African Journal of Biotechnology Vol. 10(71), pp. 15989-15997, 14 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2346
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Immobilization of raw starch digesting amylase on silica gel: A comparative study

Tochukwu N. Nwagu[1]*, Bartho N. Okolo[1] and Hideki Aoyagi[2]

[1]Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka.
[2]Life Sciences and Bioengineering, Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan.

Accepted 19 September, 2011

To stabilize the raw starch digesting amylase from fungus *Aspergillus carbonarius* (Bainier) Thom IMI 366159, the enzyme was immobilized on an inorganic porous support silica gel using different methods. Immobilization was carried out by spontaneous adsorption and crosslinking (reticulation), initial physical adsorption followed by crosslinking or conjugation on a silica gel activated with glutaraldehyde or polyglutaraldehyde. Concentration of glutaraldehyde, pH and duration of enzyme immobilization greatly influenced immobilization yield. A shift of optimum pH from pH 5 to 6 was observed for reticulated raw starch digesting amylase (RSDA), while other immobilized derivatives remained the same as the soluble enzyme. Immobilized enzyme exhibited increased activity at alkaline pH 8 to 9. Glutaraldehyde and polyglutaraldehyde activated RSDA showed lower activity at acidic pH 3.5 to 4 as compared to the crosslinked enzyme derivatives which had above 75% activity. The temperature optimum for the reticulated derivative was remarkably broadened from 30 to 60°C. All immobilized derivatives were more active and stable at higher temperatures to varying degrees. Soluble amylase lost 30% of its activity after 2 h incubation at 65°C, while 2.9% loss was recorded for reticulated derivative, 6.2% loss was recorded for physically adsorbed and crosslinked derivative, and 1.9 and 10% loss was recorded for polyglutaraldehyde and glutaraldehyde activated derivatives, respectively. Immobilization led to a slight decrease in K_m for all the derivatives. However, spontaneous adsorption and crosslinking (reticulation) of RSDA to silica gel with glutaraldehyde gave the best overall stability results.

Key words: Raw starch digesting amylase, immobilization, crosslinking, starch, stability.

INTRODUCTION

Starch, the most abundant staple in the world, is lost yearly especially in the developing countries of the world due to unavailable resources and technologies for proper storage or conversion to value added products (Dock et al., 2008; Omemu et al., 2005). Starch can be hydrolyzed to dextrins or simple sugars used as raw materials for a number of products. Some of the available starch hydrolyzing enzymes includes α-amylases, β-amylases and glucoamylases. Starch hydrolyzing enzymes and their products are utilized in a wide range of industrial processes including food, paper, textile, detergent, fuel, medical and bioanalytical processes (Saxena et al., 2007). However, most of these enzymes are incapable of degrading raw starch; as a result, gelatinization of starch is a major step in starch hydrolysis.

Raw starch digesting amylases (RSDAs) are starch hydrolyzing enzymes capable of catalyzing the hydrolysis of raw starch to dextrins or simple sugars. RSDA offers additional advantage of cost effectiveness coupled with conservation of time since gelatinization is not required prior to enzymic hydrolysis of starch. Gelatinization incurs extra cost of energy used for heating and cooling the starch before and after gelatinization (Goyal et al., 2005).

Although a number of reports exist on the production of RSDA from different bacteria and fungi (Abe et al., 1988; Burhan et al., 2003; Demirkan et al., 2005), the application of these enzymes in industrial processes remain limited (Liu and Ziu, 2008). According to a report by Robertson et al. (2005), RSDAs are unstable and are

*Corresponding author. E-mail: tochynwagu@yahoo.com. Tel: 2348036740060.

inhibited by their hydrolysis products (glucose and maltose). Also, they are unable to degrade a wide range of raw starches and their utilization could lead to product contamination due to the low temperature employed during processing.

Immobilized enzymes are widely used in biotechnological processes for substrate conversion to value added products (Cao et al., 2005), and they provide various advantages over free enzymes including increased catalytic, storage and operational stability. Immobilization provides a two-phase medium capable of minimizing competitive or non-competitive enzyme inhibition by products of enzymic hydrolysis (Pessela et al., 2003). Biocatalyst immobilization also affords easy product separation and convenient handling (Cao et al., 2005). Important factors influencing enzyme immobilization include properties of carrier, enzyme surface charges and immobilization conditions (Bryjak et al., 2007). Enzyme immobilization on porous supports may prevent enzyme proteolysis and aggregation during processing with a resultant promotion of enzyme stability (Mateo et al., 2000). Inorganic supports, such as silica gel, are thermally and mechanically stable, non toxic and highly resistant to microbial attacks (Reshmi et al., 2007). Amylases contain a number of reactive lysine groups on their surfaces which are capable of reacting with the aldehyde groups from glutaraldehyde for enzyme rigidification (Silva et al., 2004). Glutaraldehyde is a bifunctional agent which could also serve as a spacer arm in enzyme immobilization. Although there are various methods of immobilization, the use of a spacer arm facilitates greater rigidification of the enzyme structure and enhances multipoint attachments (Mateo et al., 2007).

A number of works exist on the immobilization of amylase on silica gel and these are mostly based on adsorption, conjugation or entrapment in sol gel or in agents mixed with silica gel (Afsahi et al., 2007; Ajitha and Sugunan, 2009; Bellino et al., 2010; Clarence et al., 1979; Hon and Reilly, 1979; Kvesitadze and Dvali, 1982; Leng et al., 2003; Rajagopolan and Krishnan, 2008; Trevisan et al., 2000); however, much remains to be done towards the stabilization of RSDAs for their use in industrial application. Major factors considered during immobilization are the price of the final immobilized enzyme and the simplicity of the immobilization procedure (Adamczak and Krishna, 2004).

