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Immobilization of α- Amylase on Mesoporous Silica KIT-6 and Palm Wood Chips for Starch Hydrolysis

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Abstract

α-Amylase was immobilized on highly ordered mesoporous silica KIT-6 and palm wood chips (PWC) by physical adsorption. The activity of the immobilized enzyme for starch hydrolysis was investigated at different solution pH and enzyme concentration for both supports. The thermal stability of the immobilized enzyme was also investigated for both supports. The result indicates that the maximum activity, in terms of glucose concentration, for both supports was observed close to the isoelectric point (PI = 7.00) of the enzyme. Activity increased with increasing concentration of the enzyme solution for both supports. The glucose to support ratio of 48 was obtained for KIT-6 compared to a value of 0.6 obtained for PWC. KIT-6 shows superior thermal stability. The exceptional performance of KIT-6 relative to PWC can be ascribed to its high adsorptive capacity as well as its excellent mass transfer characteristics arising from its very high surface area, large pore diameter and highly ordered pores.
Keywords: Mesoporous silica, KIT-6, Palm wood chips, Enzyme immobilization, α-amylase, Starch hydrolysis

1. Introduction

Enzymes are biocatalysts that are gradually making in-roads in various fields of chemical engineering, where chemical synthesis routes are being replaced by enzymatic ones. Enzymatic routes offer advantages in terms of selectivity with its concomitant high yield and exclusivity towards the desired product (Lilly, 1994). Commercial application of enzymes is however fraught with the difficulty of separation from the solution and their inactivation by organic solvent and extreme pH or temperature. Immobilized enzymes are being increasingly used owing to their ease of separation and enhanced thermal or pH stability (Wang et al., 1992; Leckband and Langer, 1991; Pierre and Crichton, 1988). The immobilized enzyme molecules may also be stabilized against denaturing agents that promote unfolding processes that can destroy the active site (Mozhaev, 1993). Thus, immobilization reduces loss of enzyme and offers the opportunity to use a continuous reactor with a reuse of the enzyme for many reaction cycles to give an economic advantage (Posorske, 1984).

Amylases are endo-enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units. These enzymes are of great significance in present day biotechnology with applications ranging from food, baking, brewing, fermentation, detergents, textile designing, and paper industries to analysis in medicinal and clinical chemistry (Ahmed et al., 2008). Like any other enzyme, α-amylase has to be immobilized to improve its industrial applications.

Various solid support materials have been studied for the immobilization of enzymes such as clay/modified clays (Naidja and Huang, 1997,1996; Lopez Santin et al., 1983), silica (Pierre and Crichton, 1988; Martins and Cruz, 1987), zeolite (Pifferi et al., 1982), amorphous aluminium phosphate (Felipa et al., 1998), zirconia (Reshmi et al., 2007), calcium alginate beads (Roy and Gupta, 2004), palm wood chips (Bello and Ogunbayo, 1986, 1993, 2011), mesoporous silicas MCM-41 and SBA-15 (Pandya et al., 2005; Zou et al., 2010).

Palm wood chips (PWC) are medium-sized solid material made by cutting or chipping large pieces of wood. Its main constituent includes cellulose and lignin. PWC offer advantages in terms of high adsorptive capabilities and retention of enzymes for a long time (Bello and Ogunbayo, 1986, 1993; Egwin and Oloyede, 2008). Enzymes have been successfully immobilized on PWC and used for the hydrolysis of starch (Bello and Ogunbayo, 1993; Egwin and Oloyede, 2008).

Mesoporous silicas are interesting solids for studying enzyme immobilization due to their relatively uniform pore structure, large surface area and biocompatibility. It is well known that pore size, pore volume and pore structure of mesoporous materials have influence on the immobilization of enzymes (Wei et al., 2008). However, 1-D and 2-D structure, lower pore size and pore volume of the early mesoporous materials such as MCM-41 and SBA-15 restrict enzyme diffusion and transportation, thus limiting their use in the field of enzyme immobilization. Mesoporous silica KIT-6 has unique 3-D interconnected network which provides a highly open porous host with an easy and direct access for guest species, thus facilitating inclusion or diffusion throughout the pore system without pore blockage (Guo et al., 2010).

