluminescence based image analysis using charge coupled device

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Abstract

A novel method based on immuno-chemiluminescence and image analysis using charge coupled device (CCD) for the qualitative detection of methyl parathion (MP) with high sensitivity (up to 10 ppt) is described. MP antibodies raised in poultry were used as a biological sensing element for the recognition of MP present in the sample. The immuno-reactor column was prepared by packing in a glass capillary column (150 μl capacity) MP antibodies immobilized on Sepharose CL-4B through periodate oxidation method. Chemiluminescence principle was used for the detection of the pesticide. Light images generated during the chemiluminescence reaction were captured by a CCD camera and further processed for image intensity, which was correlated with pesticide concentrations. K3 Fe(CN)6 was used as a light enhancer to obtain detectable light images. Different parameters including concentrations of K3Fe(CN)6, luminol, urea H2O2, antibody, addition sequence of reactants and incubation time to obtain best images were optimized. The results obtained by image analysis method showed very good correlation with that of competitive ELISA for methyl parathion detection. Competitive ELISA method was used as a reference to compare the results obtained by CCD imaging.

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Keywords: K3Fe(CN)6; Methyl parathion; Egg yolk antibodies; Charge coupled device; Chemiluminescence; Immuno-sensor

1. Introduction

Environmental protection in connection with water and agriculture needs urgent attention for human health and safety. Major health problems occur due to the use of contaminated water and food. Application of organochlorine and organophosphorous pesticides in agriculture has been practiced in many countries. As a result of the increased use of pesticides in agriculture in the last few decades, ground water, raw food materials and processed food are becoming contaminated with pesticide residues. The pollution monitoring and protection agencies require rapid and sensitive tools or methods for the analysis of these pollutants. The commonly used analytical methods for pesticide analysis include liquid chromatography, gas chromatography and ELISA methods (Skerritt et al., 2003; Lehotay, 2002; Sasaki et al., 1987; Fernandez et al., 2001).

These conventional methods are sensitive up to ppb level, time consuming, laborious, and require skilled technicians and expensive instrumentation. Biosensor is an alternative tool to detect the pesticides rapidly and economically. Literature on the applications of biosensor for rapid qualitative detection of pesticide is scanty. Analytical methods for the rapid detection of pesticides are not available. A dipstick immuno-assay format for atrazine and terbutylazine analysis in water samples has been developed by (Mosiello et al., 1998). In their method, antibodies were immobilized on a nylon membrane and the detection limit was 1.2–10 μg/l (ppb level) using reflectometer. The main problem in this
2. Materials and methods

Horse radish peroxidase (HRP), luminol, urea H₂O₂, glutaraldehyde, Bovine serum albumin (BSA), gelatin, fish gelatin were procured from M/s Sigma chemicals, USA. Sepharose CL 4B was procured from Amersham Pharma
cia Biotech, Sweden. Sodium cyanoborohydride was pro
cured from Janssen Chimica, Belgium. All other reagents were of analytical grade and procured from standard sources.

MP stock solution (1000 ppm) was prepared in methanol and further dilutions (1000 ppm to 10 ppm) were made every day by appropriate serial dilution in phosphate buffer saline (PBS). 10 mM Luminol was prepared by dissolv
ing 17.5 mg of Luminol in 1.5 ml of 0.1 M NaOH and the volume was made up to 10 ml in 0.2 M Tris buffer. 10 mM urea H₂O₂ stock was prepared in distilled water; further dilutions were made from the stock. MP-HRP dilu
tions were made in PBS-BSA. HRP activity was deter
dined by adding 1 mM luminol (100 μl); 0.1 mM urea H₂O₂ (150 μl) into a luminometer cuvette and liberated light sig
dals during the biochemical reaction were measured using a luminometer (Luminoscan TL Plus, Thermo Lab Systems Finland).

2.1. ELISA method for detection of pesticide

1 μg of MP-IgY antibody was taken in ELISA well with 100 μl of sodium bicarbonate buffer (pH 9.0). The antibod
ies were coated on to the plate and incubated overnight at room temperature. After incubation, the plate was washed thrice with PBS wash buffer having Tween 20 (pH 7.4) to remove unbound antibodies. The unbound IgY sites were blocked with 1% BSA. Different concentrations of methyl parathion and MP–HRP conjugates were added (100 μl) to each well and allowed to bind with the antibody for 1 h at room temperature. The plate was further washed thrice with wash buffer to remove excess pesticide/MP–HRP complex. Substrate solution (150 μl) was added and the reaction was allowed to proceed for 30 min at room temperature. Stop solu
tion (1 M H₂SO₄) was added to arrest the reaction, at which instance the color of the reaction mixture turns yellow from blue. The plate was read at 450 nm on an ELISA plate reader. All analysis were done in duplicates.

2.2. Synthesis of hapten for the production of IgY

2.2.1. O-(4-aminophenyl)-O,O-dimethyl thiophosphate (I)

O,O-dimethyl chlorothiophosphate (7.9 ml, >0.05 m) was added to 4-aminophenol (5.46 g, 0.05 m) dissolved in acetone (250 ml) followed by anhydrous potassium carbonate (10 g, >0.05 m) and 4-N,N-dimethylan
yliminopyridine (DMAP) (0.5 g) catalyst. The mixture was refluxed for 30 min. A con
trol was prepared by refluxing O,O-dimethyl chlorothio
g phosphate with other reagents in the same proportion without 4-aminophenol. The reaction was monitored by thin-layer chromatography by the method of Pasha et al. (1996) and the product formation was confirmed. The product forma
tion was also confirmed by comparing with the product from reduction of methyl parathion by using iron + hydrochloric acid.

2.2.2. 4-[(4-

{\text{Dimethoxyphosphorothioyl} \text{oxyphenylamino})-4-

oxobutanoic acid (II)

O-(4-aminophenyl)-O,O-dimethyl thiophosphate (1.16 g) was dissolved in acetonitrile (45 ml). Succinic anhydride (0.5 g) was added to the solution followed by DMAP (0.4 g). The mixture was heated to 60–80°C with stirring for 2 h. Water (500 ml) was added and the mixture was extracted with dichloromethane (3 x 50 ml), the organic layer was separated, washed with brine and dried over anhydrous magnesium sul
fate, the solvent evaporated off to obtain a residue that was analyzed by TLC by the same method as mentioned earlier. Formation of the product was confirmed.

2.2.3. O-[4-[(4-\text{Di\text{m}eth\text{oxyphosphorothio}loxyphenylamino})-4-

oxobutanoic acid]amino]phenyl][O,O-dimethylthiophosphate (active ester of the hapten) (III)

O-[4-[(4-\text{Dimethoxyphosphorothio}loxyphenylamino})-4-

oxobutanoic acid (260 mg) was dissolved in dry dichloro-
methane (8 ml). N-hydroxysuccinimide (67 mg) was added to the solution and the mixture was cooled to 0°C and stirred on a magnetic stirrer. N,N′-dicyclohexylcarbodiimide (DCC) (137 mg) and DMAP (6 mg) were added and stirring was continued overnight. The dicyclohexylurea was removed by filtration and the solvent evaporated off to obtain the active ester that was stored desiccated below 0°C. The scheme of reaction is given in Fig. 1.

2.3. Conjugation of the hapten to protein

The hapten–protein conjugate was prepared as follows: ovalbumin (OVA) (40 mg) was dissolved in phosphate buffer pH 9.1 (10 ml) and the solution was cooled to 0°C. O-[4-{4-[4,5-dioxopyrrolidin-1-yl]oxy}-4-oxobutanoyl]amino[phenyl] O,O-dimethyl thiophosphate (25.7 mg) dissolved in dimethylformamide (DMF) (2.5 ml) was added to the solution slowly with swirling. The mixture was stored at ca. 8°C overnight and dialyzed using 50 mM phosphate buffered saline against three changes.

2.4. Conjugation of hapten to horse radish peroxidase

The methyl parathion hapten-horse radish peroxidase enzyme conjugate was prepared as follows: horse radish peroxidase (HRP) (5 mg) was dissolved in phosphate buffer pH 9.1 (2 ml) and the solution was cooled to 0°C. O-[4-{4-[4,5-dioxopyrrolidin-1-yl]oxy}-4-oxobutanoyl]amino[phenyl] O,O-dimethyl thiophosphate (10.7 mg) dissolved in DMF (107 μl) was added to the solution slowly with swirling. The mixture was stored at ca. 8°C overnight and dialyzed using 50 mM phosphate buffered saline against three changes. The protein conjugates were apportioned into different volumes and stored below 0°C and the HRP conjugate was stored at ca. 8°C.

2.5. Production of IgY

The use of antibodies from the egg yolks of hyperimmunized hens (IgY antibody) for immuno-logical procedures overcomes some serious limitations associated with the polyclonal antibodies produced in rabbit and monoclonal antibodies, and provides a continuous supply of large quantities of consistent, high titer specific and sensitive antibody which can be easily collected and stored. The immunization protocol comprises periodic intra-muscular immunization of the poultry birds with respective hapten-protein conjugate (1–5 mg) in the breast muscle (Indian Patent No. NS/108/02).

Individual poultry (White Leghorn birds) were immunized initially with the immunogen conjugate in Freund’s complete adjuvant (FCA), followed by booster injections. The first three boosters were given in Freund’s incomplete adjuvant (FICA) with immunogen at time intervals of 2, 3 and 5 weeks, respectively. The fourth and fifth boosters were given at 5 weeks interval. The antibodies were harvested from egg yolk.

The eggs were collected and stored at 4°C until further use, after precipitation of the lipid from egg yolk, antibodies were isolated and purified using different methods like ammonium sulphate, change in pH, chloroform, DEAE-Sephacl column, Dextran sulphate, gums, phosphotungstic acid and the patented method with polyethylene glycol. The patented method gave good quantity of sensitive antibodies.
2.6. Immobilization of IgY-MP antibodies on sepharose CL-4B

MP antibodies (MP Ab) were immobilized on sepharose CL-4B matrix through periodate oxidation method (Hermanson et al., 1992). 200 μg of antibodies per ml of activated sepharose were incubated at 4 °C for 2 h with intermittent mixing. Schiff’s bases were reduced using sodium cyanoborohydride by incubating at 4 °C overnight. The immobilized antibody preparation was thoroughly washed with distilled water and PBS at 4 °C and stored at 4 °C until further use. These immobilized sepharose beads were packed in immuno-reactor column.

2.7. Experimental set up and chemiluminescence assay procedure

A glass capillary column (immuno-bioreactor) 150 μl capacity was packed with MP Ab immobilized on sepharose. This column was equilibrated with PBS (50 mM pH 7.4) for 5 min by passing PBS at a flow rate of 50 μl/min. 50 μl of MP sample was passed through the column, recirculated for 5 min. During this, PBS was used as running buffer and unbound pesticide was excluded. Column was washed with PBS-Tween 20 (0.02%) followed by PBS for 5 min with a flow rate of 50 μl/min. Subsequently, 50 μl of MP–HRP conjugate (1:5000) was passed through the column, 0.1% BSA-PBS (PBS-BSA) was used as running buffer and recirculated for 5 min at a flow rate of 50 μl/min. The unbound conjugate was eluted with PBS. The immobilized matrix having pesticide and MP–HRP conjugate was taken out using PBS–BSA and used for the analysis. In case of control, instead of pesticide sample, PBS buffer (50 μl) was passed; subsequently, the conjugate was run and recirculated in the immuno-bioreactor column for 5 min (Fig. 2). Finally, unbound conjugate was excluded from the column and matrix was collected using PBS-BSA. Using this matrix CL reaction was carried out by taking into ELISA strip wells. Light produced thorough this reaction is of very low intensity. To get enhanced light intensity, the electron mediator K3FeCN6 (0.5%) was added. A peristaltic pump (ALITEA, Sweden) was used to maintain a constant flow rate of buffers and other reagents through the immuno-bioreactor column. The residence time of the reactants in the bed was controlled by adjusting the flow rate.

2.8. Development of detection device for chemiluminescence and detection method

A charged coupled device based light detection system was developed in the laboratory and was employed for chemiluminescence detection. The light generated through the chemiluminescence reaction was captured using a CCD ‘WAT 202D’ Digital camera (WATEC, Japan) and further processing was done using the computer, which was interfaced with CCD using a color frame grabber card with BNC connection for video and trigger inputs. A 25mm focal length CCD camera lens was employed. Grabbed images were further processed and analyzed using a custom software by employing digital image processing tools. Fundamental algorithms for color to gray conversion, thresholding, filtering, segmentation were implemented using Turbo C++ programming language with Microsoft Visual Basic 6.0 as front end.

Fig. 2. Flow chart of the qualitative determination of methyl parathion.
2.8.1. Principle of the pesticide detection

During the analysis, the reaction was allowed for 5 min at room temperature after the addition of urea H$_2$O$_2$ to the matrix, then luminol and K$_3$Fe(CN)$_6$ were simultaneously added followed by the capture of images immediately by a CCD in dark. The images were enhanced using histogram stretching and the intensity values (I) were determined. The image intensities of the pesticide sample were more as compared to that of the control. Chemiluminescence reaction was carried out in black coloured ELISA strips so as to prevent the light passing from one well to another.