We report the immobilization of RSDA capable of degrading a wide range of cereal and tuber starches from Aspergillus carbonarius (Bainier) Thom IMI 366159, previously isolated by Okolo et al. (1995) on silica gel. The fungus was capable of producing large amounts of extracellular RSDA, which was relatively stable under extreme conditions of pH and temperatures with glucose and maltose as hydrolytic products (Okolo et al., 2001). RSDA was stabilized by immobilization through spontaneous adsorption and crosslinking (reticulation) on silica gel. Immobilization was also carried out on silica gel activated with glutaraldehyde or polyglutaraldehyde, or through adsorption on silica gel followed by crosslinking with glutaraldehyde, in order to determine the best suitable method for RSDA stabilization using silica gel. Effect of immobilization on the catalytic activity, storage and operational stability and kinetic parameters of the enzyme was evaluated.

MATERIALS AND METHODS

Raw potato starch was prepared in our laboratory according to the method outlined by Okolo et al. (2001). Dinitrosalicylic acid (DNS) was purchased from Lancaster, England. All other chemicals were of analytical grade and were purchased from Wako pure chemicals, Japan.

Enzyme isolation

The pre-inoculum culture was prepared by inoculating two loopfuls of profuse growth into 500 ml Erlenmeyer flasks, each containing 100 ml of sterile fermentation medium. The fermentation medium comprised 20 gL^{-1} raw corn starch, 2 g yeast extract, 10 g L^{-1} $(NH_4)_2HPO_4$, 1 g L^{-1} NaCl and 1 g L^{-1} $MgSO4.7H_2O$ in de-ionized water. Cultures were incubated at 30°C with rotary shaking at 100 rpm for 24 h. After 24 h, 10 ml of the culture was used to inoculate a 500 ml flask containing 100 ml of the aforementioned fermentation medium. The culture was cultivated for 96 h at 30°C, after which mycelial pellets were separated by filtration through sterile Whatman No 1 filter paper. The resultant cell-free filtrate was purified through affinity chromatography using raw corn starch. The partially purified RSDA was crystallized using 80% $(NH_4)_2SO_4$ as precipitant. The solution was stirred at 4°C for 4 h, while the excess $(NH_4)_2SO_4$ was removed by decantation, and the saturated solution was kept for 36 h at -21°C. The crystals formed were suspended in citrate-phosphate buffer pH 6.0 containing 0.1% starch solution at -21°C until they were used.

Preparation of soluble polyglutaraldehyde solution

Soluble polyglutaraldehyde was prepared by the method of Tanriseven and Ölcer (2008). Glutaraldehyde solution (20 ml 25% w/v, pH 10.5) was polymerized by the addition of 0.6 ml of 1 M NaOH solution at room temperature for 20 min. The reaction mixture was neutralized using an equal volume (0.6 ml) of the HCl solution.

Activation of carriers

Polyglutaraldehyde activation of carriers Prior to activation, silica gel was washed with acetate buffer and sucked dry. Polyglutaraldehyde activation of the silica gel was achieved by the reaction of 2 g of silica gel with 10 ml of soluble polyglutaraldehyde in 0.2 M citrate-phosphate buffer at pH 8 for 15 min at 25°C using a magnetic stirrer. The activated carrier was washed repeatedly with distilled water for 10 min and it reacted with glutaraldehyde (10 ml, 25% w/v) in acetate buffer (10 ml, 25 mM, pH 6) for 30 min at 30°C. The untreated glutaraldehyde was removed from the activated carrier by washing repeatedly with acetate buffer (Tanriseven and Ölcer, 2008).

Glutaraldehyde activation of carriers

Glutaraldehyde activation was carried out by treating the silica gel

with varying concentrations of glutaraldehyde (1.5 to 12%) for different durations (30 min to 6 h) at 30°C. Following the activation, carriers were thoroughly washed with distilled water and the acetate buffer was used to remove excess glutaraldehyde.

RSDA immobilization

Immobilization of activated silica gel

Activated silica support (1 g) was suspended in 5 ml of 0.2 M citrate-phosphate buffer pH 6.0 containing 0.01 g of enzyme with an initial activity of 360 U/ml and stirred gently for 24 h at 4°C. The gel was recovered by filtration and thoroughly washed with distilled water and 0.2 M phosphate buffer at pH 6.0. Immobilized enzyme derivative was reduced with sodium borohydride (0.5 mg/ml, 4°C, 20 min) as earlier reported (Rodrigues et al., 2008), whereas immobilized RSDA was stored at 4°C.

Henceforth, RSDA immobilized on glutaraldehyde-activated support will be referred to as glutaraldehyde silica-RSDA derivative (GSil-RSDA), while that immobilized on polyglutaraldehyde will be referred to as polyglutaraldehyde silica-RSDA derivative (PGSil-RSDA).

Immobilization of inactivated support

Prior to immobilization, silica gel was properly washed with distilled water, equilibrated with 0.2 M citrate-phosphate buffer, with pH 6.0, and sucked dry, unless otherwise stated.

