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Adsorption and Stabilization of a Raw Starch Digesting Amylase on Micro Bead Silica Gel 300 A

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Authors' Contribution

B.N. Okolo and H. Aoyagi co-supervised the study. T.N. Nwagu designed the study and H. Aoyagi wrote the protocol. T.N. Nwagu managed the analyses of the study and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To produce a robust starch hydrolyzing enzyme (improved catalytic and non-catalytic properties) by the adsorption of the soluble enzyme on micro bead silica gel.
Place and Duration of Study: Department of Life Sciences and Bioengineering, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba-shi Ibaraki-ken, Japan between July 2009 and August 2010.
Methodology: Ten types of Micro Bead silica gel with pore sizes ranging from 0.4-100 nm were screened to determine the best support for the immobilization of a microbial raw starch digesting amylase (RSDA). The micro bead which gave the highest yield was selected for further studies. Properties of the immobilized enzyme were compared to the free type to determine the effect of immobilization on catalytic, storage and operational stability.
Results: Micro Bead 300 A gave the highest yield and the optimum condition for adsorption of the RSDA was at pH 5, 25°C for 24 h. Optimum pH of the immobilized enzyme shifted from 5 to 4.5 and optimum temperature from 30 to 50°C. The immobilized

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amylase retained over 70% of its initial activity after 12 h incubation at 70°C in 0.2 M citrate phosphate buffer pH 5 whereas free enzyme lost 92% initial activity under same conditions. Immobilized enzyme retained 95% activity after 10 batch reactions of 30 min each and 100% activity after storage for 6 weeks at room temperature.
Conclusion: Immobilized RSDA was marginally more pH and temperature stable compared to the native type. It also exhibited storage stability and could be re-used repeatedly without considerable desorption during washing. The kinetic and stability features combined with the properties of the support make this process appealing for industrial application.

Keywords: Amylase; stabilization; adsorption; immobilization; starch; micro bead silica gel.

1. INTRODUCTION

Raw starch digesting amylases (RSDAs) are starch hydrolyzing enzymes which can catalyze the degradation of raw starch to simple sugars. RSDAs vary from other amylases in their special affinity and interaction with the microcrystalline structures of the raw starch molecule, through the starch binding domain, SBD (Sarikaya et al., 2000). This is unlike other starch hydrolases which only act on gelatinized starch. RSDAs are produced from numerous plants and microorganisms and these enzymes vary in their substrate specificity, optimum pH, temperature of activity and hydrolytic products (Abe et al., 1988; Balkan et al., 2010; De Silva et al., 2009; Forgarty and Kelly, 1990; Horváthová et al., 2004; Matsubara et al., 2004). Some of these enzymes are starch liquefying where as others are saccharifying. RSDAs are often produced together with the non raw starch digesting type and proteolytic enzymes whose activity may lead to the digestion of the SBD and a resultant loss of raw starch hydrolyzing activity (Hasan et al., 2008). This is partly responsible for the poor stability reported for raw starch digesting amylases. RSDAs find potential application in food, paper, detergent, textiles, bioanalytical, biopharmaceutical and biomedical industries (Liu and Xu, 2007).

Though enzymes are produced from plants, insects and mammals, microbial enzymes offer advantages of improved yield, process dynamics, ease of processing, specificity of reaction, product stability and favorable economics (Bozic et al., 2011; Goyal et al., 2005; Mamo and Gessesse, 1999; Nwagu and Okolo, 2011; Pothiraj et al., 2006; Yu-Na et al., 2010). However for industrial application, enzymes are required to function, retain their stability and selectivity and even specificity in conditions which may be totally different from their physiological environment (Betancor and Luckarift, 2008; Hernandez and Fernandez-Lafuente, 2011). The labile natures of enzymes and inability to function under extreme conditions or conditions which vary from their natural environments have limited their utilization in industrial processes (Iyer and Ananthanarayan, 2008). Immobilization is an age old method of enzyme stabilization. Immobilization when properly designed leads to the production of a biocatalyst which is more stable under process conditions, resistant to substrate inhibition and the reaction product is not contaminated with enzyme (Chiyanzua et al., 2010; Dove and Madamwar, 2006; Hamerska and Dudra et al., 2007; Mateo et al., 2004). Other properties of a robust immobilized enzyme include reduction of product inhibition, longer half life and predictable decay rate (Cao et al., 2005; Reshmi et al., 2006). Immobilized enzymes are also preferred over the free type due to their multiple and

repetitive use which offset the high cost of enzyme production and their ability to promote process/reactor designs and operations (Iyer and Ananthanarayan, 2008; Roger, 2007; Sanjay and Sugunan, 2005). Stabilization is achieved through multipoint covalent/non-covalent interactions between enzyme and support groups, multi subunit immobilization (Fernandez-Lafuente, 2009), proper orientation of enzyme to support, prevention of autolysis/proteolysis (Mateo et al., 2007) and steric exclusion of substrate/product from the inhibition site (Cowan and Fernandez-Lafuente, 2011).