The intensity of light produced mainly depends upon the urea H$_2$O$_2$ concentration in the reaction mixture prior to the addition of K$_3$Fe(CN)$_6$. The urea helps in increasing the stability of the substrate H$_2$O$_2$ as well as the shelf life of the substrate solution. The urea H$_2$O$_2$ concentration utilized during the reaction varies with the amount of conjugated HRP bound to the immobilized antibody matrix. Hence, during the reaction, incubation was required to degrade the urea H$_2$O$_2$ by the conjugate. At high pesticide concentration, there was more of unreacted urea H$_2$O$_2$ as less of MP–HRP is present on the immobilized matrix. K$_3$Fe(CN)$_6$ reacts with this H$_2$O$_2$, produces more light, which can be captured by CCD camera and the intensity of the light produced is directly proportional to the pesticide concentration. For the reaction between the conjugated HRP present on the immobilized Ab and urea H$_2$O$_2$, the incubation time and mixing of the reactants were necessary before addition of K$_3$Fe(CN)$_6$. After the addition of urea H$_2$O$_2$ to the matrix (which contained Ab + Ag–HRP complex) the reaction mixture was incubated for 5 min at room temperature (28±2°C) with intermittent shaking. Similar mixing was also done after the addition of luminol. Finally, at the end of 5 min K$_3$Fe(CN)$_6$ was added and immediately the light images were captured in dark condition.

3. Results

The clarity of CCD image depends on the concentrations of the reactants such as luminol, urea H$_2$O$_2$ and K$_3$Fe(CN)$_6$ present in the chemiluminescence reaction. At higher concentration of these reactants, background noise was generated which overlaps/interferes with the analysis. Optimizing the concentrations of the reactants can minimize this background noise. Hence, in the present study K$_3$Fe(CN)$_6$, luminol and urea H$_2$O$_2$ concentrations were optimized. To achieve clear images between different pesticides concentrations parameters like immobilized antibody concentration, reagents addition sequence and reaction time were also optimized.

3.1. Effect of K$_3$Fe(CN)$_6$ concentration

In the chemiluminescence reaction, light is produced due to reaction between H$_2$O$_2$ and luminol in the presence of HRP. As these low level light signals cannot be either visualized by naked eye or captured by ordinary CCD camera, a very sensitive photo multiplier tube or luminometer would be essential. But in the present investigation, we observed that addition of appropriate concentration of K$_3$Fe(CN)$_6$ lead to an enhanced light image produced by CL reaction, which can be captured by CCD. In order to enhance the light intensity (through which visual differentiation is possible) K$_3$Fe(CN)$_6$ was used. To obtain optimal detectable light signal (CL) studies on the optimization of K$_3$Fe(CN)$_6$ were carried out in the concentration range of 0.1–2% (w/v) without any HRP. At higher concentration (2%), K$_3$Fe(CN)$_6$ gave very bright light signal (I = 195.75) which produces more background noise; hence, it was not convenient for the qualitative determination. Table 1 tabulates the image intensity obtained for different concentrations of K$_3$Fe(CN)$_6$. It was found that by using 0.5% K$_3$Fe(CN)$_6$, the best visual differentiation with low background noise was obtained. A very dull image was found using 0.1% K$_3$Fe(CN)$_6$ (Table 1); hence, for further studies 0.5% concentration of K$_3$Fe(CN)$_6$, was selected.

3.2. Effect of urea H$_2$O$_2$

Varying concentrations of H$_2$O$_2$ were studied and 10 mM concentration was found to the optimal. Further optimal volume of urea H$_2$O$_2$ was evaluated by varying volume from 15 to 100 µl. It was observed that with increasing volume, the light intensity increased. 25 µl of urea H$_2$O$_2$ gave good difference between two concentrations of urea H$_2$O$_2$ and background noise was also less. Hence, 25 µl of 10 mM urea H$_2$O$_2$ was considered as optimum for the CL reaction (Table 2).

3.3. Effect of luminol

Different volumes of 10 mM luminol, 100, 50, 25 and 15 µl were tried and found that using 25 µl of luminol showed less background and optimum light signal production was observed which is suitable for capture of the images through the CCD camera (Table 3). 25 µl luminol gave reasonable

<table>
<thead>
<tr>
<th>Volume of urea H$_2$O$_2$ (µl)</th>
<th>Image intensity (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>124.27</td>
</tr>
<tr>
<td>25</td>
<td>132.97</td>
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<td>50</td>
<td>138.85</td>
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</table>

<table>
<thead>
<tr>
<th>K$_3$Fe(CN)$_6$ concentration (%)</th>
<th>Image intensity (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>100.6</td>
</tr>
<tr>
<td>0.5</td>
<td>148.23</td>
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<tr>
<td>1</td>
<td>172.51</td>
</tr>
<tr>
<td>2</td>
<td>195.75</td>
</tr>
</tbody>
</table>
Table 3

<table>
<thead>
<tr>
<th>Effect of urea H₂O₂</th>
<th>Volume of luminol (μl)</th>
<th>Image intensity (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>86.96</td>
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<tr>
<td></td>
<td>50</td>
<td>88.74</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>94.22</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>119.1</td>
</tr>
</tbody>
</table>

3.4. Effect of antibody concentration

The clear gradation between the pesticide samples mainly depends on the concentration of antibody present on sepharose. To evaluate the optimum antibody concentration, different concentrations (50–500 μg Ab/ml of sepharose) were immobilized. Among these, 200 μg Ab/ml sepharose was found as the optimum antibody concentration for the better images. The CCD image intensities were not distinguishable at remaining concentrations of antibody as shown in Fig. 3.

3.5. Effect of addition sequence of reactants

The addition sequence of the reactants during chemiluminescent reaction showed a considerable impact on light production and differentiation. When the reactants were added in the sequence: matrix–urea H₂O₂–luminol–K₃Fe(CN)₆, the difference between the sample and control images was found to be best. At different addition sequences the quality of light is described in Table 4.

3.6. Effect of reactants incubation time

Incubation time of reactants showed a considerable impact on the gradation of images between the pesticide and without pesticide samples. Poor light intensity difference was observed without mixing. 5 min incubation was found to be optimum after the addition of urea H₂O₂ to the immobilized matrix and good images were obtained (images not shown). With either increase or decrease in incubation time, the gradation was poorer.

At optimized conditions, good images were obtained as shown in Fig. 4. The light intensities were linearly proportional with the concentration of methyl parathion with an R-value of 0.9892 (Fig. 5). It was possible to detect the presence or absence of pesticide in the range of 10 ppt to 1000 ppb. Below 10 ppt and above 1000 ppb images were not clear with even the enhanced image intensities showing no appreciable difference from the control. Also, above 1000 ppb, all images were showing almost the same intensity and below 10 ppt, images were very faint and not distinguishable from the control.

Table 4

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Luminal + urea H₂O₂ + K₃Fe(CN)₆ + matrix</td>
</tr>
<tr>
<td>2</td>
<td>Matrix + K₃Fe(CN)₆ + urea H₂O₂ + luminol</td>
</tr>
<tr>
<td>3</td>
<td>Matrix + urea H₂O₂ + luminol + K₃Fe(CN)₆</td>
</tr>
<tr>
<td>4</td>
<td>Matrix + luminol + K₃Fe(CN)₆ + urea H₂O₂</td>
</tr>
<tr>
<td>5</td>
<td>Luminal + urea H₂O₂ + K₃Fe(CN)₆ + matrix</td>
</tr>
</tbody>
</table>

Fig. 3. Image intensities as a function of antibody concentration.

Fig. 4. Gradation in light intensity with different pesticide concentrations.

Fig. 5. Image intensity as a function of methyl parathion.
3.7. Competitive ELISA

Using the competitive ELISA, IC50 value of IgY chicken antibody was found to be 2.2 ppb (Fig. 6) and the minimum detection limit of methyl parathion was 220 ppt.

3.8. Comparison of CCD Imaging with ELISA method for detection of methyl parathion

Recovery studies were conducted to study the results obtained by the two methods. The results obtained by CCD imaging method showed good agreement with the ELISA method as is evident from Fig. 7.

4. Discussion

In general, enzymatic chemiluminescence reactions for the luminol oxidation HRP are widely used. However, the light signal intensity produced is so low, it is detected by naked eye in dark room condition or even by an ordinary CCD camera. Therefore, it became necessary to increase the light intensity to a desirable level to be able to be grabbed by the camera. For this purpose, a well known electron mediator, K3Fe(CN)6 was used. It is reported that metal ions possessing oxidation states requiring a single electron transfer are capable of promoting the chemiluminescence reaction between peroxide and luminol in water at alkaline pH (Seitz and Hercules, 1973). Fe(II)-containing compounds such as hemin and heamoglobin, copper(II), as well as mixed Cu(II)-persulphate and Cu(II) hemin solutions have been employed in the luminol reaction. Cobalt (II), Fe(II) [Fe(CN)6]3− and SbCl6− have been cited as reagents capable of producing chemiluminescence in the presence of luminol and hydrogen peroxide (Bostick and Hercules, 1975).

The use of both HRP and K3Fe(CN)6 for enhanced light signal production coupled with image enhancement enables sensitive detection of pesticides even at sub-nanomolar concentrations. In our experiments, HRP/MP-HRP conjugate alone did not produce sufficient light signals which was sufficient to detect pesticide at ppt level. There are some reports, Ramanathan et al. (2002) which use K3Fe(CN)6 or HRP alone as an electron mediator. Also reports are not available on the use of image analysis technique for the detection of MP.

The light intensity produced during this process was directly proportional to the pesticide concentration. This phenomenon can be interpreted as follows. When there is less amount of pesticide in the sample, more conjugates bind to immobilized antibodies, and therefore more MP–HRP would be available for reaction with H2O2 leading to its higher degradation. Hence, less H2O2 is available for K3(FeCN)6, and thereby resulting lesser amount of light. Similarly, when there is more pesticide, more light is produced.

To obtain distinctive light signals for image processing, the different parameters were optimized. K3Fe(CN)6 played a key role in light production and 0.5% (w/v) was found to be the optimum concentration. With higher concentration, more background noise was observed. Low concentration of K3Fe(CN)6 led to very poor images with very low light intensities. Luminol and urea H2O2 were also optimized, and in both the cases, with increasing levels, light intensity also increased and lead to more background noise. 25 μl of 10 mM luminol and urea H2O2 were found to be optimum.
Efficient gradation and sensitive level detection depends on the antibody loading on the sepharose. Among the Ab concentrations tried for this purpose 200 µg/ml sepharose exhibited good light images at different pesticide levels. 500 µg/ml sepharose exhibited poor light images due to the greater binding of conjugate to the immobilized antibody and degradation of urea H₂O₂ was very fast and higher. Hence, very low level of urea H₂O₂ was available to react with K₃Fe(CN)₆, which finally led to less light production. With low Ab concentration, more light production was observed to lesser binding of conjugate and degradation of H₂O₂, and thereby resulting in higher light intensities. However, it lead to poor and inconsistent gradation.

Reaction time showed greater impact on light images. After addition of H₂O₂ to immobilized matrix, the reaction mixture was allowed to react for 5 min, simultaneously luminol and K₃Fe(CN)₆ were added which gave good images having distinguishable difference between images of different pesticide concentration. With higher or lower incubation times, the gradation was adversely affected. In this case, urea H₂O₂ first reacts with MP–HRP and maximum oxidation takes place. Addition sequence of reactants also showed a considerable impact on images. When immobilized matrix, urea H₂O₂, luminol and K₃Fe(CN)₆ were added in a sequence, the light images were showing distinct difference between pesticide samples and control. In case of other sequences, reproducibility and gradation between images was very poor because the entire reaction takes place before addition of matrix and also K₃Fe(CN)₆ reacts very fast with urea H₂O₂ than the conjugated HRP.

Comparing the two methods for detection, the chemiluminescence imaging method was found to be highly sensitive for the detection of methyl parathion even at ppt level. In ELISA, it was possible only to detect the analyte concentration up to 2.2 ppb level. At lower levels, the image background noise dominates and requires higher enhancement with digital filtering. As the lower limit for detection by ELISA was only 2.2 ppb, the CCD imaging results were confirmed by recovery studies. As no other reference method was available for cross-validation, it can be reasonably concluded based on the results obtained that semi-quantification of MP is possible with CCD imaging.

5. Conclusion

Rapid detection of pesticides at sub-nanogram level with high accuracy and reliability is a challenging task. Immunosensors based on chemiluminescence is an attractive alternative analytical tool. The results in this study strongly indicate that the current chemiluminescence-based method is highly reliable, fast and sensitive for the qualitative detection of methyl parathion. Combining photoenhancement by electron mediators, image enhancement techniques and software for image processing, provide a convenient means to minimize the signal noise and enhance the low level light signals produced due to chemiluminescence. Once the protocol is standardized, this technique does not require much technical knowledge to the user. It was also possible to distinguish the light images in the dark, which enables the user to decide the presence or absence of the pesticide, and hence the present method is highly field applicable. Further studies are in progress towards the development of software for the quantitative estimation using this method through image processing analysis, which is matter of next research communication.

