Studies on Development of a Microbial Biosensor for Biomedical Application.

By

Izhar Ali

163-FBAS/MSBT/F-14

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

(2017)

Biomedical engineering.

Microbe.

Determination of caffeine.

Pseudomonas aeruginosa

Studies on Development of a Microbial Biosensor for Biomedical Application.

Researcher

Izhar Ali

163-FBAS/MSBT/F-14

Supervisor

Dr. Bashir Ahmad

Assistant Professor

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

(2017)

"In the name of ALLAH The Most Gracious and The Most Beneficial"

Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad

Dated: _____

FINAL APPROVAL

It is certified that we have read the final thesis entitled "**Studies on Development of a Microbial Biosensor for Biomedical Application.**" submitted by **Mr. Izhar Ali** and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

COMMITTEE

External Examiner

DR. SAADIA ANDLEEB
Assistant Professor
Atta ur Rahman School of Applied Biosciences NUST

Internal Examiner

_____

Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad

Supervisor

Dr. Bashir Ahmad

Assistant Professor

Department of Bioinformatics and Biotechnology

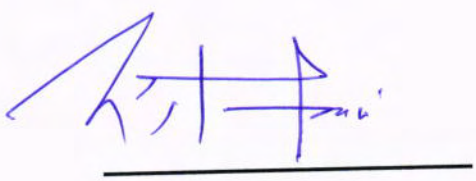
International Islamic University, Islamabad

Chairman

Prof. Dr. Asif Mir

Department of Bioinformatics and Biotechnology

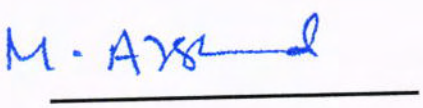
International Islamic University, Islamabad



Dean, FBAS

Professor Dr. Muhammad Arshad Zia

International Islamic University, Islamabad



A thesis submitted to Department of Bioinformatics and Biotechnology,

International Islamic University, Islamabad as a partial fulfillment of requirement of the award of the

Master in Sciences of Biotechnology

(MSBT)

Dedicated

To

My beloved

Parents,

Brother, Sisters

And

Teachers

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Studies on Development of a Microbial Biosensor for Biomedical Application"** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated _____

Izhar Ali

163-FBAS/MSBT/F-14

LIST OFTABLE

LIST OF FIGURES

ABBREVIATIONS

(CLSM)	Confocal scanning laser microscopy
CM	Calmodulin
CV	Cyclic voltammograms
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and statistical manual of mental disorder
GECE	Graphite epoxy composite electrode
Gr	Graphene
l-GLDH)	l-Glutamate dehydrogenase
l-GLOD)	l-Glutamate oxidase
MALDI TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
ml	Milliliter
NIST	National institute of standards and technology
NLEA	Nutrition labeling and education act
PDMS	Polydimethylsiloxane
SEB	Staphylococcal enterotoxin B
SEM	Scanning electron microscopy
Sp.	Specie
UV	Ultra violet
SPR	Surface plasmon resonance
HUVECs	Human umbilical vein endothelial cells

ACKNOWLEDGEMENTS

All praises and thanks to **ALLAH Almighty**, The Lord of all creation, The Beneficent, The most Merciful and the most Compassionate, Who created man and taught him manner and is the source of entire knowledge and wisdom endowed to mankind. All respect and admiration for The Holy Prophet (PBUH) whose teachings are complete guidance for the mankind.

Sincere thanks from the depth of my heart to my supervisor Dr. Bashir Ahmad for his valuable suggestion, consistent help in research, experimental work and compilation of this thesis. A special thanks to Dr. Syed Ali Imran Bokhari for providing the help during my research work.

I acknowledge Mr. Muhammad Faheem, Syed Waseem Shah, Shamsul Ghani, and Hanifullah for providing help to carry out this project. I am very thankful to all my classmates and friends especially, Fiaz Ahmed, Aftab Rafiq, Amir Atlas, Hazrat Usman, Muhammad Waqas Manzoor, Syed Waqas Umar, Afra Siab, Ali Nawaz and Tahir Aman.

At last, my family is one of the greatest blessings of ALMIGHTY ALLAH that gave me strength to persevere during difficult times. Without endless love of my father and mother, I have no doubt that success in any capacity would be unattainable for me. I am very grateful to my parents, brother and sisters.

Izhar Ali

ABSTRACT

We have utilized a microbe, which can degrade caffeine to develop a biosensor for determination of caffeine in solutions. Successful 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. Whole cells of *Pseudomonas aerogunosa* having the capability to degrade caffeine were immobilized on a cellulosic materials (wooden toothpicks and DE lignified sawdust peices) and inorganic material like glass slide. The biosensor strip was efficient to detect caffeine in solution over a detectable concentration range with read-times as short as 15 minutes. We also optimized the temperature 30 °C, cells volume 0.125 mg/ml and pH 7.0 for the better performance of the *Pseudomonas* immobilized cells strip. 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. According to the results, it was obvious that caffeine in the soft drinks can be sensitively determined with the developed bio strip.

INTRODUCTION

1. INTRODUCTION

Caffeine, a methyl xanthine molecule is the most extensively used up psychoactive substance in the world. This is present in more than sixty plant classes, with important levels in coffee beans, tea, and cocoa, and may be present in the drinks like coffee, tea and soda. The English term caffeine comes from the French (Spanish and Portuguese) term for coffee: cafe. Because of its stimulatory nature, it was used as a cardio-tonic till the end of 19th century (Wijhe, 2002). Other two vital alkaloids of the xanthine copied group are theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) and are found in coco beans, mate leaves, cola nuts, tea leaves and guarana seeds. Caffeine is well recognized as an inducer of human hepatic cytochrome P450 enzymes, and as a probe drug for monitoring broad-spectrum detoxification functionsof the liver (Bechtel *et al.*, 2001).

1.1.1 Sources of caffeine

Caffeine (1,3,7-trimethylxanthine) and related methyl xanthines are extensively dispersed in many plant species. Caffeine is also a chief human nutritional ingredient that can be found in common drinks and foodstuffs, such as coffee, tea, and sweeties (Dash *et al.*, 2006). Caffeine content (dry matter basis of green coffee bean) varies prominently between species and inside species. *C. canephora* (robusta coffee), *C. brevipes* and *C. stenophylla* are the coffee species with the maximum caffeine content, while *C. pseudozanguebariae* and *C. humblotiana*are almost caffeine-free (Campa*et al.*, 2005). Coffee and tea floras are the major sources of normal caffeine and related mixtures such as theophylline and theobromine are produced by many floras species belonging to many types, relations, and instructions. It is believed that methyl xanthine-producing plants collect these substances as part of a chemical defense system in contrast to pests and herbivores. Stimulatingly, a very large quantity of the non-alcoholic drinks used in social locations hold caffeine. The most important beverages and foods containing caffeine are coffee, guarana, mate, tea, cola drinks, cocoa, cola nuts, chocolate, yaupon and yoco. Created on dry weight, the maximum amounts are found in guarana (4-7%). Tea leaves contain around 3-5% caffeine, coffee beans 1.1-2.2%, cacao beans 0.03%, and cola nuts 1.5% (Bogo and Mantle, 2000).

Caffeine too arises in positive soft drinks, energy drinks, and so called "smart" drinks, as well as in remedial medications. In these cases, though purified or manufactured caffeine has frequently been added to the foodstuffs.

1.2 Mechanism of action of caffeine

The stimulating result of caffeine is supposed to be due to rise in adrenaline release, which might excite the understanding nervous system, but the mechanism is not exclusively unspoken. Caffeine increases Acetylcholine release in the hippocampus in vivo by a careful contact with adenosine A1 receptors (Carter *et al.,* 1995). It is recognized that caffeine is accountable for numerous effects on the human body. Facts of the mechanism of caffeine at cellular and tissue level can give an awareness into how this element affects different functions in the body and the details for opposing effects on the body. Caffeine stimulates the Central nervous system, increases the shrinkage power of the heart, enlarges the vessels of heart, kidney and the skin and displays bronchiolitis and dietetical actions. In mammals, consumed caffeine is rapidly immersed, metabolized, and excreted in the urine as methyl xanthine derivatives.

Apart from being a stimulating to the central nervous system, if spent in excess it causes mutation; it is teratogenic, causes inhibition of DNA repair, inhibition of cyclic AMP phosphodiesterase activity and inhibits seed germination (Friedman and Waller, 1983a and b). It causes complications in pregnant women, aging, the major cause of cancer, and heart diseases (Fenster*et al.,* 1991).

1.3 Caffeine and health problems:

1.3.1 Gastrointestinal problems

Many people experience a red-hot sensation in their stomach after consumption of coffee because coffee rises the excretion of hydrochloric acid leading to an enlarged risk for ulcers (James and Stirling 1983). Coffee decreases the pressure on the valve between the esophagus and the stomach so that the highly acidic insides of the stomach pass up to the esophagus leading to heartburn and gastro-esophageal reflux disease.

1.3.2 Caffeine in pregnancy

Caffeine crosses the placental barrier so exposing the growing fetus to maternally ingested caffeine. The effect of this is potentiated in the last trimester when caffeine elimination from the mother is reduced about threefold. Caffeine intake has been associated with low birth weight and spontaneous abortion in some studies. Also, caffeine withdrawal at birth can induce clinical effects, including apnea, in newborns. Overall, it is evident that high levels of caffeine (more than 300 mg/day, equivalent to three or more cups of cove) during pregnancy are potentially harmful (Hughes *et al.*, 1993).

1.3.3 Heart diseases

Researchers at Johns Hopkins Medical Organization found heavy coffee drinkers (five or more cups per day) were two to three times more likely to have coronary heart disease than were nondrinkers. This suggestion was correct uniform when accounting for other vital risk factors such as age, serum cholesterol, smoking habits, and blood pressure (Waring*et al.*, 2003).

1.3.4 Cancer

In period between the 1950s and 1970s many believed that caffeine can be a serious cause of cancer in humans since of studies in plants showing chromosome breaks, formation of chromatin bridgesand inhibition of mitosis after high-dose caffeine treatment. More recent evidence does show a capacity for caffeine to worsen the mutagenicity of ionizing radiation and other cancer-causing agents through interfering with cell cycle control (Kaufmann *et al.*, 1997).

1.3.5 Platelets regulation

Regulation of platelets accumulation is also reported to be effected with consumption measure of caffeine due effect of platelets response to adenosine. A2A receptor on the platelets outward is affected when intake is made increase which in turn prevents platelets aggregation. Caffeine acts mainly via barrier of adenosine receptors, which have been classified into A1, A2A, A2B, and A3 subtypes. We determined whether frequent caffeine administration (750 mg/d for 1 week) up-regulates the human platelet A2A adenosine receptor and is attended by sensitization of platelet responses

(increase in cAMP buildup and reduction in platelet aggregation) to selective stimulation of the A2A receptors (Varani *et al.*, 1999).

1.3.6 Neuro-muscular activity

Beside numerous additional health effects data shows that caffeine increases the skill to excellently activate skeletal muscle with efforts to produce maximal voluntary knees extension. This increase in activation was associated with a significant increase in force. Therefore, caffeine may improve the ability to produce MVC in subjects who are otherwise unable to maximally activate the vastuslateralis voluntarily (Kalmar *et al.*, 1999).

1.3.7 Blood sugar swings

Caffeine assembles intracellular sugars and induces a impermanent surge in blood sugar which is then followed by an overproduction of insulin which causes a blood sugar crash in hours. It is also known as weight loss agent and principals to the rise in weight due to its hyperglycemic effect which excites insulin's message to the body to stock excess sugar as fat (Lee *et al.*, 2005).

1.3.8 Other effects of caffeine

Caffeine administered acutely increases diuresis (urination). Caffeine regularly increases energy metabolism throughout the brain while decreasing cerebral blood flow and there is no tolerance for these effects. Vasoconstriction due to 250 milligrams of caffeine can decrease central blood flow by 20-30%, which is why caffeine has been used to treat migraine headache. Because blood glucose is usually more than ample for cerebral metabolism the combination of increased metabolism & decreased blood flow would be more likely to induce hypoxia than ischemia. But if caffeine increases oxygen intake by broncho-dilation or increases sensitivity to carbon dioxide in the medulla, then there may be compensation. (Both hypoxia and caffeine elevate plasma adenosine.) The consumption of fewer than four cups of coffee daily during pregnancy is not deemed to endanger the child (Leviton and Cowan 2002).

1.4 Dosage intake

In difference to effects originate from usual caffeine intake, there are reports that have established bad effects when very big quantities are given or sensitive groups (e.g. anxiety disorders patients) were studied. In this situation caffeine, has been shown to increase anxiety and damage sleep. Some indication that fine motor control may be decreased as a function of the increase in anxiety. Concentration measurement of caffeine is significant in both serum and foods of the individuals as foolish use can leads to severe problems in likely individuals. Lengthy ingesting of caffeine results in fatigue, headache, adrenal stimulation, irregular muscular behavior and apathy. (Smith *et al.*, 2002).

1. 5 Importance of food nutrients value in human life

To show, good nutrition information on packages of products the Nutrition Labeling and Education Act (NLEA) of 1990 (HR 3562) recognized in the United States. Therefore, it has developed crucial that methods and well-characterized position materials be developed to help recover the accuracy of nutrition labeling information to assist customers in preserving a healthy food and to provide trace ability for food spreads needed for receiving in many foreign markets. National Institute of Standards and Technology (NIST) development characterization of food founded materials with values allocated for nutrients that could be used for number of required nutrients values in unlike food stuffs (Thomas *et al.*, 2004).

1.6 Caffeine as a model drug of abuse

While, caffeine is actual usually consumed licit stimulant drug. (Ford *et al.*, 1998). Normal caffeine use has mostly not been measured as a case of drug exploitation, and is not so classified in DSM-IV (Diagnostic and Statistical Manual of Mental Disorder). But some years ago, it was keen out that caffeine may be a potential drug of abuse and more recently caffeine has been labeled as "a model drug of abuse (Holtzman *et al.*, 1990).

1.7 Biosensor

A biosensorbasically consists of a transducer in conjunction with a biologically active molecule that converts a biochemical signal into a quantifiable electric response.Enzymes are typically used in the biosensor creation due to their high specific actions and analyte understandings. Greatest of the enzymes used in the biosensor applications are unstable and so expensive for routine analysis of the target analytes (Akyilmaz *et al.,* 2005).

Biosensing elements may be an enzyme, antibody, nucleic acid, bacteria or even whole tissues of higher organisms while the transducer converts the signal derived from the catalytic or binding event. The specificity of the biosensor depends on the selection of the biomaterial enzymes, antibodies, DNA, receptors, organelles, microorganisms as well as animal and plant cells or tissues have been used as biologic sensing materials.

The display systems can be different, for example as electrochemical, optical, piezoelectric or thermal (Byfield and Abuknesha, 1994a). Compared with chemical sensors, biosensors are more specific because evolution has optimized biomolecules to operate at ambient temperatures and in dilute aqueous media at around pH 7 (Byfield and Abuknesha 1994b).

1.7.1 Whole Cell Biosensor

Entire cell finding system is of most status as whole cells carry the inside intact. Entire cell can be genetically modified and as well as without genetic modification can be used for the development of a microbial biosensor.

For fast and exact quantification of targeted analyte attention of microbial cells are genetically engineered conferring to typical molecular biological techniques. Some of the genetically engineered microbial strains *Shewanella onedensis* use for arsenic finding (Webster *et al.,* 2014), Based on *Shewanella oneidensis* outer membrane protein complexes microbial biosensor is designed (Goltish *et al.,* 2013). Whole cell without genetic engineering are also used for development of biosensor as *Shewanella onedensis* MR-1 cells for detection of fumarate in foods (Si *et al.,* 2015).

1.7.2 Enzyme based biosensor

Compensations of enzymes are based on their specificity and rapid response time, but maximum of the enzymes used in the biosensor are so expensive for routine analysis and unstable (Akyilmaz and Dinçkaya, 2005).

