Effect of Temperature towards Immobilised Tyrosinase Enzyme on Magnetic Chitosan

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***Abstract*— Bisphenol A (BPA), a compound that is traceable in wastewater and packaging material can bring detrimental effect to human health when it is consumed. BPA has been linked to particular diseases related to endocrine system, such as breast cancer among women and defective reproductive system among men. In this modernised world, there is limitation of method to detect the presence of the compound in the environment as the available techniques are usually expensive, cannot be conducted in-situ, and require experienced technicians to operate effectively. In this study, the tyrosinase enzyme is employed to identify the presence of the BPA compound and to degrade the BPA into its monomer because the method of preparation is simple and relatively cheap compared to other existing techniques used to identify the presence of BPA in a solution. In this research, only effect of temperature is studied towards the ability of the enzyme to degrade the phenolic compound. The tyrosinase enzyme is immobilised onto magnetic chitosan via simple crosslinking process with the aid of glutaraldehyde as the crosslinking medium. Other parameters that influence the enzyme behaviour, like pH, hydrophobicity and electrostatic interactions, are not investigated due to time constraints and lack of necessary equipment and assumed to be similar from one set of experiment to another by maintaining stirring rate, amount of chemicals used in preparation steps. Based on the results obtained, the tyrosinase managed to degrade highest amount of BPA at temperature, T = 20 ˚C.**

Keywords*— *Tyrosinase, magnetite chitosan, enzymes immobilisation, bisphenol A, temperature.

I. INTRODUCTION

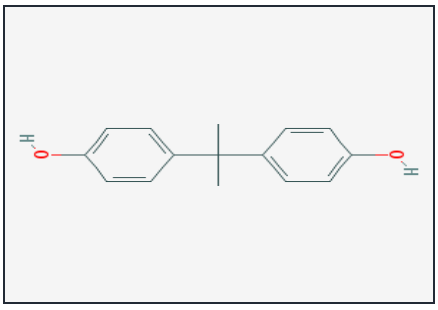


Fig. 1: 2-Dimensional Chemical Structure of Bisphenol A. Retrieved from CAMEO Chemicals (2016).

According to Vandenberg *et. al.*, (2007), Bisphenol A (BPA) or 2,2-bis(4-hydroxyphenyl) propane ($C_{15}H_{16}O_2$) with chemical structure shown in Figure 1, has found wide applications in the industries, such as in packaging industries, medical applications, safety equipment and many more. BPA (Figure 1) has been recommended by the necessary organisations, such as U.S Food and Drug Administration, to be used to manufacture many subjects of interest due to its properties such as lightweight, strong resistance towards breakage, durability, sustainability, good thermal stability and easy to be sterilised.

Although it has influenced the industries in many good ways due to its mentioned properties, BPA and its other derivative compounds have been reported to leach out easily into the environment and pollute the drinking water as well as food sources. Consequently, this type of compound has been traced in the human bodies and has been linked to certain critical conditions such as the impaired of regular functionalities of the organ liver, heart diseases because of damaged endocrine system, cancer and many more (Reza *et. al.*, 2015). BPA is reported to behave as agent that can disrupt the regular functions of the endocrine system by acting as an oestrogen agonist and androgen antagonist which results in precursors for breast cancer and low sperm count and activity (Flint et al., 2012).

According to Guerra *et al.*, (2015), the authority of Canada deduced that the presence of BPA in the environment may pose danger towards human beings health due to its alarming concentration that has been increased significantly over the years. The ubiquity of BPA in the surrounding may be justified by the released of this chemical substance during industrial manufacturing processes as waste or products in use. For example, the highest average amount of BPA detected was approximately equal to 4.55ng/m^3 in several cities of India (Bombay and Chenay) due to the burning of plastics for household applications (Michalowicz, 2014). BPA is also reported to contaminate some of water resources nearby with industrial and commercial locations as it is discharged into the aquatic environment, generally from wastewater treatment plants and landfill sites. Based on the article by Huang *et al.*, (2012), it was investigated that BPA concentration in southern Taiwan of China reaches about 16 µg/L in wastewater from a developing industrial area, which at that time, exceeds 16 times the concentration of BPA level normally found worldwide.