Irrespective of the potentials of a robust immobilized enzyme (improved catalytic and non-catalytic properties), immobilization offers specific challenges which if not met leads to the loss of stability, activity and in some cases specificity of the enzyme. In cases of large substrates, improper orientation of enzyme to support could lead to the distortion of critical areas of the protein or block the active center of the enzyme causing severe activity loss (Hernandez and Fernandez-Lafuente, 2011). Methods employed for enzyme immobilization include adsorption, cross linking, covalent modification, encapsulation and entrapment (Bryjak et al., 2007; Dey et al., 2003; Hasirci et al., 2006; Milosavic et al., 2007; Torres et al., 2004).

Adsorption is the oldest and simplest immobilization technique (Brady and Jordan, 2009). Binding is based on weak forces such as van der Waals forces, ionic, hydrophobic and hydrogen bonds and multiple salt linkages. Depending on the available charges on the support polymer and the protein orientations, strongly bound but not distorted immobilized enzyme could be formed. Immobilization by adsorption enables desorption of enzymes at the end of their active life, to permit the regeneration and re-use of the carrier. Amylases have been adsorbed on nitrocellulose membranes (Tanyolac et al., 1998) and chitosan beads (Noda et al., 2001) and on Cu^{2+} chelated polytheylene glycol dimethacrylate-n-vinyl imidazole matrix (Kara et al., 2005).

RSDA from *Aspergillus carbonarius* (Bainier) Thom IMI 366159 is capable of one step hydrolysis of a wide range of tuber and cereal starches to simple sugars (glucose and maltose), under a range of pH and temperature conditions (Okolo et al., 2001). However, in spite of these desirable qualities the enzyme has instability issues which adversely affect its utilization in industrial processes.

Micro Bead (MB) Silica gel consists of a range of spherical porous inorganic silica beads with uniform diameters and high surface area derivatized with normal and reversed phase groups such as C18, C8, C4, Diol, NH_2 etc. M B silica gels are inert and stable at elevated temperatures.

In this study RSDA from *A. carbonarius* was adsorbed to M B silica gel. The effect of immobilization on the activity and stability of the amylase was evaluated.

2. MATERIALS AND METHODS

2.1 Enzyme and Materials

RSDA was produced from *A. carbonarius* and partially purified according to the method of Nwagu et al. (2012). Micro bead silica gels of varying particle sizes (10-200 µm) and pore sizes (0.4-100 nm) were purchased from Fuji Silysia Chemical Company, Japan. Raw potato starch was prepared in our laboratory using the method outlined by Okolo et al. (2001). 3, 5-

Dinitrosalicylic acid (DNS) was purchased from Lancaster, England. All other chemicals were of analytical grade and purchased from Wako pure chemicals, Japan.

2.2 Enzyme Adsorption

Prior to immobilization, micro bead silica gels were properly washed with distilled water equilibrated with 0.2 M citrate-phosphate buffer, pH 6.0, and sucked dry. Equilibrated beads (1 g) were suspended in 5 mL of 0.2 M citrate-phosphate buffer pH 6 containing the enzyme protein and stirred gently for 24 h at 25°C. The support was repeatedly washed with distilled water and 0.02 M sodium acetate buffer, pH 5.6, and the amount of adsorbed enzyme was estimated by difference after measuring the non immobilized protein. Adsorbed RSDA was recovered by filtration and stored at 4°C.

2.3 Optimization of Immobilization Method

To determine the optimum conditions during the immobilization, the following parameters were studied in a stepwise manner: Temperature (4, 10, 25°C.), incubation period (4 - 24), pH (4.5-7.5) and enzyme concentration (0.01-0.1 g/ml).