1.8 Importance of Biosensor

Globally the annual total sales of biosensor devices in the 1990s has surged in value from $US35M, in 1990, to around $US510M by 1996, and is projected to exceed $US890M by year-end 2000. At its glance this could indicate that biosensors are a robust and nourishing commercial industry. While this is true for the glucose sensor, it does not hold for the whole of the biosensor industry. In 1996, the blood glucose sensor accounted for $US485M of the $US510M total. The next most successful biosensor product, BIACORE™, is used more as a research tool for pharmaceutical design rather than as a chemical sensor. The success of the glucose sensor is due to several factors, including: a large and increasing demand for blood glucose monitoring worldwide, the stability and cheapness of glucose oxidase (Wilson and Turner 1992) and mass-manufacturability of the sensor element. Research and development activity has been devoted to preparing compact analytical devices comprising a bioactive sensing element integrated with a suitable transducing system. The development and application of new caffeine detection methods remain an active area of investigation, particularly in food and clinical chemistry. In pharmaceuticals, caffeine is used generally as a cardiac, neurological, and respiratory stimulant, as well as adiuretic (Dash *et al.*, 2006).

1.9 Biosensor for toxin detection

The toxic and harmful effects of DON (trichothecenes) on animal and human health have been subject to various studies in the past. Recently, high contamination levels of DON have been detected in wheat and in wheat containing food products in the Netherlands. Children (1-4 year) have the highest wheat intake when expressed as g per kg body weight per day, i.e. 4.5 – 8.5 g/kg body weight per day. The toxic effect of DON in experimental animals is growth reduction therefore fast growing children will be vulnerable to this type of toxic effect. The Ministry of Public Health, Welfare and Sports (VWS) requested RIVM/CSR to carry out a risk evaluation on DON. For

1.10 Biosensor for Bacterial infections

Plague is a characteristic zoonosis dispersed in Asia, Africa and America and it was reestablishing by the World Health Organization (WHO) as a recurring transferable disease due to the increase outbreak of plague around the world. Microbial strains in the genus yarsenia are the etiological mediators of the plague a typical zoonosis. Plague occurs mostly in three different forms, that is pneumonic, bubonic, and septicemic defending on the way of exposure to pathogen. A bite of flea that caused bubonic plague has before fed on ill rodent and this infection can spread to lungs (Gage *et al.*, 2004).

1.11 Biosensor for determination of different components concentration in foods

Amines contents level in food must not exceed the dangerous restrictions for human beings. Some toxicological characteristics and eruptions of food poisoning are related with tyramine and histamine. To evade toxic effects of amines in food, their level in European sausages (Ansorena*et al.*, 2002) and Brazilian beer (Gloria *et al.*, 1999) were measured and bio-sensing methods were optimized. Fumarate attendance showed in drinks, backing powders, apple juices and candy is the result of microbial metabolism of food additives. Beside, an oncometabolite, fumarate is a good food spoilage indicator. Kvasnička and Voldřich 2000).Nearby, ethanol finding in blood for the diagnosis (determination of acute intoxication and suspected drunk driving) to treat various disorders (Liden*et al.*, 1998)). It is important too in engineering and biotechnological process such as products (Boujtita*et al.*, 1996), pharmaceutical, cosmetic and alcoholic beverages production.

1. 12 Caffeine and microorganisms

Caffeine manufacture by plants is recognized to be an evolutionary version to ward off predators, insects and microorganisms. Caffeine found in florae at amounts found in *C. 9rabica* is poisonous to a diversity of fungi and insects. Caffeine is known to be strongly allelopathic to numerous florae, microorganisms and insects. Caffeine is known to constrain the development of plants and bacteria near the developing seeds. (Friedman and Waller 1983a) showed that caffeine prevents mitosis in many plants roots and thus reduces access to nutrients. Since caffeine decreases root development and

murders most bacteria, it efficiently kills off any nearby opposition for vital nutrients as well as defends itself from being spent by microbes (Friedman and Waller, 1983b). Precise knowledge about the nature and characteristics of coffee pulp natural micro-flora is of extreme importance for its additional operation in the development of bio decaffeination process for caffeine covering materials and in the separation and documentation of potent caffeine degrading strains Caffeine's solubility in water provides for simple conveyance into the close soil where inhibition of close organisms will occur. High attentions are compulsory for bactericide action, though some microorganisms can produce in the presence of caffeine and existence would be connected to their volume to degrade the alkaloid. Variety of microbial populations during the maturing and natural processing (sun-dried) of coffee fruits during two successive years. A total of 754 separates of bacteria, yeast and fungi were found. Bacteria were the major microorganisms. Fermentative bacteria and yeast, cellulolytic bacteria, and pectinolytic bacteria, yeast and filamentous fungi were recognized amongst 626 microorganisms. (Silva *et al.*, 2000).

Caffeine (1,3,7-trimethylxanthine) and related methylamines are extensively dispersed in many plant class. Caffeine is also a main human dietary element that can be originate in common drinks and food products, such as coffee, tea, and chocolates. In medicines, caffeine is used generally as a neurological, cardiac, and respiratory stimulant, as well as a diuretic. Hence, caffeine and related methylamines enter soil and water easily through decomposed plant resources and other income, such as wastes from coffee- and tea-processing services. So, it is not amazing that microorganisms proficient of degrading caffeine have been isolated from numerous natural environments, with or deprived of enhancement procedures. Bacteria use N-demethylating and oxidative pathways for catabolism of caffeine (Yu *et al.*, 2009).

1.13 Caffeine in energy drinks

Care issues related with feeding of commercially available energy drinks is highlighted using caffeine. Greatest energy drinks contain usual product caffeine as much as 80 to 300 mg as in Pimp Juice, cocaine, Red Bull, Spike Shooter, and Pimp Juice. No reports were recognized of bad effects related with others arrangements of

energy drinks as taurine and guarana ginseng. Usually stated opposing effects seen with caffeine in the amounts present in most energy drinks are headache, insomnia, tachycardia, and nervousness (Clauson *et al.*, 2008).

Aim and Objectives

- Selection and identification of the efficient micro-organisms for caffeine degradation.
- Induction of microbe for efficient caffeine degradation.
- Immobilization of whole cells for bio-decaffeination.
- Optimization of different parameters for efficient bio-decaffeination by the immobilized cell.
- Detection of caffeine in different soft drinks through optimized bio-strip.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Chou *et al.,* (1996) developed a coupled enzyme based ampherometric biosensor for aspartame quantification in food stuffs. Enzymes α-chymotrypsin and alcohol oxidase were immobilized with gluteraldhyde on a dissolved oxygen electrode. During enzymatic reaction aspartame is converted to ammonium which is detectable ampherometrically. Aspartame is a low calorie artificial sweetener and less harmful than cancer causing agent saccharin. Conditions are optimized for the linear range of the substrate, pH, temperature and interference ingredients.

Liden *et al.,* (1998) developed whole cell ampherometric biosensor for biomedical applications. Different bacterial strains *Candida boidinii*, *Pichia pastoris*, and *Hansenula polymorpha* were used to optimize the activity of enzyme alcohol oxidase (AOD) carried by these microorganisms. Studies showed that *P. pastoris* based biosensor can determine efficiently the concentration of ethanol and methanol in natural fluids such as blood and urine when combined with an optimize CLC (column liquid chromatography) system. To make the surface of the reagent electrode smooth it is rubbed with glass piece and covered with protective membrane (electro polymerized layer) orthophenylenediaamine. Enzymatically formed hydrogen peroxide in its oxidation phase is detected from AOD using horse radish enzyme (HRP) in a succession of reactions.

Pizzarillo *et al.*, (1999) used 3',5'-cyclic phosphodiesterase (CPDE) enzyme in combination with a pH electrode to develop a new bio sensing method. The (CPDE) was extracted from the bovine heart by methylxanthine and used for detection of caffeine in coffee. The CPDE activator calmodulin (CM) played a most important role in the performance of the sensor. The addition of 10 U/ml of CM to the assay solution resulted in a more than threefold increase in the hydrolytic activity of CPDE. Determination of caffeine was carried out for standard solution and in espresso coffee. The potential response changes of the sensor were proportional to the concentration of caffeine in the range 0±4mg/ml. The response time was about 2±4min. The standard deviation of measurements of a 0.2 mg/ml caffeine solution was _7.1mg/ml. The electrode gave a detection limit of 0.6mg/l caffeine. This electrode exhibits advantages such as fast

response, short conditioning time and low cost of the instrumentation used.

Nedelkov *et al.,* (2000) experimented detection of bacterial toxins in food samples at low to sub nano gram in per gram food to evaluate the sensitivity of the two-step approach. Binding of toxins to immobilized enzymes on surface of sensor chip was performed using techniques such as bimolecular interaction analysis mass spectrometry (BIA–MS) surface Plasmon resonance (SPR). The second step measure the captured toxins via its unique mass by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOP MS). 10 mM concentration of acetate buffer at 4.8 pH was prepared and dilution of antibiotics to its working concentration was prepared while *Staphylococcal* Enterotoxin B (SEB) stock solution were prepared in HBS EP buffer for 200, 20 and 2 ng/ml at pH 4.7 by serial dilution. *Staphylococcal* enterotoxin B (SEB) and noxious shock syndrome toxin-1 (TSST-1) were analyzed in milk, milk products and mushroom sample at level of 0.5-1.0 mg/ml.

Pasco *et al.,* (2001) designed a novel biosensor for rapid analysis of biochemical oxygen demand and noxiousness measurement. To obtain the best result, during quantifying the toxin, the traditional oxygen as a co substrate is replaced with synthetic co substrate which is more soluble than traditional oxygen. The amount of ferricyanide was analyzed coulometrically that was reduced by the microorganisms by determining the change required for its re oxidation in a bulk electrolysis cell. Ratio of the coulombs, recorded in presence of toxin affords a complete guide of the toxin inhibition. The technique is a rapid method reducing the five-day biochemical oxygen mandate assay (BOD 5) incubation time to 1 hour that is 60 minutes. A 100-fold MICREDOX technique was used for reducing the time for substrate conversion. It was stated for three standard toxic materials 3, 5-dichlorophenol, zinc sulphate and sodium lauryl sulphate.

Kim *et al.,* (2004) constructed an immunosensor immobilizing four types of anti-*Pseudomonas aeurogenosa* antibodies were chemisorbed onto gold electrodes made up of piezoelectric quartz crystals. The polyvalent antibodies (I–III) respond to *Pseudomonas aeurogenosa* group specifically. The frequency response of quartz crystal and the operational features of the antibody sensor system were first assessed at different NaCl concentrations and ionic strength of the buffer solution. Resonant frequency responses

were checked according, to flow rates, antibody and *Pseudomonas aeurogenosa* strain. The biosensor retorts to varying concentrations of the *P. aeruginosa* cells ranging from 1.4×10^9 to 1.9×10^8 CFU/ml were determined as 17–176 Hz. The outcome of cell density on biosensor response was assessed with an antibody (IV) and *Pseudomonas aeurogenosa* KCTC 2413 suspension.

Pasco *et al.,* (2004) developed a rapid technique MICRODOX for measuring BOD by removing oxygen instead of quantifying an equivalent biochemical co-substrate (redox mediator) demand. *Proteus vulgaris*, cells, either as free or immobilized in Lentikat disks were incubated with an excess of redox mediator (potassium hexacyanoferrate(III) and organic substrate for 1 h at 37°C without oxygen. Accumulation of the reducing mediator, which was reoxidized at a working electrode, generating a current quantifiable by a coulometric transducer. Electrolysis was performed at 37 °C and was terminated when the current diminished to 1 percent of its initial value. Stoichiometric converged efficiencies for oxidation of standard substrate by *P. vulgaris* were typically 60 percent for free cells and 35 to 50 percent for immobilized cells. This substantially reduced the incubation time, from 5 days to 1 h, for the biological oxidation of substrates equivalent to those observed using the standard BOD5 test.

May *et al.,* (2004) constructed an innovative whole cell biosensor for sensing the potentiometric response of histamine as a model toxin. Human umbilical vein endothelial cells (HUVECs) were attached to an ion selective cellulose triacetate (CTA) membrane. Iodophors cocktail was added to the second layer of the cellulose triacetate (CTA) membrane to make it selective for potassium. Permeability of the ions changed when the human umbilical vein endothelial cells (HUVECs) confluent monolayer exposed to histamine toxins on the specific ion electrode (SIE). It is concluded that overall response increases with increasing histamine concentration. L histidine toxin was checked in further experiments but the sensor response remained unchanged.

Luca *et al.,* (2005) carried out research for the advancement of oxidase based electrochemical biosensor composed of carbon film electrode. Modified with Prussian blue (PB) the resistor electrode is covered with a layer of covalently immobilized oxidase

enzyme. These carbon film enzyme electrodes were cast-off to sense the substrate of the oxidase (ethanol, glucose, lactate, glutamate) with the reduction of hydrogen peroxide at +25 mV versus Ag/AgCl in minute micro molar range. Ampherometric and voltammetric measurements were carried out using an ABD ampherometric detector. A UV–vis spectrophotometer was castoff for the spectrophotometric measurements a UNICAM 8625 Biosensor was used to analyze food samples such as commercial wine and yoghurt and response times were measured 1–2 min.

Radke *et al.*, (2005) constructed a biosensor for detection of pathogenic bacterial strains such as *Escheresia coli* and *Salmonella infantis*. To obtain accurate results, bacterial strains were immobilized on the interdigitated gold electrodes while analyte specific antibodies were immobilized to the oxides between the electrodes to create a biological sensing surface. To validate the fabrication of the biosensor atomic force microscopy (AFM), scanning electron microscopy (SEM), and confocal scanning laser microscopy (CLSM) were performed for the purpose. The change in impedance of the sensor is directly proportional to the number of bacteria immobilized on the sensor surface. The impedance caused by bacteria was found to increase linearly with the number of cells present in solution. The biosensor was able to discriminate between different cellular concentrations from 104–107 CFU ml (colony-forming units per milliliter) in solution.

Akyilmaz *et al.*, (2005) developed microbial biosensor, based on the alcohol oxidase enzyme of *Candida tropicalis* yeast cells for the assessment of ethanol. Ethanol was assessed based on variance in the respiratory technique by using *Candida tropicalis* cells which were immobilized with gelatin glutaraldehyde. YSI MODEL 57 oxygen meter, YSI 7539 MODEL dissolve oxygen probe and high sensitive Tephlon membrane were used in the objectives of the biosensor. The steady-state current represents the oxygen diffusion through the composed membrane and reflects the endogenous respiration of the microorganisms. Assay conditions optimization of the microbial biosensor were carried for the effect of the amount of *Pseudomonas aeurogenosa* on the biosensor response, pH, temperature, Interference effects of some substances on ethanol determination, substrate specificity, working and storage stability.

Ngundi *et al.,* (2006) carried out his research work on detecting concentration of *Fusarium mycotoxin* in foods and feeds. DON biotin conjugates were immobilized on the NeutrAvidin coated optical wave guide using polydimethylsiloxane (PDMS) and DON monoclonal antibody are fluorescently labeled in the samples. The NeutrAvidin-functionalized slides were rinsed with PBS and either used immediately or stored in PBS at 4 °C. DON was immobilized in arrays onto the NeutrAvidin functionalized waveguide using polydimethylsiloxane (PDMS). Extraction of DON from Food Samples barley, wheat, oats, cornmeal, and cornflakes were blended to a fine texture (average diameter estimated at 100-500 m) and assayed without cleanup or pre-concentration.

Yan *et al.*, (2006) carried out research for quantitative detection of *Yersinia pestis* using up-converting phosphor technology (UPT-LF) immunoassay system based biosensor technology. The rapid identification of *Yersenia pestis* is basic for controlling the spread of flauge Sensitivity testing was conducted to determine the detection limit of the UPT-LF immunoassay for *Y. pestis* by using pure cultures of *Y. pestis* EV76 as a model. Microbial strains in the genus *Yersinia* are the etiological agents of the plague zoonosis. Microbial strains in the variety *Yersenia* are the etiological operators of the torment a run of the mill zoonosis. Plague occur predominantly in three different forms, that is, bubonic, pneumonic and septicaemic defending on the route of exposure to pathogen. Bubonic plague caused by a bite of flea that has previously fed on infected rodent and this infection can spread to lungs (pneumonic plague). Surface modifications and activations of UPT particles are require to realize covalent coupling of antibodies to them initially, UPT particles were coated with a thin layer of silica by using TEOS. Then the silica-coated surface of UPT particles can be functionalized with amino-, aldehyde-functional groups using APES, polyoxyethylene bis-amino and glutaraldehyde. Eventually these aldehyde-functionalized UPT particles can be conjugated to the antibody directly in a 4 °C-pre-chilled Na2CO3–NaHCO3 buffer (50 mM, pH 9.5) under stirring. We observed a good linearity between the ratio and log CFU/ml of *Y. pestis* above the detection limit, which was approximately 104 CFU/ml. Test voltage TV and control voltage CV are the analytical lines on the strip and the ratio VT/VC is directly proportional to the number of *Y. pestis* in a sample.

