Quantitative Caffeine Analysis in Robusta Coffee Utilizing Amperometric Biosensing Technology

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Abstract

Consuming caffeine in inappropriate amounts can disrupt various aspects, especially health. Controlling intake by knowing the caffeine levels in coffee is necessary to reduce the potential negative impacts. This research focuses on the detection of caffeine in Robusta coffee at two different concentrations (1:10 and 1:20 g/mL) and its relationship with yeast metabolism. An amperometric biosensor with a transimpedance amplifier to measure caffeine levels is used which has the advantages of sensitivity, cost-effectiveness, real time monitoring, biocompatibility, and reliable measurements. The data were statistically analyzed using ANOVA and visualized using Principal Component Analysis (PCA). The results revealed a concentration-dependent decrease in biosensor readings as caffeine levels increased (0.1, 0.5, 1, 1.5, and 2 mM), indicating caffeine’s ability to inhibit yeast oxygen consumption and oxygen-dependent metabolic processes. The sensitivity of the biosensor in detecting caffeine is 36.66 mV/mM. PCA uncovered complex patterns, relationships, and variations within the caffeine data. PC1 and PC2, the first two principal components, collectively explained 86.3% of the data’s variance. Eigenvalues for both PCs were greater than 1, highlighting their significance in understanding the dataset’s complexity. This research enhances our understanding of caffeine content in Robusta coffee and its effects on yeast metabolism, providing valuable insights for the coffee industry. This use of yeast biosensors offers efficiency, and adaptability that make that biosensor valuable in a variety of scientific and industrial contexts.

Keywords: amperometric biosensor, caffeine, PCA, robusta, yeast

1. Introduction

Caffeine, a natural stimulant found in various plant species, is a pivotal compound that influences the flavor, aroma and stimulating effects of coffee [1]. Caffeine consumption has been associated with health benefits such as a reduced risk of type 2 diabetes and migraine relief, and it can improve mood within the recommended limits. However, excessive consumption of caffeine can cause various health problems such as restlessness, anxiety, insomnia, and an increased heart rate [2]. In terms of recommended caffeine intake, guidelines vary based on factors such as age, health status, and individual tolerance. In general, moderate caffeine usage (up to 400 mg per day) is considered safe for most individuals, which is equivalent to the caffeine content in four cups of coffee [3].

Among the different coffee species, Coffea canephora, commonly known as Robusta coffee, stands out as a prominent source of caffeine due to its distinct biochemical composition [4]. Robusta coffee holds significant importance in the industrial domain, offering the potential to enhance agriculture, substitute the cultivation of illicit substances, and broaden income avenues for farmers [5]. The detection of caffeine levels in Robusta coffee holds significance as the concentration of caffeine plays a role in taste, sensory attributes, and has relevance for consumer choices and health implications [6]. The capacity to accurately identify and measure caffeine content in Robusta coffee not only ensures quality control but
also assists in comprehending the biochemical composition of different coffee types. Consequently, the development of precise detection systems is required.

The variability of caffeine detection methods in coffee has been widely carried out by Akinbile [7], extensively employed spectroscopic techniques to detect caffeine variability in coffee as well as in certain packaged drinks. This research shows that caffeine can be extracted better at boiling temperatures and the UV/Vis spectrophotometry method provides higher caffeine concentrations than the HPLC method. Moreover, Tugnolo [8] utilized Near-Infrared Spectroscopy (NIR) to identify caffeine in green coffee, ground coffee, and roasted beans, and compared two near-infrared spectrometers to evaluate coffee matrices. The results show that both devices provide similar results and can be used for real-time characterization of coffee. Wang [9] employed Liquid Chromatography-Mass Spectrometry (LC-MS) for caffeine detection in Liberica coffee. Nevertheless, these studies have limitations, primarily stemming from intricate sample preparation procedures that render them time-intensive and prone to contamination concerns that could potentially compromise the accuracy of the results. In addition, these methods also have high complexity and require specialized expertise and substantial financial investment in both equipment acquisition and maintenance.