Acknowledgments

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References

Immobilised tyrosinase-based biosensor for the detection of tea polyphenols

K. S. Abhijith - P. V. Sujith Kumar - M. A. Kumar - M. S. Thakur

Abstract
An amperometric principle-based biosensor containing immobilized enzyme tyrosinase has been used for detection of polyphenols in tea. The immobilized tyrosinase-based biosensor could detect tea polyphenols in the concentration range 10–80 mmol L\(^{-1}\). Immobilization of the enzyme by the crosslinking method gave good stable response to tea polyphenols. The biosensor response reached the steady state within 5 min. The voltage response was found to have a direct linear relationship with the concentration of polyphenols in black tea samples. Enzyme membrane fouling was observed with number of analyses with a single immobilised enzyme membrane. The tyrosinase-based biosensor gave maximum response to tea polyphenols at 30°C. The optimum pH was 7.0. This biosensor system can be applied for analysis of tea polyphenols. Variation in the biosensor response to black tea infusions gave an indication of the different amounts of theaflavins in the samples, which is an important parameter in evaluating tea quality. A comparative study of the quality attributes of a variety of commercially available brands of tea were performed using the biosensor and conventional analytical techniques such as spectrophotometry.

Keywords
Biosensor - Tyrosinase - Catechin - Amperometric - Crosslinking - Black tea

Introduction

Diet is increasingly considered a decisive factor of protection against serious diseases such as cardio and cerebro-vascular diseases and tumours [1]. Tea is one of the beverages rich in anti-oxidants whose health benefits have been actively pursued in recent times [2]. Tea leaves contains many polyphenols, catechins being particular prolific. Tea leaves have been found to contain seven major groups of catechins namely (+)-catechin, (+)-gallocatechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, and (−)-gallocatechin gallate. The catechin content of tea leaves goes up to 30% of the dry weight. During fermentation in the presence of the enzyme polyphenol oxidase (−)-epicatechin (EC) and (−)-epigallocatechin (EGC) are oxidized to form polymers of catechin called theaflavins and thearubigins [3].

Many biosensors have been developed using the catalytic activity of the redox enzymes for phenol determination. Biosensors have been developed with enzymes such as tyrosinase, peroxidase, laccase, etc. [4]. There is also a report of the determination of the antioxidant capacity of different polyphenol-containing foodstuffs using a tyrosinase-based superoxide dismutase biosensor [5]. Amperometric biosensors based on electrical reduction of o-quinones liberated by the enzyme catalytic reaction have been reported [6]. There are also reports of a tyrosinase-based biosensor for detection of phenols present in water [7], but there are no reports of biosensors for detection of polyphenols in tea.

The enzyme tyrosinase (polyphenol oxidase EC 1.14.18.1) belongs to the group of polyphenol oxidases commercially extracted from the mushroom Agaricus bisporus. Tyrosinase catalyzes the oxidation of diphenols to o-quinones. Tyrosinase takes part only in the initial formation of quinones from...
catechins (diphenols); the quinones formed will then poly-
merize to form theaflavins and thearubigins.

There is a relationship between the polyphenol content
and quality of tea [8, 9], so there is a need for a technique
for rapid detection and quantification of polyphenols.
Biosensors for tea polyphenols enable rapid detection for
quantification of polyphenols in different grades of tea. In
analysis of a sample with a biosensor it is particularly
desirable to produce the largest possible detectable signal
from a small quantity of substrate and catalyst. Conven-
tional methods such as spectrophotometry and HPLC are
labourious and time-consuming. Professional tea tasters
currently grade tea by sensory evaluation. This method
has some disadvantages, as the analyses are subjective and
may also be biased so there is always a chance of
discretion. It is necessary to develop objective methods to
identify tea quality. Biosensors, having the advantage of
both biological and electronics components, will be a good
alternative to sensory evaluation. The biosensor for tea
polyphenols which is being developed in our laboratory
will help to grade tea more accurately on the basis of the
polyphenol content of different samples. The principle of
operation of the tea biosensor is the enzymatic transforma-
tion of polyphenols with consumption of oxygen in the
reaction. The change in the dissolved oxygen concentration
correlates with the concentration of catechins, which is a
measure of tea quality in terms of polyphenol content. The
biochemical reaction is represented by:

\[
QH_2 + \frac{1}{2}O_2 \rightarrow Q + H_2O
\]

where \( QH_2 \) and \( Q \) are the reduced and oxidized forms
of the phenols. In terms of electron transfer, the reaction can
be written as:

\[
O_2 + 4H^+ + 4e^- \rightarrow 2H_2O
\]

The depletion of oxygen at the electrode caused by the
biochemical reaction also involves consumption of elec-
trons, resulting in an electrochemical signal which is
proportional to the concentration of polyphenols in the
sample. This signal is conditioned, amplified, and monitored
by use of an amperometric detector system.

Fig. 1 Schematic diagram of
the biosensor system for poly-
phenols detection

1. Air bubpler
2. Electrode
3. Immobilized enzyme membrane
4. Buffer
5. Signal conditioning System
6. AC/DC Converter
7. Peripheral Interphase
8. Micro controller for Data read out and storage
9. Display
10. Sample injector
Materials and methods

Apparatus and reagents

Studies carried out using a Shimadzu (Japan) 1601 UV–visible spectrophotometer. All biosensor work was carried out with a tea biosensor developed at CFTRI (Mysore, India). A two-electrode system procured from Century Electrodes (India) was used for construction of the amperometric probe.

Tyrosinase (Agaricus bisporus) having specific activity 6050 IU mg⁻¹, lysozyme, glutaraldehyde, catechin, and catechol, were procured from Sigma (USA). A cellophane membrane with a molecular weight cut off of 6–8 kD was from Spectra/por. An oxygen-permeable Teflon membrane was from WTW (Germany). All other chemicals and reagents were of analytical grade and were procured from Qualigens (India).

Methods

Enzyme immobilisation

Tyrosinase was immobilised by cross-linking in accordance with Ref. [10], modified slightly. Tyrosinase (6050 IU, 1 mg) was dissolved in 1 mL sodium phosphate buffer (pH 6.8) and lysozyme (30 mg) was dissolved in 1 mL sodium phosphate buffer (pH 6.8). Glutaraldehyde solution (4% v/v) was prepared by appropriately diluting 70% glutaraldehyde.

On a 2 cm²×2 cm cellophane membrane about 100 IU enzyme and 30 μL lysozyme solution were placed and mixed thoroughly. To this 30 μL glutaraldehyde solution was added and again mixed thoroughly so that enzyme and stabilizing agent were distributed evenly over the membrane. After drying for 1 h the enzyme membrane was washed three times with 0.1 mol L⁻¹ phosphate buffer (pH 6.8) to remove excess glutaraldehyde.

Fabrication of detector system

A schematic diagram of the biosensor for polyphenol is shown in Fig. 1 and the immobilized enzyme-based bioelectrode used for construction of the biosensor for polyphenols is shown in Fig. 2. The biosensor device (Fig. 1) comprises a

![Schematic diagram of the biosensor](image)

Table 1 Quantitative estimation of tea polyphenols by use of the biosensor and by the spectrophotometric method

<table>
<thead>
<tr>
<th>Black tea sample</th>
<th>Biosensor response (mmol L⁻¹)</th>
<th>Spectrophotometric response (mmol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.01</td>
<td>7.02</td>
</tr>
<tr>
<td>B</td>
<td>6.56</td>
<td>9.51</td>
</tr>
<tr>
<td>C</td>
<td>6.36</td>
<td>9.21</td>
</tr>
<tr>
<td>D</td>
<td>5.98</td>
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</tr>
<tr>
<td>E</td>
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<td>F</td>
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</tr>
<tr>
<td>G</td>
<td>2.61</td>
<td>4.23</td>
</tr>
</tbody>
</table>
Clark-type amperometric electrode, air pump, an immobilized enzyme membrane system consisting of hydrophobic polymer (Teflon membrane) to avoid leakage of the electrolyte, and a hydrophilic polymer (cellophane membrane) with immobilized tyrosinase. The dissolved oxygen electrode is immersed in a reaction cell containing suitable buffer. A signal-conditioning unit consisting of a current-to-voltage converter circuit is used for processing of the electrode signal which is digitized using a 12-bit analogue-to-digital converter and a microcontroller unit for data processing with 4×4 keyboard and LCD display. The microcontroller unit has an RS-232 serial interface, keyboard, display, printer interface, and 4 kB flash random-access memory (RAM), with software for data-acquisition, processing, calibration, and report generation.

When substrate is introduced into the reaction cell using the sample injector the enzyme reaction proceeds resulting in depletion of oxygen in the vicinity of the enzyme membrane thereby yielding an electrochemical signal (decreasing voltage). This signal is suitably conditioned using the signal-conditioning unit which is digitized using a 12-bit ADC and further processed using the microcontroller for quantification of the polyphenols. The acquisition and processing parameters are programmable. The system provides for a two-point calibration for quantification of polyphenols. The biosensor system further comprises immobilized tyrosinase, resulting in depletion of oxygen, which is measured by the amperometric O₂ electrode as discussed above.

**Enzyme electrode construction and operation**

The enzyme electrode was constructed as reported in an earlier paper [11]. In brief, a Clark’s electrode with an amperometric detection system was used for detection of tea polyphenols. This Clarke-type electrode consists of a gold cathode and a reference Ag/AgCl electrode covered with saturated KCl electrolyte enclosed within a Teflon membrane. The immobilized enzyme membrane was secured to the electrode using an “O” ring. The electrode containing the enzyme membrane was dipped in a sample cell containing pH 6.8 phosphate buffer (2 mL). The sample cell was continuously saturated with oxygen using an aerator. The electrode was connected to the detector system developed by the Central Food Technological Research Institute, Mysore, India. During biochemical reactions, O₂ is consumed which was monitored as a decrease in current. This decrease in current was converted into a voltage by the system and the

![Fig. 3 Calibration of immobilized tyrosinase-based biosensor using catechin standard (10–100 mmol L⁻¹). The regression value (R²) was 0.9955. All the concentrations were measured in triplicate and mean values are reported with percentage error ±3%](image1)

![Fig. 4 Calibration for standard catechin by the spectrophotometric method. The absorbance of catechin standard (1–10 mmol L⁻¹) was measured at 765 nm. It showed R² (linearity) of 0.9817. All the concentrations were measured in triplicate and mean values are reported with percentage error ±5%](image2)
response in voltage, which is directly proportional to the concentration of the analyte, was displayed.

Measurement of tea polyphenols through biosensor is carried out by:

1. incubating the biosensing element for 5–10 min in a sample cell containing air-saturated buffer (2 mL) to obtain a steady sensor signal response;
2. drawing 25–100 μL samples from polyphenol-containing solutions in the concentration range 5–100 mmol L⁻¹ catechin or green/black tea samples, and injecting the same into the sample cell;
3. recording the response of the biosensor in terms of the decrease in the amount of dissolved oxygen after 5 min, detected by the amperometric detection system (volts) for a period of 1–5 min.

Validation

Different parametric studies such as linearity and pH studies were carried out with catechin as substrate. All the experiments were carried out at 25±1°C unless mentioned.

Linearity

Linearity studies were performed with catechin concentrations ranging from 10–90 mmol L⁻¹. The response was recorded for 5 minutes at 30 seconds intervals.

pH studies

For the pH studies sodium phosphate buffer with pH in the range 5.5–8.0 was used. After measuring the initial activity at pH 6.8, the membrane was incubated for 30 min at different pH and then readings were taken at the same pH.

Temperature studies

For temperature studies, the response to catechin (50 mmol L⁻¹) was studied with the immobilized tyrosinase membrane-based biosensor, keeping the working buffer in a sample cell in a water bath maintained at different temperatures in the range 25–50°C.

Operational stability of the immobilized enzyme membrane

The general problem for many biosensors is the lack of necessary operational and storage stability. Our experiments involved the storage of membranes at room temperature and at 4°C either dry or in phosphate buffer. The membranes were stored up to 30 days and the activity was checked daily by

Validation

Different parametric studies such as linearity and pH studies were carried out with catechin as substrate. All the experiments were carried out at 25±1°C unless mentioned.

Linearity

Linearity studies were performed with catechin concentrations ranging from 10–90 mmol L⁻¹. The response was recorded for 5 minutes at 30 seconds intervals.

pH studies

For the pH studies sodium phosphate buffer with pH in the range 5.5–8.0 was used. After measuring the initial activity at pH 6.8, the membrane was incubated for 30 min at different pH and then readings were taken at the same pH.

Temperature studies

For temperature studies, the response to catechin (50 mmol L⁻¹) was studied with the immobilized tyrosinase membrane-based biosensor, keeping the working buffer in a sample cell in a water bath maintained at different temperatures in the range 25–50°C.

Operational stability of the immobilized enzyme membrane

The general problem for many biosensors is the lack of necessary operational and storage stability. Our experiments involved the storage of membranes at room temperature and at 4°C either dry or in phosphate buffer. The membranes were stored up to 30 days and the activity was checked daily by
injecting 50 μL 50 mmol L⁻¹ catechin and the response recorded as drift in voltage. In order to improve the stability of the immobilized tyrosinase-based biosensor system the tyrosinase enzyme (100 IU) was immobilized with different protein-based stabilizing agents (PBSA) such as lysozyme, BSA, and gelatin. Each PBSA (2 mg) was cross-linked with tyrosinase using 5% glutaraldehyde. The biosensor response was measured for repeated analyses using catechin (50 mmol L⁻¹) as substrate.