The main aim of this work was to investigate the activity and stability of α-amylase immobilized on ordered mesoporous silica KIT-6 for starch hydrolysis in comparison with the performance of α-amylase immobilized on palm wood chips (PWC). Performance was expressed in terms of concentration of glucose produced.

2. Materials and Methods

2.1 Materials

α-amylase (Brand: Termanyl, 120 L, Type L) in sodium phosphate buffer, palm wood chips, dinitrosalicyclic acid, corn starch powder, acetic acid, sodium hydroxide, distilled water, acetic acid, aluminium foil, filter papers. Mesoporous silica KIT-6 was synthesized and characterized as reported in our previous work (Usman et al., 2012). Other reagents used in this work were of analytical grade and used as-received.

2.2 Preparation of Enzyme

Two sets of enzyme samples were prepared for this study. The first set of five samples were prepared by adding 10, 20, 30, 50, and 60 ml of the enzyme in sodium phosphate buffer to 90, 80, 70, 50 and 40 ml of distilled water respectively, in a conical flask, to give concentration range of 0.1 – 0.6 v/v. The pH of each solution was 7.00 ± 0.4. The second set of three samples was prepared at varied pH but constant concentration of 0.5 v/v. This was achieved by adding 2 ml of 0.1 M, 0.5 M NaOH to the 1st and 2nd conical flask containing enzyme of concentration 0.5 v/v with corresponding pH 8.20 ± 0.2, 10.00 ± 0.40 respectively, 2 ml of 0.5 M acetic acid with pH 6.7 ± 0.1 in the 3rd conical flask.

2.3 Evaluation of Enzyme Activity

The prepared enzyme solutions were immobilized on PWC and KIT-6 and used for the hydrolysis of starch. For the immobilization on PWC, 2 g of wood chips each was used to immobilize the enzyme at concentrations of 0.1 – 0.6 v/v and at solution pH of 7.04, 8.40, 10.40 and 6.80 (all at a constant concentration of 0.5 v/v), which gave a total setup of 10 conical flasks. The resulting mixture was covered with aluminum foil, continuously shaken in a water bath at 100 shakes/min at 27.2 ^{o}C for 4 hrs. The treated PWC were washed three times with distilled water to remove excess enzyme on the surface and subsequently used for starch hydrolysis.

For the immobilization on KIT-6, 50 mg of the highly ordered mesporous siliceous material each was used to immobilize the enzyme at concentrations 0.1v/v, 0.3v/v, 0.5 v/v, 0.60 v/v (all at PH 7.00) and solution PH of 6.70, 7.02, 8.20 and 10.20 at constant enzyme concentration of 0.5 v/v, these gave a total set of 8 conical flasks. The resulting mixture was covered and also agitated. After 4Hrs, each KIT-6 setup was centrifuged at 3500 rpm for 10 mins, cooled in ice water at intervals of 2 mins. The liquid on top was decanted and the resulting loaded material was dried at room temperature and was subsequently used for starch hydrolysis.

The starch hydrolysis was carried out by preparing a starch solution of 0.025 g/ml of distilled water. 2 g (2000 mg of the loaded wood chips) was immersed in 50 ml of starch solution (40:1) and 40 mg of the loaded KIT-6 organosilica in 1 ml of starch solution (40:1) and hydrolysis in each flask allowed for 40 mins at 27.2^{0}C and 100 shakes/min in a water bath.

2.4 Thermal Stability Experiment

The prepared enzyme sample, 0.1 v/v concentration and pH 7.00, was immobilized on PWC and KIT-6 and used for this study. For the KIT-6, 350mg of the carrier was immersed in a conical flask containing 100 ml of the enzyme solution for 4 hours. After immobilization, the solution was centrifuged at 3500 rpm for 5 minutes to separate the immobilized KIT-6 from the solution. The KIT-6 was left to dry in air overnight. For the PWC, the carrier was boiled in water at 80^{o}C for 30 minutes. The boiled chips were then immersed in 100 ml of the enzyme solution for 4 hours. The chips were then removed and rinsed with water to remove excess enzyme.