Biosensors offer a highly suitable solution for caffeine detection due to their exceptional sensitivity, specificity, and rapid response capabilities. By utilizing specific biorecognition elements, Saccharomyces cerevisiae biosensors can selectively detect caffeine even in complex matrices like coffee. Their ability to provide real-time or rapid results with minimal sample preparation makes them advantageous for both quality control in coffee production and on-site testing. The use of yeast biosensors is a highlight of this research. Detection of caffeine with a biosensor combined with the yeast Saccharomyces cerevisiae has previously been carried out by Štukovnik [10] where in this research the yeast biosensor was proven to be able to respond to caffeine with the benefits of saving time, durability, and low cost.

In this research, caffeine detection in Robusta coffee was carried out at two concentrations of 1:10 and 1:20 g/mL, which were evaluated based on the metabolic results of the yeast S. cerevisiae expressed in dissolved oxygen. Measurements were made with an amperometric biosensor integrated with a transimpedance amplifier. The results of the data will be analyzed using the analysis of variance (ANOVA) method and visualized using the principal component analysis (PCA).

2. Methods

2.1 Yeast Microorganism and Medium

The yeast culture Saccharomyces cerevisiae FNCC-3049 utilized in this study was obtained from Gadjah Mada University and propagated using potato dextrose broth (PDB) medium, HiMedia M403, at a temperature of 30°C and pH 6.2. This active culture was employed as the inoculum. The yeast strain was inoculated at 1% (v/v) into 250 mL Erlenmeyer flasks containing 100 mL of PDB medium. This medium consisted of 4 g/L potato extract and 20 g/L dextrose (pH 5.6) and was autoclaved at 121°C for 15 minutes. The viable cell concentration is a key characteristic that needs to be monitored during the development and operation of the biosensor [11]. One of the methods to calculate cell concentration is by using a hemocytometer, observed with a microscope at 10X magnification. The unit of measurement for cell concentration is cells/mL [12].

2.2 Analyte Preparation

The caffeine test samples (molecular weight: 194.19) used in this study were prepared as solutions with varying concentrations: 0.1, 0.5, 1, 1.5, and 2 mM, corresponding to common caffeine levels found in coffee.

![Figure 1. Robusta coffee samples from Temanggung.](image)

These caffeine solutions were prepared by measuring the mass concentrations using a digital scale, where the mass for caffeine at concentrations of 0.1, 0.5, 1, 1.5, and 2 mM were 0.97 mg, 4.85 mg, 9.71 mg, 14.56 mg, and 19.42 mg, respectively.
The coffee batch used for measurement samples was 100% Arabica from Temanggung. Each 100g pack of coffee beans was opened just before brewing to prevent oxidative damage. The coffee was ground to a medium consistency using a Coffee Electric Grinder and roasted to a medium level. Coffee solutions were prepared with sample-to-solvent (deionized water) ratios of 1:10 and 1:20 (g/mL). The coffee extract from the solution was filtered using a Whatman Grade 598 paper filter (8-10µm) to remove insoluble particles [13].

2.3 Amperometric Biosensor

An electronic module and biosensor software were utilized for testing the measurement data of dissolved oxygen (DO) levels in yeast cells. A biosensor module was employed, which simultaneously processed data from a biochip, as illustrated in Figure 2. This module is integrated with a TIA (transimpedance amplifier) circuit designed based on the principles of three-electrode amperometry. The electrodes included in this module consist of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE) [14].

This op-amp component consists of an op-amp buffer and a current to voltage converter or what is commonly known as an I/V converter (Figure 2a). The reference electrode in the amperometric biosensor is connected to an op-amp buffer, where this op-amp buffer functions as a comparison for the potential values on RE and WE, so that WE will produce a potential value that is the same as the potential that has been set on RE with the condition of comparing $U_{out}$ with $U_{in}$ must be worth one. The potential value used on the WE, which ranges from 0.6 V to 0.9 V, is determined based on the specific redox reaction or electrochemical process.