Adsorption of RSDA followed by crosslinking with glutaraldehyde (SiRSDA-CROSS)

Equilibrated silica gel support (1 g) was suspended in 5 ml of 0.2 M citrate-phosphate buffer at pH 6 containing 10 mg of enzyme protein and stirred gently for 12 h at 25°C. The RSDA adsorbed into the silica gel was recovered by filtration. This was followed by crosslinking using varying concentrations of glutaraldehyde at 30°C for 60 min. Crosslinked RSDA on the gel was filtered out from the solution and was thoroughly washed with 0.2 M phosphate buffer (pH 6.0). However, immobilized RSDA was stored at 4°C.

Spontaneous adsorption and crosslinking of RSDA (Sil-RSDA-Ret)

To 1 g of equilibrated agarose support, 2.5 ml of 0.2 M citrate-phosphate buffer (pH 6.0) containing 10 mg of enzyme protein and 2.5 ml of glutaraldehyde was added. This was followed by incubation at 30°C for 2 h. Immobilized amylase was thoroughly washed with 0.2 M phosphate buffer and stored at 4°C.

Optimization of immobilization method

Glutaraldehyde activation of carriers and RSDA immobilization of enzyme protein was performed at varying levels (pH 5 to 7.5) at 25°C temperature for different durations (6 to 36 h), followed by assaying of enzyme activity to determine the optimal condition for immobilization on each carrier.

Enzyme assay

The RSDA 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 of the enzyme solution, and incubated at 40°C for 10 min in a bioshaker for homogeneity. 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 1 µmol of reducing sugar per minute under the assay conditions.

Estimation 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 bovine serum albumin (BSA) as the standard (Bradford, 1976) and/ or using the spectrophotometer at a wavelength of 280 nm.

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 pH 3.0 to 9.0 at a temperature of 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 pH 9.0 for 2 h at room temperature. Afterwards, the residual amylase activity was estimated as earlier described.

The effect of temperature on the activity of the immobilized enzymes was determined by incubation at temperatures ranging from 30 to 80°C for 20 min. Amylase activity was determined after incubation. Thermal stability of the enzyme preparations was determined by incubation in 0.2 M citrate-phosphate buffer of optimum pH at a temperature of 60°C for 120 min. After incubation, the enzyme preparation was cooled and the residual activity was determined. Kinetic parameters of the RSDA preparations (K_m and V_{max}) were calculated from Lineweaver-Burk plots.

The effects of metal ions, surfactants and inhibitors were examined by incubating immobilized RSDA preparations with these substances for 120 min at room temperature. Thereafter, the residual activity was determined. Operational stability test was carried out by repeated 10 batch experiments using the method for activity determinations. Storage stability was tested after 30 days of keeping the immobilized enzyme preparations at 4°C. Nonetheless, all experiments were done in duplicates.

RESULTS

Optimization of immobilization parameters

To optimize conditions for RSDA immobilization, the effect of the concentration and duration of glutaraldehyde activation or crosslinking was evaluated. The optimum concentration of glutaraldehyde for spontaneous adsorption and crosslinking (Sil-RSDA-Ret) was 1.5%, while for adsorption followed by crosslinking (Sil-RSDA-CROSS), it was 3%; however, the optimum concentration of glutaraldehyde needed for the activation of silica beads was 10% (Figure 1). A short period of 30 min was required for the preparation of Sil-RSDA-Ret, and 60 min was needed for optimum activation of silica gel with glutaraldehyde prior to enzyme immobilization (GSil-RSDA). For SilRSDA-CROSS preparation, crosslinking of the physically adsorbed enzyme with glutaraldehyde for

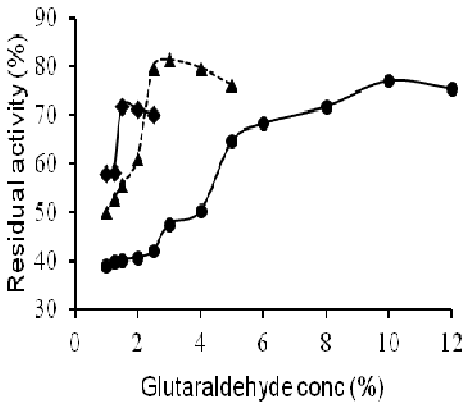

Figure 1. Effect of glutaraldehyde concentration on the activation of Sil-RSDA-Ret (◆), SilRSDA-CROSS (▲) and GSil-RSDA (●).

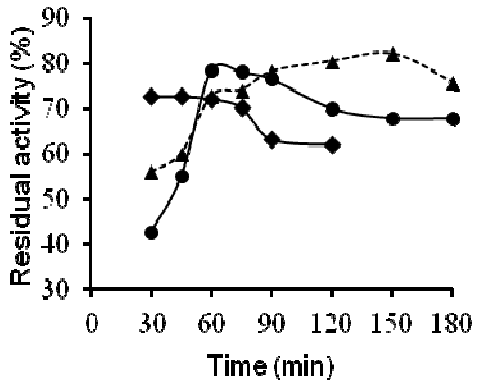

Figure 2. Influence of the duration of glutaraldehyde activation on Sil-RSDA-Ret (◆), SilRSDA-CROSS (▲) and GSil-RSDA (●).

150 min gave the highest enzyme residual activity (Figure 2) while 6 h incubation of RSDA with polyglutaraldehyde / glutaraldehyde activated silica gave the highest immobilization yield; 18 h incubation was necessary for the optimal enzyme adsorption on non-activated silica gel prior to crosslinking for preparation of SilRSDA-CROSS (Table 1). Nevertheless, immobilization on silica gel was optimal when carried out at the pH range of 6.0 to 7.5 as shown in Table 2.