Due to their high toxicity, reliable and highly sensitive analytical methods are required to monitor the presence of such compounds in specified environment to reduce the effects of contaminations by these pollutants. At the present time, there are tools available to detect BPA namely as high performance liquid chromatography, chemiluminescence immunoassay, enzyme-linked immunosorbent assay (ELISA) technique and so on. These techniques have been deployed because they have been able to produce decent outputs with good accuracy, low detection limit and high sensitivity as well as selectivity. Regardless of that, these

procedures require highly skilled operators as they are relatively complex, sample preparation can be tiresome, very costly and lacks of quick and real-time detection (Kochana *et al.*, 2015). Therefore, electrochemical biosensors has recently gained much interests to overcome the limitations of using previously stated approaches due to its capability to allow in situ analysis of phenols, it is also particularly responsive to changes, simplicity and effectiveness.

A biosensor is defined as the device incorporated with a bio-receptor and a transducer components to detect specific compounds and monitor the presence, reaction activity and concentration of the analytes. The bio-receptor is a dynamic biomolecule that binds the desired and specific chemical compound while the transducer will aid in converting the recognition event into a measurable signal. In this research, the enzyme that acts as bio-receptor is the tyrosinase enzyme, which its ability to degrade phenolic compounds into quinone compounds will be investigated under effect of varying temperature. Whereas, the target substrate that will be used for the catalytic reaction is bisphenol A (BPA) compound. In this research, ability of immobilised tyrosinase enzyme to magnetic chitosan by crosslinking approach to degrade the BPA compound based on different effect of temperature will be investigated.

Enzymatic activity is affected by many factors such as the mass transfer site for catalysis reaction to occur (diffusivity and adsorption of substrates into and out of the porous substance), polarity and surface charge of the substrate and enzyme, orientation of the enzymes' specific groups, concentration of enzyme used, enzyme's kinetic activity, presence of inhibitors, pH and temperature.

In this research, magnetic chitosan composites as solid support will be bonded to the enzyme for immobilisation method. Only effect of temperature towards the immobilised enzyme will be investigated due to time constraints and limitation of resources such as the availability of equipment and raw material. The other parameter that affects the behaviour of tyrosinase enzyme, like electrostatic interactions, is assumed to be constant by maintaining definite experiment parameter, such as stirring rate. To study the efficiency and effectiveness of the enzyme to convert the bisphenol A compound into is respective monomers, the activity of immobilised enzyme on chitosan at different temperature is investigated with spectrophotometer analysis under wavelength of 465nm. The absorbance readings will be correlated with the extent of degradation of bisphenol A compound. The lowest value of absorbance at particular temperature will represent the most conversion rate of bisphenol A into its monomers quinone compound. The experiment will be carried out at neutral pH with temperature range between 20 to 40°C.

II. METHODOLOGY

A. Material

Tyrosinase (Sigma Aldrich), Bovine Serum Albumin pH 7 (Vivantis), Chitosan (Sigma), Acetic Acid (Merck), Magnetite Nanoparticles Iron II,III Oxide 95% (Sigma), Bisphenol A (R&M Chemicals), Distilled water, Ethanol 95% (HmBG), NaOH Pellets (R&M Chemicals), Glutalaraldehyde 50% aqueous solution (R&M Chemicals), Dipotassium Hydrogen Phosphate (R&M Chemicals), Potassium Dihydrogen Phosphate (R&M Chemicals).

B. Equipment and Apparatus

Beaker, Measuring Cylinder, Magnetic Stirrer Heating Plate, Magnetic Stir Bar, Test Tubes, Cuvette, Vials, Pipette, Blender, Spatula, pH meter, Fume, Hood, Centrifuge, Weighing Machine, Rotary Shaker, Spectrophotometer (SECOMAM UvLine 9400).

C. Preparation of 1M Sodium Hydroxide Solution

40 g of sodium hydroxide pellets from R&M Chemicals (molecular weight = 40g/mol) are added into 1 litre of distilled water and mixed well to create 1M sodium hydroxide solution.

D. Preparation of 1 L 0.5 mM Phosphate Buffer

Solid powder of Dipotassium Hydrogen Phosphate and Potassium Dihydrogen Phosphate attained from R&M Chemicals are mixed and dissolved in 800ml of distilled water. The pH of the solution was calibrated to neutral pH (pH = 7.2) by dropping solution of 1M sodium hydroxide carefully. Then, distilled water was added to the solution until 1 Litre of phosphate buffer was achieved.

E. Preparation of Crosslinking Medium 5% Glutaraldehyde Solution

For the immobilisation part, only 5% glutaraldehyde solution was required. Glutaraldehyde 50% aqueous solution was purchased from R&M Chemicals. Next, 10.5 ml of the stock solution was diluted with 89.5ml of the 0.5mM phosphate buffer to obtain 5% glutaraldehyde solution.