Hydrolysis of starch using immobilized α-amylase on PWC was performed according to the following procedure. 2 %w/v of starch solution was prepared by dissolving 4 g of starch in 200 ml of hot water. 70 ml of the prepared starch solution was placed in a conical flask and 2 g of the immobilized α-amylase on PWC added to it. The flask was immersed in the water bath at 30^{o}C for 40 minutes and agitated at the speed of 100 shakes per minute for hydrolysis to take place. Thereafter, the PWC were removed to stop the hydrolysis. The procedure was repeated at temperatures of 40, 50 and 60^{o}C respectively.

Hydrolysis of starch using immobilized α-amylase on KIT-6 was carried out as follows. 2 % w/v of starch solution was prepared by dissolving 4 g of starch in 200 ml of hot water. 5 ml of the prepared starch solution was placed in a conical flask and 20 mg of the immobilized KIT-6 was added. The mixture was heated to 30^{o}C and agitated using a magnetic stirrer for 40 minutes. Hydrolysis was stopped by heating the solution in boiling water. The procedure was

repeated at temperatures of 40 and 50 ^{o}C.

2.5 Analysis
The dinitrosalicyclic acid (DNSA) method was used for estimating the reducing sugar content (glucose) of the product of the experimental runs. DNSA was mixed with the hydrolysed product(P) in the ratio of DNSA:P of 1:2 and after heating in the water bath for 5 mins , the yellow colour of the mixture change to deep red; an indication of the reaction between DNSA and the hydrolysed product. The maximum absorbance of each sample was measured in a colorimeter at 660 nm and the corresponding pure glucose concentration was obtained from a standard glucose curve at 660 nm.

3. Results and Discussion

3.1 Evaluation of Enzyme Activity

3.1.1 Effect of Enzyme Concentrations.
As shown in Figure 1, there is an initial rise in glucose concentration with increasing enzyme concentration for both KIT-6 and PWC, though sharper for KIT-6. This indicates a high affinity of the supports for α-amylase molecules. With increasing α-amylase concentration, the glucose concentration increases which means that the amount of α-amylase absorbed on both supports increase.
However, the glucose concentration attains a maximum value of 1.91g/l for KIT-6 and 1.24g/l for PWC for both supports at 0.50 v/v. This trend may be due to the fact that the protein molecules may be adsorbed on the solid supports in various distinct orientations (Vinu et al., 2005). At low enzyme concentrations, the α-amylase molecules may be adsorbed with a side-on- type configuration perpendicular to the supports. When the enzyme concentration is high, the molecule may be adsorbed with an end-on-type configuration that helps the molecule land close to each other, which reduce the increasing electrostatic repulsion between the protein molecules resulting in higher α-amylase adsorption. Moreover, the high concentration of the enzyme helps close packing because of the decreased hydrophobic interactions between the protein and the support upon adsorption of some α- amylase molecules. Therefore, the maximum glucose concentration was observed at 0.5v/v for both supports.