The resulting potential value will be transferred to the electrolyte solution, the potential value is converted into current. The resulting current will be injected by a companion electrode into the electrolyte solution. This is done so that the reference potential remains in a constant state. The magnitude of the change in current will be read as a change in the DO level in the electrolyte solution by WE.

Meanwhile, Figure 2(b) shows the port for connecting the module to the biochip which serves as the sensing electrode for DO measurement. The biochip used is Biochip-D from Celasys GmbH with DO parameters. The DO sensor operates according to the Clark sensor principle and is composed of a platinum electrode (Pt) and an Ag/AgCl electrode, and covered with a PTFE membrane that allows the permeation of oxygen. The acquired data readings will be displayed in the form of graphs by interfacing the biosensor module with the Ch-Biosensor software.

2.4 Biosensor Caffeine and Coffee Measurement

Variations in caffeine concentration were introduced to S. cerevisiae yeast cells to observe the metabolic response generated by the biosensor. The yeast cells were immobilized within the sensor electrode chamber with a volume of 150 µL. Measurements were taken until the yeast reached a steady state, following which an analytical sample of 150 µL was added to the chamber. The experimental setup is depicted in Figure 3. Subsequently, the caffeine concentration from coffee, which contains varying levels of caffeine for each type, was measured. The measurements were replicated five times (n=5) for each sample. Clustering of the results will then be performed using PCA based on caffeine concentration and the variation among the coffee types used.

Figure 3 shows the caffeine and Robusta measurement setup using a biosensor system. The components applied in this measurement include a microcontroller (µC), a digital to analog converter (DAC), an analog to digital converter (ADC), a multiplexer (MUX), an amplifier (A1), and a biochip-D integrated to obtain the digital output of changes in caffeine concentration. The measurement flow is that the caffeine sample along with the yeast bioreceptor is inserted into the biochip-D, where the biochip-D is activated from the microcontroller voltage supply, which is then converted into analog by the DAC to the biochip-D. Subsequently, the output generated by biochip-D due to the reaction between yeast and caffeine is passed to the transimpedance amplifier and the multiplexer. The output from the multiplexer is still in analog, so it needs to be converted to digital by the ADC to be readable by the microcontroller. The digital data received by the microcontroller due to the reaction between yeast and caffeine is displayed in real-time as voltage over time using a computer.
2.5 Statistical Analysis
Statistical analysis was performed using Minitab 16 software. One-way analysis of variance (ANOVA) was employed to analyze the differences in caffeine concentration used in this study, based on their effects on yeast cell metabolism. Subsequently, the Tukey method was utilized to determine confidence intervals \((\alpha = 0.05)\) for all paired differences between factor means [15]. The biosensor measurement data was analyzed using the PCA method to classify and group data that generated trends and outliers for each caffeine concentration.

2.6 Characteristics Biosensor
Optimizing the use of microbial biosensors has been developed by investigating the ideal operating circumstances of the biosensor, where the characterisation of the biosensor was observed based on several parameters such as accuracy, precision, and sensitivity. Accuracy and precision are determined based on the average value, standard deviation (SD) and coefficient of variation (CV), while the sensitivity of the device in response to caffeine is shown in the caffeine concentration (mM) and voltage (mV) curves.

3. Results and Discussion
3.1 Yeast Cell Density Calibration
The measurement begins with the calibration of yeast with varying densities of \(11.8 \times 10^6\), \(21.4 \times 10^6\), \(31.2 \times 10^6\), and \(44.5 \times 10^6\) cells/mL.
The testing is carried out at room temperature of 30°C (86°F) with a pH of 5-6. The yeast volume immobilized in the biosensor biochip chamber is 150 µL using an Eppendorf pipette. The calibration results are shown as in Figure 4. The density of yeast cells in the culture can affect various aspects of its growth, behaviour, and metabolic activities [16]. At lower cell densities, more oxygen is generally available for aerobic respiration. As the cell density in the culture increases, oxygen becomes limited [17].