Reusability of the immobilised enzyme membrane

To check reusability, standard samples were analysed with the immobilised tyrosinase-based biosensor over a period of six days. The response to catechin (50 mmol L⁻¹) was measured until 50% of the initial activity was lost.

Determination of polyphenols in black tea samples

Seven different brands of black tea were purchased from a local market. Extraction of polyphenols was performed as described elsewhere [12]. In brief, the black tea sample (2.4 g) was infused in 100 mL boiling water and kept at 90°C for 20 min. The infusion was rapidly filtered through Whatman No 1 filter paper and the filtrate (100 μL) was injected into the enzyme electrode containing the immobilised tyrosinase membrane. The response was measured in millivolts. The same tea samples were also analysed for total polyphenols by the FC reagent method [14]. A sample (0.1 mL) was made up to 5 mL with distilled water. To this solution, 0.2 mL Folin–Ciocalteau reagent and 0.5 mL saturated sodium carbonate was added. The volume was finally made up to 10 mL. This final mixture was incubated for 60 min and samples were then analysed by spectrophotometry at 765 nm (Table 1).

Results and discussion

Studies were performed with catechin concentrations ranging from 10 to 90 mmol L⁻¹. The steady-state voltage response at the end of 5 min was recorded and plotted.
against catechin concentration (Fig. 3). Very good linearity is indicated ($R^2=0.9955$). The linearity was best from 10 to 80 mmol L$^{-1}$. The linearity achieved with the biosensor is good agreement with the spectrophotometric method (Fig. 4).

The effect of pH on sensor response was studied after measuring the initial activity at pH 6.8. The membrane was incubated for 30 min in sodium phosphate buffer at different pH in the range 5.5–8.0 and readings were taken at that pH. In the free state the optimum pH was 6.0 for tyrosinase enzyme. Our optimization studies demonstrated that the optimum pH for the immobilized tyrosinase membrane was 6.5–7.0. The shift in the optimum pH of the enzyme may be due to the immobilization (Fig. 5). For our further studies we used pH 6.8 sodium phosphate buffer.

To study the effect of temperature on biosensor response, the steady-state voltage response to catechin (50 mmol L$^{-1}$) was studied with the immobilized tyrosinase membrane-based biosensor, keeping the sample cell in a water bath maintained at different temperatures in the range 25–50°C. Figure 6 demonstrates the effect of temperature, indicating 30–35°C to be the optimum temperature for measurement using the biosensor. At 40°C the enzyme retained 75% of its activity but the activity declined sharply with further increase in temperature.

It is reported that protein-based stabilizing agents (PBSA) help to enhance the operational stability of immobilised enzyme-based biosensors [11]. Using this approach, the tyrosinase enzyme (100 IU) was immobilized with different PBSAs such as lysozyme, BSA, and gelatin. Each PBSA (2 mg) was cross-linked with tyrosinase using 5% glutaraldehyde. The biosensor response was measured for repeated analyses using catechin (50 mmol L$^{-1}$) as substrate over several days with several analyses. Figure 7 shows the...
effect of PBSA on the operational stability of the enzyme membrane. Among the three PBSAs used, lysozyme was found to be the best, followed by BSA and gelatin. For tyrosinase immobilised with lysozyme it was possible to analyse 80 samples. The response gradually decreased with number of analyses, reaching 50% of the initial activity after 80 analyses. This may be due to accumulation of products formed, causing membrane fouling, and can probably be attributed to the strong interaction between polyphenols and the enzyme which has been reported to be irreversible [13]. Protein-polyphenol binding forms a strong complex over the membrane surface, which in turn decreases the response of the biosensor. With BSA and gelatin it was possible to analyse 60 and 50 samples, respectively. Another significant observation from Fig. 7 is that tyrosinase immobilized with gelatine and BSA lost its activity rapidly when compared to tyrosinase immobilized with lysozyme.

A general problem for many biosensors is lack of necessary operational and storage stability. Our experiments involved the storage of membranes at room temperature and at 4°C either dry or in phosphate buffer in a refrigerator. The membranes were stored up to 30 days and the activity checked daily by injecting 50 μL 50 mmol L⁻¹ catechin and recording the response as drift in steady state voltage response on each day. The enzyme membrane retained 50% of its initial activity after 10 days and 60 analyses (Fig. 8).

Seven different brands of black tea (samples designated A, B, C, D, E, F, and G) were purchased from a local market. Tea decoction filtrate (100 μL) was injected into the enzyme electrode containing the immobilised tyrosinase membrane and the steady-state voltage response was measured. The same tea samples were analysed for total polyphenols by the FC reagent method [14].

Figure 9 shows the biosensor response to different commercial black tea samples. The biosensor response to the samples was distinguishable. This may be because of the variation in total polyphenol content in the respective samples. Figure 10 shows the total polyphenol content of the above black tea samples as estimated by the FC method. The results showed that the samples with the highest polyphenol content resulted in the maximum biosensor response. This implies there is a direct relationship between the polyphenol content of the sample and the biosensor response (Table 1). This result will be helpful in quantification of tea polyphenols using tyrosinase-based amperometric biosensor.

Conclusions
Polyphenols play a very important role in the quality of tea. The health benefits of tea are largely attributed to the polyphenol content. Sensitive and rapid detection of polyphenols is therefore very much desired. Available methods, such as spectrophotometric and HPLC, are laborious and time-consuming. Also tea tasting by sensory assessment may not be accurate all the time, as it may sometimes be subjective. The tyrosinase-based biosensor reported in this work provides a very good alternative to conventional methods. It can also be used for grading tea on the basis of the polyphenol content, which can be a very efficient tool for pricing of tea in the market. With the biosensor developed in our laboratory we can analyse up to 80 samples with a single enzyme membrane.

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References
Development of a biosensor for caffeine

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Abstract

We have utilized a microbe, which can degrade caffeine to develop an Amperometric biosensor for determination of caffeine in solutions. Whole cells of Pseudomonas alcaligenes MTCC 5264 having the capability to degrade caffeine were immobilized on a cellophane membrane with a molecular weight cut off (MWCO) of 3000–6000 by covalent crosslinking method using glutaraldehyde as the bifunctional crosslinking agent and gelatin as the protein based stabilizing agent (PBSA). The biosensor system was able to detect caffeine in solution over a concentration range of 0.1 to 1 mg mL⁻¹. With read-times as short as 3 min, this caffeine biosensor acts as a rapid analysis system for caffeine in solutions. Interestingly, successful isolation and immobilization of caffeine degrading bacteria for the analysis of caffeine described here was enabled by a novel selection strategy that incorporated isolation of caffeine degrading bacteria capable of utilizing caffeine as the sole source of carbon and nitrogen from soils and induction of caffeine degrading capacity in bacteria for the development of the biosensor. This biosensor is highly specific for caffeine and response to interfering compounds such as theophylline, theobromine, paraxanthine, other methyl xanthines and sugars was found to be negligible.

Although a few biosensing methods for caffeine are reported, they have limitations in application for commercial samples. The development and application of new caffeine detection methods remains an active area of investigation, particularly in food and clinical chemistry. The optimum pH and temperature of measurement were 6.8 and 30±2°C, respectively. Interference in analysis of caffeine due to different substrates was observed but was not considerable. Caffeine content of commercial samples of instant tea and coffee was analyzed by the biosensor and the results compared well with HPLC analysis.

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Keywords: Caffeine; Biosensor; Amperometric; Immobilization; Microbial biosensor; High-performance liquid chromatography

1. Introduction

Demand for biosensors has increased markedly in recent years, driven by needs in many commercial and research sectors for specific sensors that are capable of rapid, reliable measurements [1]. Development of biosensors is of interest for diverse applications ranging from biochemical profiling of normal and diseased cells (metabolomics), clinical diagnostics, drug discovery and biodefense, to more straightforward analyses such as fermentation, process monitoring, environmental testing and quality control of foods and beverages. Caffeine (1,3,7-trimethylxanthine) is a natural alkaloid occurring in coffee, cocoa beans, cola nuts and tea leaves. It is mildly stimulating and is used as a therapeutic agent [2]. It is a white compound, moderately soluble in water and organic solvents like ethanol, ethyl acetate, methanol, benzene, etc.

While being a stimulant to the central nervous system, it can have some adverse effects on health. If consumed in excess it can cause adverse health effects [3,4]. It is teratogenic, causes inhibition of DNA repair [5], inhibition of cyclic AMP phosphodiesterase activity [5] and inhibits seed germination. It can be a cause of cancer, heart diseases [6] and complications in pregnant women and aging [7].

Coffee is one of the most popular beverages across the world and its caffeine content has an important role in determining the quality of coffee beverages. Further, on account of the harmful effects of caffeine its efficient measurement is relevant. In this context, the development of a sensitive, rapid and cost effective method for monitoring caffeine is greatly needed.

Conventionally, high-performance liquid chromatography (HPLC) separation [8] and UV-spectrophotometric detection [9], methods are applied to both regular and decaffeinated
green and roasted coffee beans for caffeine content determinations. Other methods such as capillary electrophoresis [10], thin layer chromatography (TLC) [8] and gas chromatography (GC) [11], are used for separation of caffeine in the analysis of mixtures, combined with detection methods such as mass spectrometry [11] and FTIR spectrophotometry [12]. However, expensive instrumentation, highly skilled technicians and complicated and time-consuming procedures are required for such methods. Another possible technique is flow injection immunoassay using a solid phase reactor, which makes the assay faster because no separation step is needed [13]. However, the time and cost for monoclonal antibody production and purification, and the need for their manipulation with extreme care, are the disadvantages of this approach. A biosensor based on inhibition of 3,5-cyclic phosphodiesterase (CPDE) from bovine heart in combination with a pH electrode for the detection of caffeine in coffee was reported by Pizzariello et al. [14]. The development and application of new caffeine detection methods remains an active area of investigation, particularly in food and clinical chemistry. Significant research and development activity has been devoted to preparing compact analytical devices comprising a bioactive sensing element integrated with a suitable transducing system, known as biosensors, for determination of various inorganic, organic and biological substances. The main advantages of these devices are their specificity, sensitivity and ease of sample preparation, and the fact that no other reagents besides a buffer and a standard are usually required [15]. With these advantages in view, investigations have been carried out in this work to develop a microbial biosensor for the estimation of caffeine in food and beverage samples.

1.1. Principle of microbial based biosensor for the detection of caffeine

Oxidase enzymes utilize molecular oxygen for oxidation of substrate. This oxygen consumption can be monitored when these enzymatic reactions are brought about in the vicinity of a dissolved oxygen electrode. In microorganisms, the enzymatic degradation of caffeine is brought about by sequential demethylation by an oxygenase, into theobromine or paraxanthine. A stoichiometric relation exists between the amount of caffeine converted by the microorganisms and the amount of oxygen consumed based on which, the amount of caffeine in the sample can be determined.

Caffeine $+ \text{O}_2$ $\rightarrow$ Product

2. Materials and methods

Caffeine, theophylline, theobromine, gelatin, glutaraldehyde and para chloro mercuri benzoic acid (Ultra Pure) were obtained from Sigma Chemicals, St. Louis, U.S.A. Analytical grade, di-nitro phenyl hydrazine, polyvinyl alcohol and polyvinyl pyrrolidone were procured from Sisco Research Laboratory Chemicals, Mumbai, India. Dehydrated nutrient agar and nutrient broth (AR) were obtained from M/s Himedia Labs, Mumbai, India. All other chemicals were of high purity and were procured from standard sources.

A microbe previously isolated and characterized in our laboratory as Pseudomonas alcaligenes MTCC 5264 [16], which was found to have potent caffeine degradation capability was used for the studies. The isolate was cultivated in a modified nutrient broth containing peptone (1.5 g L$^{-1}$), beef extract (5 g L$^{-1}$), yeast extract (1.5 g L$^{-1}$), sodium chloride (5 g L$^{-1}$) and caffeine (0.3 g L$^{-1}$) adjusted to pH 7.2 to obtain biomass.

2.1. Induction of the organism for caffeine degradation

The caffeine degrading enzyme system has been reported to be highly inducible [17]. The induction was effected by harvesting the biomass and incubating in M-9 medium [18], which was modified by incorporating 0.1% (w/v) caffeine.

The microbial cells accumulated in the nutrient broth were harvested towards the end of logarithmic phase of growth (about 90 h) by centrifugation at 16,000 $\times$ g and 4°C for 30 min. The microbial cells accumulated in the nutrient broth were harvested towards the end of logarithmic phase of growth (about 90 h) by centrifugation at 16,000 $\times$ g and 4°C for 30 min.

The microbial cells were harvested towards the end of logarithmic phase of growth (about 90 h) by centrifugation at 16,000 $\times$ g and 4°C for 30 min. The microbial cells harvested were then washed thrice with cold phosphate buffer (100 mM), pH 7.2 and aseptically transferred into a 500-mL flask containing 100 mL of caffeine liquid medium (CLM) containing 1 g L$^{-1}$ of caffeine. This was incubated at 30°C in an orbital rotary shaker (150-rpm) for a period of 48 h. The cells were then harvested by centrifugation for 30 min at 16,000 $\times$ g and 4°C. The pellet was washed 4–5 times with phosphate buffer and stored at 4°C until further use.

2.2. Construction of the caffeine biosensor

The biosensor comprises the biological sensing element, the transducer, amplification and detector systems as shown in Fig. 1, which was earlier reported [19].

