Original Research Article

Impact of metal ion substitution on the activity and stability of saccharifying raw starch digesting amylase from *Aspergillus carbonarius*

Running Title: Effect of Metal ions substitution on enzyme activity and stability

ABSTRACT

Though raw starch digesting amylases can be utilized in numerous bioprocesses, poor activity and stability remain a limiting factor. In this study, the effect of metal ion substitution on the activity and stability of the RSDA from *Aspergillus carbonarius* was investigated. The amylase was inactivated using the chelating agent ethylene di aminotetraacetic acid (EDTA) and reactivated using different metal ions. The effect of different metal ions on the reactivation of the amylase activity was investigated. Impact of the metal ions on the stability of the amylase was also studied. Kinetic constants of the native enzyme were compared to the metal reactivated holoenzyme. Most efficient was 5 mM concentration of Co^{2+} with 94.6% activity recovery. Others included 5 mM Zn^{2+} (77.7%) and 5 mM Ca^{2+} (68.7%). Incubating the Co^{2+} activated amylase in 10 mM Mn^{2+} further stimulated the activity of the amylase to 136.7%. Compared to the metal ions tested, Mn^{2+} had the most stabilizing effect on the amylase; the amylase exhibited 148.2% and 136.5% activity at 70 ^{o}C and 80 ^{o}C respectively in the presence of 5 mM Mn^{2+}. Ca^{2+} inhibited the amylase activity and inhibition rate increased with increasing concentration of Ca^{2+} concentrations. K_m of the reactivated amylase was 0.18 mg/ml.

Keywords: metal ion substitution; raw starch hydrolysis; activity; stability; amylases; re-activation

1. Introduction

Amylases are widely used in the food industries for starch liquefaction or saccharification to sugar syrups used in the production of sweets, baked goods, ice cream, tomato ketchup etc. They can also be used in the hydrolysis of starch for production of alcoholic and non-alcoholic beverages such as beer, wine and fruit juices. Amylases are also used as food and feed additives to accelerate the rate of digestion of starchy foods in animal and human diet [1]. The scope of application of these hydrolases continues to expand with advances in the field of biotechnology. The raw starch digesting type of amylase affords greater possibilities of time, energy and cost savings by cutting off gelatinization and liquefaction; two major steps in starch hydrolysis. However, poor activity and stability remains the limiting factor in the application of raw starch digesting amylases [2].

In most enzymes metals are very vital as they are not only found in the active sites of enzymes but play crucial roles in maintaining its catalytic structure [3]. Around forty percent of all enzymes are metalloenzymes and some of these need multiple metal ions to function; these can either be the same metal or different metals [4]. Amylases belong to the group of metalloenzymes and therefore, require metals for proper functioning [5, 6]. Metals aid in allosteric activation [7] and maintain structural integrity of the protein by binding domains A and B to form the substrate binding site [8]. The catalytic cleft in amylases is formed through the bridging of the $(\beta/\alpha)_8$ barrel of the domain A and the β-pleated sheets of domain B on the NH_2 terminus of the enzyme molecule by metal ions [9]. This constitutes the primary metal binding site or in case of calcium, the primary calcium binding site.

Metal ions play a major role in enzyme stabilization by promoting extra energetically favorable interactions within the enzyme structure [10, 11]. D'Amico *et al*. [12] reported a large conformational change in the α-amylase from *Pseudoalteromonas haloplanktis* following binding of chloride in the active site. According to the authors, the enzyme conformation shifted towards a more compact and organized structure by a process analogous to induced fit mechanism.

Metal ions have different specificities and any alteration results in the change of enzyme activity, kinetic stability, thermodynamic stability [8,13] or even region and stereo specificity of an enzyme molecule [14, 15]. This is owing to the fact that metal ions form co-ordinate covalent bonds with specific residues (ligands) on the enzyme molecule which alter the enzyme conformations often in a subtle form [16].

Amylases have been stabilized using protein engineering [17], immobilization [2] and chemical modification [18]. Reports exist on amylase activation and stabilization when incubated with different metal ions [19,20]. It is therefore apparent that metal ion substitution can be used as a simple method of amylase activation and stabilization.

The raw starch digesting amylase (RSDA) from *Aspergillus carbonarius* can degrade a range of starches including cereal and tuber starches. This work was targeted towards the activation and stabilization of this amylase through metal ions substitution.

2 MATERIALS AND METHOD

2.1 Cultivation of Microorganism and Crude Enzyme Preparation

RSDA was obtained from culture filtrate of *Aspergillus carbonarius* (*Bainier*) *Thom* IMI 366159 grown in submerged culture [2]. The pre-inoculum culture was prepared by inoculating two wire loops of profuse growth into 500 ml Erlenmeyer flasks each containing 100 ml of sterile fermentation medium. The fermentation medium comprised of (g/l) 20 raw corn starch, 2 yeast extract, 10 $(NH_4)_2HPO_4$, 1 NaCl and 1 $MgSO_4.7H_2O$ in de-ionized water. Cultures were incubated at 30 ^oC 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 fermentation medium. The culture was cultivated for 96 h at 30 ^oC, after which mycelial pellets were separated by filtration through Whatman No 1 filter paper. The resultant cell-free filtrate was used as crude amylase and stored at 4 ^oC.

