Fibre-optic biosensor for the detection of xanthine for the evaluation of meat freshness

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Abstract. Xanthine is produced by guanine deaminase and xanthine oxidase from guanine and hypoxanthine, respectively. It serves as an indicator of spoilage of meat and various pathological conditions. An optical biosensor has been fabricated for the detection of xanthine by absorption transmission approach. The bioassay principle used in this study is conversion of xanthine to uric acid and H₂O₂ by XO. Due to the production of uric acid, pH of the reaction decreases and falls in range of 7.5 - 6. The absorptive dye phenol red has been screened to visualize the change in colour. The co-immobilization of XO and dye phenol red indicator was done with sol-gel method onto the circular plastic discs for the development of biosensor. Volume of sample required for detection has been miniaturized to 10µl. The linear range of concentration and limit of detection were 0.5µM – 150µM and 0.5 µM, respectively, have been achieved for xanthine. The designed biosensor was successfully employed for the detection of xanthine in chicken meat sample. Present study leads to the development of a fast and dependable biosensor with miniaturized sample volume and also mass scale screening possible through colour visualization.

1. Introduction
Xanthine (C₅H₄N₄O₂) is a heterocyclic nitrogen containing compound which is one of the products of ATP degradation pathways[1]. It is produced by guanine deaminase and xanthine oxidase from guanine and hypoxanthine, respectively. Further, it is oxidized to uric acid by the action of XO[2,3]. It is present in most of the body fluids and tissues with concentration range of 40-160µM in urine and 0.5 – 2.5µM in blood plasma[4]. Elevated levels of xanthine serve as indicator of several pathological conditions such as hyperuricemia, gout, xanthinuria, cerebral ischemia or renal failure. Thus, the levels xanthine serve as potential marker in diagnosis of various clinical conditions[5,6].
In addition, xanthine also act as freshness marker of meat or fish[7]. Immediately after the death of animal, ATP starts degrading to xanthine and continue throughout storage. As the shelf-life of meat goes on decreasing, the concentration of xanthine increases[8, 9]. Determining the levels of xanthine as freshness indicator and also increase in its concentration is a sign of spoilage of meat[10]. Because of this reason, food industries mainly used the levels of xanthine as quality index where freshness of meat or fish is indispensable for manufacturing superior quality products. Xanthinedetection is highly significant in food industries, pathological analysis and medical diagnosis[11, 12]. Various techniques have been described for the identification of xanthine such as HPLC [13-15], capillary electrophoresis [16], chromatography [17], amperometry[5, 18-21], voltammetry [22,23].
These methods are expensive, require pre-treatment of sample which is a time consuming and complicated process and produce undesirable or harmful waste products. Sensory evaluation is subjective and expensive in many cases. Microbial methods only estimate spoilage caused by bacteria and chemical methods in determining early post-mortem spoilage encounter problems [24]. That is why simple and rapid methods for estimating meat freshness are required. Optical biosensors are simple, cost effective, produce fast response, highly sensitive and selective which enables low detection, wide concentration range, reproducibility and better stability. The present study focussed on fabrication of fibre optic tip with miniaturized sample volume and mass scale screening through colour visualization.

2. Experimental

2.1. Materials and Methods –
Xanthine, Tetra Methyl Ortho Silicate (TMOS), Phenol red and other reagents used, were of analytical reagent grade. All solutions were prepared in double distilled water. Maya 2000 series spectrometer with fibre optic probe (OceanOptics) has been used.

2.2. Partial purification of Xanthine Oxidase –
Enzyme xanthine oxidase was purified from cow milk [25,26]. Fresh cow milk was kept at refrigeration temperature (4°C) overnight. EDTA (3%) and Toluene (2mM) added milk was blended at high speed at room temperature for 20 min and again cooled down the sample to 4°C. The sample was brought to 20% saturation with solid ammonium sulphate salt, centrifuged at 10,000 rpm for 25 min at 4°C. The collected supernatant was brought to 30% saturation by the addition of ammonium sulphate. The supernatant was again centrifuged at 12000 for 35 min at 4°C. Formed precipitate were collected and dissolved in 0.1 M Tris-HCL buffer (pH 7.6). The redissolved precipitate (partially purified) extracted from cow milk was stored at 4°C for fabrication of optical biosensor.

2.3. Assay of xanthine oxidase -
Xanthine oxidase catalyses the oxidation reaction of xanthine and produce uric acid as in figure (1). The rate of formation of uric acid is determined by monitoring the change in absorbance at 292 nm because of uric acid production.