Fig. 1. Schematic diagram of the whole cell electrode used for the biosensor.
2.3. Immobilization of whole cells

Immobilization of microbial cells was done with three immobilizing agents namely gelatin [19], polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) [21]. using the following steps.

The harvested microbial cells were washed 4–5 times. A 10% (w/v) suspension of the induced cells was prepared in phosphate buffer (pH 6.8).

Immobilization of the cells using gelatin was done by pipetting 50 μL of cell suspension on to a cellophane membrane (2 cm × 2 cm) and 50 μL of a 10% (w/v) of gelatin and mixing well with a fine glass rod by avoiding air bubbles. To this cell matrix mixture 25 μL of 5% aqueous solution of glutaraldehyde was added and mixed well. The cells were allowed to crosslink with the immobilizing agent (gelatin) on the membrane by incubation at room temperature for 1 h. The immobilized cell membranes were then washed thoroughly with distilled water and phosphate buffer (pH 6.8) and stored at 4°C till further use.

For immobilization of the cells using PVP and PVA, 50 μL of the cell suspension and 100 μL of PVA and PVP, respectively, were added to the membrane and mixed with a glass rod to form a fine layer of the cells. The membranes were allowed to set at room temperature for 1 h. These membranes were preserved in buffer at 4°C till further use.

2.4. The biological sensing element

In order to construct the biosensor for caffeine, immobilized cells having caffeine degrading activity sandwiched between two semi permeable membranes were fixed on to the electrode surface by using an ‘O’ ring shown in Fig. 1 [19]. Gas permeable teflon membrane (WTW, Germany) was used as the inner membrane in the sensor element and cellophane membrane (3000–6000 MWCO; Gambro, Lund, Sweden) was used as the outer membrane. Electrode poisoning due to electrochemically interfering compounds like metal ions and ascorbic acid and metal chelating agents like citric acid, is avoided chemically interfering compounds like metal ions and ascorbic acid and metal chelating agents like citric acid, is avoided chemically interfering compounds like metal ions and ascorbic acid and metal chelating agents like citric acid, is avoided chemically interfering compounds like metal ions and ascorbic acid and metal chelating agents like citric acid, is avoided during real sample analysis due to the selective permeability of the teflon membrane to gases only. A 10 mL glass container with 2.5 mL phosphate buffer was used as the sample cell. Air was continuously bubbled using a simple aquarium pump to keep the contents mixed as well as oxygen supplied continuously.

2.5. Construction of the transducer-amplifier-detector system

In order to apply the potential to the electrode and to process the signal from the electrode, a Clarke type dissolved oxygen (DO) sensor (EDT Instruments, UK) was used. The reduction of oxygen at the cathode due to the biochemical reaction gives an output voltage/current, which is proportional to the oxygen concentration in solution, which can be correlated with the analyte concentration.

2.6. Measurement of response by immobilized microbial cells for caffeine

Microbial (immobilized) cells having caffeine degrading activity were taken for construction of microbial biosensor for caffeine analysis in tea and coffee samples. Immobilized membrane was tied on the tip of the DO probe and bubbled continuously. Initially, the electrode kept in the sample-cell containing buffer was polarized for 1 h. Then 100 μL of the sample containing caffeine (0.1%, w/v) was injected and the decrease in DO with time was recorded.

The response of the DO electrode as decrease of DO at steady state was plotted against the caffeine concentration (% w/v) on X-axis and electrode response (Δ %DO) on Y-axis. The immobilized cell membranes retained 50% of the initial activity after 30 days when stored at 4°C.

2.7. Response of xanthine oxidase to caffeine

The enzyme involved in the first step of degradation of caffeine has not been characterized, but reports are available on the further degradation of caffeine to xanthine, xanthine oxidase, uricase, allantoinase, allantoinase, glyoxylate dehydrogenase and urease [5]. To check for the interference of xanthine oxidase activity (XOD) in the determination of caffeine, XOD was immobilized and its response to caffeine, theobromine and paraxanthine (0.1%, w/v) was recorded.

2.8. Optimization of parameters for the biosensor

2.8.1. pH

Effect of pH on the activity of the immobilized cells was studied by incubating the membranes in buffers in the pH range of 5.0–8.0 with an increment of 0.5 pH units and response of the membrane to 1% (w/v) of caffeine was recorded as depletion of oxygen.

2.8.2. Temperature

The optimum temperature for the caffeine response was determined by incubating the immobilized membranes at different temperatures (32, 35, 40, 45 and 50°C) in shaking water (Julabo bath) and the response to 1% (w/v) of caffeine was recorded.

2.8.3. Interference of different sugars in caffeine estimation

The response of immobilized (P. alcaligenes) to different sugars viz. sucrose, glucose and fructose was studied by injecting the samples (1%, w/v) and the response was recorded.

2.8.4. Studies on inhibition of glucose uptake by the immobilized cells

Three known glucose uptake inhibitors viz., P-chloromercuribenzoate, sodium bisulphate and Dinitrophenyl hydrazine at 0.1% (w/v) concentration were injected into the sample cell during analysis of caffeine and the response of the biosensor to caffeine was recorded.
2.8.5. Calibration for caffeine using HPLC and biosensor

Calibration graphs for caffeine were plotted for the analysis of caffeine in samples by the biosensor and by HPLC. Calibration graph for caffeine analysis by the biosensor in the concentration range of 0.01–0.1% (w/v) was plotted by injecting 100 μL of standard caffeine solution at concentrations of 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1% (w/v) of caffeine and recording the decrease in dissolved oxygen (%DO drop) concentration of the sample. A graph of concentration versus decrease in dissolved oxygen was plotted with caffeine concentration on X-axis and %DO drop on Y-axis.

Calibration graph for caffeine analysis by HPLC in the concentration range of 0.01–0.1% (w/v) was plotted by injecting 10 μL of standard caffeine solution at concentrations of 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1% (w/v) of caffeine and recording the peak area of the sample. A graph of concentration versus peak area was plotted with caffeine concentration on X-axis and peak area on Y-axis.

2.8.6. Analysis of caffeine in real samples by HPLC

HPLC analysis of caffeine was done using a Shimadzu-LC 10A fitted with a C18 Column (300 mm × 4.6 mm) with acetonitrile and water (10:90%) as mobile phase set at a flow rate of 1 mL min⁻¹ and a UV detector set at 273 nm. Real samples (coffee and tea sample) were analyzed for their caffeine content. Samples of tea and coffee were prepared as described below and the concentration was determined by recording the peak area and comparing with the calibration graph.

2.8.7. Preparation of real samples for determination of caffeine

Instant coffee and tea were prepared by dissolving 0.5 g of each in 125 mL boiling water [20] and were kept for 30 min on a magnetic stirrer and filtered using a Whatman filter Paper No.1. A 100 μL of the sample was then injected and the response was recorded. The commercial cola samples were injected without any treatment. For HPLC analysis 10 μL of the sample was injected and the peak area was recorded.

In order to reduce the interference of glucose present in the sample, the sample was passed through a packed column containing glucose oxidase enzyme immobilized on glass beads prior to injection to the biosensor.

The caffeine content in the samples was calculated using the Eqs.(2) and (4) for biosensor and HPLC, respectively.

3. Results and discussion

3.1. Construction of caffeine biosensor

3.1.1. Immobilization of whole cells

For immobilization of cells, different agents viz. gelatin, polyvinyl pyrrolidone and polyvinyl alcohol were tried. The biosensor gave good response for caffeine (1%, w/v) showing a steady state depletion of 53.2% DO concentration as shown in Table 1. It was observed that polyvinyl alcohol is a better immobilizing agent in terms of a higher response and reusability, which may be due to a higher oxygen permeability and diffusivity [21]. With PVA as stabilizing agent, about 15–25 analyses could be done with a single membrane.

### Table 1

<table>
<thead>
<tr>
<th>Stabilizing agent</th>
<th>%DO depletion shown by the biosensor after 3 min (1%, w/v)</th>
<th>No. of analyses with one sensor element</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>Polyvinyl pyrrolidone</td>
<td>1.6</td>
<td>2</td>
</tr>
<tr>
<td>Polyvinyl alcohol</td>
<td>53.2</td>
<td>15</td>
</tr>
</tbody>
</table>

3.1.2. Response of biosensor to caffeine

The response of the biosensor to 1% (w/v) of caffeine is shown in Fig. 2.

For the determination of caffeine content in the samples using the immobilized cells based biosensor, a calibration graph for caffeine in the concentration range of 0.01–0.1% (w/v) was plotted with a regression value of 0.996 using HPLC.

The concentration of caffeine present in the sample can be calculated by using Eq. (4) derived from the calibration curve:

\[ y = 4453x - 20.323 \]  
\[ x = \frac{y + 20.323}{4453} \] (4)

Fig. 2. Response of the biosensor to 1% (w/v) of pure caffeine.
where ‘y’ is the % dissolved oxygen consumed and ‘x’ is the concentration of caffeine in the sample.

3.1.3. Optimization of parameters for the biosensor

The following parameters were studied to develop the biosensor for caffeine estimation.

The immobilized cell had a maximum activity at pH 6.8 (Fig. 3). Therefore, phosphate buffer (100 mM) at pH 6.8 was used for further studies on the biosensor for caffeine. The optimum pH for raising the biomass was 7.2. At this pH, maximum biomass accumulation was observed. Therefore, the cells were induced for caffeine degradation at pH 7.2. However, maximum caffeine degrading activity was observed at pH 6.8 as the optimum pH for activity of caffeine degradation enzymes is 6.8 (results not shown).

The optimum temperature for the caffeine response was found to be 32°C. The response of the immobilized cells based biosensor gradually increased up to a temperature of 35°C, but above this temperature the activity gradually decreased and the cell membrane was inactivated at 50°C (Fig. 4).

3.1.4. Interference of different compounds in caffeine estimation

Glucose was found to interfere with the estimation of caffeine whereas the other sugars sucrose and fructose showed less interference (Table 2). So elimination of glucose in real samples is needed for the efficient estimation of caffeine. Introduction of an immobilized glucose oxidase membrane can be a good method for the elimination of interference due to glucose.

Immobilized xanthine oxidase did not respond when caffeine was injected, but there was a notable response when theobromine and theophylline were injected (Fig. 5). This proved that the degradation of caffeine was not brought about by xanthine oxidase as reported earlier [22], but another oxygenase enzyme, which is supposedly caffeine demethylase, is responsible for the degradation of caffeine.

Table 2

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Interfering agent (1%, w/v)</th>
<th>Response (% DO drop)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Theophylline 2.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Theobromine 5.50</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Sucrose 4.00</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Glucose 5.00</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fructose 0.20</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Glycine 1.20</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Tyrosine 0.60</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Histidine 1.10</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Ascorbic acid 1.50</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Catechin 1.20</td>
<td></td>
</tr>
</tbody>
</table>

*Response to 1.0% (w/v) caffeine = 20.6.

3.1.5. Analysis of caffeine in real samples by biosensor and by HPLC

Glucose was found to interfere with the estimation of caffeine whereas the other sugars sucrose and fructose showed less interference (Table 2). So elimination of glucose in real samples is needed for the efficient estimation of caffeine. Introduction of an immobilized glucose oxidase membrane can be a good method for the elimination of interference due to glucose.

Immobilized xanthine oxidase did not respond when caffeine was injected, but there was a notable response when theobromine and theophylline were injected (Fig. 5). This proved that the degradation of caffeine was not brought about by xanthine oxidase as reported earlier [22], but another oxygenase enzyme, which is supposedly caffeine demethylase, is responsible for the degradation of caffeine.

Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Caffeine concentration (% w/v)</th>
<th>%Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instant coffee</td>
<td>0.056 (0.0015) 0.052 (0.004)</td>
<td>(+) 07.69</td>
</tr>
<tr>
<td>Commercial coffee sample 1</td>
<td>0.030 (0.01) 0.033 (0.002)</td>
<td>(-) 10.09</td>
</tr>
<tr>
<td>Commercial coffee sample 2</td>
<td>0.035 (0.0035) 0.034 (0.004)</td>
<td>(+) 02.94</td>
</tr>
<tr>
<td>Commercial tea sample 1</td>
<td>0.056 (0.0035) 0.038 (0.002)</td>
<td>(-) 18.26</td>
</tr>
<tr>
<td>Commercial tea sample 2</td>
<td>0.076 (0.0043) 0.072 (0.003)</td>
<td>(+) 05.55</td>
</tr>
<tr>
<td>Cola drink 1</td>
<td>0.042 (0.003) 0.047 (0.002)</td>
<td>(-) 10.63</td>
</tr>
<tr>
<td>Cola drink 2</td>
<td>0.046 (0.021) 0.050 (0.002)</td>
<td>(+) 08.00</td>
</tr>
</tbody>
</table>

Figures in parenthesis represent the standard deviation of the sample analysis.
Analysis of caffeine contents of real samples was done by using HPLC and microbial biosensor and presented in Table 3. All the analyses have been done in triplicates and the average of the three analysis is presented in the Table 3. The biosensor results were in correlation with HPLC results. The spectrophotometric results showed a high error% indicating matrix interferences. The samples need prior treatment for analysis by HPLC and spectrophotometric methods. The error% was calculated taking HPLC as standard.