Properties of the immobilized enzyme

Sil-RSDA-Ret was most insensitive to pH change and it retained 77% of its activity at pH 3.5; although the highest residual activity (93.5%) at pH 9 was also recorded for this derivative (Figure 3). The pH optimum shifted from pH 5 (of the soluble enzyme) to 6 for Sil-RSDA-Ret, but no change in pH optimum was observed for PGSil-RSDA, SilRSDA-CROSS and GSil-RSDA. The immobilized RSDA derivatives were more stable in pH than the soluble RSDA as shown in Figure 4. The highest pH stability results were observed for GSil-RSDA (83.5%) and Sil-RSDA-Ret (78.7%) after 2 h storage in a buffer of pH 3.5 at 10°C. However, the immobilized enzyme was more pH stable and it retained 94.8 (GSil-RSDA) and 90% (Sil-RSDA-Ret) activities as compared to 70% activity of the soluble RSDA.

Interestingly, while immobilization led to a positive shift of 10°C for GSil-RSDA and SilRSDA-CROSS, from the optimum of 30°C observed for the soluble RSDA (Figure 5), the optimum temperature remained unchanged for Sil-RSDA-Ret and PGSil-RSDA. However, the optimum temperature was remarkably widened for Sil-RSDA-Ret, with retention of 100% activity in the range of 30 to 60°C. Moreover, only 6.8% of its activity was lost after incubation at 30 to 80°C. Investigation of the thermostability of the immobilized and soluble RSDA showed that the immobilized enzyme was remarkably more thermostable than the soluble enzyme (Figure 6). The highest stability was recorded for Sil-RSDA-Ret and PGSil-RSDA (97.1 and 98.1%) respectively, with the residual activities after 2 h incubation at 60°C.

To evaluate the operational stability of the immobilized enzyme, starch hydrolysis by the immobilized RSDA was carried out in batches with the enzyme activity assayed, and the immobilized enzyme was washed prior to each re-run. Sil-RSDA-Ret retained 90.2% activity after 10 batches, PGSil-RSDA retained 96.0%, SilRSDA-CROSS retained 92% and GSil-RSDA retained 94.1% as shown in Figure 7. After storage for 30 days at 4°C, Sil-RSDA-Ret and GSil-RSDA retained 100% activity and PGSil-RSDA retained 106.3% activity, while SilRSDA-CROSS and soluble RSDA lost 10 and 30% activity, respectively as shown in Table 3.

Immobilization led to a decrease in the K_m of RSDA (Table 4). K_m of the soluble RSDA was 0.35 x 10^{-1} mg/ml and those of Sil-RSDA-Ret, PGSil-RSDA, SilRSDA-CROSS and GSil-RSDA were 0.28, 0.31, 0.35 and 0.21 x 10^{-1} mg/ml, respectively. The use of 0.5% Tween 80 and Triton X-100 stimulated the activity of the immobilized enzyme to varying degrees as shown in Table 5. Immobilized enzyme was also stable in the presence of metal ions and inhibitors; although 5 mM concentration of CaCl, SDS, EDTA and sodium deoxycholate had no effect on the activity of Sil-RSDA-Ret (Table 5).

DISCUSSION

According to Saudagar and Singhal (2004), optimization studies are necessary for proteins that are not widely studied yet. To optimize the immobilization procedure, a number of variables which might influence the interaction between enzyme and support were investigated using the trial and error method, reported as the dominant method

Table 1. Effect of incubation time on immobilization of raw starch digesting amylase on silica beads.

Bound RSDA	[a]Expressed activity (%)				
	6 h	12 h	18 h	24 h	48 h
PGSil-RSDA	86.2	85.1	85.5	75.7	69.2
SilRSDA-cross	60.9	77.9	83.6	75.7	71.2
GSil-RSDA	72.5	62.8	60.0	60.5	59.7

[a]Expressed activity (%) = Actual activity of the derivative/expected activity considering the immobilized enzyme. Load in all experiments was 360 U/ml protein.

Table 2. Influence of immobilization pH on raw starch digesting amylase immobilization on silica beads.

Bound RSDA	[a]Expressed activity (%)				
	pH 4.5	pH 5.0	pH 6.0	pH 7.0	pH 7.5
Sil-RSDA-Ret	51.7	52.6	68.1	59.4	55.3
PGSil-RSDA	70.1	70.6	74.2	80.4	78.1
SilRSDA-CROSS	68.3	70.7	83.0	65.4	62.4
GSil-RSDA	62.5	64.8	65.0	7.48	66.7

[a]Expressed activity (%) = Actual activity of the derivative/expected activity considering the immobilized enzyme. Load in all experiments was 360 U/ml protein.