F. Preparation of Substrate Bisphenol A (BPA) at Different Concentrations

Bisphenol A powder was obtained from R&M Chemicals. 100 ppm of BPA stock solution was first prepared by diluting 0.1 g of BPA powder in 1 L of distilled water. Then, it was diluted again to 3 different concentrations of BPA solutions (2, 4 and 5 ppm BPA in distilled water).

G. Preparation of Tyrosinase Solution

The mushroom tyrosinase (polyphenol oxidase) was purchased from Sigma Aldrich and stored at 4°C. The specific activity for the enzyme was quoted as 2687 units per mg where one unit corresponds an increase in absorbance at 280nm of 0.001 per minute in a reaction mixture containing 0.1 mM L-tyrosine at pH 6.5 at 25°C. Tyrosinase stock solution of 0.1% (w:v) was prepared by dissolving 1mg of enzyme in 100 mL phosphate buffer of neutral pH. 25 mL of Bovine serum albumin (BSA) solution obtained from Vivantis was added to 50 ml of the tyrosinase stock solution as a stabilising agent for the enzyme (Matyholo, 2011). The BSA solution was made by dissolving 0.1 g of BSA powder into 25 mL of the phosphate buffer.

H. Preparation of Magnetic Nanoparticles Entrapped in Chitosan Gel

Magnetic chitosan gel was prepared according to article by Pospiskova and Safarik (2013), with slight modification of the required mass of the chitosan gel. Chitosan in powder form and the magnetic nanoparticles Iron II,III Oxide 95% were obtained from Sigma Aldrich. The chitosan powder was solubilised completely in 0.2M Acetic Acid, as seen in below Figure 2. Then, the black magnetite nanoparticles were added to the solution and was mixed well (Figure 3). Gel magnetite chitosan was attained by mixing the solution with 1M of sodium hydroxide solution which can be observed in Figure 3.2. The material was washed several times with distilled water. Next, the magnetic chitosan gel was cut with blender to homogeneity in terms of size before it was stored at 4°C.

Fig. 2: Dissolution of Chitosan Solution with Acetic Acid.

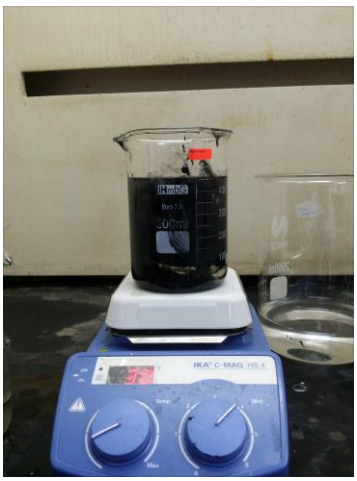


Fig. 3: Preparation of Magnetite Chitosan Gel.

I. Immobilisation of Enzyme to the Magnetic Chitosan Composites

About 3 g of magnetic chitosan composites (wet weight) was mixed with 50 ml of glutaraldehyde 5% solution. The mixture was put inside a rotary shaker at 20rpm for 3 hours under temperature of 25°C. After that, the particles were filtered from remaining solution and washed carefully several times with distilled water. 50 ml of tyrosinase solution was then added to the solid support and shaken for 20h at 60 rpm at room temperature. The particles were then filtered and dried with filter paper for subsequent analysis (Figure 4).

Fig. 4: Production of Immobilised Tyrosinase on Magnetic Chitosan (Left) and Magnetic Chitosan Gel (Right).

J. Tyrosinase Enzyme Assay

Blank consisting of only enzyme solution was first measured with spectrophotometer at wavelength 475nm. Standard curve of bisphenol A compound was made by measuring absorbance values at different concentrations (1, 2, 3, 4 and 5 ppm), each about 2ml inside cuvette with spectrophotometer under similar wavelength. The BPA solutions at varying concentrations were then mixed with the solid enzyme-magnetic chitosan particles under different set of temperature (T=20, 25, 30, 35, and 40°C) for about 6 hours (Figure 5). Then, the samples liquid layer were analysed under spectrophotometer at wavelength 465 nm (Figure 6), collected and analysed with the BPA standard curve.

Fig. 5: Spectrophotometer Analysis of Product from Biodegradation of Bisphenol A Compound by Immobilised Tyrosinase Enzymes.