2.4 Assay of Enzyme Activity

Amylase activity was assayed using a reaction mixture containing 0.2 ml of 1% raw potato starch in 0.2 M citrate-phosphate buffer (pH 6.0) and 0.2 mL (34 U) of enzyme solution, incubated at 40°C for 10 min in a bioshaker at 100 rpm and 12 mm diameter for homogeneity (unless otherwise stated). Reducing sugars released after incubation were estimated by the DNS method of Miller (1959). One unit of amylase was defined as the amount of enzyme, which liberated 1µ mol of reducing sugar per minute under the assay conditions.

The immobilized enzyme activity was assayed by incubating at 40°C for 10 min (unless otherwise stated) an appropriate amount of suction dried derivate (≈10 mg) with 1 mL of 1% (w/v) solution of soluble starch in 0.2 M sodium citrate-phosphate buffer (pH 6.0) with constant stirring. Sample of the reaction mixture was withdrawn and assayed for reducing sugar as earlier described.

2.5 Determination of Protein

The amount of protein immobilized was estimated by subtracting the amount of protein in supernatant after immobilization from the total amount of protein used for immobilization. Protein content was determined by the Bradford method using BSA as the standard (1976) and/ or using the spectrophotometer at a wavelength of 280 nm.

2.6 Immobilization Efficiency

The immobilization yield was defined as

Immobilization (%) = Protein in control suspension – Protein in the supernatant of the immobilization suspension/ Protein in control suspension x 100.

2.7 Properties of the Enzyme Preparations

Optimum pH of the RSDA preparations was determined by incubating the RSDA preparations in 1% raw potato starch solution prepared in buffers of pH ranging from 3.0-7.0 using 0.2 M citrate-phosphate buffer and at pH 8.0-9.0 using 0.1 M Tris/HCl buffer at 40°C. The pH stability of the RSDA of the soluble and immobilized RSDA was studied by storing the enzyme in appropriate buffers of pH values ranging from pH 3.0 to 9.0 for 12 h at 10°C. After, the residual amylase activity was estimated as earlier described at optimum pH recorded for the free and immobilized amylase respectively.

The effect of temperature on the activity of the immobilized enzyme was determined by incubation with substrate in 0.2 M citrate-phosphate buffer pH 5 at temperatures ranging from 30 to 80°C for 20 min. Amylase activity was determined after incubation by DNS method. Thermal stability of the enzyme preparations was determined by storing the RSDA in 0.2 M citrate-phosphate buffer pH 5.0 at temperatures 50 and 70°C for 12 h. The enzyme preparation was cooled, incubated with starch solution at optimum temperature for 20 min and the activity determined.

Operational stability test was carried out by repeated 10 batch experiments using the method for activity determinations. Immobilized enzyme was incubated with 2% raw potato starch in 0.2 M citrate-phosphate buffer pH 5 for 30 min, samples were collected and reducing sugar tested using DNS method. Bound enzymes were then thoroughly washed and re-used. Storage stability was tested after keeping the immobilized enzyme preparations at 4°C for 6 weeks.

To determine enzyme stability during substrate conversion, the free and immobilized enzymes were incubated in 1% starch solution suspended in 0.2 M citrate-phosphate buffer pH 5 for 60 min. Samples were collected every 10 min and assayed for enzyme activity.

To determine the kinetic parameters, enzyme activity was assayed in reaction mixtures containing different concentrations (0.2-1 mg ml^{-1}) of raw potato starch in 0.2 M citrate-phosphate buffer, pH 5.0. The kinetic constants (K_m/ V_{max}) were estimated by double reciprocal plots of the data according to Lineweaver and Burk.

All experiments were done in triplicates.

3. RESULTS AND DISCUSSION

3.1 Screening Test to Determine Best carrier for the Immobilization

Enzyme adsorption on support polymer is dependent on ionic strength, pH, surface area, porosity and physical characteristics of both the enzyme and the support, such as the number and type of nucleophilic and reactive groups on the enzyme and support surfaces, respectively. Different types of micro bead silica gel were screened to determine the best carrier for the immobilization of the raw starch digesting amylase. Table 1 shows that the lowest immobilization yields were obtained using 15N (46%), followed by 20N (50%) and 10N (44%) whereas 300A (88%), 500 (84%) and 800A (76%) gave the highest yield. Micro bead silica gel 300A (M B 300A) has a specific surface area of 90 m^2/g, pore size (30 nm), and particle size ranging from 75-300 µm. It is possible that the M B 300A has the smallest

pore size which permitted the highest loading of the enzyme. Further studies were therefore carried out with micro bead silica gel 300 A.