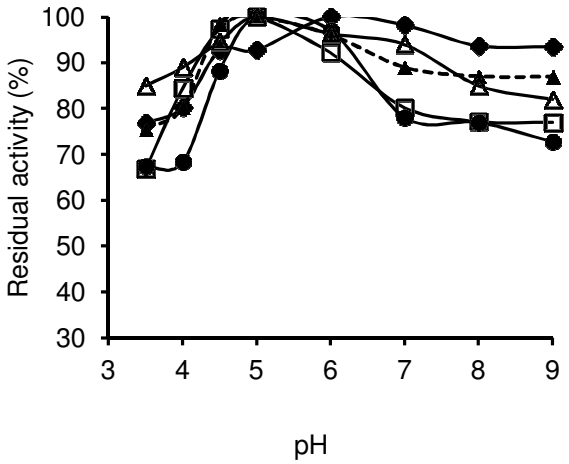

Figure 3. pH activity profile of Sil-RSDA-Ret (◆), PGSil-RSDA (□), SilRSDA-CROSS (▲), GSil-RSDA (●) and soluble RSDA (△). Activity assays were performed using RSDA immobilized gel incubated at 40°C at different pH with appropriate buffer solutions. The enzyme activity at optimum pH was considered as 100%.

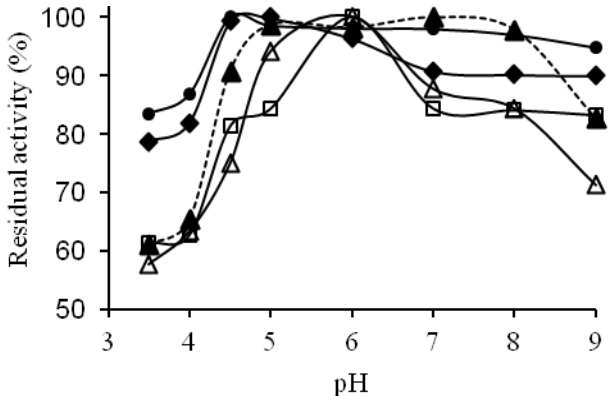

Figure 4. pH stability profile of Sil-RSDA-Ret (◆), PGSil-RSDA (□), SilRSDA-CROSS (▲), GSil-RSDA (●) and soluble RSDA (△). Activity assays were performed using RSDA immobilized gel prepared with buffer of varying pH and stored at 25°C for 120 min. Incubation was in a bioshaker for 20 min at 40°C. The enzyme activity at optimum pH was considered as 100%.

for selection of appropriate immobilization protocol (Bryjak et al., 2007). The concentration of glutaraldehyde, time and pH of enzyme incubation or carrier activation greatly influenced the immobilization efficiency. Sil-RSDA-Ret had the lowest activity after immobilization, though protein concentration was similar to that of Sil-RSDA-CROSS and GSil-RSDA. Immobilization yield was highest at a neutral range and this may be attributed to the effect of both the pH activity of the enzyme and the reactivity of glutaraldehyde. Glutaraldehyde was reported to be most reactive in neutral or basic conditions (Tanriseven and Olcer, 2008). Immobilization often times lead to a shift in pH either towards the acidic side (Kahraman et al., 2007) or towards the alkaline side (Li et

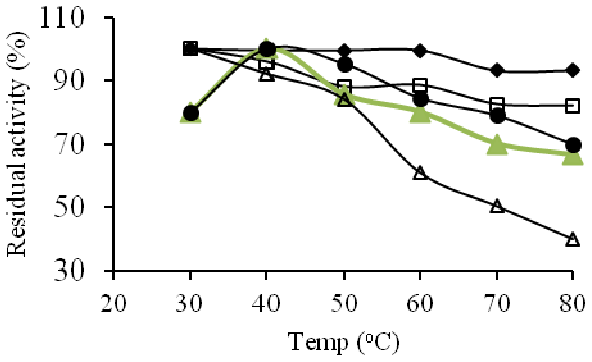

Figure 5. Temperature activity profile of Sil-RSDA-Ret (◆), PGSil-RSDA (□), SilRSDA-CROSS (▲), GSil-RSDA (●) and soluble RSDA (△). Activity assays were performed using RSDA immobilized agarose gel prepared with phosphate buffer of optimum pH, incubated in a bioshaker for 20 min at varying temperature. Optimum temperature was considered as 100%.

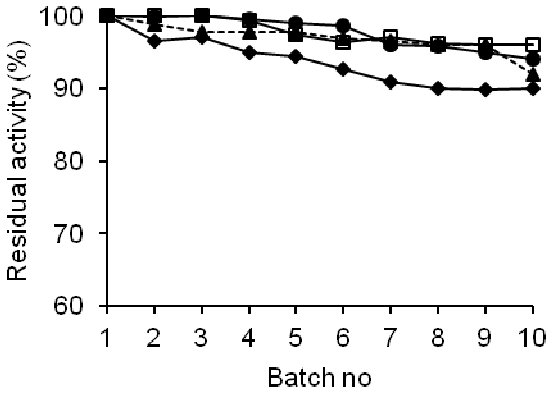

Figure 7. Repeated batch hydrolysis with Sil-RSDA-Ret (◆), PGSil-RSDA (□), SilRSDA-CROSS (▲) and GSil-RSDA (●).