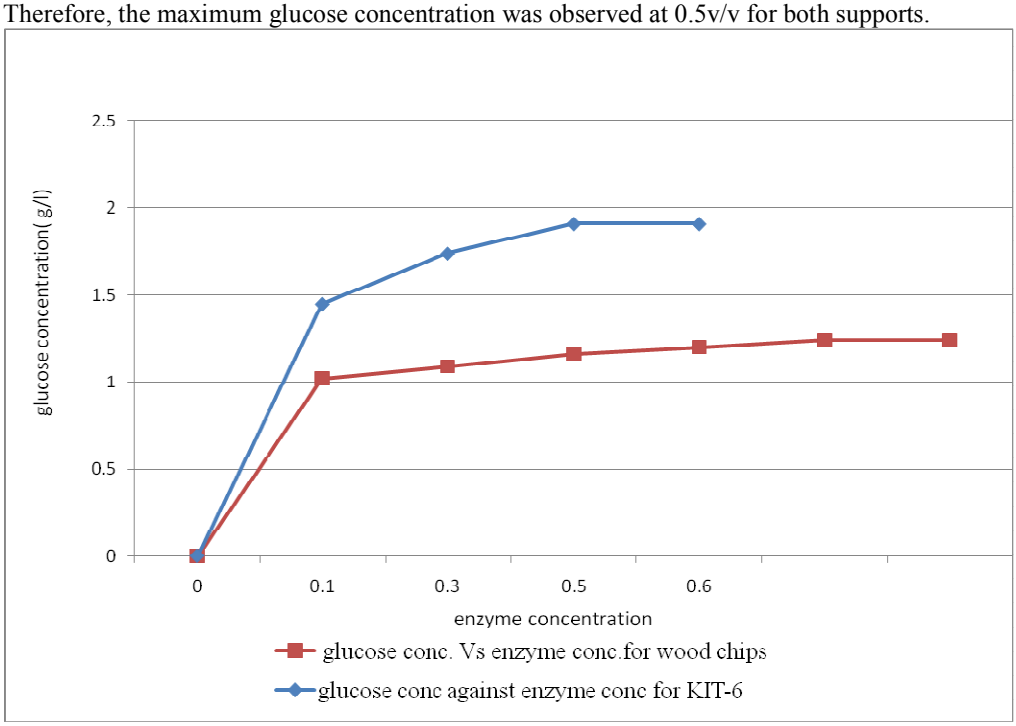

Figure 1: Glucose Concentration (g/l) vs Enzyme Concentration (v/v) .

3.1.2 Effect of Support Material

To ensure that the glucose concentration will be a function of only the acivity of the enzyme, the temperature was kept constant at 27.2^0C. As shown in figure 2, as the concentration of the enzyme increases, the glucose/support ratio of both support materials increases before attaining a near contant value at 0.5v/v. Interestingly, the trend shows that at an enzyme concentration of 0.1 v/v, the ratio is 36:1 this implies that 1g of KIT-6 (with immobilized enzymes) will be required to produce 36g of glucose, while for PWC (with immobilized enzymes) 1g will be producing just 0.51g of glucose. Generally 40mg of α-amylase/KIT-6 produced 1.44g glucose/l at an enzyme concentration of 0.1v/v, while 2g α-amylase/PWC produced 1.02g glucose/l. At an enzyme concentration 0.3 v/v, more α-amylase molecules are active. 40mg of α-amylase/KIT-6 produced 1.76g of glucose/l (increment of 0.32g) and 2g of wood chips produced 1.16g of glucose (increment of 0.14g). Finally, at 0.5 v/v, 40mg KIT-6 produced 1.91g of glucose (an increment over the previous value which is twice that of PWC) and 2g of PWC produced 1.24g of glucose. Hence, activity increases as enzyme concentrations increases up to 0.5 v/v and KIT-6 is shown to be a superior support compared with PWC.

The reason for this high adsorption capacity and activity of the enzyme on KIT-6 is due to its well- ordered, large pore diameter and pore volume. Besides, it was explained earlier that KIT-6 has a 3-D pore network with two intersecting pore systems which allows the α-amylase to access the adsorption sites from all the three dimensions, unlike PWC which is a relatively poorly ordered support.

It was also observed during experiment that the absorbances in the 1st and 3rd hours of starch hydrolysis are approximately the same for KIT-6, while an increase was observed when PWC were used. Thus, hydrolysis of starch using α-amylase on KIT-6 support is accomplished with a shorter time than with PWC. This advantage has commercial appeal and lends credence to the earlier assertion that KIT-6 support are more elegant house and better than PWC.