Table 1. Screening of micro bead silica gel for enzyme immobilization

Micro bead	Immobilization yield (%)
300A	87.6
4B	62.3
1000A	64.3
3A	40.1
10N	44.5
800A	75.6
500A	84.3
20N	50.0
15N	45.6
5D	62.7

3.2 Influence of Temperature and Time on Immobilization

Immobilization yield was influenced by temperature and the duration of immobilization as shown in Table 2. From the table, the reaction was observed to progress rapidly in all cases and immobilization yield reached 50% within the first 3 h. However, the highest immobilization yield was obtained after 24 h at 25°C. In order to establish multipoint interaction between the enzyme and support groups, a properly activated support operated under suitable immobilization conditions is needed. At moderately high temperature, there may be increased vibration of enzyme and support molecules which facilitates greater interaction and formation of enzyme-support linkages. Moreover, temperature changes during immobilization create poor solvency as a result of the reduction or elimination of steric repulsion, thereby promoting protein polymer interaction and higher immobilization yield (Hamerska-Dudra et al., 2007). Immobilization yield and enzyme stabilization is correlated to the number of enzyme-support linkages formed (Mateo et al., 2007). Other factors which directly affect enzyme loading on a porous carrier include shape of enzyme particles, distribution in size and shape of pores, depth of pores, total volume of pores with respect to particle volume and the depth and tortuosity of the route to the pores which the enzyme encounters (Chaplin and Bucke, 1990). During immobilization, higher volumetric concentration of the enzyme is rapidly attached towards the exterior of the support. However, prolonged incubation enables the penetration of enzyme into the pore spaces and promotes the number and strength of bonds formed.

Table 2. Effect of temperature and duration of incubation on the immobilization yield

Duration (h)	Immobilization yield (%)		
	Temperature (^{o}C)		
	4	10	25
3	55.7	66.2	60.8
6	59.5	68.2	70.0
12	77.3	74.7	84.0
18	84.0	76.1	88.1
24	90.3	77.5	94.8

3.3 Influence of pH on Immobilization Yield

The pH of the immobilization is one of the factors known to affect enzyme coupling (Dey et al., 2002). To determine the optimum pH for immobilization, enzyme was immobilized at different pH. Fig. 1 shows that the maximum yield was obtained when immobilization was carried out at pH 5. Earlier Semba and Dobashi (2001) reported a pH optimum of 5.5 for the immobilization of S-hydroxynitrile lyase using micro bead silica gel 300 A. Also considering the low pI of this RSDA, pH 5 could be the pH value where reactive groups on both enzyme and support surfaces are most suitable to permit higher possibility of achieving multipoint interaction between the enzyme and support groups.