Kirgoz *et al.*, (2006) optimized a coupled enzyme biosensor for detection of aspartame in commercial cock and ethanol concentration in commercial beer and wine samples. Carboxyl esterase and alcohol oxidase were immobilized on gelatin membrane and then integrated onto the surface of a graphite epoxy composite electrode (GECE). Aspartame and ethanol concentration were monitored chrono ampeormetrically by tracing oxygen consumption during enzymatic reaction. Detection showed aspartame concentration in the range of 2.5- 400 M and ethanol concentration in the range of 2.5-25 M. The optimum enzyme amount was arranged and the effect of pH on the biosensor response was investigated by using phosphate buffer. The current density increases significantly from pH 6.0 to 7.0 but at pH higher than 7.0 decreases of current was investigated. The biosensor is design as which can detect ethanol right after aspartame by using the same biosensor in separate samples. Response time for analyte aspartame was measured 170 and for ethanol it was measured 70s.

Basu *et al.*, (2006) experimented that the coupled enzyme system based on l-glutamate oxidase (l-GLOD) and l-glutamate dehydrogenase (l-GLDH) for estimation of MSG l-glutamate in food. Monosodium glutamate is a food flavor enhancing additives used mostly in Chinese food and reported as a neuro toxic amino acid involve in several behavior patterns. Biosensor based estimation of monosodium glutamate in food are mostly based on the L-glutamate oxidase and L-glutamate dehydrogenase enzymes immobilized on electrodes. Studies showed that signal amplification increase in the presence of ammonium ion and response time was measured 2 min. The detection limit of the coupled enzyme system was 0.02 mg/L, whereas under non recycling condition only 0.1 mg/L concentration can be detected. The *K*m value of the single system as well as coupled enzyme system in immobilized condition have also been determined and *K*m values 1.9222 and 0.4451 mM were found, respectively. The sensor had a half-life of over 60 days, with about 50 assays.

Babu *et al.*, (2007) utilized a whole cell of *Pseudomonas alcaligenes* MTCC 5264 to construct a microbial biosensor for caffeine estimation in instant tea and coffee samples. If consumed in excess it can cause adverse mutation effects, It is teratogenic, causes restraint of DNA repair and hindrance of cyclic AMP phosphodiesterase activity.

It can be a reason for tumor, heart sicknesses and inconveniences in pregnant women's and aging. Caffeine degrading bacteria utilize caffeine as a sole source of carbon and nitrogen. Bacterial strain *Pseudomonas alcaligenes* MTCC 5264 were immobilized using glutaraldehyde as the cross linker on a cellophane membrane. Immobilized bacterial cells having caffeine degrading activity, sandwiched between two semipermeable layers were fixed on the cathode surface. Temperature for the biosensor was optimized by incubating immobilized membranes at different temperatures 32 °C, 35 °C, 40, 45 °C and 50 °C. Impact of the pH on the immobilized cells was considered by incubating the membranes in buffer in pH range 5.0 to 8.0. Response of the different interfering substances in the samples was not only negligible but also not considerable. Detection time for the biosensor was optimized as 3 min and detectable concentration range in solution approximately 0.1 to 1 mg/ml.

Rodrigues *et al.*, (2007) studied the detection of caffeine and organic acids in food sample with traditional technique reversed-phase UV high performance liquid chromatography (RP/UV-HPLC. Robust detection of caffeine is important to avoid overdosing, where individuals are sensitive to it. Robusta coffee demonstrated the highest content of caffeine. Caffeine was separated with a mobile phase of 85% tri-ethylamine phosphate buffer with 15% acetonitrile, pH 4.3, at a flow-rate of 1.0 mL/min, at room temperature. Injection volume was 20 ml and detection was carried out at 270 nm. Total organic acid content varied with coffee type and also with the geographic origin of the green coffee.

Liu *et al.*, (2011) carried out his research work on cell based electrochemical biosensor for measurement of phenol and nitrophenol. As many scientists have focused on the development of technologies to enforce environmental monitoring and homeland security. New technique not only requires the sensitive response to chemicals but also needs to reflect the effects that the chemicals may have on human health. *Pseudomonas fluorescens, Pseudomonas putida, Trichosporon cutaneum, Escherichia coli, Enterobacter cloacae* and *Alcaligenes faecalis* bacterial strains are used in the study but the best sensitive inhibitory response was showed by *Pseudomonas florescence*. By comparing the inhibition concentration (IC50) values, *Pseudomonas fluorescens* bacterial

strain was the most sensitive one response to 3,5-Dichlorophenol (DCP) toxicity, under the optimum conditions (solution pH 7.0 and together with the standard glucose–glutamic acid (GGA)). Microorganisms which belongs to the same bacterial family had simmiller responses to inhibition concentration.

Sun *et al.*, (2011) studied an enhanced sensing platform based on fabricated Nafion–Gr/GCE was for the sensitive determination of caffeine. Caffeine sensitivity is associated with many health problems such as anxiety with panic disorder, sudden infant death syndrome, cancer and many others health problems therefore its rapid concentration demonstration is essential. The Nafion–Gr composite film was described with electrochemical. Nafion is a cation trade polymer with properties of magnificent antifouling limit, synthetic dormancy and high porousness to cations. Caffeine can be adequately aggregated at Nafion–Gr/GCE and deliver a delicate anodic crest due to electro synergist conduct of Gr which is ascribed to its extraordinary physical and synthetic properties. To look at the strength and repeatability of the proposed technique, the immediate electrochemistry of Nafion–Gr/GCE was explored by CV (Cyclic voltammograms). The results are in agreement with the values obtained with UV–vis absorption spectrophotometry and HPLC. According to the results, it was obvious that caffeine in the soft drinks can be sensitively determined with the developed method.

Gummadi *et al.*, (2012) reviewed different techniques for rapid caffeine degradation, effective in producing useful by-products besides offering advantages in decaffeination. Immobilized agar matrix using *P. putida* C3024 showed caffeine removal at a rate of 0.25 mg caffeine/h mg cell in approximately 6 days. It was reported that *Pseudomonas alcaligenes* CFR 1708 entrapped with agar matrix has shown caffeine degradation with initial concentration of 1 g/l as compared with the other matrices viz. gelatin, κ-carrageenan, agar, sodium alginate. Large scale decaffeination by immobilized cell debris of *P. alcaligenes* CFR 1708 and designed a packed bed reactor for continuous decaffeination process. They showed that 1 g/L of caffeine was completely degraded in 4–6 h after optimization of parameters like pH, temperature, amount of inoculum and caffeine concentration. Production of high levels of caffeine demethylase in bioreactors where a maximum specific productivity of caffeine demethylase of 2,214 U/g cell dry

weight was obtained at 0.27 vvm, 700 rpm and pH 7.0. The volumetric oxygen transfer coefficient was 74.2 h−1 implying that caffeine demethylase production by Pseudomonas sp. was highly oxygen-dependent..

Goltish *et al.*, (2013) engineered *S. oneidensis* wild type bacterial cell, with useful electron transfer rate toward anode. Construction of the *Shawenella onedensis* mutant type. Linearization of the plasmid merging of the fragments, membrane preparation and sensor development were the focused studies in the research work. The L-arabinose biosensor presented in this study proves the principle of an outer membrane complex based sensing method which could be easily modified to different specificities by a simple change of the regulatory elements. It was tested first whether and to what extent MtrC and MtrF can be overexpressed in the OMC strain.

Webster *et al.*, (2014) experimented whole cell microbial biosensor and engineered arsenic responsive circuit in plasmid of bacterial strain *Shawenella onedensis*. Studies relied on *Shawenella onedensis* mtrB complementation strategy and a bioelectrochemecal system (BES) was developed in a single chamber glass electrochemical reactor. Bioelectrochemical systems (BESs) output a direct electric signal, which can be used for remote environmental monitoring. *Shawenella onedensis* exposed to low level of oxygen continue to express Mtr pathway at functional levels and do not lose electrode respiration capabilities. Studies showed that, BES-based biosensor has a detection limit of 40 μM arsenite with a linear range up to 100 μM arsenite.

Liu *et al.*, (2015) carried out his research work on cell based electrochemical biosensor for measurement of phenol and nitrophenol. *Pseudomonas fluorescence*, *Pseudomonas putida*, *Trichosporon cutaneum*, *Escherichia coli*, *Enterobacter cloacae* and *Alcaligenes faecalis* bacterial strains are used in the study but the best sensitive inhibitory response was showed by *Pseudomonas fluorescence*. By comparing the inhibition concentration (IC50) values. *Pseudomonas fluorescense* bacterial strain was the most sensitive one responded to 3,5-Dichlorophenol (DCP) toxicity, under the optimum conditions (solution pH 7.0 and together with the standard glucose–glutamic acid (GGA)). A microorganism which belongs to the same bacterial family had similar

responses to inhibition concentration.

Si *et al.*, (2015) experimented rapid quantification of fumarate, for its importance as an uncometabolite and food spoilage indicator. Abnormal accumulation of fumarate due to fumarate hydratase (FH, responsible for hydration of fumarate to malate, also served as tumor suppressor) mutation is one of the main causes of hereditary leiomyomatosis and renal cell cancer the top 10 most common malignancies. Therefore, it is considered to be an oncometabolite and might be a potential biomarker for early diagnosis of FH-associated cancer diseases. Fumarate reductase directly linked with bacterial outer membrane redox proteins, therefore the inward electron flow can be used for fumarate quantification via its reduction. *Shewanella onedensis* MR-1 cells were used to develop a whole cell electrochemical biosensor which provides a simple, cost-effective, fast and robust tool for fumarate quantification. biosensor limit of detection (LOD, defined as 3 times of baseline noise determined was 0.83 μM (S/N¼3), while the limit of quantification (LOQ, defined as 10 times of baseline noise determined was 1.2 μM (S/N¼10). Compared with the conventional physic-chemical methods, the LOD obtained by this system is about 20 times lower than chemometric method and about 2 times lower than HPLC method. There was no significant detectable output was obtained when applied to the fresh apple juice and mice kidney samples without fumarate addition (control samples), which indicated the possible interference from food and kidney tissue samples. When the synthetic spoiled food or tumor samples were applied, the fumarate concentration was quantified by the developed bio-sensing system and HPLC, respectively.

Canbay *et al.*, (2015) experimented a biosensor for the determination of lactic acid and pyruvic acid by immobilizing lyophilized *Lactobacillus delbrueki* strain on a platin electrode with polypyrole. Potassium ferricyanide (1.0 Mm) used as a mediator and lactate concentration was measured (+0.2 v) on the cathodic peak and pyruvate concentration is determined (+0.1 v) on anodic peak CV responses of bare pt/ppy electrode were not prominently detected while the pt/ppy-MO electrode with polypyrole formation and substrate addition showed detectable responses at cathodic and anodic peaks. The results indicated clearly the enzymatic reaction like *L. delbruecki* which

immobilized on the electrode surface. Optimization studies were carried out for amount of microorganism, polypyrole concentration, pH and temperature.

3. MATERIALS AND METHODS

3.1 Materials

Materials and equipment's like glass slide, cover slip, spirit lamp, microscope, glass slides, autoclave, wire loop, inoculating loop, sawdust pieces, toothpicks, petri plates, static incubator, shaking incubator, spectrophotometer and Erlenmeyer flasks were utilized in this study.

3.1.1 Chemicals used

Caffeine (N99%), acacia glue (Gum Acacia), Nutrient Agar and all others analytical grade chemicals were obtained from Sigma Aldrich.

3.1.2 Collection of samples

Pseudomonas sp. was isolated from the soil samples, which were collected from IIUI cafeterias, affected with detritus of tea discarded side soil. All the soil samples were stored in sealed plastic bags for further analysis in the laboratory.

3.2 Serial dilution of samples

Serial dilution method was used to dilute all the samples up to concentration of 10^{-6}.A total of seven dilutions were made by labeling the test tubes from 10 to 10^{-6}. To each of the test tubes 9 ml distil water was added. One gram soil sample was taken from each sample, transferred to the test tube labeled with 10 and mixed well with vigorous shaking and stirring. One milliliter solution was then transferred to the test tube labeled as 10^{-1}. Different dilutions of 10^{-1} to 10^{-5}were prepared from by repeating the same process of transferring one ml solution to the test tubes already labeled.

3.2.1 Isolation and purification of caffeine degrading microorganisms.

Enrichment culture technique was used to isolate Caffeine degrading microorganisms from the soil samples, by using a medium consisted of five gram caffeine, 2 gram K_2HPO_4' 1 gram NaCI, 0.5 gram $MgSO_4$' $7H_2O$, 05 gram yeast extract and l gram nicotinic acid in 1 liter of tap water (PH 7.0). Addition of MgS04 in 0.002 % concentration and fructose 1.0 can increase the production of caffeine 0.5 g/liter' h. Enrichment culture of soil bacteria completely consumed caffeine in 24 hours days when it was incubated in caffeine medium. This culture was further enriched by successive transfers to fresh caffeine medium. After three subcultures, several morphologically

different colonies were isolated from caffeine agar. One of these colonies, designated strain CBB5, was identified as *P. aerogenosa.*

3.2.2 Purification of bacterial culture

Streaking, also known as purification was carried out by isolating pure culture of selected strain of microorganism. Bacterial colony was picked by using a sterile inoculation loop, streaked on nutrient agar media. Incubation at 30°C was performed for 24 hours. Then a single colony of bacteria was picked from the nutrient agar plates having Caffeine (carbon source), transferred to a fresh plat containing the same nutrient agar medium with Caffeine as carbon source. In this way pure culture of *pseudomonas* was obtained and maintained on nutrient agar medium.

3.2.3 Identification of *Pseudomonas aeruginosa* sp. for caffeine degrading

The pure strains were cultivated in the presence of oxygen for 15 hours at 28°C on a medium containing 3 gram caffeine, 5 gram K_2HP0_4, 0.02gram $FeS0_4$ and $7H_20$.An isolate of bacteria was chosen from the soil, as the best the bromine producer. Microscopic observation, Gram staining and biochemical analysis were performed to identify *Pseudomonas* sp.

3.2.3.1 Gram staining

A small drop of distilled water was put on a clean glass slide. With the help of a sterile loop a single bacterial colony was picked from the pure culture, smear was made on another clean glass slide. The glass slide was then gently moved over the spirit lamp to fix the culture on the slide. A drop of violet dye was put; smear was made and left for one minute. Followed by adding gram iodine, left undisturbed for one minute and washing was done with distil water. Then decolorizing agent was added, left for 5 seconds, followed by a gentle watercourse with distilled water. At last, a drop of Safranin was introduced for covering the smear and left unstirred for one minute.After gram staining, the glass slide was rinsed with distilled water and was analyzed through a microscope.

3.2.3.2 Biochemical investigation

Biochemical tests including catalase and coagulase tests were performed for identification.

3.2.3.3 Catalase test

Bacterial species that can release catalase enzyme can be identified by Catalase test. A clean glass slide was taken and a single drop of Hydrogen peroxide was put on it. By using sterile loop, a single bacterial colony was picked from the pure culture and mixed with hydrogen peroxide. For indication of catalase positive bacteria bubbling was noted on slide.

3.2.3.4 Coagulase test

This test is performed to identify bacterial cultures which produce coagulase enzyme. A clean glass slide was taken, a single drop of blood plasma and distilled water were placed separately at each end. A full wire loop of the pure culture was immersed with plasma and water, gently mixed and left for 10 seconds. After 10 seconds clumps were observed. Plasma wasn't mixed with water in order to avoid any clumping of organism from the actual coagulase clump.

3.3 Inoculum preparation

For inoculum preparation 0.9 % saline solution was used. To prepare 10 % stock solution 10 gram NaCl was added to 100 ml of distilled water. Concentration of 0.9 ml was taken from the stock solution with the help of a graduated cylinder, 100ml distilled water was added in order to get a 0.9 % saline solution. Followed by autoclaving Saline solution at 121 °C and 15 psi pressure for a minimum time of 15 minutes for sterilization. Five ml sterilized saline solution was added to the nutrient agar slant, the bacterial strain were picked with a clean loop and mixed. The prepared bacterial suspension was then used as inoculum for immobilization processes using organic and inorganic support materials.