This calibration is performed to understand the effect of the density of yeast cells used on the sensor readings, expressed in output voltage (mV). This value serves as a baseline for subsequent measurements. These measurements are carried out for the same yeast treatment and are repeated three times (n=3) to ensure consistent results. As the density of yeast cells increases, the availability of oxygen becomes limited due to the reduced surface area for oxygen diffusion and increased competition among cells. This can result in a situation where some cells experience oxygen limitation, leading to a decrease in the remaining oxygen that the sensor detects. This is proportional to the increase in voltage values, as illustrated in Figure 4.

The cell density used in this study was 21.4 x 10^6 cells/mL at 4 hours, during which the yeast cells were transitioning towards the stationary phase. This time point represents the optimal concentration for use as a biosensor sample. Subsequently, the measurement was conducted using concentrated caffeine that had been prepared beforehand, as in the analytical sample preparation.

### 3.2 Caffeine Measurement

The measurement was set at a yeast cell density of 21.4 x 10^6 cells/mL, resulting in an output voltage value of ±1913 mV. This yeast was then combined with varying concentrations of caffeine: 0.1 mM, 0.5 mM, 1 mM, 1.5 mM, and 2 mM, as illustrated in Figure 5. The measurements were conducted at room temperature of 30°C, with three repetitions for each measurement. The measurement results for caffeine concentrations of 0.1, 0.5, 1, 1.5, and 2 mM are shown in Table 1 below.

The impact of caffeine on yeast oxygen consumption is characterized by inhibiting oxygen-dependent metabolic processes in a concentration-dependent manner. At lower concentrations, caffeine can simply reduce oxygen consumption due to its disruption of enzymes crucial for cellular respiration [18]. As caffeine concentration increases, its inhibitory effects become more pronounced, leading to a significant reduction in yeast’s ability to consume oxygen during aerobic respiration.

Previous research, Kuranda [19] has investigated the effect of caffeine on yeast, where caffeine can have a toxic effect on yeast cells, causing cell death. This effect is likely due to the inhibition of TOR (target of rapamycin) kinase activity by caffeine, which is can disrupt various cellular processes and cause cell death in yeast [20].
Table 1. Results of measuring concentrated caffeine

<table>
<thead>
<tr>
<th>Yeast density, pH, and temperature</th>
<th>Caffeine conc. (mM)</th>
<th>Voltage (mV)</th>
<th>%CV</th>
<th>F</th>
<th>R</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.4 x 10^6 cells/mL, pH = 5.7 30°C</td>
<td>0.1</td>
<td>1904.4 ± 0.89</td>
<td>0.05</td>
<td>5183.62</td>
<td>0.96</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1879.8 ± 1.78</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1860 ± 0.70</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1847 ± 1.58</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1832.2 ± 2.38</td>
<td>0.13</td>
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</tbody>
</table>

Babu [21] also conducted research on measuring DO levels for caffeine detection using amperometric biosensors to see the effect of temperature on the microorganisms used.

The system’s linearity was obtained in the concentration range from 0.1 mM to 2 mM with R^2 of 0.998 (Figure 5). Based on the regression of measurement results using pure caffeine, \( U = U_o + a(x) \), where \( U_o = 1902.08 \), \( a = 36.66 \) and \( x \) denotes concentration, the caffeine levels obtained in Robusta. The sensitivity of the biosensor in detecting caffeine is 36.66 mV/mM.

The standard deviation for each data is < 2.38 which indicates that the values in the data set are relatively consistent and do not vary widely from the mean. Additionally, the coefficient of variation (%CV) represents the relative variability of a data set compared to the mean, expressed as a percentage. Whenever the coefficient of variation is low, it indicates that the data set's values are relatively stable and do not deviate much from the average. This can be interpreted as a sign of stability or reliability in the data [22]. These results reflect the high precision of the biosensor in measuring caffeine concentration.