![Figure 1. Oxidation of xanthine catalysed by Xanthine oxidase.](image)

The assay mixture contained 1.9 ml of 50 mMTris-HCl buffer (pH 7.6), 1 ml xanthine (0.15 mM) and 0.1 ml xanthine oxidase as described by [27] with modifications. The change in absorbance at 292 nm was noted down against blank in a UV-VIS spectrophotometer. The activity of enzyme was calculated using formula:

\[
\text{Units/ml} = \frac{\Delta A/\text{min} \times 3 \text{ ml}}{12.2 \times 0.1 \text{ ml}}
\]

Extinction coefficient of uric acid = 12.2 mM.

2.4. Screening of absorptive dye –
Xanthine oxidase catalyse the conversion of xanthine to uric acid and hydrogen peroxide. Due to production of uric acid, pH of the reaction decreases and falls in the range of 7.5 – 6. The absorptive
dye phenol red has been screened to visualize the change in colour [32]. Because its colour exhibits a gradual change in colour from bright pink to yellow over the change in pH from 8 to 6.

2.5. Construction of bio-component –
The enzyme immobilization was done by hydro sol-gel method by mixing 200 µl of alcohol, 20 µl of Tetra Methyl Ortho Silicate (TMOS), 5 µl of NaOH (50mM), 10µl of phenol red (1.25mg/ml in 50% Ethanol) and 20 µl of partially purified xanthine oxidase as described by [28] with modification. The mixture (10 µl) was introduced onto the transparent circular plastic discs(diameter 5 mm). Prepared discs were kept for immobilization for 1hr at 4°C. These discs were used for the detection of xanthine at different concentrations.

2.6. Determination of xanthine in meat sample -
Various methods have been proposed for the preparation of meat samples using different extraction solutions containing deionised water, perchloric acid, hydrochloric acid and phosphate buffer[29-31]. Fresh chicken was bought from a local meat shop and stored at 0°C prior to use. The meat sample was thawed at room temperature and allowed to degrade for 5 days. Finely chopped meat sample (5g) was homogenized in 15ml of deionised water for 30 min, centrifuged at 5000 rpm for 15 min and collected supernatant was analysed.

3. Results and Discussion

3.1. Characterization of bioassay principle –
An optical biosensor has been developed in the present study based on a bioassay principle of conversion of xanthine to uric acid by enzyme xanthine oxidase(figure 1) which thereby lowers down the pH of the reaction mixture. The fall in pH can be traced optically with the use of pH indicator phenol red. Because phenol red exhibits a colour change from red to yellow over the change in pH 7.5 to 6 (figure 2).

![Image of colour change](image1)

**Figure 2.** Picture showing the change in colour of the bio-component from red to yellow before (left) and after (right) the reaction, respectively.

![Image of instrument](image2)

**Figure 3.** Picture showing the instrument Maya 2000 series spectrometer with fibre optic probe.

The absorption spectra of phenol red was scanned on Maya 2000 series spectrometer at different pH (figure 3) and 555nm was found to be $\lambda_{\text{max}}$. According to literature [32], at 555nm phenol red molecules showed a very high absorption value that can be reduced by decreasing the pH of the solution. The linear relation was obtained between xanthine concentration and absorbance. Increase in xanthine concentration resulted in uric acid production thereby decrease in absorbance at 555 nm ($\lambda_{\text{max}}$
of phenol red at 7.5). At first the absorbance was monitored for 10 minutes but after 5 minutes, no significant change was noticed. Therefore optimized response time is 5 minutes.

3.2. Analytical performance of the xanthine biosensor -
It is believed to be another achievement of the study, in the developed biosensor the sample volume could be miniaturized to a level as low as 10 µl. The biosensors showed a linear relation between concentration of xanthine and absorbance in the samples. Increase in concentration of xanthine from 0.5 to 150 µM leads to rise in uric acid production, decrease in absorption from 0.92 ± 0.01 to 0.86 ± 0.01 (for 0.5 µM) and 0.43 ± 0.02 to 0.20 ± 0.02 (for 150 µM) at 555 nm for 5 minutes (table 1). Though a linear range of concentration was found to be 0.5 µM to 150 µM with a correlation coefficient $R^2 = 0.9961$ and linear equation $y = -4.3514x + 0.8344$ (Figure 4). The limit of detection (LOD) was calculated to be 0.5 µM based on the equation: $\text{LOD} = (\sigma/S)*3.3$.