2.2 Purification of Raw Starch Digesting Amylase

2.2.1 Affinity Chromatography

This was done according to a slight modification of the method of Najafi & Kembhavi [21]. The crude enzyme filtrate was incubated on ice for 45 min with continuous shaking to achieve a homogenous temperature. The raw corn starch was washed with 0.2 M citrate-phosphate buffer pH 6 for 10 min at room temperature to remove all soluble starch. The starch pellet was kept on ice for 20 min and then mixed with the chilled supernatant. The suspension was incubated on ice and swirled slowly for about 2 h at 4 ^{o}C. After, the suspension was centrifuged at 4 ^{o}C for 15000 x *g* for 20 min. This was followed by washing of the pellet with chilled NaCl (0.5 M) for 5 min on ice. The suspension was again centrifuged at 15000 x *g* for 20 min. Citrate-phosphate buffer pH 6 was added to starch pellet and incubated at 30 ^{o}C for 20 min. After incubation, the suspension was centrifuged and supernatant checked for enzyme activity and protein concentration.

2.2.2 Ammonium Sulphate Concentration of the RSDA

The crystalline ammonium sulphate was slowly added over a period of 1 h while stirred with magnetic stirrer to a total of 80% concentration. After continuous stirring for another 2 h the supernatant was decanted and kept at -21 ^{o}C for 36 h for crystallization to occur [2]. Approximately 0.05 g of the enzyme crystals was dissolved in 5 ml 0.2 M citrate-phosphate buffer pH 6.0 and incubated with 1% starch solution to confirm amylase activity, initial activity was 360 U/ml. Amylase crystals were recovered from the solution and stored in minimum concentration of 0.1% starch solution at -21 ^{o}C till further use.

2.3 RSDA 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 enzyme solution, incubated at 40 ^{o}C for 10 min in a bio-shaker for homogeneity. Reducing sugars released after incubation were estimated by the DNS method of Miller [22].

One unit of amylase was defined as the amount of enzyme, which liberated 1 **µ**mol of reducing sugar per minute (glucose) under the assay conditions.

2.4 Determination of the Metal Requirement for RSDA Activity and Stability

The enzyme solution prepared in buffer was thoroughly dialyzed (3x) against 100 vol of 50 mM MES buffer (pH 5) containing 5 mM EDTA for 24 h and then excessively dialyzed against 50 mM MES, buffer pH 6 to remove excess EDTA. The enzyme was used to study the metal ion requirement for the activity of the enzyme.

About 1 ml of 5 mM or 10 mM each of various metal ions was added to 0.2 ml of RSDA solution and incubated with 0.2 ml 1% raw starch solution for 20 min at 40 ^{o}C. After RSDA activity was determined.

To determine the metal ion responsible for enzyme stabilization, the RSDA was stored in 5 mM or 10 mM solution each of various metal ions for 2 h. After, the mixture was incubated with 1% raw starch solution for 20 min followed by the determination of the residual RSDA through DNS method.

3 RESULTS AND DISCUSSION

3.1 Reactivation of RSDA with different metal cations

Similar to other bio-catalysts, amylases require co-factors such as metal ions for their proper functioning. Metal ions assist in binding and holding the substrate in the active site, and in maintaining the tertiary structure of the enzyme molecule [23]. Ethylene di-aminotetraacetic acid (EDTA), a chelating agent was used to inactivate the RSDA by chelating its metal ion component. Treatment of the RSDA with 5 mM EDTA for 24 h led to the loss of over 95% of its activity, this corresponded with previous report by Okolo *et al*. [24]. However, the inactivation was reversible as further incubation in solutions containing various cations led to the recovery of its activity. Figure 1 shows the reactivation of RSDA using various metal ions. Most effective for the reactivation of the RSDA was 5 mM concentration of Co^{2+} ions which reactivated 94.6% of the initial activity of the RSDA, this was followed by 5 mM Zn^{2+} ions (77.7%), 10 mM Mn^{2+} ions (77.7%) and 5 mM Ca^{2+} ions (68.7%).

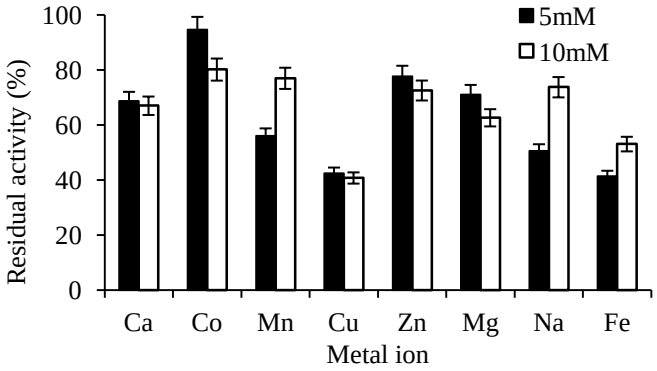

Figure 1. Metal ion reactivation of the apoenzyme. *Activity of native RSDA was 360 U/ml, this was considered as 100% activity.*