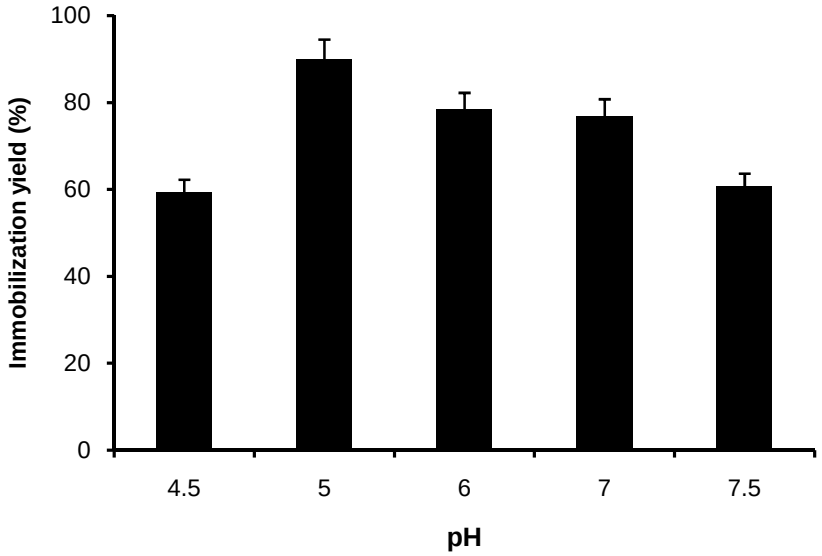

Fig. 1. Effect of pH on immobilization yield

3.4 Effect of Enzyme Concentration on Immobilization Yield

Fig. 2 shows the effect of enzyme concentration during the immobilization coupling stage. At lower concentrations of enzyme, immobilization progressed rapidly and attained a limiting value at 0.05 g/ml. After this point, higher loading of carrier led to a reduction in enzyme activity. This could be owing to the fact that at the later stage of carrier loading the enzyme molecules permeate into the pore spaces and are mostly inaccessible during catalysis. This is because during biocatalysis which involves bound enzyme, reaction predominantly involves the external enzyme molecules. Internal enzyme molecules may not be accessible to the substrate or not receive it because it has been modified by external enzyme molecules (Dey et al., 2003). This ensures high biocatalytic stability since there is conservation of enzyme molecules in the pores until those in use are denatured or lose their specific activity with time.

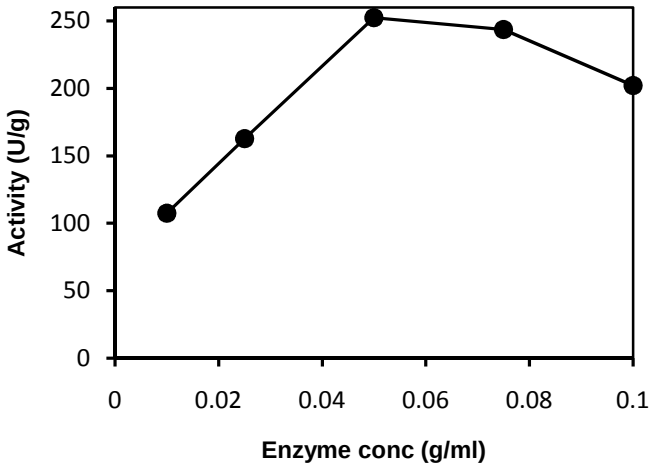

Fig. 2. Effect of enzyme concentration during the immobilization coupling stage

3.5 Characterization of the Immobilized Enzyme

To determine the efficacy of the immobilization process a comparative study of the properties of the free and adsorbed enzyme was conducted.

3.5.1 Influence of pH on the activity and stability of the free and immobilized enzyme

The optimum pH for the activity of the immobilized enzyme shifted by 0.5 pH unit towards the acidic region compared to the pH optimum of 5 recorded for the free enzyme (Fig. 3). Similar results were reported for RSDA immobilized through glutaraldehyde cross linking on agarose (Nwagu et al., 2012), amylase immobilized on chitosan beads (Tripathi et al., 2007), and pectinase from *Aspergillus niger* immobilized on polyacrylonitrile membrane (Delcheva et al., 2007). The pH of the microenvironment compared to that of the bulk solution is dependent on charges on the support and influenced by the partitioning of the hydrogen ions in and out of the enzyme matrix. This in turn affects enzyme activity and substrate binding (Shewale and Pandit, 2007; Tumturk et al., 2007). Negatively charged supports displace pH activity towards higher pH values and vice versa (Morana et al., 2006). Immobilized enzyme was observed to be more active at extremes of pH, an indication that immobilization broadened the pH activity profile of the enzyme. The free enzyme was not pH stable compared to immobilized enzyme. Free enzyme had only 45% of its initial activity at pH 3.5-5 and even less activity (< 20%) at pH 8-9.

Immobilized enzyme was more stable at a wide range of pH and over 88% of the initial activity was retained at pH 4.5-9 (Fig. 4). The broader pH stability profile allows the application of the immobilized enzyme in a range of liquefaction and saccharification processes under acidic and alkaline conditions. The pH stability observed is an indication of the complementary nature of the enzyme and support surfaces which must have ensured formation of strong unstrained interactions by preventing enzyme molecules from interacting with each other.

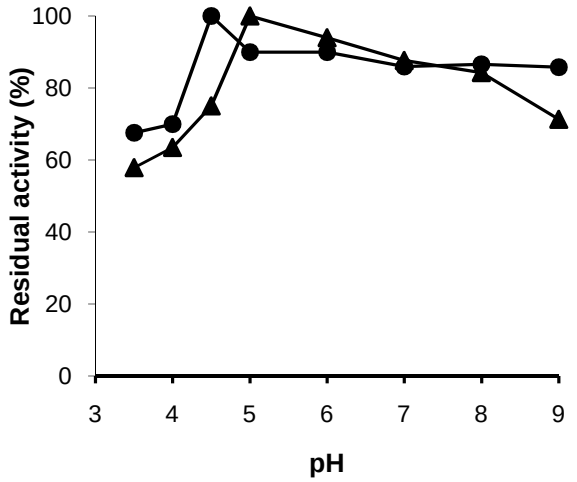

Fig. 3. Influence of pH on the activity of the free and immobilized amylase.
Symbols: triangle- free enzyme; circle-immobilized enzyme