III. RESULTS AND DISCUSSION

A. Preparation of Magnetic Chitosan Composite

Multiple attempts had been produced to create the magnetic chitosan composites by referring to different journals, to each of those papers claim to produce the magnetic chitosan successfully as a solid support for enzyme immobilisation. Obviously, each of these references are different from one another in terms of method of preparation (raw material and equipment), the simplicity of the techniques applied, and final form of the magnetic chitosan particles. Despite all of the efforts that had been made, only one method defined by Pospiskova and Safarik (2013) to construct the magnetic chitosan particles was effective. In their paper, they described the simplest approach to produce the target substance by only entrapping commercial magnetite nanoparticles inside chitosan gel-like solution. In the experiment, the effect of

separating the magnetised particles could not be observed as the gel was too viscous. In spite of that, weak magnetic force was felt when a magnet was put near to the beaker containing the magnetised chitosan gel substance prior to enzyme immobilisation onto the solid support. The reason magnetic nanoparticles are used as solid support for the tyrosinase enzyme is to allow effective separation of immobilised enzymes from other substances during washing procedure, thus enabling the reusability of the enzymes. Reusability of enzymes is important in the industry mainly because of the cost.

B. Immobilisation of Tyrosinase by Crosslinking onto Magnetic Chitosan Compound

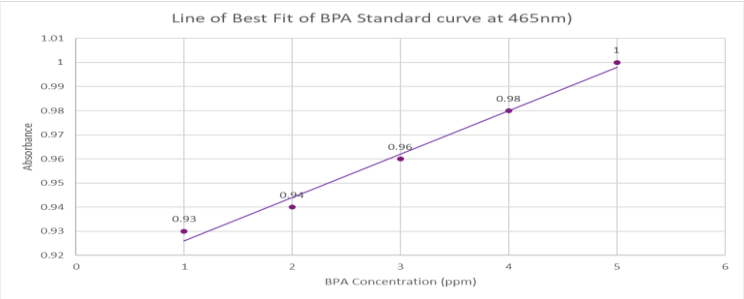
The enzyme tyrosinase was bonded covalently onto the magnetised chitosan gel with the aid of glutaraldehyde solution. The glutaraldehyde compound is believed to assume the role of crosslinking medium between the enzyme tyrosinase and the magnetic chitosan gel. The glutaraldehyde behaves as a stabilising agent between the enzymes and the magnetic chitosan composite. This immobilisation step was done simply by shaking the mixture solution composed of enzyme solution, solid magnetite chitosan and glutaraldehyde 5% solution at 60 rpm for 20 hours to ensure as much as the enzymes in the solution bind onto the magnetic chitosan solid particulates. Then, the particles were washed out carefully with distilled water several times to remove any unbound enzyme particles and remaining glutaraldehyde solution.

C. Enzyme Assay and Data Analysis

The bisphenol A (BPA) standard curve was plotted by measuring 2 ml of BPA solution at different concentrations after a blank solution consisting of only enzyme solution was measured by the spectrophotometer at wavelength 465nm. The values of the absorbance for different concentrations were collected and shown in Table 1 below. These values were then used to plot The BPA standard curve, which can be observed from the subsequent Graph 4.1 by producing a line of best fit. This curve will indicate the initial absorbance values of BPA at different concentrations before any degradation process.

BPA (ppm)	Absorbance (A) (465nm)
1	0.93
2	0.94
3	0.96
4	0.98
5	1.00

Table 1: Standard BPA curve at Different BPA Concentrations.



Graph 4.1: Plot of BPA Standard Curve at Different Concentrations of BPA.

Then, the immobilised enzymes were tested on different temperatures (T= 20, 25, 30, 35 and 40°C) for 6 hours prior to measuring their activity in term of absorbance with a spectrophotometer. The solid magnetised chitosan-enzyme complex was put equivalently (about 6mg) into plastic vials and mixed with 2mL of different BPA concentrations. Eventually, the remaining solution in each of the vials was measured in term of absorbance to determine the extent of degradation of BPA compound at different temperature. The absorbance values were collected and presented in below Table 2 to Table 6:

Temperature (°C)	Absorbance (465nm)	% BPA Conversion (1 ppm BPA)
20	0.066	92.903
25	0.619	33.440
30	0.782	15.914
35	0.610	34.409
40	0.704	24.301

Table 2: Absorbance Values of Unconverted BPA Compound at Different Temperature when 1 ppm of BPA was used.

Temperature (°C)	Absorbance (465nm)	% BPA Conversion (2 ppm BPA)
20	0.105	88.830
25	0.574	38.936
30	0.573	39.425
35	0.630	32.979
40	0.559	40.532

Table 3: Absorbance Values of Unconverted BPA Compound at Different Temperature when 2 ppm of BPA was used.

Temperature (°C)	Absorbance (465nm)	% BPA Conversion (3 ppm BPA)
20	0.735	23.438
25	0.560	41.667
30	0.610	36.458
35	0.611	36.354
40	0.644	32.917

Table 4: Absorbance Values of Unconverted BPA Compound at Different Temperature when 3 ppm of BPA was used.