3.4 Culture conditions

Culture conditions for the caffeine consumption by *Pseudomonas* sp.were analyzed. Nitrogen and Carbon sources like fructose, was the most suitable carbon sources for the cell proliferation and production of theobromine from caffeine. The advantageous concentration of fructose was 1.0, although glucose did not. Upon adding Caffeine to the call suspension, certain metabolites were detected including Xanthine, Theobromine, uric acid and 7-methylxanthine, indicating the degradation of Caffeine by

N-demethylation process, just like seen in using the nutrient agar media provided with caffeine of *Pseudomonas* culture.

3.5 Standard and sample solutions preparation for caffeine determination

Both the standard and sample solutions were prepared and these solutions were subjected for spectrophotometric assay and the absorbance was taken at 273 nm. The samples produce highest and lowest concentration of caffeine was determined.

3.5.1 Standard solution preparation

0.2 Gram of caffeine were taken and dissolved in 1000 ml of distill water in order to prepare a stock solution of caffeine. Then 10 ml from the prepared solution was taken, diluted with distilled water up to 100 ml. From the stock solution, different dilutions were obtained by using following concentration.

(1). 5 µg/ml dilution was prepared by taking one ml stock solution and mixed in 40 ml of distilled water in measuring flask.

(2). 10 µg/ml dilution was prepared by taking one ml stock solution and mixed in 20 ml distilled water in graduated flask.

(3). 15 µg/ml dilution was prepared by taking one ml stock solution and integrated in 13.33 ml distilled water in a measuring flask.

(4). 20 µg/ml dilution was prepared by taking one ml stock solution, dissolved in a 10 ml of distill water in a graduated flask.

(5). 25 µg/ml dilution was prepared by taking one ml stock solution, mixed in 8 ml distilled water in a flask.

(6). 35 µg/ml dilution was prepared by taking one ml stock solution, immersed in 5.7 ml distilled water in a measuring flask.

(7). 40 µg/ml dilution was prepared by taking one ml stock solution, mixed with 5ml distilled water in a volumetric flask.

3.5.2 Samples solution preparation

Commercially available soft drink (Pepsi, Coca cola, Sprite, 7up, Dew, Fanta and Marinda) samples were degassed and studied by UV-VIS spectrophotometry. The samples were then treated with immobilized *pseudomonas aeruginosa* cells strip

biosensor to find out the presence concentration of stimulant caffeine. The result obtained was compared with samples which were not treated with immobilized cell strip. One ml of all the beverages sample was diluted up to 100 ml by distilled water and absorbance was taken at 273 nm. Absorbance is shown in the table and absorbance (OD) value converted to concentration (μg/ml) following the principles of Beer-Lambert law.

3.5.3 Determination of wavelength

The wavelength was measured by performing scanning of the standard solution in the 190 to 400 nm range. The maximum absorbance was noted on 273 nm. The resulted data of UV-spectrophotometer for the various dilutions of the standard stock solution was used to form a calibration curve by plotting the absorbance against varying concentrations.

3.6 Immobilization of *P. aeruginosa* cells.

P. aeruginosa was grown in agar medium containing caffeine as the only source of carbon and mean of energy. Harvesting of cells was carried out by centrifugation at 10,000 rpm for 10 minutes at 40^0C during exponential growth stage. Cells were immobilized with use of natural (purchased from local market) and as well as laboratory used synthetic (powder) acacia glue (purchased from sigma) and polyethyleneamine (purchased from sigma). Two types of solid carriers used in our research work are cellulosic materials (wooden toothpicks and De-lignified sawdust) and inorganic material like glass slide. The characterization of the support surfaces was done after carrying out each of the two alternative support materials.

3.6.1 Cells amount

The combining step may include immersing the receptor. Cells were loaded on the support materials and cells volume of microbes was optimized on the basis of mg/ml. After immobilization, the microscopic slides and sawdust loaded carriers were freeze thawed in order to boost its mechanical strength. Cellulosic supports showed improvement in validity of strip upon freeze thawing as compared to microscopic glass slide support.

3.6.2 Polyethyleneimine

Microscopic slides of 25 were surface washed with chromic acid (for effective adsorption of the cells and then treated with polyethyleneimine for less than one minute. The clean Glass slides were incubated in a 0.2% PEI (pH 7.0) medium for 2 hours, excess of PEI solution was discarded and the glass slides dried in open air. Saline Phosphate buffer (PBS), with 10mM Sodium Phosphate (pH 7.3) and 15 mM NaCl, was used to wash the Bacterial pellets for once. The slides coated with PEI were then treated with pseudomonas cells (I gram) in a 20 ml water for 20 minutes and washing was done through tap water. Polyethylene-amine also shows effective adsorption of the cells on support material like cellulosic (wooden toothpicks and delignified sawdust).

3.6.3 Glue

Dissolving solid *Acacia* Gum in distilled water. The curing step may include stirring the suspension. When the powder form is dissolved in boiling hot water until a 50 percent colloidal solution is formed, the mucilaginous solution is autoclaved for 15 minutes. The resulting solution becomes increasingly viscous as the water evaporates, becoming a solid at room temperature.

The glue attachment of cells was good on microscopic glass slide but poor when applied to cellulosic materials (wooden toothpicks and delignified sawdust). The attachment with natural glue was poor and this effect may be attributed to the presence of impurities in the natural glue, however the maintenance life of the strip was good with natural glue.

3.6.5 Microscopy

Repeated microscopy was done for morphological examination of the attachment and surface structure of pseudomonas cells. The glass slides with a negative surface charge, which were used as model system, might be replaced by less expensive support materials like sand and glass pieces.

3.7 Optimization of different parameters for caffeine degradation by *Pseudomonas aeruginosa*

The development of Bio decaffeination processes stresses high enzymatic activity stated by the organism during the course of the fermentation. So, assemblage optimum

conditions like pH, temperature, cells volume and response time were determined in this study against the different soft drinks for maximum enzyme production in the cells. Therefore, studies on the optimization of conditions for decaffeination by the organism were carried out.

3.7.1 Effect of pH on caffeine degradation

The pH plays a significant part on the growth and activity of different enzymes compulsory for caffeine degradation. The optimization studies were carried out with respect to the maximum rate of caffeine degradation by different pH against different beverages. The highest and lowest pH supporting the caffeine degradation were determined and shown in figure and table.

3.7.2 Effect of temperature on caffeine degradation

Temperature is a vital parameter for realizing efficient bio decaffeination and has a severe effect on the activities of the enzymes involved in bio decaffeination. The immobilized *Pseudomonas aeruginosa* was incubated with different temperature to determine their effect on caffeine degradation. The favorable temperature supporting caffeine degradation was also analyzed.

3.7.3 Effect of Response time on caffeine degradation

Response time also play a vital role so the effect of Response time was also studied to determine caffeine degradation. The Response time supporting highest degradation was selected.

3.7.4 Effect of cells volume on caffeine degradation.

Increased and decreased in cells specific immobilized volume on a support material definitely alter the consequences of an experiment. The response time and the pH relation with cells volume is of prime importance.

RESULTS

4. RESULTS

4.1 Isolation and purification of caffeine degrading microorganisms

The soil samples were collected from the tea wastes discarding sides of IIUI cafeteria's and were brought to the laboratory in sealed shopping bags. All the soil samples were serially diluted up to 10^{-6} concentration by serial dilution method. Caffeine-degrading microorganisms were isolated from soil samples by using a medium containing 5g caffeine, 2g K_2HPO_4, 1g NaCI, 0.5g $MgSO_4$, $7H_2O$, 05g yeast extract, 1 gram nicotinic acid in 1 liter of tap water (PH 7.0). The isolated strain was purified by plate streaking method. The purified strain was maintained on nutrient agar slant and preserved in glycerin solution containing 2 ml of pure culture inoculum and 2 ml of pure glycerin. The preserved bacterial culture was refreshed at the interval of 3 weeks to avoid contamination. Purified single colonies were obtained after fourth step of purification are shown in (figure 4.1).

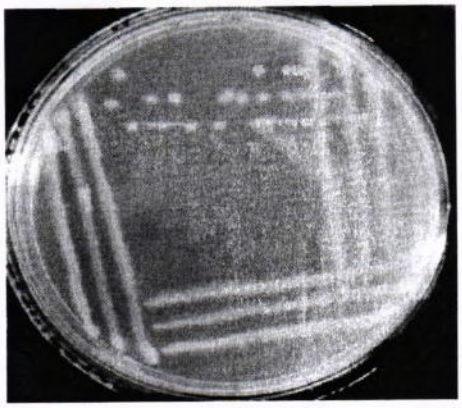


Figure 4.1 *Pseudomonas aeruginosa* purified colonies

4.2 Identification of *Pseudomonas* sp.

The selected purified strain was identified as *Pseudomonas sp.* Through microscopic observation, gram staining, catalase and coagulase tests. The microscopic observation shown that the selected strain was gram-negative, rod-shaped, motile organism and was identify as *Pseudomonas aeruginosa.*

4.2.1 Gram staining

The Gram staining results showed that the *Pseudomonas aeruginosa* was gram negative rod shaped.

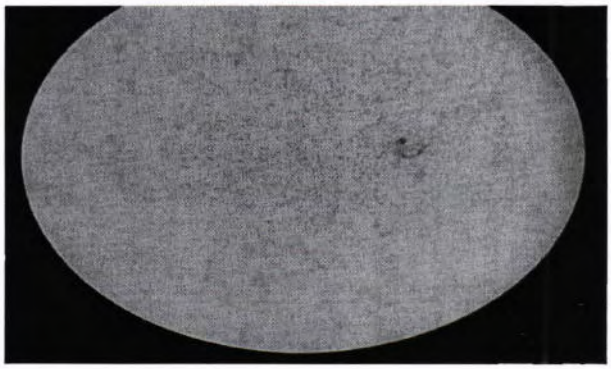


Figure 4.2 *Pseudomonas aeruginosa* gram negative rod shape

4.2.2 Catalase test

The catalase test results showed that the *Pseudomonas aeruginosa* was catalase positive.

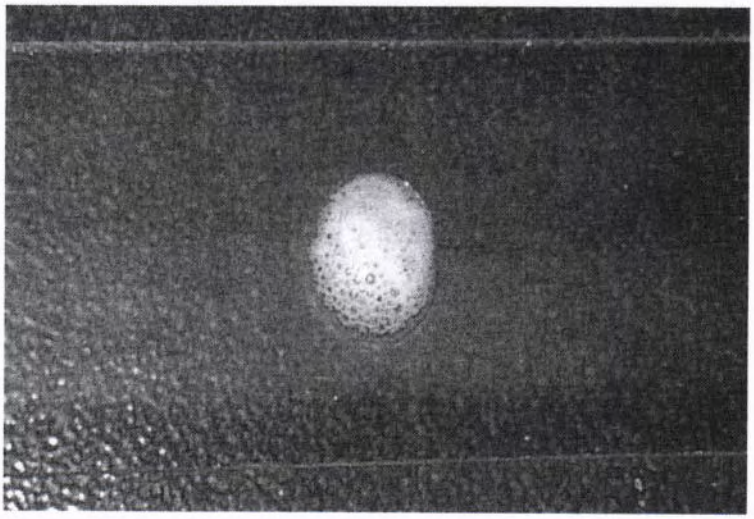


Figure 4.3 *Pseudomonas aeruginosa* catalase positive

4.2.3 Coagulase test

The coagulase test results showed that the *Pseudomonas aeruginosa* was coagulase test negative.

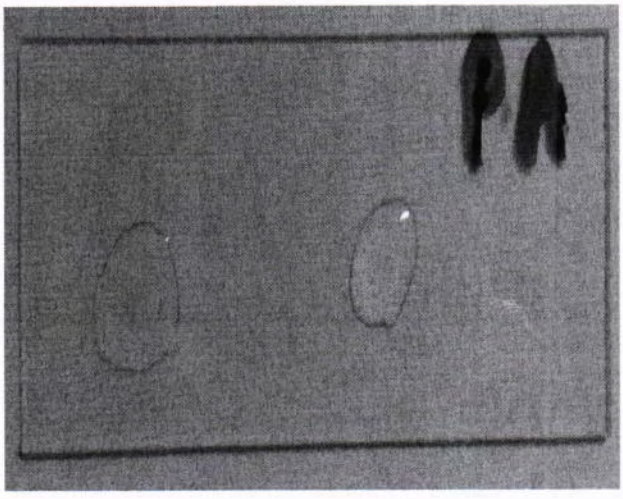


Figure 4.4 *Pseudomonas aeruginosa* (P.A) negative Coagulase test

4.3 *Pseudomonas* sp. Identification for caffeine degradation

Isolated strains were when cultivated aerobically at 28°C for 15 h on a medium containing 3 g caffeine, 5 g K2HP04, 0.02g FeS04 '7H20. After 15 h the growth was appeared on caffeine media which identify that the selecting *Pseudomonas sp.* Were capable of caffeine degradation.

4.4 Caffeine degradation optimization

4.4.1 Effect of pH on caffeine degradation

Effect of pH on the activity of the immobilized cells was studied by incubating the cells loaded strips in buffers in the pH range of 4.0–9.0 with an increment of 0.5 pH units and response of the strip to 1% (w/v) of caffeine was recorded as depletion of oxygen. The pH stability was observed more on inorganic matarials glass slide than the organic materials like wooden toothpicks and sawdusts. The loosing of the pH stability

on organic support materials seems to be due to interaction of pH buffer with that of carbon particles in wooden toothpicks and sawdust pieces. The pH 7 was chosen as optimized pH for the performance of whole cell immobilized cells strip. The concentration values obtained with pH 7 correlates with value labeled 100 mg/l of caffiene in the commercial soft drinks of 500 ml.

Coca Cola (157.53 µg/mL), Sprite (24.88 µg/mL) and Mountain Dew (56.99 µg/ml) results were nearest to that of commercially labeled amount of caffeine 100 ml/l in these soft drinks. While our results showed contrasts with that of commercially labeled amount of caffeine when applied to 7up (75.78 µg/ml), Pepsi (161.56 µg/ml), Fanta (126.44 µg/ml) and Marinda (87.38 µg/ml)

Table 4.1 Effect of pH on caffeine concentration in Pepsi

pH	Absorbance	Concentrations (µg/ml)
4	0.023	45.94
5	0.183	171.32
6	0.154	143.11
7	0.175	161.56
8	0.166	154.32
9	0.162	149.83

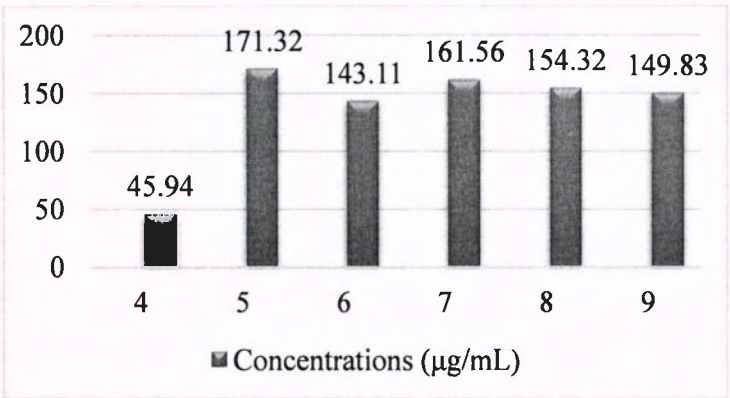


Figure 4.5 Effect of pH on caffeine concentration in Pepsi

Table 4.2 Effect of pH on caffeine concentration in Coca Cola

pH	Absorbance(OD)	Concentrations (µg/ml)
4	0.232	240.55
5	0.182	171.99
6	0.121	119.56
7	0.165	157.53
8	0.177	168.54
9	0.142	137.42