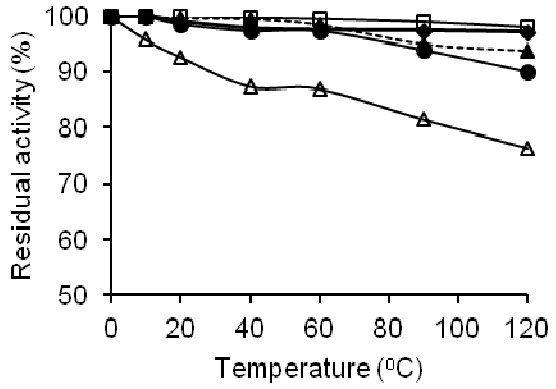

Figure 6. Thermoinactivation kinetics of Sil-RSDA-Ret (◆), PGSil-RSDA (□), SilRSDA-CROSS (▲), GSil-RSDA (●) and soluble RSDA (△) at 60°C.

al., 2007). A shift of one unit towards the basic side was observed for Sil-RSDA-Ret; although the pH of the other immobilized derivatives remained at pH 5, same as the soluble type. Similar alteration in pH was reported for RSDA immobilized on agarose using spontaneous crosslinking method with glutaraldehyde as a cross linker (Nwagu et al., 2011). Factors which influenced the pH of immobilized enzyme include surface and residual charges on solid matrix and the enzyme bound pH in the micro-environment as compared to the bulk environment. Strong interactions between enzyme and support affected intra-molecular forces responsible for main-taining the enzyme conformation leading to a resultant change in enzyme activity. Immobilized derivatives, GSil-RSDA and SilRSDA-Ret in particular, were remarkably more stable in pH than the free enzyme. Increased stability of immobilized RSDA over a wide range of pH (3.5 to 9.0) is an indication of greater insensitivity of the enzyme to changes in environmental pH, brought about by conformational changes following immobilization (El-Batal et al., 2005). The stability of immobilized enzyme at neutral pH is an added advantage as this will promote storage of RSDA for longer periods. Sil-RSDA-Ret was remarkably insensitive to temperature changes in the range of 30 to 80°C, and it retained over 95% of its activity after incubation at 80°C for 20 min. Some researchers reported a shift in optimum temperature by 10°C (Kilinc et al., 2006; Li et al., 2008; or 20°C (Kahraman et al., 2007), or no change (Tanriseven and Olcer, 2007) was reported at all following immobilization. It is remarkable that in this case the optimum temperature did not shift but was broadened by 30°C, showing increased temperature activation of the enzyme as a result of immobilization by spontaneous adsorption and crosslinking (reticulation) on silica gel. This indicates greater rigidity of the immobilized RSDA leading to an enzyme molecule more resistant to unfolding at higher temperatures than the free form. The stability of Sil-RSDA-Ret at low pH and its increased activity at high temperatures implies that the enzyme can be applied in starch hydrolysis at low pH and moderately in high temperatures of 60°C and above (below gela-tinization temperature) to minimize contamination during processing (Kaneko et al., 2005; Nwagu et al., 2011). Considering the high activity at very low pH, during processing, the RSDA can also be used in a mixed reaction with another saccharifying amylase to facilitate the release of reducing sugars. In such situation, the RSDA will serve as a liquefying as well as a saccharifying amylase. Enzyme re-use is one of the greatest

Table 3. Immobilization efficiency of RSDA on silica beads

Parameter	Amylase activity (U/ml)	Protein concentration (mg)	Immobilization[a] yield (%)	Expressed activity (%)
Sil-RSDA-Ret	276.3	8.8	ND[c]	74.5
PGSil-RSDA	329.6	9.6	98.3	89.4
SilRSDA-CROSS	300.3	8.3	85.1	74.8
GSil-RSDA	287.5	8.6	90.0	80.0
Soluble	360.0	10.0	ND[c]	ND[c]

[a]Immobilization yield (%) = Activity of control suspension – Activity of the supernatant of the immobilization suspension/Activity of the control suspension; [b]Expressed activity (%) = Actual activity of the derivative/Expected activity considering the immobilized enzyme. Load in all experiments was 360 U/ml protein.

Table 4. Kinetic parameters of free and immobilized RSDA on silica beads.

RSDA	Km (10^{-1}) (mg ml^{-1})	Vmax (U mg^{-1})
Sil-RSDA-Ret	2.79	28.40
PGSil-RSDA	3.13	20.01
SilRSDA-CROSS	3.48	26.10
GSil-RSDA	2.13	37.91
Soluble enzyme	3.54	23.82

advantages of immobilization due to the high cost of biocatalysts. The immobilized enzyme derivatives are operationally stable as shown in the experiments and over 90% of the residual activity is maintained after 10 batch runs, in all cases. Sil-RSDA-Ret had the lowest residual activity (90.1%) as compared to the other derivatives. The reason for this is not very clear; however, it is known that during spontaneous adsorption and crosslinking of enzyme on support, the enzyme molecules which are not bound to support could aggregate (form crystals). Enzyme washing and re-use could lead to the loss of these enzyme aggregates. It was observed that there was a steady loss of activity as the enzyme was re-used; however, after the 7th cycle, the enzyme activity was stabilized. Immobilization also led to an increase in the storage stability of the RSDA, where α-amylase immobilized on silanized silica particles lost its activity at the rate of 10% every 12 days of storage (Leng et al., 2003). Kahraman et al. (2007) reported that immobilized amylase on functionalized glass beads had 80% residual activity after 25 days of storage. In this study, all derivatives with the exception of SilRSDA-CROSS retained 100% of their activity after storage at 30 days. Immobilization is often times accompanied by alteration of the kinetic parameters of the enzyme. A decrease in K_m was observed for all immobilized derivatives obtained in this study. Reduction of K_m signifies similar or better affinity of the immobilized RSDA toward starch and is an indication of reduced diffusional resistance to mass transfer due to lack of steric hindrance (Sanjay and Sugunan, 2005). The activation of immobilized enzyme with surfactants Tween 80 and Triton X-100 is an added advantage towards the utilization of the immobilized enzyme. The stimulation of the enzyme activity as a result of addition of surfactants or additives has previously been reported (Yoon and Robyt, 2005). The stability of the immobilized enzyme in the presence of inhibitors, anionic surfactant and metal ions is also worthy of note. Some of these agents, for example, the anionic surfactant sodium dodecyl sulphate (SDS), could lead to the unfolding of the enzyme structure (Svensson et al., 1996). From the results in the foregoing, Sil-RSDA-Ret was the most stable, in terms of catalytic, operational and storage stability. This may be attributed to the rapid enzyme adsorption and cross-linking favored by the high porosity of silica gel enabling permeation of the enzyme molecules into the pores of the matrix. Glutaraldehyde also served as a spacer arm preventing intense rigidification and unfavorable conformational changes in the enzyme structure.