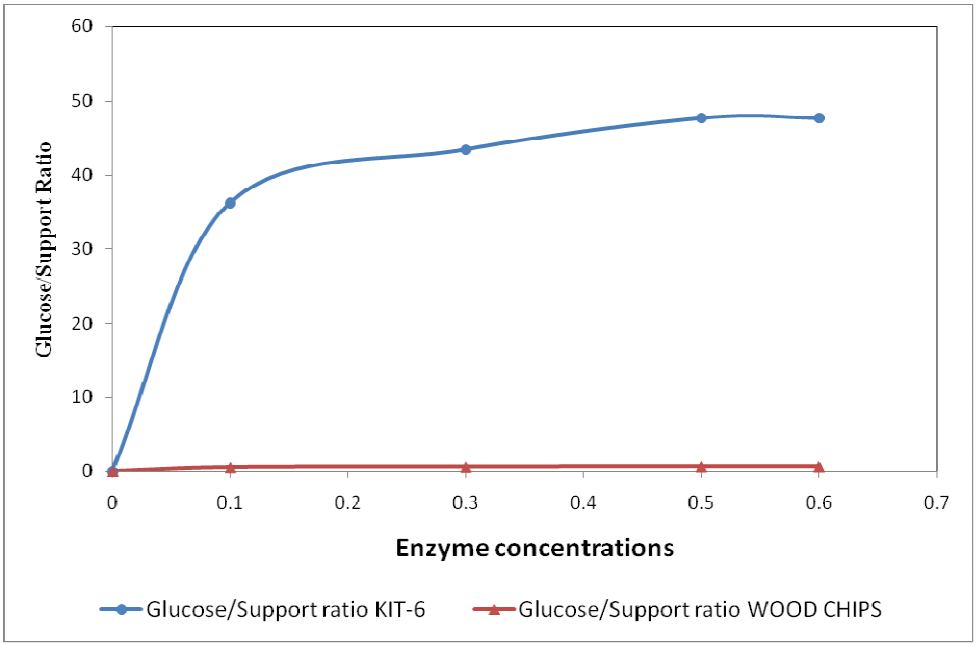

Figure 2: Glucose/support ratio vs enzyme concentrations for KIT-6 and PWC.

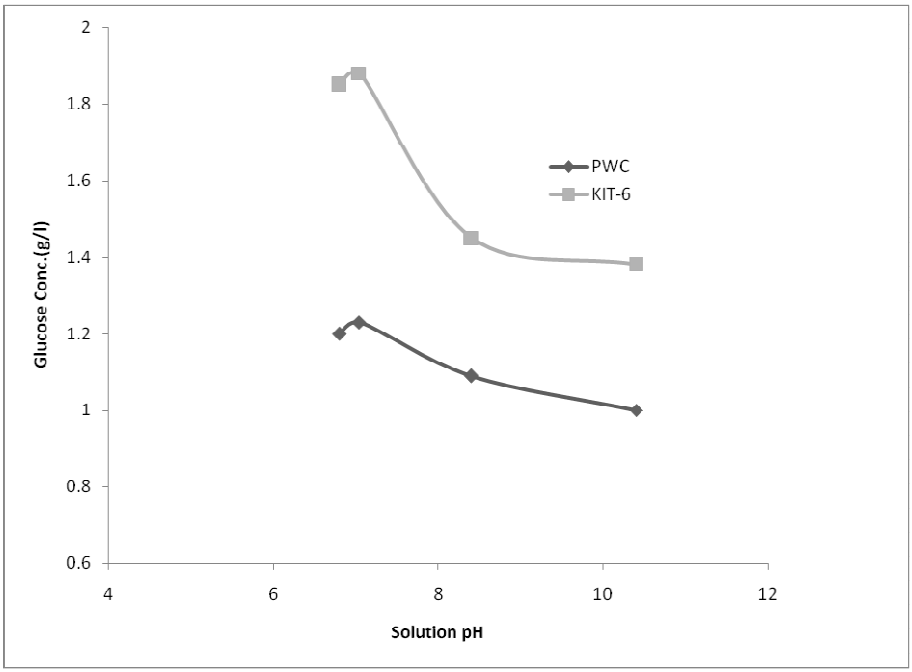

Figure 3: Effect of solution pH for both supports (PWC and KIT-6).

3.1.3 Influence of Solution pH

Figures 3 show that the glucose concentration increases with increasing solution pH; from pH 6.80 reaches a maximum at 7.04 for wood chips and pH 6.70 reaches a maximum at 7.02 for KIT-6 and then decreases in both cases.

Their corresponding glucose concentrations are1.23g/l and 1.88g/l for wood chips and KIT-6 respectively. These amounts are recorded near the isoelectric point of α-amylase in Na_2PO_4 buffer pH=7.00. This trend also shows that α-amylase molecules work relatively well in a acidic medium (Acetic acid) than in basic meduim. At the isoelectric point, the net charge of the enzyme is low, coulombic repulsive force between and (or) within the protein molecule will be minimal and closer packing of the protein molecule will be minimal (Vinu et al, 2008).