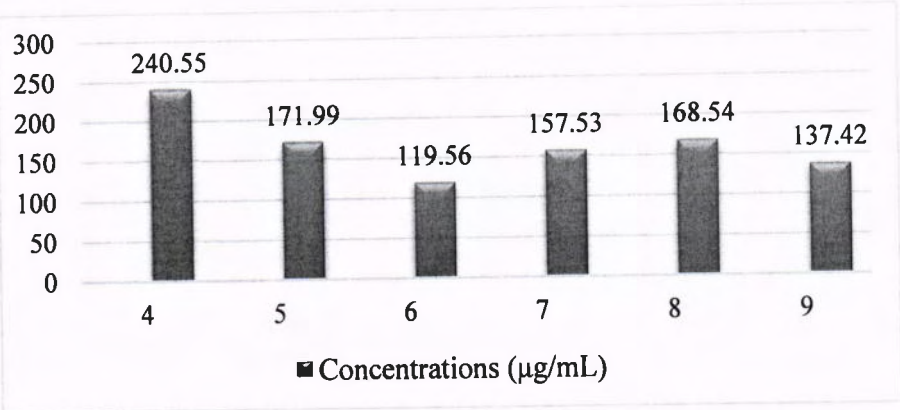


Figure 4.6 Effect of pH on caffeine concentration in Coca Cola

Table 4.3 Effect of pH on caffeine concentration in Sprite

pH	Absorbance (OD)	Concentrations (µg/ml)
5	0.148	140.55
6	0.028	18.77
7	0.039	24.88
8	0.122	138.66
9	0.066	58.33
10	0.123	119.76

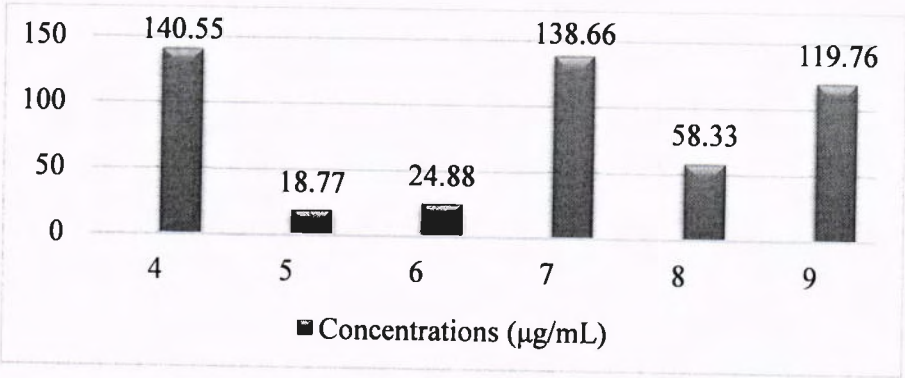


Figure 4.7 Effect of pH on caffeine concentration in Sprite

Table 4.4 Effect of pH on caffeine concentration in 7up

pH	Absorbance(OD)	Concentrations (µg/ml)
4	0.149	132.88
5	0.062	54.80
6	0.086	66.73
7	0.086	75.78
8	0.133	127.66
9	0.063	61.34

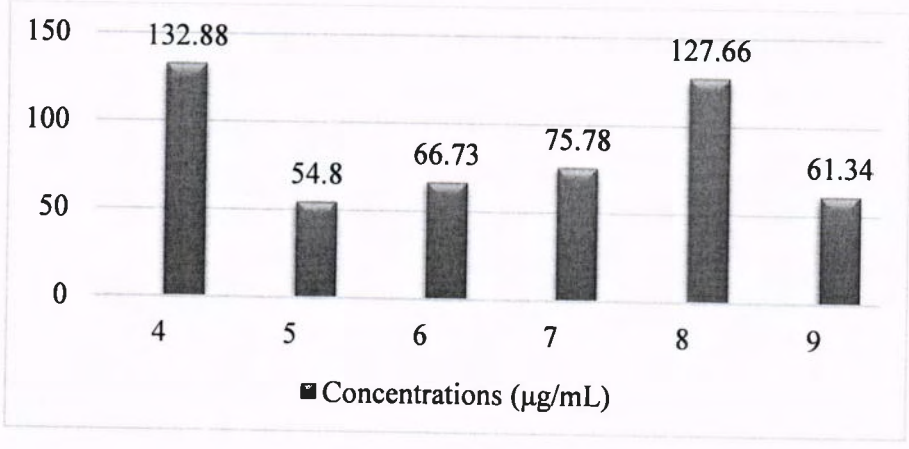


Figure 4.8 Effect of pH on caffeine concentration in 7up

Table 4.5 Effect of pH on caffeine concentration in Dew

pH	Absorbance(OD)	Concentrations (µg/ml)
4	0.177	160.55
5	0.183	175.44
6	0.087	73.54
7	0.068	56.99
8	0.084	77.64
9	0.254	245.63

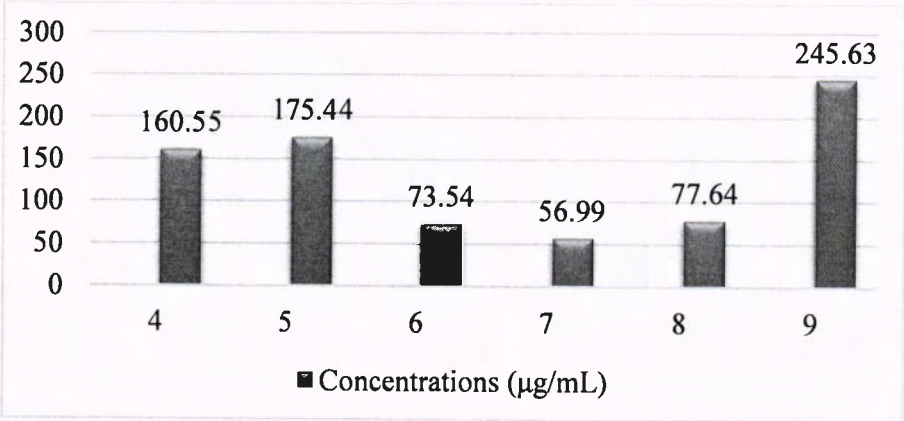


Figure 4.9 Effect of pH on caffeine concentration in Dew

Table 4.6 Effect of pH on caffeine concentration in Fanta

pH	Absorbance(OD)	Concentrations (µg/ml)
4	0.146	131.55
5	0.068	49.65
6	0.094	88.67
7	0.138	126.44
8	0.075	66.43
9	0.071	62.99

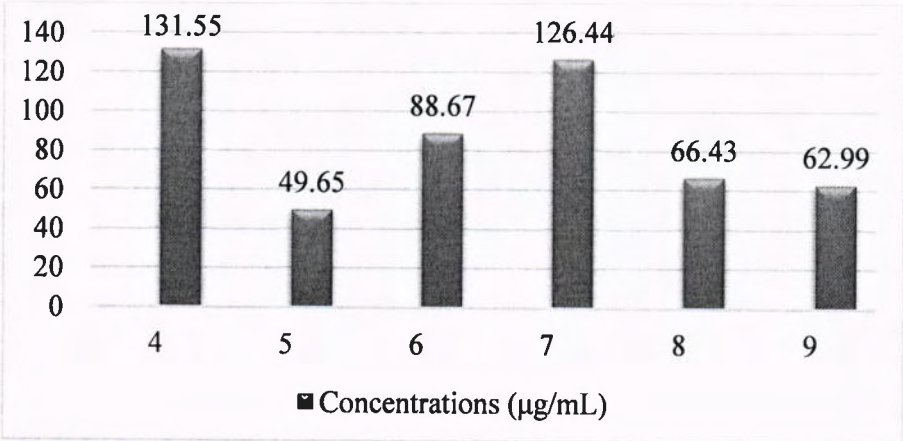


Figure 4.10 Effect of pH on caffeine concentration in Fanta

Table 4.7 Effect of pH on caffeine concentration in Marinda

pH	Absorbance(OD)	Concentrations (µg/ml)
4	0.163	157.61
5	0.183	176.64
6	0.069	57.88
7	0.099	87.38
8	0.049	38.54
9	0.164	153.79

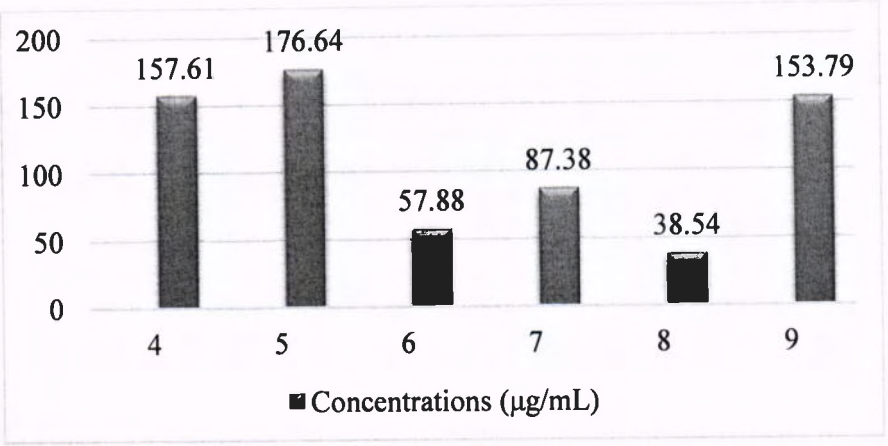


Figure 4.11 Effect of pH on caffeine concentration in Marinda

4.4.2 Effect of temperature on caffeine degradation

In our study temperature was optimized for better performance of the immobilized cells strip and the result was confirmed following the standard curve plot. The result shows deviation from the mean value of the standard curve on 20 °C, 25 °C, 30 °C, 35 °C, 40 °C and 45 °C for the caffeine amount labeled on the commercial soft drinks. Immobilized cells strip showed temperature stability on organic support materials like toothpicks and saw dust pieces, while temperature on inorganic support materials fluctuates due to its rapid cooling and heating nature. The biosensor shows prominence and better degradation when operated at 30 °C. Soft drinks Pepsi (153.11 µg/ml), coca cola (185.45 µg/ml), sprite (27.52 µg/ml), 7up (17.55 µg/ml) and dew (61.41 µg/ml) bottles of are satisfactory similar results for detection of caffeine to that of commercially soft drinks on the 30 °C. Fanta (58.22 µg/ml) and marinda (78.36 µg/ml) results although deviate from the mean values labeled commercially on these soft drinks.

Table 4.8 Effect of temperature on caffeine concentration in Pepsi

Temperature	Absorbance(OD)	Concentrations (µg/ml)
20	0.123	105.44
25	0.162	174.1
30	0.154	153.11
35	0.188	165.23
40	0.172	144.32
45	0.160	169.88

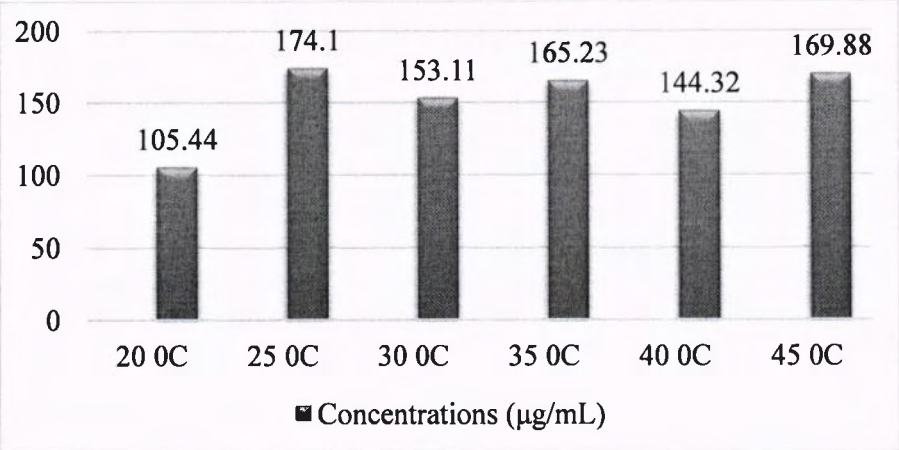


Figure 4.12 Effect of temperature on caffeine concentration in Pepsi

Table 4.9 Effect of temperature on caffeine concentration in Coca Cola

Temperature	Absorbance	Concentrations (µg/ml)
20	0.134	153.28
25	0.152	173.61
30	0.161	185.45
35	0.102	115.21
40	0.124	137.08
45	0.146	167.92

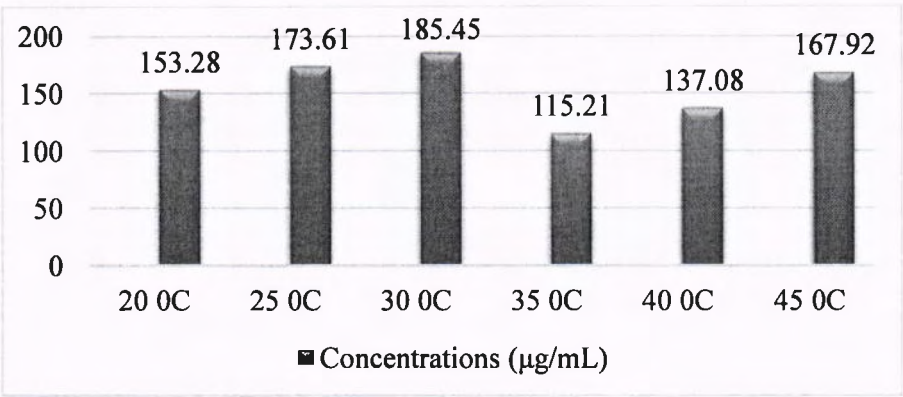


Figure 4.13 Effect of temperature on caffeine concentration in Coca Cola

Table 4.10 Effect of temperature on caffeine concentration in Sprite

Temperature	Absorbance (OD)	Concentrations (µg/ml)
20	0.048	30.49
25	0.027	17.93
30	0.045	27.52
35	0.022	14.66
40	0.043	24.42
45	0.023	15.24

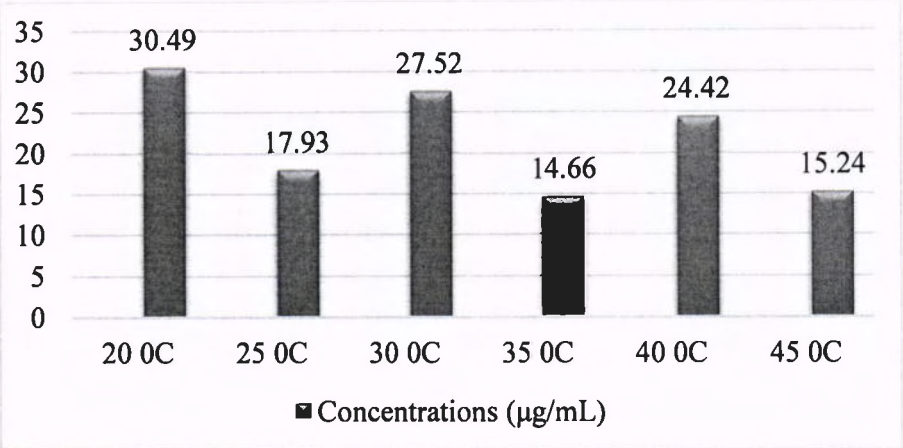


Figure 4.14 Effect of temperature on caffeine concentration in Sprite

Table 4.11 Effect of temperature on caffeine concentration in 7up

Temperature	Absorbance (OD)	Concentrations (µg/mL)
20	0.039	21.59
25	0.042	24.40
30	0.027	17.55
35	0.017	13.00
40	0.49	47.57
45	0.053	51.88

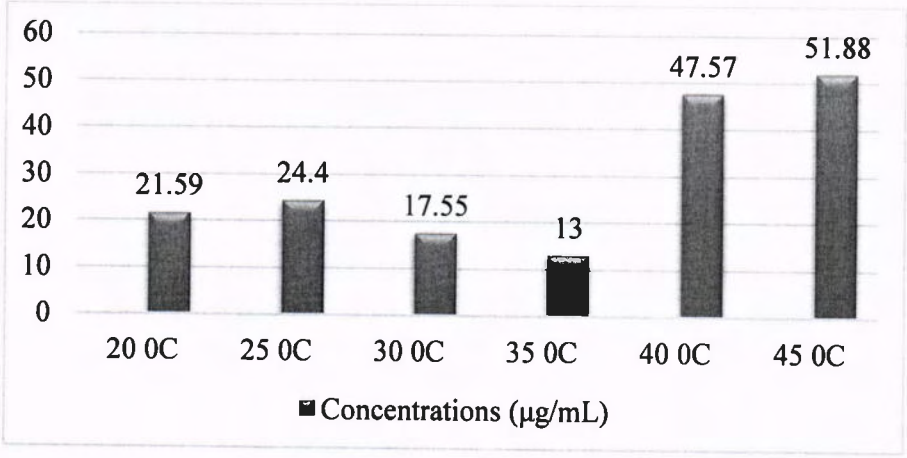


Figure 4.15 Effect of temperature on caffeine concentration in 7up

Table 4.12 Effect of temperature on caffeine concentration in Dew

Temperature	Absorbance (OD)	Concentrations (µg/ml)
20	0.088	91.52
25	0.179	147.82
30	0.073	61.41
35	0.147	122.66
40	0.093	97.44
45	0.217	210.77