### Table 1. Absorbance of different samples at 555 nm taken up to 5 minutes

| Concentration (µM) | Absorbance at 555 nm at time (in minutes) |
|-------------------|------------------------------------------|
| 0.5               | 0.92 ± 0.01 0.90 ± 0.01 0.88 ± 0.01 0.86 ± 0.01 0.86 ± 0.01 |
| 1                 | 0.91 ± 0.01 0.88 ± 0.02 0.86 ± 0.01 0.84 ± 0.02 0.84 ± 0.01 |
| 5                 | 0.90 ± 0.01 0.88 ± 0.01 0.84 ± 0.02 0.81 ± 0.01 0.81 ± 0.02 |
| 10                | 0.85 ± 0.01 0.83 ± 0.01 0.81 ± 0.01 0.80 ± 0.02 0.78 ± 0.01 |
| 30                | 0.79 ± 0.02 0.76 ± 0.02 0.71 ± 0.02 0.70 ± 0.01 0.69 ± 0.01 |
| 60                | 0.70 ± 0.01 0.66 ± 0.02 0.62 ± 0.01 0.58 ± 0.02 0.56 ± 0.01 |
| 90                | 0.63 ± 0.02 0.54 ± 0.01 0.52 ± 0.02 0.45 ± 0.01 0.43 ± 0.02 |
| 120               | 0.56 ± 0.01 0.45 ± 0.02 0.39 ± 0.02 0.32 ± 0.01 0.31 ± 0.01 |
| 150               | 0.43 ± 0.02 0.36 ± 0.01 0.31 ± 0.01 0.25 ± 0.02 0.20 ± 0.02 |
| Meat sample (day 1)| 0.89 ± 0.01 0.87 ± 0.02 0.84 ± 0.02 0.82 ± 0.01 0.81 ± 0.01 |
| Meat sample (day 5)| 0.78 ± 0.01 0.74 ± 0.02 0.70 ± 0.02 0.65 ± 0.01 0.63 ± 0.01 |

![Figure 4](image-url)  
**Figure 4.** Standard curve illustrating the linear relationship between absorbance and xanthine concentration from 0.5 µM to 150 µM.

Biosensors for xanthine reported earlier includes construction of a Chemiluminescent biosensor by [33] for the detection of xanthine using enzymes xanthine oxidase and peroxidase. A linear range of
concentration 3.1-316 µM for xanthine and detection limit of 0.55µM was obtained. Different biosensor such as XOD/P(GMA-co-VFc0/REGO-Fe3O4 with linear range of 2.36 µM [34], XOD/ZnO-NPs-PPy/Pt with LOD of 0.8 µM and linear range of 0.8-40µM [35], XOD/PVF/Pt with linear range of concentration 0.43-28.4 µM and LOD 0.52 µM [36]. Our developed biosensor is comparable and much more sensitive.

Table 2. Real sample analysis of xanthine in chicken samples by proposed method.

| Sample          | Detected (µM) | Added (µM) | Found (µM) | Recovery (%) |
|-----------------|--------------|------------|------------|-------------|
| Meat Sample     | 5            | 2          | 6.6        | 94.2        |
|                 | 5            | 10         | 14.5       | 96.6        |

3.3. Meat sample analysis
The critical issue regarding quality of meat in the meat industry is to protect consumer health. In this concern, the levels of xanthine were analysed for quality assessment. The xanthine content of the meat sample was measured during the storage from day 1 to day 5. After the death of animal, increase in xanthine content cause a decrease in meat quality. The designed biosensor was applied to monitor xanthine in chicken meat samples collected from local market. Extraction of xanthine from meat is achieved by taking finely chopped 5 g of meat and homogenised in 15 ml of deionised water for 30 minutes, followed by centrifugation at 5000 rpm for 15 minutes. The supernatant was taken and filtered and 10 µl of filtrate was introduced on to the plastic disc for the study. The results obtained at different time interval at day 1 and day 5 are summarised in table 1 and found more xanthine content after 5 days than day 1. The concentrations of xanthine in meat samples (day 1 and 5) were around 5µM and 44µM, respectively. Dependability of the proposed method was tested using spiked sample. The indicated that the recovery of spiked sample were between 94.2% to 96.6% (Table 2). Therefore, the developed optical method could be effectively used to determine xanthine in meat samples.

4. Conclusion
To conclude, an optical biosensor has been fabrication for xanthine detection and successfully applied to real meat sample. The enzyme xanthine oxidase was co-immobilized with phenol red indicator by sol-gel approach onto the circular plastic disc. The detection of xanthine was done by absorption transmission approach. Volume of sample required for detection has been miniaturized to 10µl. The linear concentration range of 0.5µM – 150µM with detection limit of 0.5 µM for xanthine has been achieved. The study developed a fast, reliable biosensor with miniaturized sample volume and mass scale screening possible through colour visualization.

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