

## Development of a biosensor for caffeine

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### Abstract

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

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

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

### 1. Introduction

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

moderately soluble in water and organic solvents like ethanol, ethyl acetate, methanol, benzene, etc.

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

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

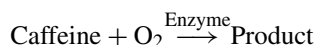
Conventionally, high-performance liquid chromatography (HPLC) separation [8] and UV-spectrophotometric detection [9], methods are applied to both regular and decaffeinated

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

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

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



## 2. Materials and methods

Caffeine, theophylline, theobromine, gelatin, glutaraldehyde and para chloro mercuri benzoic acid (Ultra Pure) were obtained from Sigma Chemicals, St. Louis, U.S.A. Analytical grade, di-nitro phenyl hydrazine, polyvinyl alcohol and polyvinyl pyrrolidone were procured from Sisco Research Laboratory Chemicals, Mumbai, India. Dehydrated nutrient agar and nutrient broth

(AR) were obtained from M/s Himedia Labs, Mumbai, India. All other chemicals were of high purity and were procured from standard sources.

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

### 2.1. Induction of the organism for caffeine degradation

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

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

### 2.2. Construction of the caffeine biosensor

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

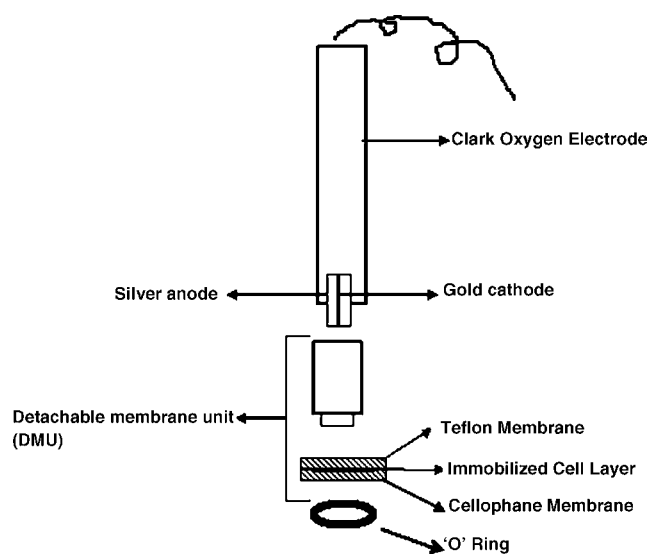


Fig. 1. Schematic diagram of the whole cell electrode used for the biosensor.

### 2.3. Immobilization of whole cells

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

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

Immobilization of the cells using gelatin was done by pipetting 50  $\mu$ L of cell suspension onto a cellophane membrane (2 cm  $\times$  2 cm) and 50  $\mu$ L of a 10% (w/v) of gelatin and mixing well with a fine glass rod by avoiding air bubbles. To this cell matrix mixture 25  $\mu$ L of 5% aqueous solution of glutaraldehyde was added and mixed well. The cells were allowed to crosslink with the immobilizing agent (gelatin) on the membrane by incubation at room temperature for 1 h. The immobilized cell membranes were then washed thoroughly with distilled water and phosphate buffer (100 mM, pH 6.8) and stored at 4 °C till further use.

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

### 2.4. The biological sensing element

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

### 2.5. Construction of the transducer-amplifier-detector system

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

### 2.6. Measurement of response by immobilized microbial cells for caffeine

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

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

### 2.7. Response of xanthine oxidase to caffeine

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

### 2.8. Optimization of parameters for the biosensor

#### 2.8.1. pH

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

#### 2.8.2. Temperature

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

#### 2.8.3. Interference of different sugars in caffeine estimation

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

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

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

### 2.8.5. Calibration for caffeine using HPLC and biosensor

Calibration graphs for caffeine were plotted for the analysis of caffeine in samples by the biosensor and by HPLC.

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

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

### 2.8.6. Analysis of caffeine in real samples by HPLC

HPLC analysis of caffeine was done using a Shimadzu-LC 10A fitted with a C<sub>18</sub> Column (300 mm  $\times$  4.6 mm) with acetonitrile and water (10:90%) as mobile phase set at a flow rate of 1 mL min<sup>-1</sup> and a UV detector set at 273 nm. Real samples (coffee and tea sample) were analyzed for their caffeine content. Samples of tea and coffee were prepared as described below and the concentration was determined by recording the peak area and comparing with the calibration graph.