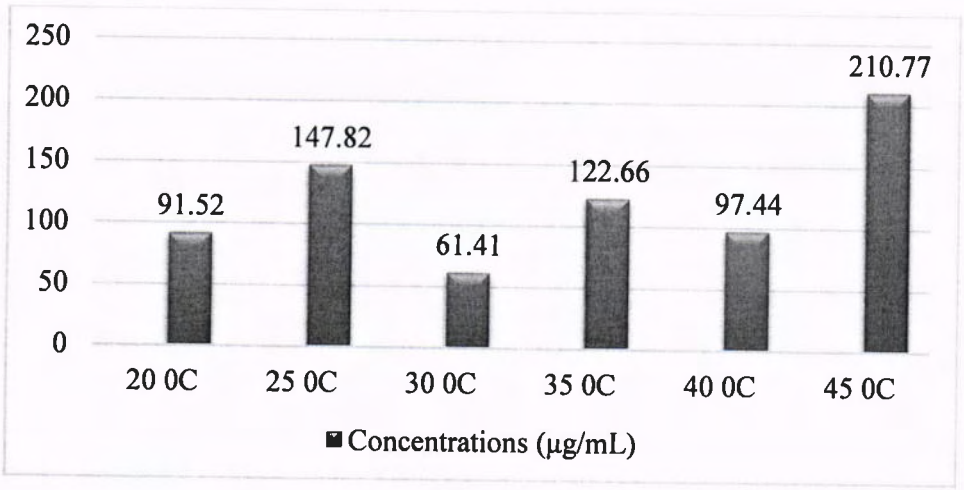


Figure 4.16 Effect of temperature on caffeine concentration in mountain Dew

Table 4.13 Effect of temperature on caffeine concentration in Fanta

Temperature	Absorbance (OD)	Concentrations (µg/mL)
20	0.153	138.47
25	0.078	55.63
30	0.085	58.22
35	0.145	121.55
40	0.121	108.48
45	0.075	52.89

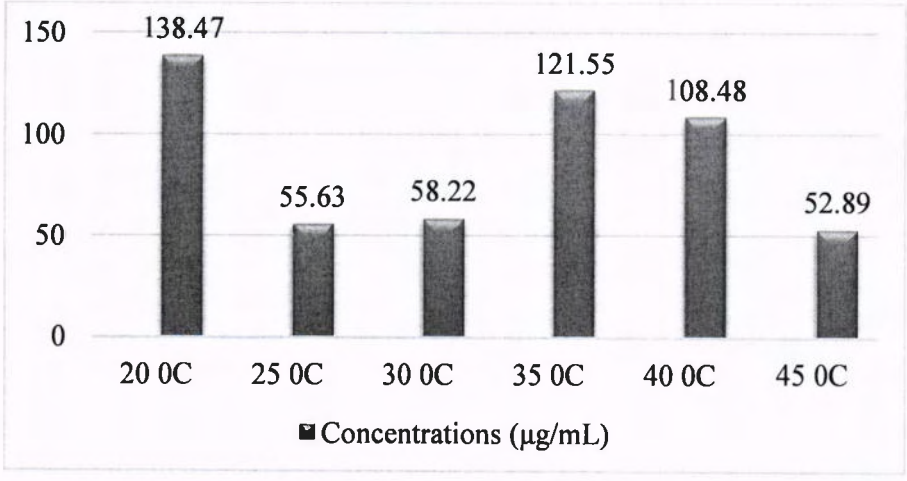


Figure 4.17 Effect of temperature on caffeine concentration in Fanta

Table 4.14 Effect of temperature on caffeine concentration in Marinda

Temperature	Absorbance (OD)	Concentrations (µg/ml)
20	0.159	147.51
25	0.193	177.53
30	0.094	78.36
35	0.086	69.45
40	0.044	33.54
45	0.064	53.99

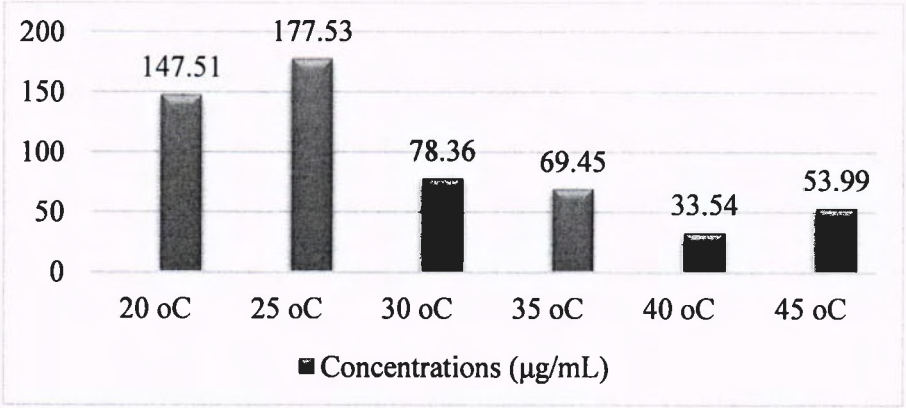


Figure 4.18 Effect of temperature on caffeine concentration in Marinda

4.4.3 Effect of Response time on caffeine degradation

In our study response time was optimized as 5, 10, 15, 20, 25 and 30 minutes for better performance of the strip. In our study we did not find any difference between inorganic support material and organic support material for optimizing response time of immobilized *Pseudomonas aerogenosa* cells strip. Coca cola 184.55 µg/ml, sprite 29.22 µg/ml, mountain dew 84.21 µg/ml and marinda 69.95 µg/ml concentration shows linearity in measurement of caffeine concentration on 15 minutes response time when compared with labeled amount of caffeine on commercial soft drinks. Pepsi (204.11 µg/ml) 7up (167.22 µg/ml) and Fanta (49.32 µg/ml) showed decrease in caffeine concentration from the commercially labeled amount of caffiene on 15 minutes response time.

Table 4.15 Effect of response time on caffeine concentration in pepsi

Response time	Absorbance	Concentrations (µg/ml)
5	0.138	144.64
10	0.114	123.31
15	0.212	204.11
20	0.188	165.23
25	0.172	144.32
30	0.062	69.83

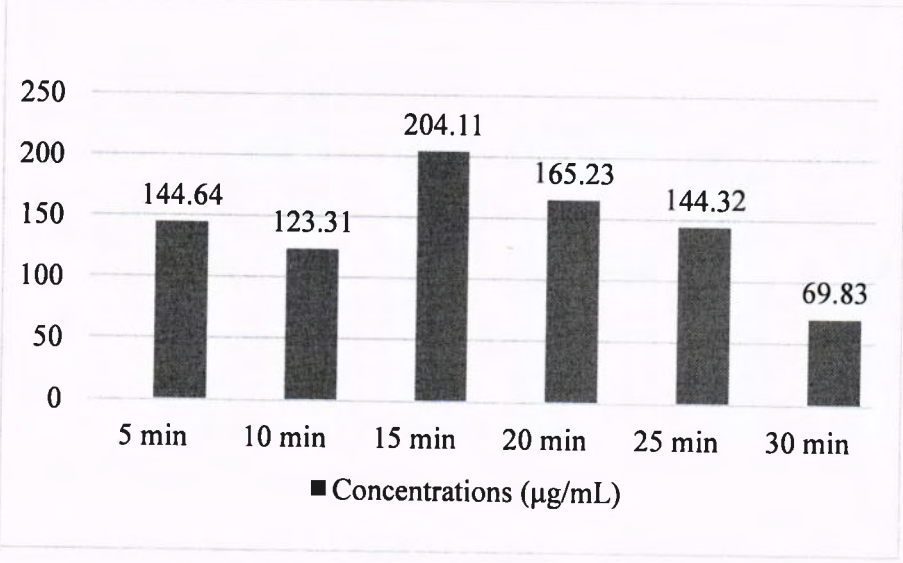


Figure 4.19 Effect of response time on caffeine concentration in Pepsi

Table 4.16 Effect of response time on caffeine concentration in Coca Cola

Response Time	Absorbance (OD)	Concentrations (µg/ml)
5	0.177	163.28
10	0.126	132.61
15	0.172	184.55
20	0.144	125.31
25	0.166	187.08
30	0.246	257.92

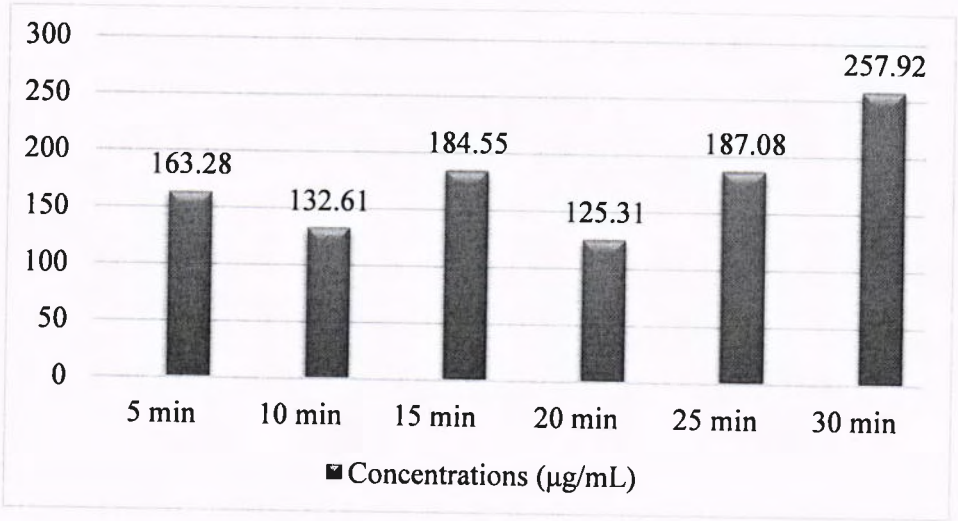


Figure 4.20 Effect of response time on caffeine concentration in Coca Cola

Table 4.17 Effect of response time on caffeine concentration in Sprite

Response time	Absorbance (OD)	Concentrations (µg/ml)
5	0.052	47.99
10	0.034	31.83
15	0.044	29.22
20	0.037	23.72
25	0.143	124.42
30	0.021	11.14

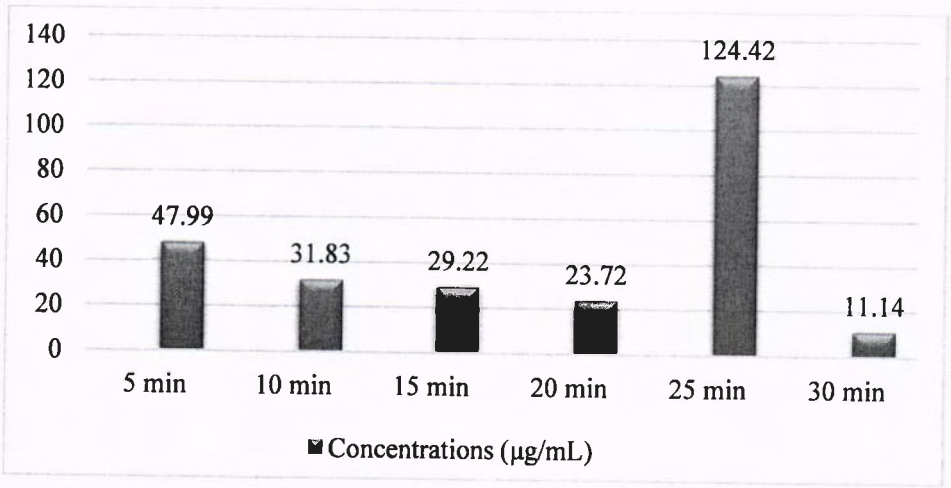


Figure 4.21 Effect of response time on caffeine concentration in Sprite

Table 4.18 Effect of response time on caffeine concentration in 7up

Response time	Absorbance (OD)	Concentrations (µg/ml)
5	0.077	51.83
10	0.055	37.31
15	0.172	167.22
20	0.062	15.57
25	0.38	31.78
30	0.043	41.66

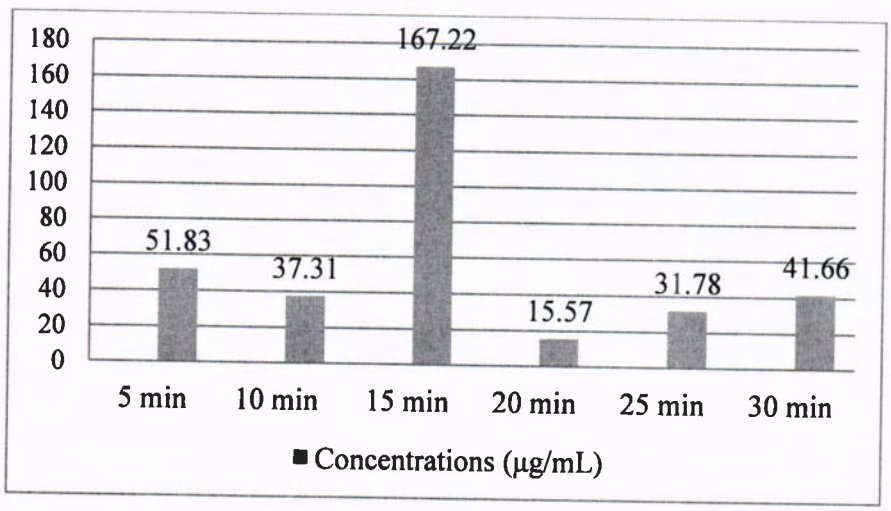


Figure 4.22 Effect of response time on caffeine concentration in 7up

Table 4.19 Effect of response time on caffeine concentration in Dew

Response time	Absorbance (OD)	Concentrations (µg/ml)
5	0.123	142.30
10	0.099	93.77
15	0.093	84.21
20	0.138	119.42
25	0.082	77.84
30	0.199	198.87

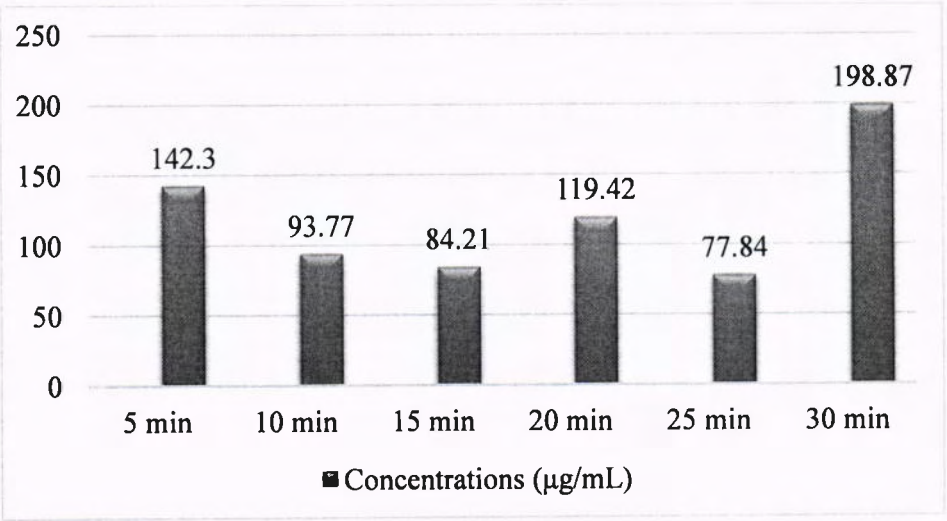


Figure 4.23 Effect of response time on caffeine concentration in Dew

Table 4.20 Effect of response time on caffeine concentration in Fanta

Response time	Absorbance (OD)	Concentrations (µg/ml)
5	0.132	126.11
10	0.071	52.33
15	0.074	49.32
20	0.138	109.67
25	0.127	138.88
30	0.065	42.98