Far above or far below this point, the glucose concentration significantly decreases and this negatively affects the stability and re-usability of the enzyme for starch hydrolysis i.e. the activity of the enzyme would significantly decrease in a highly acidic and highly basic medium.

3.2 Thermal Stability

From the Figures 4, it is seen that there is a high increase in glucose concentration for enzyme immobilized on both PWC and KIT-6 as the temperature increases from 30^0C-40^0C. Likewise, there is also an increase as the temperature increases from 40^0C-50^0C. The increase in concentration shows a drop of 65.3% -13% for PWC as opposed to 71.6%-65.2% for KIT-6. The reason for the higher drop obtained for wood chips is as a result of its pore structure, which favours the leaching of the enzyme, thus reducing its activity over time, unlike KIT-6 which maintains the attachment of the enzyme over time. Thus, it can be said that KIT-6 is a better support than PWC.

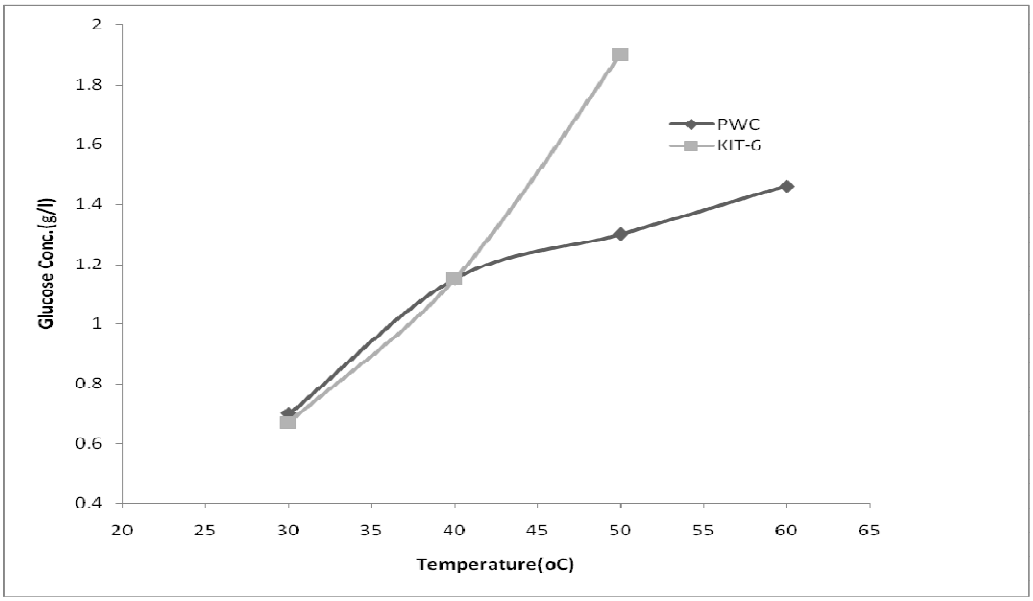

Figure 4: Effect of temperature on enzyme activity

4. Conclusion

The activity of α-amylase during starch hydrolysis has been studied with KIT-6 and PWC as supports at different enzyme concentrations and solution pH. It is evident that KIT-6 is a better support compared to PWC at these operating conditions. The maximum glucose concentration was 1.91g/l for KIT-6 and 1.24g/l for wood chips at 0.5 v/v when varying enzyme concentrations at constant solution pH = 7.00 and 27.2^{0}C. At varying solution pH, the maximum activity was observed very close to the isoelectric point of α-amylase (pH = 7.00), 7.04 for PWC and 7.02 for KIT-6 with their respective glucose concentrations 1.23g/l and 1.88g/l. More enzymes were immobilized on KIT-6 than wood chips due to its very high surface area and well- ordered, large pore, interpenetrating and interconnected channels. This gives room for a higher adsorption as well as better mass transfer characteristics thus producing a higher glucose/support ratio. Thermal and mechanical stability study also confirms the superiority of KIT-6 over PWC.

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