4. Conclusions

An amperometry based microbial biosensor for the analysis of caffeine in beverages and fermentation samples was developed using immobilized whole cells and cell debris of sonicated cells of the isolate. The biosensor was found to have maximum efficiency at a pH of 6.8 and a temperature of 32°C. However, the biosensor showed a good response at a temperature of 30 ± 2°C. Sugars like glucose and sucrose were tested for interference in the results and were found to interfere in the analysis. An approach for the removal of these sugars prior to injection in the biosensor was tested by passing the sample through a packed column of immobilized glucose oxidase enzyme. Tests on the use of glucose uptake inhibitors like 2-chloromercuri benzene, sodium sulphate and dichoro phenyl hydrazine were carried out and it was found that the inhibitors also inhibited the analysis of caffeine. Further work is to be carried out on increasing the selectivity of the immobilized system to caffeine.

4.1. Perspectives

Caffeine biosensor has lot of potential applications in tea, coffee and other beverages along with pharmaceutical caffeine based products. The biosensor devised for the analysis of caffeine with a working range of 0.01 to −2% (w/v) of caffeine is found to have large number of commercial applications. Industries such as coffee, tea and soft drinks where the concentration of the caffeine is to be monitored for safety of human health and the level of caffeine is of critical importance. In view of these facts, caffeine biosensor will have potential market world over.

This device helps overcome the problem of time consuming and tedious sample preparation and analytical procedures commonly involved in conventional analysis methods. The main advantage of this method is the fast response time, which enables the quick estimation of caffeine in real samples. Presently work is being carried out in our laboratory on the purification of the enzyme involved in the utilization of caffeine and future work would be carried out on the development of an enzyme based biosensor for the determination of caffeine. The biosensor is anticipated to be free of interferences and enable more analyses in a shorter time.

Acknowledgements

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References

Paper VIII
A Biosensor for On-line Analysis and Control of an Acetonitrile Degrading System
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Abstract
A biosensor system, based on an immobilised mixed culture of microorganisms in combination with a dissolved oxygen electrode, was developed for the purpose of on-line monitoring and control of an acetonitrile degrading process. Feedback control was used to vary the feed rate of acetonitrile. Problems that occurred were inhibition of the bacteria during too frequent analysis and clogging of filters and tubings during continuous dilution. The inhibition was reversible when buffer was introduced into the system and by using manual dilution the problem of clogging disappeared.

The biosensor showed very good performance provided it was used with a frequency of 1 sample per hour and during future work an automatic dilution step just prior to analysis can be introduced to prevent the clogging.

Introduction
Microorganisms are capable of degrading a broad range of organic compounds, including those that are toxic (Guieysse et al. 2000; Yuan et al. 2000; Lakhwala et al. 1992; Guieysse et al. 2001, Nawaz et al. 1989; Chapatwala et al. 1993; Chapatwala et al. 1993; Babu and Chetty 1994; Chapatwala et al. 1995; Ramakrishna et al. 1999; Håkansson and Mattiasson 2002). However, when dealing with toxic compounds, it is often seen that what is observed as being a substrate at a low concentration appears to be toxic for the cells at higher concentrations. This fact has limited the applicability of cells for degrading toxic compounds due to problems with dosage of the substrate to suitable concentration levels- a too low concentration will mean that very little degradation takes place and a too high concentration means that the microorganisms may die. There is thus an interesting challenge for the biochemical engineer to address if microbial reactors shall be used for degradation of toxic compounds.

Several means to improve the robustness of a bioreactor can be used. To operate with immobilised cells where the cells grow in dense populations will help to increase the survival rate to sudden spikes in concentration of toxic compounds and a well-controlled feed rate of the toxic substrate to the reactor is another mode to improve the performance of the degradation system. To reach the latter one needs proper monitoring and control. One aspect may be to use biosensors that will also react to any metabolites being produced during the degradation process.

Biosensors combine biological sensing elements (e.g. enzymes, cells) with a suitable transducer (e.g. Clark oxygen electrode) thereby converting a biochemical signal into a quantifiable electrical response. Cells can be immobilised directly on the electrode (Håkansson and Mattiasson 2004) or to separate electrode and reaction chamber
(Nandakumar and Mattiasson 1999). The biosensor record the metabolic activity of the cells in response to the sample to be analysed (Heim et al. 1999; D'Souza 2001).

Biosensors based on oxygen electrodes have been reported for toxic compounds like phenol (Orupold et al. 1995; Nandakumar and Mattiasson 1999), naphthalene (Reshetilov et al. 1997), acrylonitrile, acrylamide (Ignatov et al. 1996; Ignatov et al. 1997) and acetonitrile (Håkansson and Mattiasson 2004). When the substance to be analysed comes into contact with the immobilised cells on the electrode the cell respiration increases, thereby decreasing the oxygen concentration. The oxygen consumption is proportional to the amount of the analysed substance in the sample.

Among the different sensors that can be employed in monitoring and controlling of a bioreactor, the group of biosensors have some very attractive features. If the sensors are based on the same cells as those present in the bioreactor, then one may have a possibility to monitor the physiological status of the cells in the reactor during operation. We have earlier demonstrated the possibility to monitor acetonitrile by using a biosensor based on immobilised cells in proximity to an oxygen electrode. This work has now been extended to use the electrode to monitor and control the concentration of the compound to be degraded in the bioreactor.

**Materials and methods**

*Acetonitrile degradation*

A suspended-carrier biofilm process (Håkansson and Mattiasson 2002) was used for degradation of acetonitrile. Bacteria from activated sludge were grown as a biofilm in two reactors with different pH, 8.5 in the first reactor (A) and 7.5 in the second (B). The waste from reactor A was pumped into reactor B for further degradation.

*Biosensor*

Bacteria from reactor B were immobilised between two Teflon membranes and put on top of a Clark electrode (Håkansson and Mattiasson 2004). The biosensor was mounted in a flow cell and integrated into a flow injection system (figure 1). Phosphate buffer was continuously pumped through the flow cell at 1 ml/min.
Online analysis

The system consists of a biosensor, an electronic injection valve (Cheminert C22 31860, Valco Instruments Co. Inc, Switzerland), a pump (Ismatec, Switzerland), a potentiostate (Záta-elektronik, Sweden), and a computer. The computer was a Pentium PC equipped with Windows 98 operating system, 128 MB RAM, a PCI-MIO-16E data acquisition card (National instruments, USA), and LabVIEW 6i software (National instruments, USA).

The data acquisition was developed using LabVIEW. The software was devised for online data acquisition with programmable scan rate, storage and retrieval of data, multipoint linear calibration, base-line drift compensation and real time graph capabilities.

The pump was controlled from the software using serial communication. The software was devised to vary the flow rate, the direction of pumping and also to read the set flow rate on the pump. Using the digital outputs of the PCI-MIO-16E card, the valve actuation was controlled through the 2 position valve actuator control module.

Quantification of the data from the sensor was carried out, both in terms of peak area and peak height, employing multipoint linear calibration. The sampling from the reactor to the sensor was controlled by the computer and occurred automatically through the injection valve. The software generated reports, which were read using Microsoft Excel.

The schematic flow chart for online control of acetonitrile degradation is shown in figure 2. The program was devised for analysis of acetonitrile at user specified regular intervals and it accepts the flow rate step as desired by the user. Based on the response from the sensor the flow rate of the medium was changed either by a positive or a negative step in each run to utilise the full capacity of the two-reactor system by using feedback control.
Acetonitrile solutions in different concentrations ranging from 10-150 mg/l were injected into the sensor system. The response to these standards was used to make a calibration curve. Each standard was analysed in duplicate and between every analysis, medium was pumped through the injection loop to ensure that there was no residues left that could interfere with the next analysis.

The agreement of the sensor response with known concentrations of acetonitrile was studied by analysing the response for 75 mg acetonitrile/l, 150 mg acetonitrile/l and again 75 mg acetonitrile/l.

When using the sensor online, samples were filtrated through a MGB filter paper (Munktell filter paper, KEBO, Sweden) to prevent clogging of the tubing and the injection loop. However, often the filters were clogged instead, giving rise to a lower response of the sensor. Therefore the system was evaluated by several experiments. On-line analysis and control was evaluated by using known concentrations of acetonitrile. The starting concentration was 25 mg/l and then the concentration was increased or decreased as follows:

<table>
<thead>
<tr>
<th>Acetonitrile (mg/l)</th>
<th>Set concentration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/l</td>
<td>45-50 mg/l</td>
</tr>
<tr>
<td>75 mg/l</td>
<td>70-80 mg/l</td>
</tr>
<tr>
<td>50 mg/l</td>
<td>45-50 mg/l</td>
</tr>
<tr>
<td>0 mg/l (phosphate buffer)</td>
<td>0-10 mg/l</td>
</tr>
</tbody>
</table>

Samples were diluted manually and analysed every 5th minute to evaluate if the system would work better with a fast, time controlled dilution step instead of a continuously dilution. The process was controlled on-line by the response of the sensor. Samples were also diluted manually and analysed every 60th minute to evaluate if the sensor was inhibited when analysis was carried out too often.
Results
The sensor response for 150 mg acetonitrile/l was 152.90 ± 5.95 and the response for 75 mg acetonitrile/l was 73.22 ± 3.65 (fig. 3). This shows that the sensor response is within an acceptable range compared to the known concentration. The variation for the sensor response is depending on the activity of the cells. Problems that can occur during measurement and thus affecting the cell activity could be completely or partly clogging of the tubes due to precipitation or growth.

![Figure 3](image.png)

**Figure 3.** The known concentration compared to the concentration given by the sensor.

The biosensor system was efficient in analysis and control of known concentrations of acetonitrile. The flow rate was increased or decreased until the set concentration range was reached and stable as long as the concentration was within the set range (figure 4). The sensor response also went down to zero if only phosphate buffer was injected as sample, indicating that the sensor response was accurate.
Figure 4. The acetonitrile concentration and flow rate during on-line analysis and control of known concentrations of acetonitrile. The concentration and set concentration range was A) 50 mg/l and 45-55 mg/l, B) 75 mg/l and 70-80 mg/l, C) 50 mg/l and 45-50 mg/l and D) 0 mg/l and 0-10 mg/l.

Figure 5 shows the results during measurement of reactor A when the concentration and flow rate allowed was set to 500 - 600 mg/l and 0.75 – 1.25 ml/min respectively. Samples were taken and diluted manually every 5th minute and the mean concentration of three measurements was used for control of the flow rate. The results show that the system works better with a fast, time controlled dilution step instead of a continuous dilution, during which biofilm is formed in the tubings and the dilution chamber (data not shown). The shape of the peaks went from high narrow peaks to low broad peaks. This could be due to either that the bacteria had been acclimated to the organic load or that it was inhibiting for the immobilised bacteria, resulting in a lower sensor response and thus a lower concentration. When buffer was injected instead of sample the peak shape went back to high narrow peaks, indicating that the immobilised bacteria were reversibly inhibited.
Figure 5. The acetonitrile concentration in reactor A and the flow rate for the medium. Samples were analysed every 5th minute and the mean concentration of three measurements was used to control the flow rate. The concentration decreased even if the flow rate increased, indicating that the system was inhibited.

Figure 6 shows the results during measurement of reactor A when the concentration and flow rate allowed was set to 1000 - 14000 mg/l and 0.75 – 1.50 ml/min respectively. Samples were taken and diluted manually every 60th minute. The system reached a stable concentration 5 hours after the flow rate was stable and no inhibition of the bacteria occurred. The results also indicated that it might be enough with an analysis every 6th hour to be able to see the effects of the changed flow rate.

Figure 6. Samples were analysed every 60th minute and the flow rate was changed after every analysis if necessary. In this case there was no inhibition of the system, indicating that it is better for the bacteria when analysis is carried out more seldom.
Discussion

Biosensor controlled monitoring of a bioreactor process needs to meet certain criteria in order to become successful. The use of the same cell population when constructing the sensor as present in the biodegradation reactor is often a great advantage. However, also other needs must be fulfilled before it can be regarded as successful. The analysis must be specific in the sense that the signal registered is reflecting the concentration of the target molecule for the assay. In the case of acetonitrile, there was initially a problem in the sense that the sensor also responded to acetate, which is produced in the degradation process. In a previous study it was shown that the influence could be removed by the introduction of a specific membrane that rejected acetate (Håkansson and Mattiasson 2004). Furthermore, the sensor response must be reliable and reproducible.

Flow injection analysis is a practical method for reliable and reproducible sampling. The sample is filtered, injected into a continuous buffer stream and transported to the biosensor. However, problems may occur. Some of them are clogging of the filter thus preventing the analyte to pass through due to a change in pore size, erroneous response due to diluted/undiluted samples, lack of dissolved oxygen, microbial growth in the connection tubing and drift or deactivation of the sensor during time (Schügerl 1993, Mulchandani and Bassi 1995).

It was observed that upon very frequent assays there was a memory effect in the biosensor in the sense that the response went down successively. This was interpreted as being a result of a carry over from the previous assay to the next one. The sluggishness of cell-based biosensors may be a problem since the cell population is not ready for a new assay until the metabolites from the previous sample have been metabolised. This leads to that cell-based biosensors degrading toxic compounds may be an attractive alternative when not frequent analyses are needed, and this may be the case when more recalcitrant compounds are to be degraded. It was demonstrated in this study that the electrode had a very good performance provided it was used with a frequency of e.g. a sample per hour, while when used with a frequency of ten samples per hour there was a successive decay of the signal due to inhibition. The inhibition was reversible when switching back to buffer. This is of special value when cultivating microorganisms on toxic substrates, as dosage of substrate becomes a critical parameter; too much inhibits everything while too little gives no cell growth (Thavarungkul et al. 1991).