### 2.8.7. Preparation of real samples for determination of caffeine

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

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

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

## 3. Results and discussion

### 3.1. Construction of caffeine biosensor

#### 3.1.1. Immobilization of whole cells

For immobilization of cells, different agents viz. gelatin, polyvinyl pyrrolidone and polyvinyl alcohol were tried. The biosensor gave good response for caffeine (1%, w/v) showing a steady state depletion of 53.2% DO concentration as shown in Table 1. It was observed that polyvinyl alcohol is a better immobilizing agent in terms of a higher response and reusability, which may be due to a higher oxygen permeability and diffusiv-

Table 1

Response of the biosensor using different immobilizing agents

| Stabilizing agent     | %DO depletion shown by the biosensor after 3 min (1%, w/v) | No. of analyses with one sensor element |
|-----------------------|------------------------------------------------------------|-----------------------------------------|
| Gelatin               | 1.7                                                        | 1                                       |
| Polyvinyl pyrrolidone | 1.6                                                        | 2                                       |
| Polyvinyl alcohol     | 53.2                                                       | 15                                      |

ity [21]. With PVA as stabilizing agent, about 15–25 analyses could be done with a single membrane.

#### 3.1.2. Response of biosensor to caffeine

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

For the determination of caffeine content in the samples using the immobilized cells based biosensor, a calibration graph for caffeine in the concentration range of 0.01–0.1% (w/v) of caffeine was plotted with a regression value of 0.992.

The concentration of caffeine in the sample can be calculated by using Eq. (2)

$$y = 189.53x + 6.1134 \quad (1)$$

This can be rewritten as

$$x = y - \frac{6.1134}{189.53} \quad (2)$$

where 'y' is the % dissolved oxygen consumed and 'x' is the concentration of caffeine in the sample.

For the comparison of the results of analysis of caffeine by the biosensor with a standard method, a calibration graph for caffeine in the concentration range of 0.01–0.1% (w/v) was also plotted with a regression value of 0.996 using HPLC.

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

$$y = 4453x - 20.323 \quad (3)$$

This can be rewritten as

$$x = y \frac{-20.323}{4453} \quad (4)$$

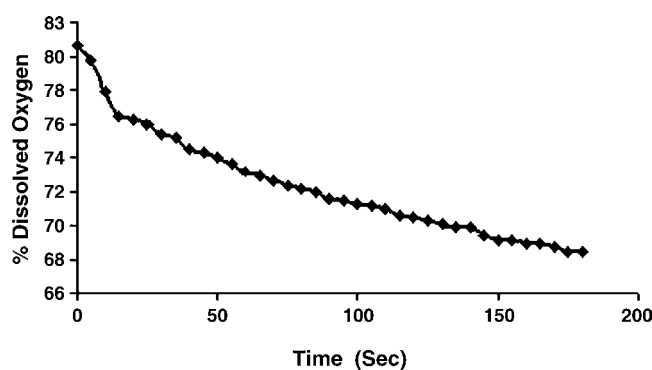


Fig. 2. Response of the biosensor to 1% (w/v) of pure caffeine.

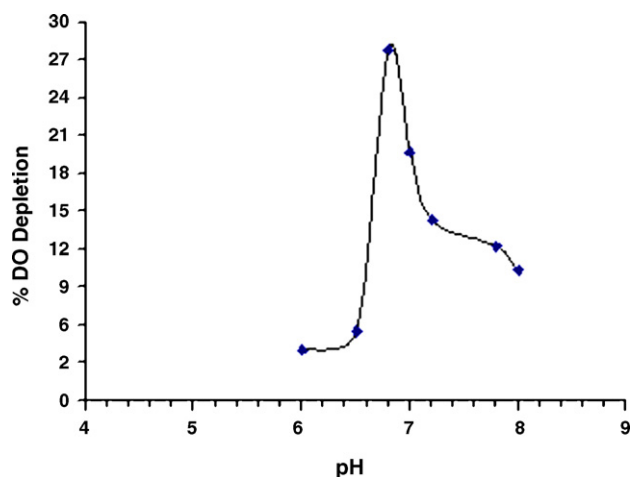


Fig. 3. Effect of pH on the response of biosensor to caffeine.

where 'y' is the % dissolved oxygen consumed and 'x' is the concentration of caffeine in the sample.