Conclusions

RSDA was immobilized on silica gel using different methods. Although the various RSDA derivatives obtained were more stable than the soluble form to varying degrees, Sil-RSDA-Ret was the most stable. Sil-RSDA-Ret preparation involved a very simple procedure as compared to all the other methods. The concentration of glutaraldehyde used and the duration of crosslinking probably helped to achieve enzyme rigidification without

Table 5. Effect of metal ions, surfactants and inhibitors on activity of immobilized RSDA on silica beads.

Agent	Concentration	Residual activity (%)			
		RA[a]	PGA[b]	CXA[c]	GA[d]
Calcium chloride	5 mM	100.0	87.5	100.0	100.0
SDS	5 mM	100.6	85.5	93.3	75.7
EDTA	5 mM	100.0	94.2	75.8	102.8
Cholic acid	5 mM	95.2	82.9	97.8	98.7
Strontium chloride	5 mM	91.7	86.9	80.1	86.3
Sodium deoxycholate	5 mM	100.0	93.3	80.0	93.2
Zinc chloride	5 mM	87.7	95.2	100.3	100.0
Tween 80	0.5%	180.4	161.8	120.8	169.8
Triton x-100	0.5%	139.0	123.1	108.2	149.2

[a]RA= Sil-RSDA-Ret; [b]PGA= PGSil-RSDA; [c]CXA= SilRSDA-CROSS; [d]GA= GSil-RSDA.

the distortion of the enzyme active sites as evident in the reduced Michaelis Menten constant. A high immobilization yield of 74.5% was recorded for the Sil-RSDA-derivative. Sil-RSDA-Ret maintained optimum activity even when temperatures were increased from 30 to 60°C and at a very low pH of 3.5. Under these conditions, starch hydrolysis can progress without risk of contamination. Immobilization is also known to reduce product inhibition. Nonetheless, application of this enzyme will be more useful and cost effective in starch hydrolysis to reducing sugars or in the numerous industrial processes which require starch hydrolyzing enzyme.

REFERENCES

Abe J, Bergmann FW, Obata K, Hizukuri S (1988). Production of the raw-starch digesting amylase of *Aspergillus* sp K-27. Appl. Microbiol. Biotechnol. 27: 447-450.

Adamczak M, Krishna SH (2004). Enzyme for Efficient Biocatalysis. Food Technol. Biotechnol. 42(4): 251–264.

Afsahi B, Kazemi A, Kheirolomoom A, Nejati S (2007). Immobilization of Cellulase on Non-Porous Ultrane Silica Particles. Scientia Iranica. 14(4): 379-383.

Ajitha S, Sugunan S (2009). Tuning mesoporous molecular sieve SBA-15 for the immobilization of α-amylase. J. Porous Materials. 17(3): 341-349.

Bellino MG, Regazzoni AE, Soler-Illia G (2010). Amylase-Functionalized Mesoporous Silica Thin Films as Robust Biocatalyst Platforms. Applied Materials Interfaces. 2(2): 360–365.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, Osman G (2003). Enzymatic properties of a novel thermophilic, alkaline and chelator resistant amylase from an alkalophilic *Bacillus* sp. Isolate ANT-6. Process Biochem. 38:1397–1403.

Cao L (2005). Immobilized enzymes: science or art? Curr. Opin. Chem. Biol. 9: 217-226.

Demirkan A, Sarikaya E, Mikami B, Adachi M, Higasa T, Utsumi S (2005). Amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli*. Process Biochem. 40: 2629-2636.

Dock C, Matthias H, Antranikian G (2008). A thermoactive glucoamylase with biotechnological relevance from the thermoacidophilic euryarchaeon *Thermoplasma acidophilum*. Appld Microbiol. Biotechnol. 78: 105-114.

D' Souza SF, Kubal BS (2002). A cloth strip bioreactor with immobilized glucoamylase. J. Biochem. Biophys. Methods. 51: 151-159.

El-Batal AI, Atia KS, Eid M (2005). Stabilization of α-amylase by using anionic surfactant during the immobilization process. Rad. Phys. Chem. 74: 96-101.

Goyal N, Gupta JK, Soni SK (2005). A novel raw starch digesting thermostable α-amylase from *Bacillus sp*. I-3 and its use in the direct hydrolysis of raw potato starch. Enzyme Microbial. Technol. 37: 723–734.

Hon CC, Reilly P (1979). Properties of β-amylase immobilized to alkyl amine porous silica. Biotechnol. Bioeng. 21(3): 505–512.