Still another problem appeared, fouling on the inner walls of the tubings and on membranes and filters. When dealing with enzyme based biosensors one can always add a bacteriocide that will prevent cell growth during the transport period. This is not a suitable approach when the same type of cells are present in the biosensor as those taken from the bioreactor and causing problems by adhesive growth on the inner walls of tubings etc. Thus, one needs some better means to prevent cell fouling without interfering with the cells in the biosensor. Furthermore, since fouling appeared in membranes and filters when cells should
be removed, it turned out to be important that one finds a good solution to handle the presence of free cells in the samples taken from the reactor. One solution to this problem could be to apply an automatic dilution step where samples are taken from the reactor just prior to analysis and automatically diluted before entering the injection loop. With such a system the tubings and filters may be washed with water or buffer between every sampling, thus eliminating microbial growth.

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References


Automated Versatile Continuous Flow System for Bioanalysis and Bioprocess Control

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Abstract

Bioanalysis may involve biosensors as sensing systems. A major requirement in bioprocess monitoring is real time information about various process parameters like concentrations of products, substrates, metabolites, inhibitors etc. Flow injection analysis provides a convenient platform for employing biosensors for monitoring bioprocesses. Depending on the application, the monitoring requirements vary resulting in need for different flow schemes for measurement. Common flow schemes employed with biosensing are FIA, SIA, BIA and multi-commuted flow. A versatile platform to employ biosensors for continuous analysis of bioprocesses with precise control of flow, volume and defined events has been developed. The system is based on National Instruments LabVIEW and employs piston, peristaltic pumps, motorized injection valves and 2-way, 3-way solenoid valves. The system supports data acquisition, controls interface with devices and visualization of data from multiple detectors used in biosensing. The system was successfully evaluated on analysis of a model analyte, human antibody IgG, employing continuous flow ELISA in a competitive mode with optical detection. The flow immunosensor showed very good linearity from 5 µg/ml to 400 µg/ml. The system had excellent reproducibility and stability with S.D. values of 0.0274. The immunosensor was also subjected to a model first order kinetics of target analyte concentration and the results from the sensor agreed well with the estimated concentration. The system being very flexible, different flow configurations are easily set up and the dynamic range of analysis can be adapted by varying different flow conditions for measurement, thereby controlling different reaction times in the biosensor system. The system is useful for online analysis and control of bioprocesses employing biosensors. Further, with automated data collection and analysis, the system provides excellent basis for cross platform data communication for integrated control.

Keywords

Online monitoring, immunoassay, biosensors, automation, ELISA, bioanalysis, bioprocess, FIA, SIA, LOV

Introduction

Flow injection analysis technique has been very popular in synergising the benefits of sampled analysis and specificity of biosensors. Flow injection is adapted for process analysis because of their suitability for automation and short response times (Schugerl, 2001). Over the years different forms of Flow injection (FIA), sequential injection (SIA), multi-commutation flow, Lab on Valve (LOV) and bead injection analysis (BIA) have evolved to address different needs of analysis employing biosensors (Scheppe et al., 1993, Shu et al., Kumar et al., 2001, Inaba et al 2003, Rui et al., 2003, Katrilik et al, 2006). Biosensors offer several advantages in monitoring bioprocess systems. They offer detection of compounds ranging from low molecular weights especially the enzyme based sensors, to determining higher molecular weights substances based on immunosensors (Mattiasson, 1990).
Further SIA and lab-on-valve concepts can be used for multi-analyte determination which is essential for bioprocess monitoring. Multi-commutation schemes are specifically employed for the analysis of pesticides and other toxic compounds of environmental importance employing biosensing elements coupled with detection techniques like chemiluminescence, absorbance measurements or amperometry. (Bucur et. al., 2005, Kumar et. al, 2006, Valdés-Ramírez et. al., 2009). The coupling of different sensitive techniques like optical and electrochemical techniques, with the selectivity of biosensors or immunosensors gives possibilities for rapid and automatic assays of complex material like food, biological and pharmaceutical samples (Perez-Olmos et. al, 2005, Nilsson et.al., 1992). Quantification of protein during production of recombinant protein to its extraction and purification is important. There is a need for sensitive, robust analytical system to accommodate the wide analysis range of protein during production to analysis of trace impurities. Immunosensing has demonstrated very sensitive assays even for analysis of trace impurities in biopharmaceutical production (Mattiasson et. al, 2010). Flow immunosensors for direct determination of canine immunoglobulin by using quartz crystal microbalance has been reported (Arce et. al., 2007). A sandwich immunoassay has been reported for determination of IgG (Rhee et. al., 1997). In situ analysis are not possible with these sensors and hence flow systems are employed to couple the sensor to the reactor for online monitoring.

Online analysis:
Bioprocess monitoring requires continuous, reproducible measurement system to minimise errors in handling and decrease analytical time. Another requirement for the biosensor is means of recalibration to extend the useful life of the sensor. The focal theme of the present work is the development of an Automated versatile continuous flow system (AVCFS) for bioanalysis which is flexible and yet robust so as to adapt to different requirements for process monitoring and control. The system was based on National Instruments LabVIEW and was tested for online analysis of antibody IgG employing a heterogeneous immunoassay in a competitive flow ELISA mode. Production of recombinant protein is also on the rise and the requirements of bioprocess monitoring and control in such processes is crucial (Surrivas et. al., 2006, Ahlqvist et. al., 2006, Kuprijanov et. al., 2009). A two step mode of immunoassay was employed. The analysis of IgG with the system showed very good reproducibility and stability (RSD of < 2.75%) and the sensor column could be reused for more than 150 injections. Being flexible, the system was easily adaptable for different flow conditions without any physical change in the flow system, resulting in different reaction times of the sample and reagents with the biocatalyst. This made it possible to operate the same immunosensor column for lower as well as higher concentration range of samples. A stability study of the sensor for continuous analysis was also performed. Further, the immunosensor was challenged for continuous analysis of the analyte by simulating a first order kinetics of the sample concentration in a stirred reactor.
Experimental Overview of the AVCFS

Bioanalysis system for bioprocess monitoring requires sampling devices, constant flow equipment, injection device, a reactor and holding coils for interaction with the biocatalyst, cell lysing system, and detection system. Further, bioanalysis involves reactor columns, holding coils and flow cells, wherein the biocatalyst (like column with immobilized enzymes, antibodies or microbial cells) is employed. There also could be a need for intracellular enzymes to be released for analysis. The control of the operation of a sonicator could be useful. Multiple measurement methods ranging from IR spectroscopy, software state estimation, physical measurements are used for monitoring a bioprocess. In all, there is a need for an integrated approach of sensing to increase the robustness of analysis and also provide a platform to interlink to other measurement and controls used in bioprocesses.

The AVCFS system has complete control of the pumps and valves. It is flexible in terms of choosing the number and type of pumps, valves and the detectors to be employed. The operations of the pump - start/stop, flow rate and direction are controlled and so are the different positions of the valves employed. The system has control over these devices and events can be set up for precise control of these devices. A friendly user interface is provided for defining flow scheme, system configuration, data visualization and analysis.

Equipment - Hardware

The automated system was setup around National instruments (NI) hardware and LabVIEW software. Two interface cards from National Instruments, Austin, Texas, USA (NI) were used to develop the system namely, NI-USB-6216, and NI 9472. Piston pumps P-500 (Pharmacia, Uppsala, Sweden), peristaltic pumps are VM4 (Watson Marlow, Falmouth, Cornwall, England), Minipuls 3 (Gilson Inc, Middleton, WI, USA) are used. The motorized valves are MV-7 and MV-8 and the solenoid valves, 24V DC are all from Pharmacia. The NI-USB 6216 is a bus-powered USB M Series multifunction data acquisition (DAQ) plug and play module with 16 analog inputs; 400 kS/s sampling rate, two analog outputs; 32 digital I/O lines, digital triggering; and two counter/timers. NI-9472 is an 8-channel, 24V logic digital output module. These cards have been interfaced with a computer with Intel Core i5 Processor, M430, 2.27 GHz, 4 GB RAM, Windows 7, 32 bit running NI LabVIEW 2009, which is an object-oriented software programming tool using a graphical programming environment. The LabVIEW based AVCFS software controls the action of the P-500 pumps using TTL (Transistor Transistor Logic) and continuous pulse signals (Frequency, 0-150 Hz) for flow rate, direction and pump operations. The flow rate of VM-4 and Minipuls 3 pumps are controlled by analog voltage signals (0-1V, DC). The motorized valves are interfaced through two 4-channel SPDT relay board RLY104 (Winford Engineering LLC, MI, USA). Power supply providing 5V, 2A and 15V, 1.5A from Elfa, Järfälla, Sweden was used for relay board and valve circuitry. 24V DC, 5.2 A from Electro-Automatik GmbH, Viersen, Germany was used for the motorized valves. The digital and analog I/O cards support USB interface.

A schematic drawing of the system is shown in figure 1.
System components of AVCFS for bioanalysis and bioprocess control:

The main device components of the developed system are categorized and listed in Table 1.

<table>
<thead>
<tr>
<th>Device Type</th>
<th>Number of devices</th>
<th>Interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pumps</td>
<td>3</td>
<td>0-1V analog and digital signals for Start/Stop, Direction – Clockwise/ Counter-clockwise 3 Pumps</td>
</tr>
<tr>
<td>Piston</td>
<td>2</td>
<td>0-150 Hz pulse for Flow rate, TTL signals for Start/Stop, Direction – Clockwise/ Counter-clockwise</td>
</tr>
<tr>
<td>Syringe</td>
<td>1</td>
<td>RS232 interface</td>
</tr>
<tr>
<td>Valves</td>
<td>4</td>
<td>24 V DC, SPDT Relay contacts for Start Stop, Open collector inputs for positions LOAD, INJECT, WASH</td>
</tr>
<tr>
<td>8 way Motorized</td>
<td>1</td>
<td>24 V DC, SPDT Relay contacts for Start Stop Open collector status for Valve positions POS1 to POS8</td>
</tr>
<tr>
<td>Solenoid Valves</td>
<td>8</td>
<td>24 V DC, 2-way or 3-way</td>
</tr>
<tr>
<td>Detectors</td>
<td>8</td>
<td>Analog 0-1 or 0-10 V or 4-20mA</td>
</tr>
<tr>
<td>Sonicator</td>
<td>1</td>
<td>220 V AC, 3A Relay contact</td>
</tr>
<tr>
<td>Mixer</td>
<td>1</td>
<td>24 V AC, Relay contact</td>
</tr>
</tbody>
</table>

Table 1 Salient device components and interface details of the Flow system
Equipment – Software

The software has primarily six modules – Hardware configuration, Flow program Configuration, Flow scheduler, Hardware driver, Data acquisition and Visualization, and Data analysis and Reporting. The hardware configuration enables to choose the main system components - pumps, valves and detectors for the required flow scheme and the parameters for data acquisition. Depending on the type of flow schemes FIA, SIA, BIA or multi-commuted flow, up to 5 pumps, 4 motorized injection valves, 1 rotary switch valve, eight 24V DC solenoid valves (2-way/3-Way) can be chosen.

The flow program module has a user-friendly interface to define the time events for the pumps, valves and the other hardware chosen. The events for each of the devices can be defined as a function of time in the program. Each event is described by the device name, time, action, flow rate and duration. The pumps have the following actions defined – start, stop, clockwise, counter clockwise. Software has features to control flow rate, operation and direction of each pump. Likewise the different positions of the MV-7 valves – Load, inject and wash can be controlled at defined times as specified by the flow program. The software controls the MV-8 valves which have eight positions for connecting any of the eight ports to the central port. The flow scheduler module, schedules the events at the predetermined times and also displays the progress of each event along with online graphical display of the acquired data. The hardware driver module controls the different pumps, valves and detectors employed through the NI-6216 and NI-9472 interface cards. Graphical visualization of the data acquired from the detector is provided for realtime view of the process. The system has post processing facilities for baseline correction, peak detection, peak height and time, graphical addition, subtraction of data recorded. The system has storage and retrieval facility of flow programs and sensor data.

Reagents

The following reagents were used: CNBr activated Sepharose (CLB-41), protein A-Sepharose gel (5 and 15µl columns), Ab-conjugate - IgG labelled with the enzyme horseradish peroxidase (HRP) (Dakocytomation, Denmark), human IgG (Sigma), Phosphate buffer 100mM, pH 7.4, NaCl 50mM) is used as carrier buffer. 100ml of substrate solution was prepared by mixing 1 ml stock solution 1 (1 ml H2O2 33% + 99 ml water, freshly prepared), 40 ml stock solution 2 (4.05 g phenol + 200 ml water), 10 ml stock solution 3 (0.25 g 4-aminoantipyrin + 100 ml water) and 49 ml carrier buffer (Vojinovic, et al., 2004). Elution buffer was 200mM Glycine – HCl, pH 2.45. De-ionized water was used for preparing all reagents.