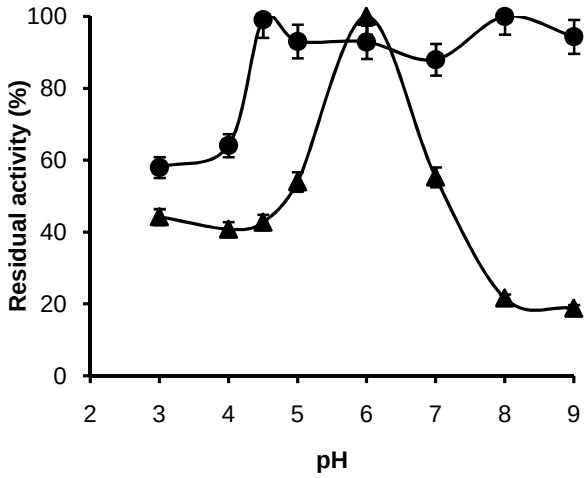

Fig. 4. Influence of pH on the stability of the free and immobilized amylase
Symbols: triangle- free enzyme; circle-immobilized enzyme

3.5.2 Influence of temperature on the activity and stability of the free and immobilized enzyme

Immobilization led to the increase in the optimum temperature of the enzyme from 30°C to 50°C as shown in Fig. 5. Similar results were reported for amylase immobilized on functionalized glass beads (Kahraman et al., 2007) and β-amylase immobilized on chitosan beads (Noda et al., 2001). Covalent immobilization of α-amylase onto poly (2-hydroxyethyl methacrylate) microspheres led to a 15°C increase in optimum temperature (Tümtürk et al., 2000).

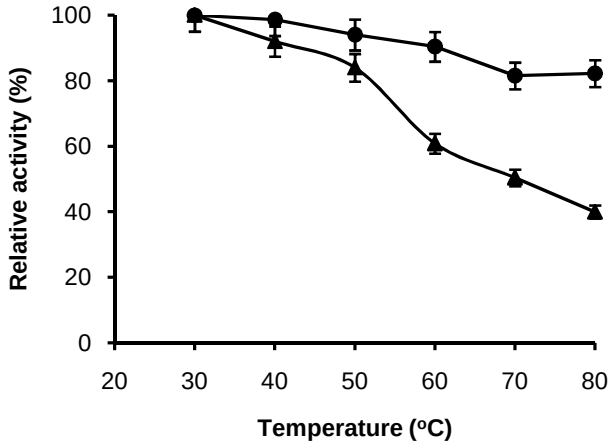

Fig. 5. Effect of temperature on the activity of the free and immobilized enzyme
Symbols: triangle- free enzyme; circle-immobilized enzyme

Storage of enzyme at 70°C in 0.2 M citrate-phosphate buffer pH 5.5 led to a drastic loss (92%) of initial enzyme activity where as immobilized enzyme lost only 30% of its initial activity (Fig. 6). The increased thermostability may be due to diffusional constraints influenced by multiple bonds formed between the amylase and support polymer and also as a result of high enzyme loading. According to Giacomini et al. (2001), the bonding mode consisting of number, nature, and position of bonds formed between the carrier and enzyme molecules greatly influences enzyme activity and stability. Due to the low temperature required for raw starch digestion, product contamination often occurs. The increased thermo activity and stability achieved through immobilization will permit its application at higher temperatures to achieve raw starch saccharification without process contamination. Furthermore due to the wide range of pH and temperature of activity and stability it is possible to use this raw starch hydrolase in a co-enzyme system involving a strictly saccharifying enzyme to facilitate complete starch solubilization and at a faster rate.