The results obtained for each caffeine concentration tested using biosensors and One-way ANOVA analysis are as in Table 1. This statistical analysis confirms that the variation in caffeine concentration between these samples is significantly different or shows a significant difference marked by a p-value < 0.05.

Clustering and visualization of caffeine measurement data using PCA resulted in a total variance of 86.3% for PC1 and PC2, with PC1 accounting for 61.9% and PC2 for 24.4%. The eigenvalues obtained from both PCs were >1. In PCA, when eigenvalues are greater than 1, it indicates that the associated principal components explain more variance in the data than a single original variable would. This implies that these principal components carry significant information and can effectively capture the underlying patterns and relationships within the dataset.

The classification of coffee using PCA was previously carried out by Barry [23] who used caffeine samples as a tool to determine active cognitive processing which resulted in a total data variance of 83.5%. Theoretically, eigenvalue calculations can be calculated based on research Mishra [24], however, in this research the eigenvalues were generated numerically from Minitab 16 software. As a conclusion, the principal components with eigenvalues greater than 1 play a crucial role in summarizing and representing the data's variability. These components are essential for reducing the dimensionality of the dataset while retaining most of its important features, allowing for more efficient analysis and interpretation [25].
3.3 Caffeine Detection in Robusta Coffee

Robusta coffee, when prepared with concentrations of 1:10 and 1:20 g/mL has been shown to effectively inhibit yeast from consuming oxygen due to its high caffeine content as shown in Figure 7. At these concentrations, the Robusta coffee provides yeast cells with a significant dose of caffeine, which interferes with their ability to carry out aerobic respiration, the process that relies on oxygen for energy production.

The reduction in this voltage value is directly proportional to the residual oxygen content within the cellular environment. For the measurements conducted at concentrations of 1:10 g/mL and 1:20 g/mL, the recorded results were 1830.28 ± 3.09 mV and 1860.14 ± 2.11 mV, respectively. The results of measurements with Robusta coffee produce measurements that are more fluctuating compared to caffeine measurements which are characterized by standard deviation due to the presence of other substances in Robusta coffee. Based on the regression of measurement results in Figure 5 the caffeine levels obtained at Robusta 1:10 and 1:20 g/mL are 1.96 mM and 1.14 mM.

4. Conclusion

This research has successfully demonstrated the effective determination of caffeine levels in...
Robusta coffee using amperometric biosensors, providing a valuable tool for analyzing caffeine content in this popular beverage. At lower concentrations, caffeine disrupts crucial enzymes involved in cellular respiration, resulting in a reduction in oxygen consumption. As caffeine concentrations increase, its inhibitory effects intensify, leading to a substantial decrease in yeast's ability to consume oxygen. Linearity was established within the concentration range of 0.1 mM to 2 mM, achieving an R² value of 0.998 and sensitivity of 36.66 mV/mM. The statistical analysis affirms a significantly difference in caffeine concentration among these samples, evidenced by a p-value < 0.05. Additionally, PCA method for clustering and visualizing caffeine measurement data resulted in a total variance of 86.3% and eigenvalue > 1. The results of caffeine measurements in Robusta coffee for 1:10 g/mL and 1:20 g/mL are 1.96 mM and 1.14 mM. This observation highlights the practical relevance of caffeine-rich beverages like robusta coffee in potentially regulating microbial activities, with potential applications in food science and biotechnology. The yeast biosensor demonstrated its capacity to react to caffeine, offering advantages in terms of time efficiency, durability, and cost-effectiveness. Overall, this research sheds light on the complex interactions between caffeine and yeast metabolism, emphasizing the potential for further research into harnessing caffeine's inhibitory properties for various industrial and scientific purposes.

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References
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