Kahraman MV, Bayramoğlu G, Kayaman-Apohan N, Güngör A (2007). UV-Curable methacrylated/fumaric acid modified epoxy as a potential support for enzyme immobilization. Reactive Functional Polymers. 67: 1385-1392.

Kaneko T, Ohno T, Ohisa N (2005). Purification and characterization of a thermostable raw starch digesting amylase from a *Streptomyces* sp. isolated in a milling factory. Biosci. Biotechnol. Biochem. 69(6): 1073-1081.

Kilinc A, Teke M, Onal S, Telefoncu A (2006). Immobilization of pancreatic lipase on chitin and chitosan. Prep. Biochem. Biotechnol. 36(2): 153-163.

Kvesitadze GI, Dvali MS (1982). Immobilization of mold and bacterial amylases on silica carriers. Biotechnol. Bioeng. 24(8): 1765–1772.

Leng HL, Douglas GM, Gordon AH (2003). Hydrolysis of starch particles using immobilized barley α-amylase. Biochem. Eng. J. 13: 53-62.

Li T, Li S, Wang N, Tain L (2008). Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. Food Chem. 109: 703-708.

Li TP, Wang N, Zhao QC, Guo M, Zhang CY (2007). Optimization of covalent immobilization of pectinases on sodium alginate support. Biotechnol. Lett. 29(9): 1413-1416.

Liu XD, Xu Y (2007). A novel raw starch digesting α-amylase from a newly isolated Bacillus sp. YX-1: Purification and characterization. Bioresour. Technol. 99: 4314-4320.

Mateo C, Abian O, Fernanadez-Lafuente R, Guisan JM (2000). Reversible enzyme immobilization via very strong and non distorting ionic adsorption on support-polyethyleneimine composites. Biotechnol. Bioeng. 8(1): 88-105.

Mateo C, Palomo Jose M, Fernández-Lorente G, Fernandez-Lafuente R, Guisán JM (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microbial. Technol. 40: 1451-1463.

Miller GL (1959). Use of dinitro-salicylic acid reagent for determination of reducing sugars. Anal. Chem. 31: 426 –428.

Nwagu TN, Okolo BN, Aoyagi H (2011). Stabilization of a raw starch digesting amylase from *Aspergillus carbonarius* via immobilization on activated and non-activated agarose gel. World J. Microbiol. Biotechnol. DOI 10.1007/s1 1274-011-0824-1.

Okolo BN, Ezeogu LI, Ebisike CO (1996). Raw starch digesting amylase

from *Thermoactinomyces -thalpophilus* F13. World J. Microbiol. Biotechnol. 12: 637-638.
Okolo BN, Ire FS, Ezeogu LI, Anyanwu CU, Odibo FJC (2001). Purification and some properties of a novel raw starch-digesting amylase from *Aspergillus carbonarius*. J. Sci. Food. Agric. 81(3): 329–336.
Omemu AM, Akpan I, Bankole MO, Teniola OD (2005). Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. Afr. J. Biotechnol. 4: 19-25.
Pessela B, Mateo C, Fuentes M, Vian A, Garcia JL, Carrascosa AV, Guisan JM, Lafuente RF (2003). The immobilization of a thermophilic β-galactosidase on sepabeads supports decreases product inhibition complete hydrolysis of lactose in dairy products. Enzyme Microbiol. Technol. 33: 199–205.
Rajagopolan G, Krishnan C (2008). Immobilization of malto-oligosaccharide forming α-amylase from *Bacillus subtilis* KCC103: properties and application in starch hydrolysis. J. Chem. Technol. Biotechnol. 83: 1511-1517.
Reshmi R, Sanjay G, Sugunan S (2007). Immobilization of α-amylase on zirconia: a heterogeneous biocatalyst for starch hydrolysis. Catal. Commun. 8: 393–399.
Robertson G, Wang D, Lee C, Wasschal K, Smith M, Orts W (2005). Native or raw starch digestion: a key step in energy efficient biorefining of grain. J. Agric. Food Chem. 54: 353-365.
Rodrigues DS, Adriano W, Mendesa A, Adriano WS, Goncalves L, Giordano RL (2008). Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. J. Mol Catal. B: Enzymatic. 51: 100–109.
Sanjay G, Sugunan S (2005). Glucoamylase immobilized on montmorillonite: Synthesis, characterization and starch hydrolysis activity in fixed bed reactor. Catal. Commun. 6: 525-530.
Saxena RK, Dutt K, Agarwal L, Nayyar P (2007). A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5 Bioresour. Technol. 98(2): 260-265.
Silva CJM, Sousa F, Gübitz G, Cavaco-Paulo A (2004). Crosslinking of proteins with glutaraldehyde, Food Technol. Biotechnol. 42(1): 51–56.
Saudagar PS, Singhal RS (2004). Curdlan as a support matrix for immobilization of enzyme. Carbohydrate Polymers. 56: 483-488.
Svensson E, Gudmundsson M, Eliasson A (1996). Binding of sodium dodecylsulphate to starch polysaccharides quantified by surface tension measurements. Colloids and Surfaces B: Biointerfaces, 6: 227-233.
Trevisan, HC, Mei LHI, Zanin GM (2000). Preparation of silica of controlled pore size for enzyme immobilization. Braz. J. Chem. Eng. 17(1): 71.
Yoon SH, Robyt JF (2005). Activation and stabilization of 10 starch – degrading enzymes by Triton X-100, polyethylene glycols and polyvinyl alcohols. Enzyme Microbiol. Technol. 37: 556-562.