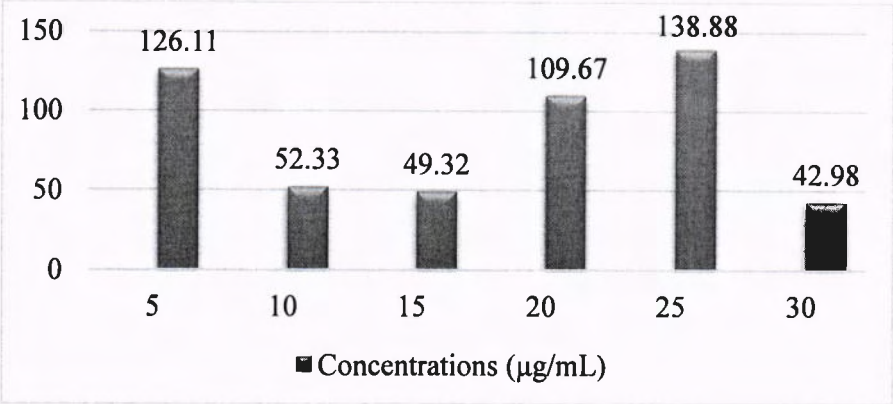


Figure 4.24 Effect of response time on caffeine concentration in Fanta

Table 4.21 Effect of response time on caffeine concentration in Marinda

Response time	Absorbance (OD)	Concentrations (µg/ml)
5	0.178	166.52
10	0.181	164.46
15	0.084	69.95
20	0.132	144.83
25	0.041	33.54
30	0.075	63.55

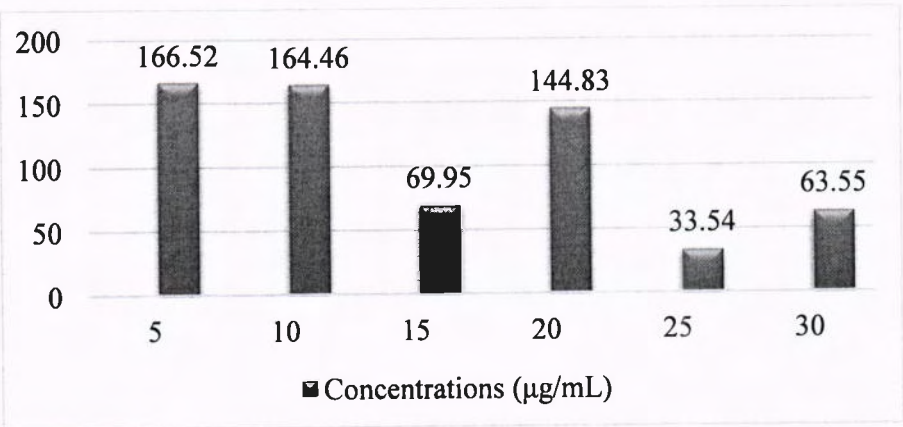


Figure 4.25 Effect of response time on caffeine concentration in Marinda

4.4.4 Effect of cells volume on caffeine degradation

Different amount of cells were examined for the proper degradation and detection of caffeine in commercial soft drinks for obtaining better results. We obtained height degradation of caffeine using 0.25 mg/ml of live microbial cells harvested in exponential phase. The result shows obvious errors when cells amount increase from 0.5 mg/ml to 2.00 mg/l onward while minimum amount of cells i.e. 0.125 mg/ml also caused failure to detect caffeine. We noticed that increase with immobilized cells amount substrate availability is strictly inhibited and minimum amount of immobilized cells also can not degrade caffeine to quantify properly. We optimized 0.25 mg/ml amount of cells which shows result for Pepsi (123.31 µg/ml), Coca Cola (124.91 µg/ml), Sprite (35.44)and in Mountain Dew (124.33 µg/ml). These values correlates with amount of caffeine mention on the commercial soft drinks bottles. However our results somewhat deviate from the mean value in the 7up (164.32 µg/ml), Fanta (122.72 µg/ml) and Marinda (158.44 µg/ml) but we still consider these values in our research work. The biosensor response time is longer at 0.125 mg/ml concentration of immobilized cells which leads to the failure of the caffeine detecting strip but this hurdle can be overcome with further studies.

Table 4.22 Effect of cells volume on caffeine concentration in Pepsi

Amount of cell	Absorbance (OD)	Concentrations (µg/mL)
0.125 mg/ml	0.177	163.55
0.25 mg/ml	0.134	123.31
0.5 mg/ml	0.147	134.88
1.00 mg/ml	0.131	126.11
1.50 mg/ml	0.067	47.36
2.00 mg/ml	0.055	49.63

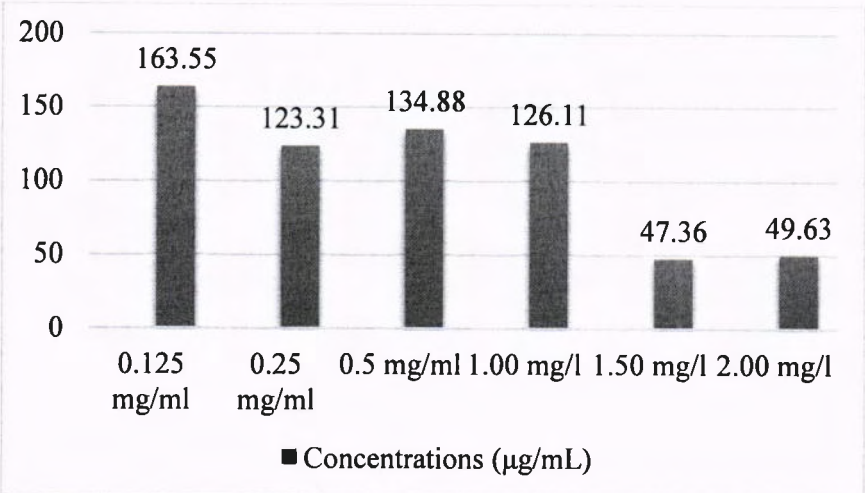


Figure 4.26 Effect of cells volume on caffeine concentration in Pepsi

Table 4.23 Effect of cells volume on caffeine concentration in Coca Cola

Cells volume	Absorbance (OD)	Concentrations (µg/ml)
0.125 mg/ml	0.162	154.73
0.25 mg/ml	0.138	124.91
0.5 mg/ml	0.247	237.82
1.00 mg/ml	0.144	125.31
1.50 mg/ml	0.066	51.88
2.00 mg/ml	0.026	13.82

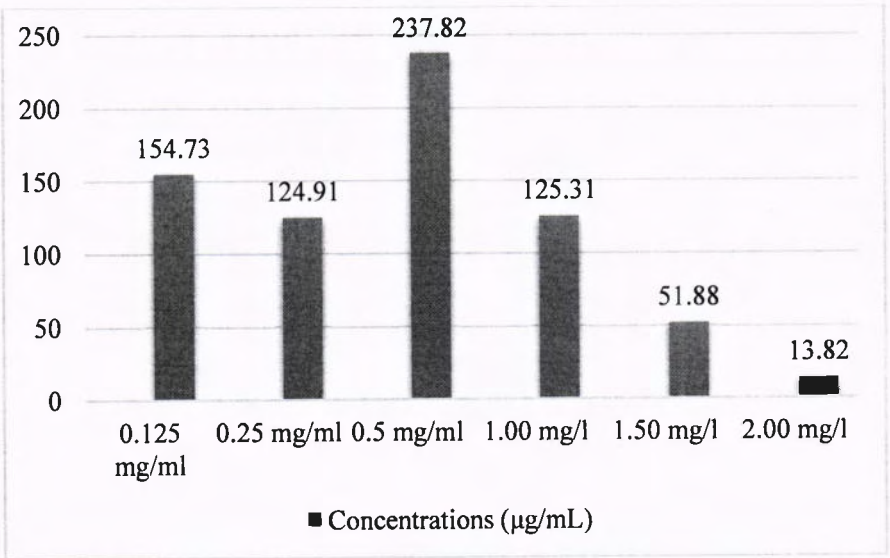


Figure 4.27 Effect of cells volume on caffeine concentration in Coca Cola

Table 4.24 Effect of cells volume on caffeine concentration in Sprite

Cells volume	Absorbance (OD)	Concentrations (µg/ml)
0.125 mg/ml	0.071	67.52
0.25 mg/ml	0.041	35.44
0.5 mg/ml	0.144	128.22
1.00 mg/l	0.031	19.49
1.50 mg/l	0.052	41.34
2.00 mg/l	0.121	111.14

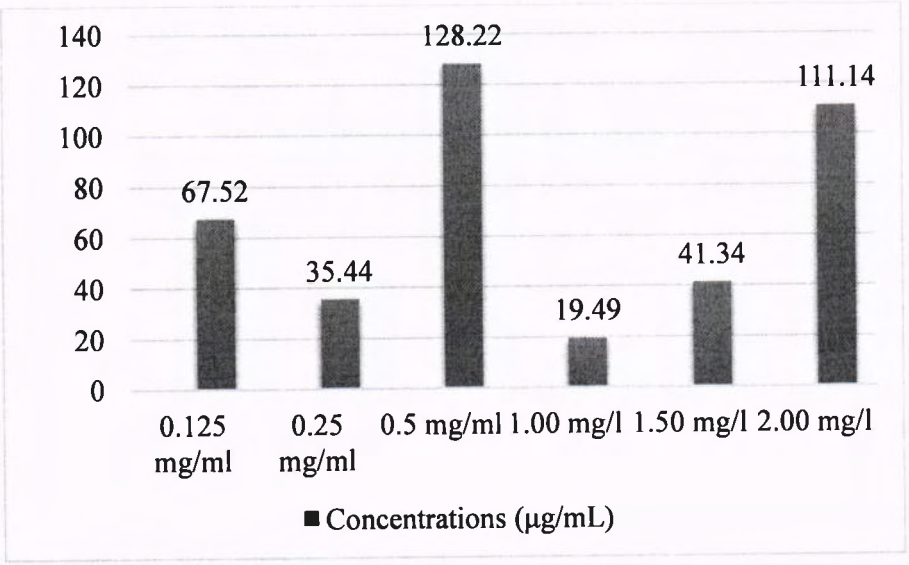


Figure 4.28 Effect of cells volume on caffeine concentration in sprite

Table 4.25 Effect of cells volume on caffeine concentration in 7up

Cells volume	Absorbance (OD)	Concentrations (µg/ml)
0.125 mg/ml	0.068	54.11
0.25 mg/ml	0.179	164.32
0.5 mg/ml	0.183	172.55
1.00 mg/l	0.067	51.66
1.50 mg/l	0.34	25.55
2.00 mg/l	0.056	42.53

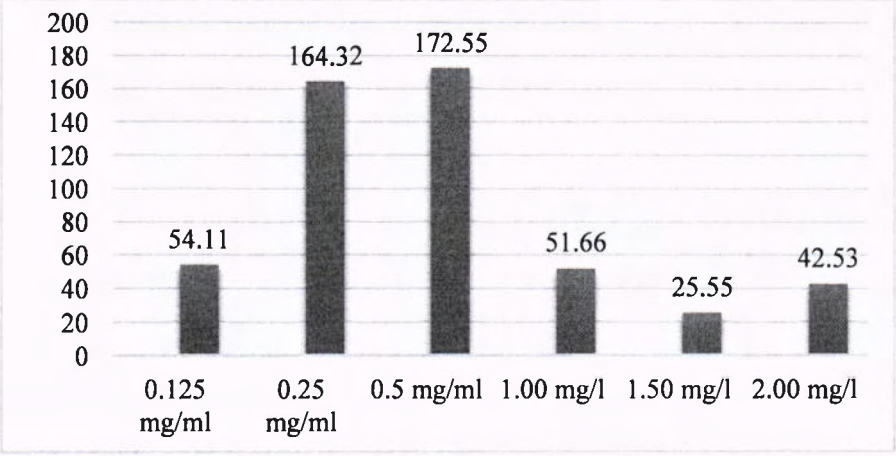


Figure 4.29 Effect of cells volume on caffeine concentration in 7 up

Table 4.26 Effect of cells volume on caffeine concentration in Dew

Cells volume	Absorbance (OD)	Concentrations (µg/ml)
0.125 mg/ml	0.065	57.22
0.25 mg/ml	0.134	124.33
0.5 mg/ml	0.0.127	116.22
1.00 mg/l	0.027	13.41
1.50 mg/l	0.187	168.44
2.00 mg/l	0.044	36.23

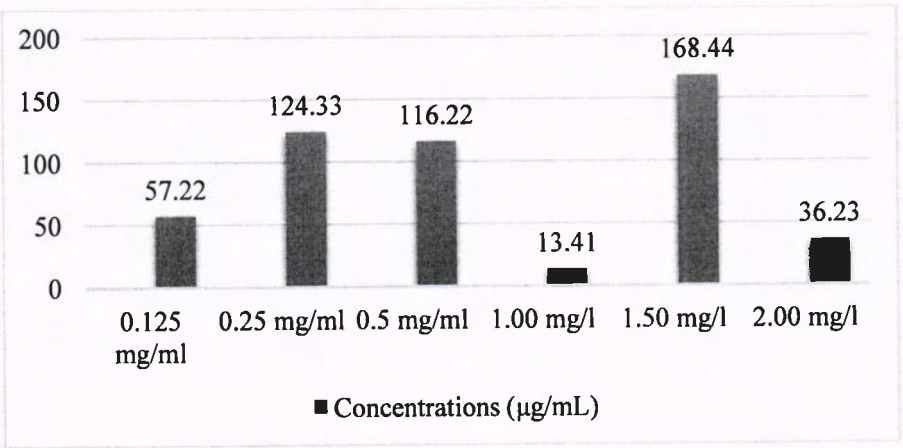


Figure 4.30 Effect of cells volume on caffeine concentration in Dew

Table 4.27 Effect of cells volume on caffeine concentration in Fanta

Cells volume	Absorbance (OD)	Concentrations (µg/ml)
0.125 mg/ml	0.132	126.11
0.25 mg/ml	0.135	122.72
0.5 mg/ml	0.084	78.32
1.00 mg/l	0.138	109.67
1.50 mg/l	0.027	17.48
2.00 mg/l	0.065	42.98

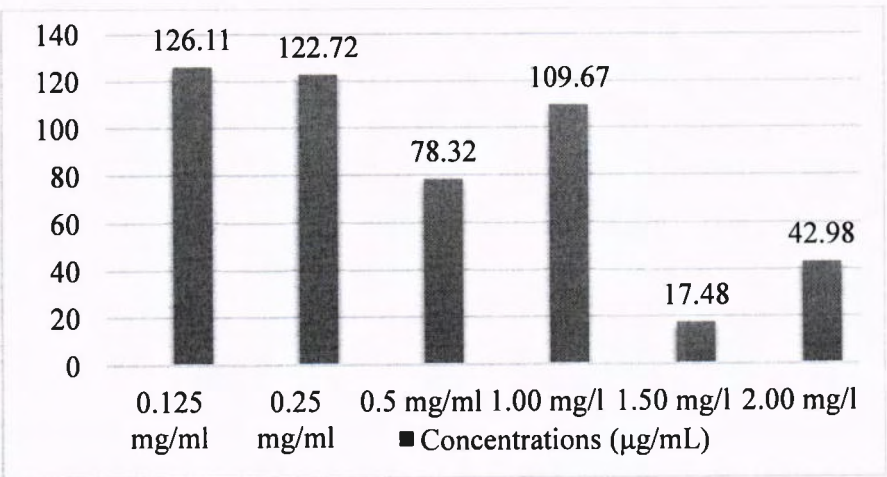


Figure 4.31 Effect of cells volume on caffeine concentration in Fanta

Table 4.28 Effect of cells volume on caffeine concentration in Marinda

Cells volume	Absorbance (OD)	Concentrations (µg/ml)
0.125 mg/ml	0.078	64.92
0.25 mg/ml	0.172	158.44
0.5 mg/ml	0.077	61.43
1.00 mg/l	0.188	194.22
1.50 mg/l	0.011	7.786
2.00 mg/l	0.084	68.25

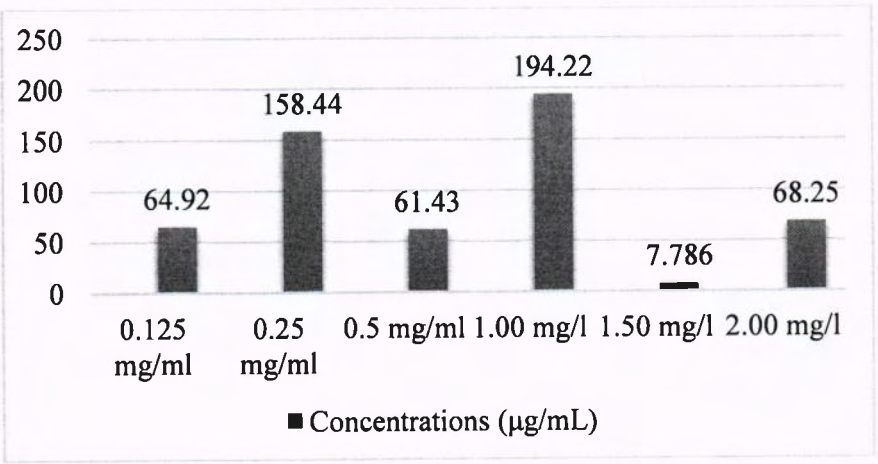


Figure 4.32 Effect of cells volume on caffeine concentration in Marinda

DISCUSSION

5. DISCUSSION

Pseudomonas strain was isolated and induced in nutrient agar medium to exploit caffeine as the sole carbon and nitrogen source. On the behalf of caffeine degrading ability the *pseudomonas* strain was further processed for the development of a bio strip.