### 3.1.3. Optimization of parameters for the biosensor

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

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

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

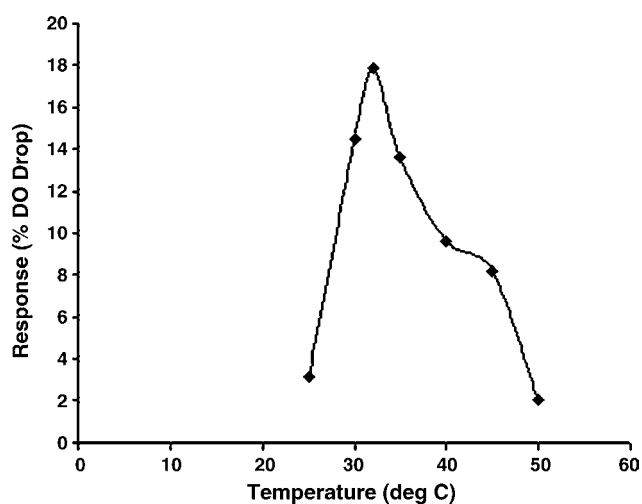


Fig. 4. Effect of temperature on the response of biosensor to caffeine.

Table 2

Effect of interfering agents on caffeine biosensor response

| Serial no. | Interfering agent (1%, w/v) | Response (%DO drop) |
|------------|-----------------------------|---------------------|
| 1          | Theophylline                | 2.00                |
| 2          | Theobromine                 | 3.50                |
| 3          | Sucrose                     | 4.00                |
| 4          | Glucose                     | 5.00                |
| 5          | Fructose                    | 0.20                |
| 6          | Glycine                     | 1.20                |
| 7          | Tyrosine                    | 0.60                |
| 8          | Histidine                   | 1.10                |
| 9          | Ascorbic acid               | 1.50                |
| 10         | Catechin                    | 1.20                |

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

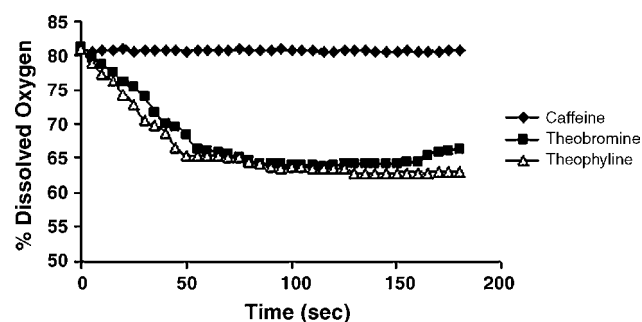


Fig. 5. Response of Immobilized xanthine oxidase to caffeine, theobromine and theophylline.

### 3.1.4. Interference of different compounds in caffeine estimation

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

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

Table 3

Analysis of caffeine in real samples by biosensor and by HPLC

| Sample                     | Caffeine concentration (% w/v) |               | %Error    |
|----------------------------|--------------------------------|---------------|-----------|
|                            | Biosensor                      | HPLC          |           |
| Instant coffee             | 0.056 (0.0015)                 | 0.052 (0.004) | (+) 07.69 |
| Commercial coffee sample 1 | 0.030 (0.01)                   | 0.033 (0.002) | (-) 09.09 |
| Commercial coffee sample 2 | 0.035 (0.0035)                 | 0.034 (0.004) | (+) 02.94 |
| Commercial tea sample 1    | 0.036 (0.0035)                 | 0.038 (0.002) | (-) 05.26 |
| Commercial tea sample 2    | 0.076 (0.0043)                 | 0.072 (0.003) | (+) 05.55 |
| Cola drink 1               | 0.042 (0.003)                  | 0.047 (0.002) | (-) 10.63 |
| Cola drink 2               | 0.046 (0.0251)                 | 0.050 (0.002) | (-) 08.00 |

Figures in parenthesis represent the standard deviation of the sample analysis.

Analysis of caffeine contents of real samples was done by using HPLC and microbial biosensor and presented in Table 3. All the analyses have been done in triplicates and the average of the three analysis is presented in the Table 3.

The biosensor results were in correlation with HPLC results. The spectrophotometric results show a very high error% indicating matrix interferences. The samples need prior treatment for analysis by HPLC and spectrophotometric methods. The error% was calculated taking HPLC as standard.

#### 4. Conclusions

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

##### 4.1. Perspectives

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

This device helps overcome the problem of time consuming and tedious sample preparation and analytical procedures commonly involved in conventional analysis methods. The main advantage of this method is the fast response time, which enables the quick estimation of caffeine in real samples.

Presently work is being carried out in our laboratory on the purification of the enzyme involved in the utilization of caffeine and future work would be carried out on the development of an

enzyme based biosensor for the determination of caffeine. The biosensor is anticipated to be free of interferences and enable more analyses in a shorter time.

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