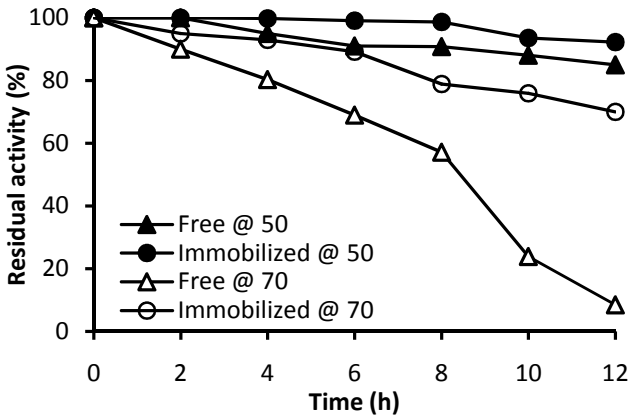

Fig. 6. Thermoinactivation kinetics of free and immobilized amylase at 50°C and 70°C

3.5.3 Enzyme stability during substrate conversion

From the result, shown in Fig. 7 it is obvious that the immobilized enzyme hydrolyzed starch over a period of time without considerable loss in activity. Free enzyme rapidly lost its activity during usage while the bound enzyme retained over 85% of its activity. Immobilization in porous matrix permits the full dispersal of enzyme without possible interaction with any external interface leading to operational stability of the enzyme (Mateo et al., 2007; Cowan and Fernandez-Lafuente, 2011). This type of operational stability functions without the structural rigidification of the enzyme structure. However, considering the previous results, it is likely that the enzyme rigidification achieved by multipoint interaction between enzyme and support groups complements the form of stabilization described earlier to further protect the enzyme nature.

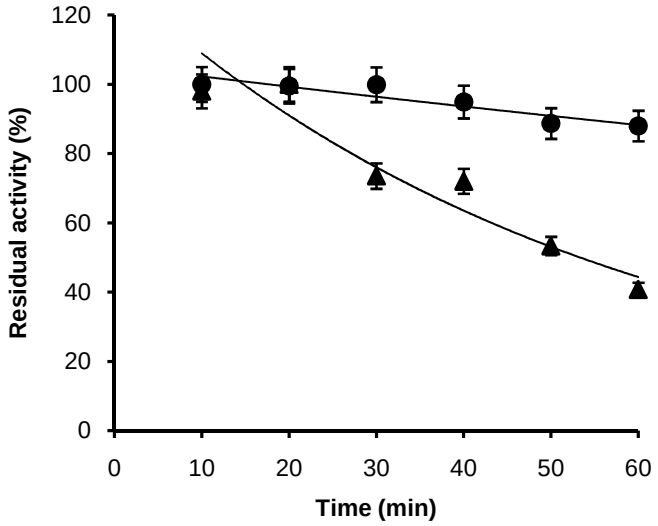

Fig. 7. Amylase stability under conditions of substrate conversion at 50°C
Symbols: triangle-free enzyme, circle-immobilized enzyme)

3.5.4 Kinetics of the free and immobilized amylase

Evaluation of the K_m (Michaelis Menten constant) and V_{max} (maximum reaction rate) of the free and immobilized RSDA showed that there was a slight alteration in enzyme kinetics as a result of enzyme immobilization (Fig. 8). K_m of free enzyme was 0.36 where as it was 0.50 $mgml^{-1}$ for the immobilized enzyme. Increase in K_m indicates a reduced affinity of the immobilized RSDA for the raw potato starch which may be attributed to steric hindrance of active site by support, diffusional resistance to solute transport near particles of the support (Agostinelli et al., 2006) or as a result of differences in ionic strength (Bayramoglu et al., 2002). A reduction in the maximum velocity of the immobilized enzyme catalyzed hydrolysis was also noted. Whereas the V_{max} of the free enzyme was 26.6, for the immobilized enzyme V_{max} was 18.0 µmol min^{-1} ml^{-1}.