In the development of biosensor support material has a preliminary role and we checked both organic (wooden toothpicks and saw dust pieces) and inorganic (glass slides) for the better performance of the immobilized *pseudomonas* cells. The pH stability was observed more on inorganic material glass slides than the organic materials like wooden toothpicks and sawdust's. Immobilized cells strip showed temperature stability in organic support materials like toothpicks and saw dust pieces, while temperature on inorganic support materials fluctuates due to its rapid cooling and heating nature. Our results showed prominence results for 0.25 mg/ml concentration of cells immobilized on organic support materials like toothpicks and saw dust pieces as compared to immobilizing *Pseudomonas aeruginosa* cells on inorganic glass slides. In our study we did not find any difference between inorganic support material and organic support material for optimizing response time of immobilized *Pseudomonas aeruginosa* cells strip.

Different Biosensor and bio strips were prepared and experienced at various temperatures to investigate the working temperature. The ideal temperature for the caffeine response of strip was found to be 30°C. The response of temperature on the immobilized cells based strip for caffeine degradation was measured maximum in the range of 25°C to 35°C, but above and below this temperature the caffeine degrading activity gradually decreased. This may be due to high heat absorption and slip nature of microscopic glass slides (as a support material) leading to spontaneous detachment of cells. While cellulosic materials (wooden toothpicks and de lignified sawdust) showed less decrease in the efficiency of cells immobilized cells strip with variation in temperature. The stability of the cellulosic materials to temperature may be the result of lesser heat absorption capacity and cells holding texture. Furthermore, increased in temperature destabilize the viability of immobilized cells.

Optimization of pH level is the one of the most important parameter for biosensor studies. Phosphate buffer (100 mM) at pH 7.0 was used for further studies in the optimization of biosensor for caffeine estimation. The optimum pH for raising the biomass was 8.0. At this pH, maximum biomass accumulation was observed. Therefore, the cells were induced for caffeine degradation at pH 8.0. However, maximum caffeine degrading activity on the strip was observed at pH 7.0 as the optimum pH for activity of caffeine degradation enzymes is 7.0. Therefore, two different pH were used for induction of cells in growth medium and for degradation of caffeine through immobilized cells strip. According to our study, when pH was increased from 6.0 to 7.0 increases for the microbial biosensor response were observed, on the other hand when pH increased from 7.0 to 10.0 decreases were recorded for bio strip response to caffeine detection. In one other study pH 6.8 was optimized for immobilized cells for caffeine degradation (Babu *et al.*, 2007)

To evaluate the performance of bio strip its analysis time should be too rapid, but in contrast in our research work we did not overcome this difficulty. The response time of our whole cell immobilized strip was measured 15 minutes which showed contradiction with others whole cells immobilized strips (Canbay *et al.*, 2015). This may be the consequence of substrates diffusion inhibition (depend on cells volume) into the immobilized cell strip. This complication in turn leading to decreasing in assimilation of substrate which is a preliminary drawback for development of caffeine concentration detecting bio-strip.

Different cells volume were examined for the proper degradation and detection of caffeine for obtaining better results. We obtained highest degradation of caffeine using 0.25 mg/ml of live microbial cells harvested in exponential phase with 10,000 rpm for 10 minutes. The result showed obvious errors when cells amount increase from 0.5 mg/ml to 2.00 mg/l onward. We noticed that increase with cells volume substrate availability is strictly inhibited. We optimized 0.25 mg/ml amount of cells which showed result for Pepsi (123.31 µg/ml), Coca cola (124.91 µg/ml), Sprite (35.44) and in mountain dew (124.33 µg/ml). These values correlates with amount of caffeine mention on the commercial soft drinks bottles. However our results somewhat deviate from the mean

value in the 7up (164.32 μg/ml), Fanta (122.72 μg/ml) and marinda (158.44 μg/ml) but we still consider these values in our research work. Beside maximum amount of caffeine degrading and detecting we find that 0.125 mg/ml was not so efficient to obtain good results because at this concentration the biosensor response time is longer than the others. Our amount of cells data shows similarity to that of obtained by (Akyilmaz *et al.*, 2005) for lactic acid quantification and (Canbay *et al.*, 2015).

Our studies on *pseudomonas* immobilized whole cell strip showed that a caffeine sensitive biosensor can be develope for caffeine sensitive people. Some reported cases of severe negative effects of caffeine in pregnancy and in anxiety with panic disorder are reported, so it is most needed to detect caffeine concentration in blood and as well as in food samples to overcome caffeine related health problems.

Conclusion

The aim of this study was mainly to develop a microbial biosensor intended for rapid determination of caffeine. Consequently, the simplicity in the bioactive material immobilization demonstrated a considerable economic advantage because of low cost, which is desirable in routine analysis. In this work, a new biosensing strip based on immobilized *Pseudomonas aeruginosa* cells for the sensitive determination of caffeine was developed. The proposed biosensor shows a good activity towards the oxidation of caffeine. Response time (15 min) showed somewhat hurdles that there is diffusion resistance for the microbial biosensor and this means that the diffusion of substrates into the cell and assimilation of substrate is a rate limiting process. All conditions including temperature 30°C, pH 7 and cells volume 0.25 mg/ml were optimized properly for development of a microbial biosensor. Further advancement can be made with more research work for the rapid detection of caffeine to overcome some behavioral effects related to caffeine consumption. Microbial biosensor system developed is a simple, suitable and efficient method for determination of caffeine in commercial soft drinks.

REFERENCES

6. REFERENCES

Akyilmaz, E., and Dinçkaya, E. (2005). An amperometric microbial biosensor development based on Candida tropicalis yeast cells for sensitive determination of ethanol. *Biosensors and Bioelectronics*, *20*(7), 1263-1269.

Babu, V. S., Patra, S., Karanth, N. G., Kumar, M. A., & Thakur, M. S. (2007). Development of a biosensor for caffeine. *Analytica chimica acta,582*(2), 329-334.

Basu, A. K., Chattopadhyay, P., Roychudhuri, U., & Chakraborty, R. (2006). A biosensor based on co-immobilized L-glutamate oxidase and L-glutamate dehydrogenase for analysis of monosodium glutamate in food. *Biosensors and bioelectronics*, *21*(10), 1968-1972.

Bogo, A., Mantle, P.G., (2000), Caffeine: also a fungal metabolite, *Phytochem,*54: p. 937–939.

Byfield, M. P., & Abuknesha, R. A. (1994). Biochemical aspects of biosensors. *Biosensors and Bioelectronics*, *9*(4), 373-399.

Carter, A.J., O'Connor, W.T., Carter, M.J., Ungerstedt, U., (1995), Caffeine enhances Acetylcholine release in the hippocampus in vivo by a selective interaction with adenosine A1 receptors. J. *Pharmacol.* Exp. Ther. 273(2): 637-42.

Campa, C., Doulbeau, S., Dussert, S., Hamon, S., & Noirot, M. (2005). Diversity in bean caffeine content among wild Coffea species: evidence of a discontinuous distribution. *Food chemistry*, *91*(4), p. 633-637.

Clauson, K. A., Shields, K. M., McQueen, C. E., & Persad, N. (2008). Safety issues associated with commercially available energy drinks. *Journal of the American Pharmacists Association*, *48*(3), p. 55-67.

Chou, S. F. (1996). Amperometric biosensor for the determination of the artificial sweetener aspartame with an immobilized bienzyme system.*Analyst*, *121*(1), p. 71-73.

Dash S. S and Gummadi S. N. (2006). Catabolic pathways and biotechnological applications of microbial caffeine degradation. *Biotechnology letters*, *28*(24), 1993-2002.

De Luca, S., Florescu, M., Ghica, M. E., Lupu, A., Palleschi, G., Brett, C. M. A., & Compagnone, D. (2005). Carbon film electrodes for oxidase-based enzyme sensors in food analysis. *Talanta*, *68*(2), 171-178.

Fenster, L., Eskenazi, B., Windham, G.C., (1991), Caffeine consumption during pregnancy and fetal growth. Am. J. Public Health. 81:458-61.

Ford, R. P. K., Schluter, P. J., Mitchell, E. A., Taylor, B. J., Scragg, R., Stewart, A. W., & New Zealand Cot Death Study Group. (1998). Heavy caffeine intake in pregnancy and sudden infant death syndrome. *Archives of disease in childhood*, *78*(1), 9-13.

Golitsch, F., Bücking, C., & Gescher, J. (2013). Proof of principle for an engineered microbial biosensor based on Shewanella oneidensis outer membrane protein complexes. *Biosensors and Bioelectronics*, *47*, 285-291.

Gummadi, S. N., Bhavya, B., & Ashok, N. (2012). Physiology, biochemistry and possible applications of microbial caffeine degradation. *Applied microbiology and biotechnology*, *93*(2), 545-554.

Holtzman, S. G. (1990). Caffeine as a model drug of abuse. *Trends in pharmacological sciences*, *11*(9), 355-356.

Hughes, J. R., Oliveto, A. H., Bickel, W. K., Higgins, S. T., and Badger, G. J. (1993). Caffeine self-administration and withdrawal: incidence, individual differences and interrelationships. *Drug and alcohol dependence*, *32*(3), 239-246.

Kalmar, J. M., & Cafarelli, E. (1999). Effects of caffeine on neuromuscular function. *Journal of applied physiology*, *87*(2), 801-808.

Kaufmann, W. F., Heffernan, T. P., Beaulieu, L. M., Doherty, S., Frank, A. R., Zhou, Y., Bryant, M. F., Zhou, T., Luche, D. D., Feinberg, N. N., Schuh, K. J., Griffiths, R.R., (1997), Caffeine reinforcement: the role of withdrawal. *Psychopharmacology*; 130:320–6

Kim, N., Park, I. S., & Kim, D. K. (2004). Characteristics of a label-free piezoelectric immunosensor detecting Pseudomonas aeruginosa. *Sensors and Actuators B: Chemical*, *100*(3), 432-438.

Kirgöz, Ü. A., Odaci, D., Timur, S., Merkoçi, A., Alegret, S., Beşün, N., & Telefoncu, A. (2006). A biosensor based on graphite epoxy composite electrode for aspartame and ethanol detection. *Analytica chimica acta*,*570*(2), 165-169.

Liu, C., Yong, D., Yu, D., & Dong, S. (2011). Cell-based biosensor for measurement of phenol and nitrophenols toxicity. *Talanta*, *84*(3), 766-770.

Lee, S., Hudson, R., Kilpatrick, K., Graham, T. E., Ross, R. (2005), Caffeine Ingestion Is Associated with Reductions in Glucose Uptake Independent of Obesity and Type 2 Diabetes Before and After Exercise Training. *Diabetes Care,* 28(3), p. 566 - 572.

Leviton, A., Cowan, L., (2002), A review of the literature relating caffeine consumption by women to their risk of reproductive hazards. *Food Chem Toxicol.* 40:1271-310.

May, K. M., Wang, Y., Bachas, L. G., & Anderson, K. W. (2004). Development of a whole-cell-based biosensor for detecting histamine as a model toxin. *Analytical chemistry*, *76*(14), 4156-4161.

Ngundi, M. M., Qadri, S. A., Wallace, E. V., Moore, M. H., Lassman, M. E., Shriver Lake, L. C., and Taitt, C. R. (2006). Detection of deoxynivalenol in foods and indoor air using an array biosensor. *Environmental science & technology*, *40*(7), 2352-2356.

Pizzariello, A., Svorc, J., Stredansky, M., & Miertus, S. (1999). A biosensing method for detection of caffeine in coffee. *Journal of the Science of Food and Agriculture*, *79*(8), 1136-1140.

Pasco, N., Joanne, H., & Webber, J. (2001). Biosensors: MICREDOX-a new biosensor technique for rapid measurement of BOD and toxicity. *Biomarkers*,*6*(1), 83-89.

Pasco, N., Baronian, K., Jeffries, C., Webber, J., & Hay, J. (2004). MICREDOX development of a ferricyanide-mediated rapid biochemical oxygen demand method using an immobilized Proteus vulgaris biocomponent. *Biosensors and Bioelectronics*, *20*(3), 524-532.

Pieters, M. N., Fiolet, D. C. M., & Baars, A. J. (1999). Deoxynivalenol. Derivation of concentration limits in wheat and wheat containing food products. *RIVM* 80(4), p. 43-47.

Rodrigues, C. I., Marta, L., Maia, R., Miranda, M., Ribeirinho, M., & Máguas, C. (2007). Application of solid-phase extraction to brewed coffee caffeine and organic acid

determination by UV/HPLC. *Journal of Food Composition and Analysis*, *20*(5), 440-448.

Sun, J. Y., Huang, K. J., Wei, S. Y., Wu, Z. W., & Ren, F. P. (2011). A graphene-based electrochemical sensor for sensitive determination of caffeine. *Colloids and Surfaces B: Biointerfaces*, *84*(2), 421-426.

Si, R. W., Zhai, D. D., Liao, Z. H., Gao, L., & Yong, Y. C. (2015). A whole-cell electrochemical biosensing system based on bacterial inward electron flow for fumarate quantification. *Biosensors and Bioelectronics*, *68*, 34-40.

Thomas, J. B., Yen, J. H., Schantz, M. M., Porter, B. J., & Sharpless, K. E. (2004). Determination of caffeine, theobromine, and theophylline in standard reference material 2384, baking chocolate, using reversed-phase liquid chromatography. *Journal of agricultural and food chemistry*, *52*(11), 3259-3263.

Webster, D. P., TerAvest, M. A., Doud, D. F., Chakravorty, A., Holmes, E. C., Radens, C. M. & Angenent, L. T. (2014). An arsenic-specific biosensor with genetically engineered Shewanella oneidensis in a bioelectrochemical system. *Biosensors and Bioelectronics*, *62*, 320-324.

Wijhe, M. V., (2002), The history of caffeine as used in anaesthesia. *Int. Congr. Ser.* 1242, p. 101–3.

Wilson R and Turner A. P. F. 1992, Glucose oxidase: and ideal enzyme. *Biosensors & Bioelectronics*,7, p.165–185.

Yan, Zhongqiang, et al. "Rapid quantitative detection of Yersinia pestis by lateral-flow immunoassay and up-converting phosphor technology-based biosensor." *Sensors and Actuators B: Chemical* 119.2 (2006): 656-663.

Yu, C. L., Louie, T. M., Summers, R., Kale, Y., Gopishetty, S., & Subramanian, M. (2009). Two distinct pathways for metabolism of theophylline and caffeine are coexpressed in Pseudomonas putida CBB5.*Journal of bacteriology*, *191*(14), 4624-4632.

Waring, W. S., Goudsmith, J., Marwick, J., Webb, D. J., Maxwell, S. R. J., (2003), Acute caffeine intake influences central more than peripheral blood pressure in young adults. Am. J. *Hypertens.* 16: 919–24.

Varani, K., Portaluppi, F., Merighi, S., Ongini, E., Belardinelli, L., & Borea, P. A. (1999). Caffeine Alters A2A Adenosine Receptors and Their Function in Human Platelets. *Circulation*, *99*(19), 2499-2502.