Immobilization of protein A on Sepharose 4B

600 mg of the dry CNBr activated Sepharose 4B was suspended in 1mM HCl and washed with 200 ml of the same solution on a sintered glass filter. 15 mg Protein A was dissolved in coupling buffer (5 ml 0.2 M NaHCO3, pH=8.3, 0.5 M NaCl) and was incubated overnight at 4ºC. Unbound Protein A was washed with enough amount of coupling buffer and the remaining active groups were blocked by incubation of the gel in 0.1 M Tris-HCl, pH=8.0 for 2h at room temperature. Finally the gel was
washed 3 times by alternating pH (0.1 M acetate buffer pH 4.0, NaCl 0.5 M followed by 0.1 M Tris-HCl, pH 8.0, NaCl 0.5 M).

The amounts of the protein A before and after immobilization and during the washing step were determined by BCA method. The amount of immobilized protein was determined to be 8.65 mg/ml of the gel. Capacity of the gel for capturing of the Human IgG was determined by incubation of an excess amount of IgG with the defined volume of the gel and the captured protein was calculated to be 28.3 mg/ml of gel.

**Flow System for immunoassay**

The AVCFS system can be used in different formats for bioanalysis like FIA, SIA, multi-commuted flow and bead injection as shown in Fig 2a-c by selecting the different valve and pump combinations.

For exemplifying the system in one of the analysis modes, a flow immunoassay was setup as shown in Figure 3. Pumps P1 and P2 are piston pumps (Pharmacia) for carrier and the elution - glycine-HCl buffers. Three motorized sample injection valves (MV1, 2 and 3) were used for sample, conjugate and substrate respectively. Peristaltic pumps P3, P4 (both Watson Marlow VM4), and P5 (Gilson Minipuls
3) were used for filling the sample, conjugate and substrate loops. All the pumps and valves were controlled by the AVCFS system. A spectrophotometer (Lambda Max Model 481, Waters Corporation, Millford, MA, USA) was used for detection of the enzyme reaction products at 510 nm.

Figure 3 Flow Immunoassay for analysis of Human IgG: P1 and P2 Piston Pumps, P3 to P5 – Peristaltic Pumps, I- Affinity reactor column, C Carrier Buffer, G- Glycine-HCl buffer, W – Waste, MV1 to 3 – Motorized Injection Valve

Measurement of Antibody IgG by Flow immunoassay

The flow immunoassay was used for determining IgG in samples. The affinysorbent, Protein-A Sepharose gel, was packed in a column with a total bed volume of 15 μl. Defined volumes (100 μl) of IgG sample, Ab-conjugate and substrate were sequentially introduced in a controlled manner at defined flow rate of carrier buffer for specific duration of time (Table 1) for interaction within the affinityreactor column. The reagents used - substrate, Ab-conjugate and sample was kept in controlled conditions in an ice pack during the experiment. The carrier buffer contained 0.5% phenol as bacteriocide. The schematic work flow for continuous analysis is as shown in Figure 4.

Figure 4 Format for continuous flow ELISA

Upon passing a sample containing human IgG through the column an amount of IgG proportional to the total content in the sample was bound to the Protein-A. Immediately after the IgG sample, the enzyme conjugate IgG-HRP was introduced and bound to the remaining unoccupied Protein-A available on the gel. After washing the column with the carrier buffer, the enzyme activity was measured by passing 100 μl of substrate to record the absorbance of the pink coloured product at 510
nm. The AVCFS provided the peak height which decreased with the increasing content of IgG in the sample. To regenerate the column the dissociation buffer of glycine – HCl (pH 2.4) was passed for 3 minutes at 1 ml/minute so as to have the affinity column ready for analysis of the next sample. To compensate for any residual conjugated HRP retained in the column in each analysis, substrate was injected prior to the IgG sample injection. A blank was also introduced as a reference. Table 2. lists the time duration for different phases of the assay.

<table>
<thead>
<tr>
<th>Assay Phase</th>
<th>Carrier flow rate (ml/min)</th>
<th>Time Duration (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Substrate (Baseline)</td>
<td>0.15</td>
<td>300</td>
</tr>
<tr>
<td>Rinse</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Blank</td>
<td>1</td>
<td>120</td>
</tr>
<tr>
<td>Substrate injection</td>
<td>0.15</td>
<td>300</td>
</tr>
<tr>
<td>Glycine</td>
<td>1</td>
<td>180</td>
</tr>
<tr>
<td>Equilibration</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Sample injection</td>
<td>1</td>
<td>130</td>
</tr>
<tr>
<td>Rinse</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Conjugate injection</td>
<td>1</td>
<td>120</td>
</tr>
<tr>
<td>Substrate injection</td>
<td>0.15</td>
<td>300</td>
</tr>
<tr>
<td>Glycine</td>
<td>1</td>
<td>180</td>
</tr>
</tbody>
</table>

Table 2 Format used for immunoassay

The system was thoroughly tested by setting up a continuous flow ELISA system for analysis of IgG. Further, it was evaluated for linearity, stability and continuous analysis of IgG in samples taken from a stirred tank reactor to simulate protein production in a bioprocess. In order to evaluate stability of analysis, a known concentration of IgG (150 µg/l) was injected repeatedly and the response was studied.

Analysis of IgG in a simulated bioreactor

An experiment was designed to use the flow immunoassay for continuous analysis of IgG from a reactor (Figure 5). A first order kinetics of concentration was set up by constantly feeding 0.5 µl/min of known concentration of IgG (750 µg/ml) in continuously stirred tank reactor containing 75 ml of carrier buffer.
Online analysis of IgG samples from a simulated stirred tank reactor using AVCFS: P1 and P2 Piston Pumps, P3 to P6 – Peristaltic Pumps, I - Affinity reactor column, C Carrier Buffer, G - Glycine-HCl buffer, W – Waste, R - Reactor, MV1 to 3 – Motorized Injection

Online analysis of IgG was carried out using the flow immunoassay as described earlier. By operating the peristaltic pumps P1 to P3, samples were drawn and analysed continuously for a period of 17 hours.

**Dynamic calibration of the sensor**

The flow setup is flexible and was changed to accommodate a rotary switching valve (MV-8) in series with the injection valve (MV-7). Reagents are filled in the holding loop and introduced to the reactor sequentially as before. The AVCFS system can accommodate injection of standards at any desired frequency so as to have a check on the calibration of the sensor (Figure 6). This is possible as the flow of different reagents and their introduction can be programmed to suit different requirements.
Results and Discussions

The AVCFS system was tested for consistent operation of all the devices employed. Flow immunoassay/affinity binding assay requires reliable flow and a stable system which has high repeatability. These aspects were tested by employing affinity reactor bed volumes of 5 and 15 µl. It was important to regenerate the column at the end of each assay cycle, when employing the system for following a process by repeated assays. Generally biosensors have problems in drift in sensitivity over time and invariably require compensation for variability in baseline. The assay format employed here accounted for this by having scope for baseline check and incorporation of a blank/standard in the assay.

<table>
<thead>
<tr>
<th>Immunoreactor Volume</th>
<th>Ab-Conjugate (Carrier buffer flow - ml/min)</th>
<th>Substrate (Carrier buffer flow - ml/min)</th>
<th>IgG Sample (Carrier buffer flow - ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl</td>
<td>1</td>
<td>0.15</td>
<td>0.5</td>
</tr>
<tr>
<td>15 µl</td>
<td>1</td>
<td>0.15</td>
<td>1</td>
</tr>
</tbody>
</table>

The differential signal obtained from the sample response and baseline was considered for calibration. Alternatively, the normalized peak height with respect to blank/standard can also be considered. Figure 7 shows the linearity of sensor with a 5µl affinity reactor column. It was seen that the linearity was very good for lower concentrations of IgG (R-value 0.9932). The experiments were carried out in duplicate. The assay time was 32 minutes per cycle, but optimization of the response time not being the focus in the study, it was not considered. The assay time can be considerably reduced based on the different flow rates employed for sample, conjugate and substrate for a given affinity reactor column capacity and needs no changes in flow setup. One can also reduce the
frequency by which calibrations and controls are carried out. Though there was response for higher IgG concentrations, linearity suffered and it was found that the maximum response was achieved for IgG concentrations around 50µg/ml. This may be because at the flow rates chosen, the column is saturated with IgG and excessive antibodies are washed out without having any Protein A binding sites on the gel to interact with. The concentration range of IgG antibodies experienced in bioreactors being high (up to 10-20 g/l), it was chosen to operate the flow immunoassay with a higher capacity column. A 15µl column was used for subsequent experiments with 1 ml/min flow rate for the sample.

Figure 8 shows the peaks obtained from the AVCS system. The system had very good repeatability. The experiments were carried out in triplicates. The calibration curve is as shown in Figure 9. The column was saturated at around 500 µg/ml. The calibration was found to be linear from IgG concentrations 50 µg/ml to 400 µg/ml as shown in Figure 10 (R-Value 0.9517).

Figure 7 Linearity of Immunoassay for low concentrations of IgG
Figure 8 Monitoring of the Assay in AVCFS

Figure 9 Calibration Curve for Flow Immunoassay
Stability and Sensitivity of sensor for continuous flow analysis

The immunosensor for continuous flow analysis needs to be stable and sensitive for the entire useful lifetime of the sensor. Ideally, it should retain the same sensitivity. Using the same setup, the sensor system was studied for any changes in sensitivity for a period of 5 days during continuous operation. The R-value was around 0.95 and there was a small change in slope value. The results in Figure 11 indicate that the sensor generally retained the same range of analysis with almost same sensitivity. However for analysis and higher accuracy, a standard can be incorporated into the assay format, instead of the blank sample and based on normalized results with respect to the standard would result in a robust format for continuous analysis. To test this, in the stability study a known concentration of IgG was introduced continuously in a cycle and the response was studied. From Figure 12 and 13, it is evident that the sensor readings recorded continuously by the AVCFS system over 16 hours had very good stability and analysis conditions were highly repeatable. The standard deviation (S.D.) values for the results for different responses are tabulated in Table 3.

<table>
<thead>
<tr>
<th>Signal</th>
<th>S.D Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.0191</td>
</tr>
<tr>
<td>Sample IgG</td>
<td>0.0576</td>
</tr>
<tr>
<td>Blank</td>
<td>0.0957</td>
</tr>
<tr>
<td>Differential (IgG – Baseline)</td>
<td>0.0533</td>
</tr>
<tr>
<td>Normalized IgG (IgG/Blank)</td>
<td>0.0274</td>
</tr>
</tbody>
</table>

Table 3. Immunosensor stability response and standard deviation

The differential response for sample with baseline had an S.D value of 0.0576. Considering the normalized response for IgG with respect to the blank, the accuracy was higher with an improved S.D value of 0.0274. Further with a Lab-on-valve setup as shown in Figure 6, the AVCFS system can be employed to automatically introduce standards at known instants to accommodate any changes in the binding capacity of the affinityreactor column. Also, it is observed that the baseline signal for initial substrate is almost constant as in seen in the graph (S.D. value of 0.0191). This shows that there is
ample scope for reduction of cycle time for assay by eliminating certain steps in the assay. Further, it was noted that the immunoreactor column employed in the continuous mode had a useful life of more than 150 analyses.

Figure 11. Study of sensitivity over continuous use

Figure 12. Stability and Repeatability of the Flow immunoassay
Stirred tank reactor and continuous IgG assay

The experiment was setup as in Figure 5 and a 15 µl affinity reactor column was employed. The concentration of the sample at the time of injection was computed by taking into consideration, the buffer volume, sample inflow and outflow for analysis. A constant flow pump for feeding IgG was utilized. The comparison of the results for concentration of IgG as measured by the sensor with the estimated value is depicted in Figure 14. There was a higher deviation in the measured value at low concentrations below 50 µg/ml (< 15%). At higher concentrations beyond 50 µg/ml the maximum deviation was < 10%. This is also evident in the non-linearity at lower concentrations as seen in Fig 8. This however can be improved by adapting dynamic switching of flow profiles of carrier buffer for sample, substrate and conjugate. The flow profiles being programmable, at lower concentration, can be setup to have lower flow rates for sample to increase sensitivity of the reactor column. Essentially it means to have different calibrations for lower and higher concentration for improved accuracy at lower concentration. The switching point for the column employed is can be a concentration value of 50µg/ml.
Conclusion

The flexible automated flow system provided a very robust operating environment for bioanalysis. The system is designed to accommodate different biosensor configurations, flow setups and detection schemes. The AVCFS system was successfully employed for continuous analysis of IgG using flow affinity binding assay. Affinity binding assay is an important tool for continuous bioprocess monitoring of higher molecular weight substances like antibodies. The competitive binding assay of IgG using Protein-A-Sepharose gel had a useful range from 50 - 400 µg/ml with good linearity (R value 0.9517).

It was also demonstrated the usefulness of automating the assay for highly repeatable and stable immunoassays. Automated analysis provides control to employ also newer approaches for sample / standard introduction thereby making it possible to improve accuracy of analysis. The continuous analysis of IgG in a stirred tank reactor with concentration kinetics using the developed system yielded consistent results with estimated values. The affinity sensor could be continuously employed for more than 150 assays. The system can be employed for other modes of bioanalysis like SIA, BIA, lab-on Valve and multi-commutation flow. The automated immunoanalysis system can be employed for online analysis of different analytes for bioprocess monitoring and control. This provides a basis for integrated process monitoring and control employing biosensors.
References