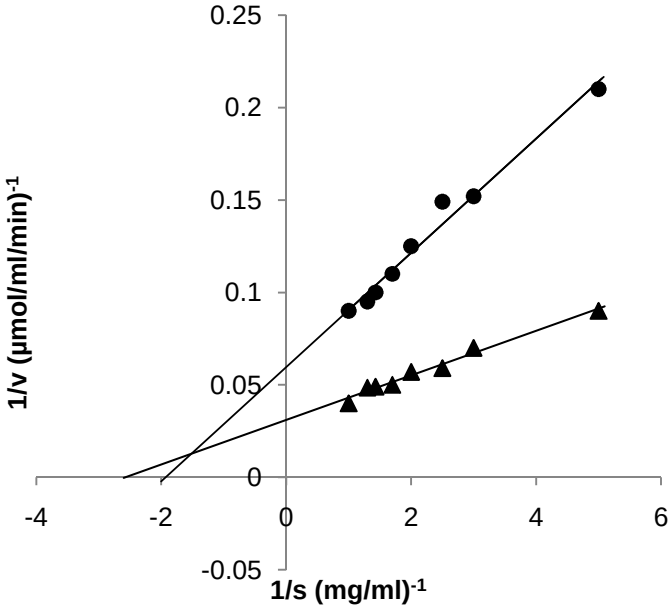

Fig. 8. Determination of Km for free enzyme (triangle) and immobilized enzyme (circle) by Lineweaver-Burk plot method at 40°C

Starch concentration varied from 0.2 to 1 mg/ml in 20 mM phosphate buffer at pH 5

3.5.5 Storage and operational stability of the immobilized enzyme

Due to high cost of enzyme production and time involved, enzyme re-use and storage stability are amongst the major advantages of enzyme immobilization. Soluble enzymes are unstable during storage and gradually lose their activity. Immobilized enzyme was storage stable and retained 100% activity after six weeks storage at 4°C (result not shown) where as free enzyme had 42% activity after storage for six weeks. To determine operational stability, each batch run constituted of 30 min incubation with 2% raw potato starch in 0.2 M citrate-phosphate buffer pH 5. Immobilized enzyme maintained 95% activity after 10 batch re-use as shown in Fig. 9. The result shows that immobilization improved the enzyme stability and enabled its re-use without considerable desorption. The major reason for enzyme immobilization is to produce a storage stable biocatalyst which can be repeatedly used by a simple and cheap method (Adamczak and Krishna, 2004).

The stabilization observed in this study could be as a result of a number of factors. As earlier stated multipoint interaction between enzyme and support groups permit enzyme rigidification. Though enzyme leaching is one of the major setbacks to the utilization of adsorption as a method of immobilization, the stability recorded in this work suggests that relatively strong bonds were formed between the enzyme and the support groups. This shows a simple and cheap method to achieve enzyme stabilization with possible regeneration of the micro bead silica gel at the end of the active life of the enzyme.

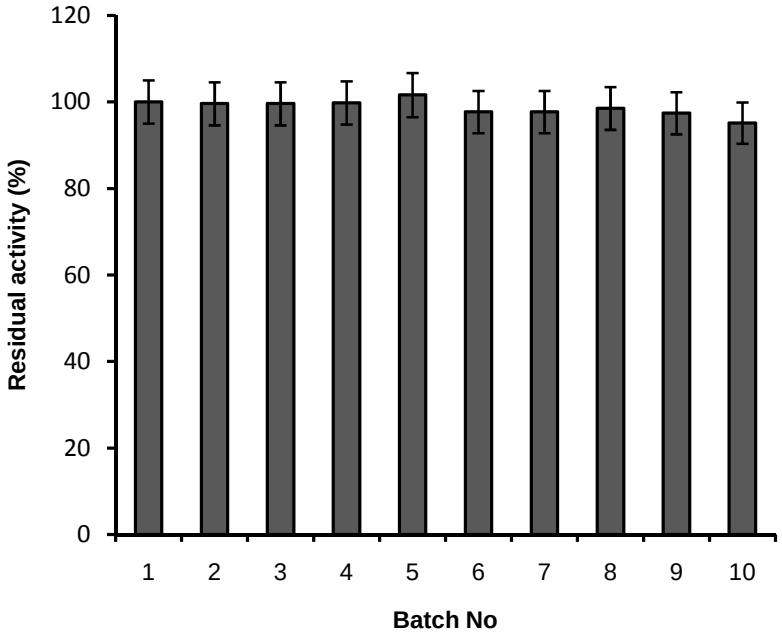

Fig. 9. Operational stability of the immobilized amylase

4. CONCLUSION

RSDA was immobilized onto micro bead silica gel 300A through adsorption. The process was optimized by alteration of variables such as pH, enzyme concentration, incubation temperature and time. Immobilized enzyme was more pH and temperature stable compared to the free enzyme. It was also quite stable under conditions of substrate hydrolysis and could be re-used repeatedly. The improved kinetic and stability features combined with the properties of the support (cheap, resistance to degradation, porosity) and the simple technique involved make this process appealing for industrial application in a wide range of biotechnological applications including food, detergent and bio-fuel production, medical and pharmaceutical applications.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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