Temperature (°C)	Absorbance (465nm)	% BPA Conversion (4 ppm BPA)
20	0.598	38.97959184
25	0.529	46.02040816
30	0.587	40.10204082
35	0.639	34.79591837
40	0.902	38.57142857

Table 5: Absorbance Values of Unconverted BPA Compound at Different Temperature when 4 ppm of BPA was used.

Temperature (°C)	Absorbance (465nm)	% BPA Conversion (5 ppm BPA)
20	0.528	47.2
25	0.666	33.4
30	0.616	38.4
35	0.791	20.9
40	0.544	45.6

Table 6: Absorbance Values of Unconverted BPA Compound at Different Temperature when 5 ppm of BPA was used.

From the gathered data of the absorbance values, a formula was used to analyse the percentage of BPA degradation under different set of temperature when a particular BPA concentration was used as substrate for the enzyme. By letting A = absorbance value of specific concentration of BPA from BPA standard curve and B = the absorbance value of enzyme assay at different temperature with the same concentration of BPA, the percentage of BPA degraded can be calculated as following:

$$BPA\ Conversion\ (\%) = \left[\frac{(A-B)}{A}\right]100\%$$

Equation 1: The Percentage of BPA Degraded at Different Temperature

Example of Calculation:

At temperature, T=20°C, when 1 ppm of BPA is used;

The value of absorbance at 1 ppm from BPA Standard Curve = 0.93

The final value of absorbance after enzymatic reaction = 0.066

$$BPA\ Conversion\ (\%) = \left[\frac{(0.93-0.066)}{0.93}\right]100\% = 92.9\%$$

From the analysis of data, it is found that at temperature, T=20°C, the result is the most promising for BPA degradation as it shows the highest amount of percentage of BPA degradation in 3 of 5 sets of the designated experiment. The absorbance obtained at final readings of the spectrophotometer correspond to the amount of BPA that is still unconverted into its monomers. Therefore, the lowest absorbance shows the highest degraded amount of BPA into its respective monomers.

Many articles state that there are readings of tyrosinase activity starting at the temperature of to 15 until 35°C, which are measured in terms of electricity due to application of biosensor enzyme to break down the phenolic compounds. As the temperature increases, so should the activity of the enzyme until optimum temperature is reached. This is because at higher temperature, the particles receive more thermal energy which then eventually transformed to kinetic energy. As the kinetic energy within the system increases, the tendency of enzymes to collide with the substrate BPA is higher. Beyond the optimum temperature, the readings usually decline possibly because of denaturation of enzyme as the protein changes its native state to adapt to new conditions. An article by Wang *et al*., (2008) states that there is a gradual increase of enzymatic response which corresponds to the degradation process. They found that temperature optimum for the tyrosinase embedded on the electrode is at 35°C.

In this experiment, the type of solid support for immobilisation process refers to magnetic chitosan. On one hand, since the absorbance readings fluctuate from 20 to 40°C, it can be inferred that immobilisation of tyrosinase into magnetic chitosan does not play significant role in enhancing the stability of the enzyme, potentially because the enzymes are not engulfed by the chitosan polymer. If the enzymes are contained in insoluble matrix beads of polymers, perhaps, the study of enzyme stability can be compared in terms of effect of temperature towards the enzymatic activity with this work.

IV. Conclusion

The magnetised chitosan composite was successfully created and bonded to enzyme tyrosinase via crosslinking procedure with the aid of glutaraldehyde compound. The approach was simply done by mixing magnetite nanoparticles with chitosan and from this magnetic chitosan gel was produced by only adding 1M of sodium hydroxide. The gel was cut into smaller pieces by a kitchen blender to form a homogenous form of the magnetic chitosan composites. Then, glutaraldehyde was added into solution mixture of tyrosinase enzyme and the solid magnetic chitosan for nearly a day, about exactly 20 hours for the immobilisation technique. Furthermore, the ability of the immobilised enzyme to degrade bisphenol A compound was studied under different temperatures.

The absorbance values measured by the spectrophotometer indicated remaining bisphenol A which was not converted by the immobilised enzyme. The extent of bisphenol A degradation was calculated using values of absorbance of specific bisphenol A concentration from generated bisphenol A standard curve. From this analysis, it is shown that at temperature 20°C, the immobilised enzyme managed to degrade the most amount of bisphenol A compound. Therefore, temperature 20°C is concluded to be the most optimum temperature for the immobilised tyrosinase enzyme in this study. Objectives of this experiment are met, thus experiment was successfully conducted.

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