The metal ion involved in catalysis is located at the active site of the enzyme and are involved in co-ordinate covalent bonds with the free amino and carboxyl groups of the enzyme to produce the active catalyst [13]. Treating the cobalt activated RSDA with 5-10 mM Mn^{2+} further activated the RSDA as seen in Figure 2. A slight stimulation (1-5%) was observed when the Co^{2+} ion activated RSDA was treated with 5-10 mM Fe^{2+} ions while Ca^{2+}, Na^{+}, Zn^{2+}, Mg^{2+} and Ca^{2+} slightly inactivated its activity. Inactivation of metalloenzymes is assumed to take place in two stages: the reversible stage and the irreversible stage. The first stage which is reversible involves the reaction between the active enzyme and the metal ion (loss of metal ion), addition of the metal ion restores the activity of the enzyme [25]. In the present work, Co^{2+} had the most re-activating effect on the EDTA denatured RSDA. Co^{2+} ions have also been reported to optimally activate the α-amylase from *Moringa oleifera* seeds [26]. Saboury [6] reported that *B. amyloliquefaciens* α-amylase had a set of 25 non-cooperative sites for cobalt binding which led to increase in the enzyme activity, though thermal stability decreased.

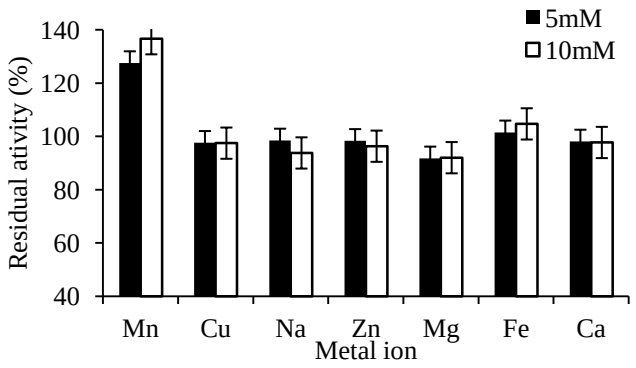

Figure 2. Further activation of the holoenzyme using various metal ions. *Activity of native RSDA was taken as 100%*

From the current work, it was observed that other ions including Ca^{2+}, Fe^{2+} and Mg^{2+} ions (not in that order) partly reactivated the RSDA. Though 5 mM Co^{2+} ions activated the amylase activity of *Bacillus* sp, Ca^{2+} ions had a higher activating effect and almost doubled the amylase activity at 10 mM concentration [27]. Report of enzyme competitive inhibition by cobalt and its activation by calcium is also available [28]. In terms of metal ions on amylase activity, alpha amylases are more widely studied and are reported to be calcium metalloenzymes. However, amylases which are not calcium metalloenzymes have been reported [5]. Alabi et al. [29] reported that Cu^{2+} and Hg^{2+} ions inhibited the activity of an amylase from *Bacillus subtilis* isolated from cassava processing site. Previous report on the amylase under study showed that it was not stimulated by Ca^{2+} ions [Okolo]. However, the RSDA was not treated with EDTA prior to incubation with metal ions, which implies that the primary metal ion binding sites would already be occupied by Ca^{2+} ions. This was further confirmed by the observation that activity loss by RSDA during immobilization on polyglutaraldehyde activated and glutaraldehyde activated chitosan beads could be restored using calcium ions [30]. Enzyme loss was thought to be a result of displacement of calcium ions from their primary binding site as a result of conformational changes during immobilization; hence activity could be restored by re-introduction of calcium ions into the amylase structure. From the Figure 2, it can be seen that though Co^{2+} ions had the most re-activating effect, it did not restore the total initial activity of the RSDA. This implies that other metal ions may be embedded in the holoenzyme. According to Nonaka *et al*. [5] metal ions at the active site of an enzyme could be up to three or even more. These metals could be bridged by a residue which is specific to the enzyme [15]. Yin and co-authors [31] observed that Zn^{2+} and Ca^{2+} binding sites in alpha amylase of Flavobacteriaceae function cooperatively to ensure protein stability.

A particular metal ion binds to different types of ligands depending on the enzyme molecule or the cultural conditions utilized for its production. Further treatment with Mn^{2+} was stimulatory to the Co^{2+} reactivated RSDA. Similarly, though proline dipeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus* required Co^{2+} for its catalytic activity, the Co^{2+}could be replaced by Mn^{2+} but not by Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} or Ni^{2+} [32]. Babu & Satyanarayana [33] also reported that while calcium inhibited the activity of the α-amylase from *Bacillus coagulans* B49, Mn^{2+} slightly stimulated the amylase activity. Mn^{2+} stimulated the amylase activity of *Moringa oleifera* seeds [26] and α-amylase from pericarp of *Borassus indica* [34]. Prakash *et al*. [35] also reported that the initial activity of soybean amylase was doubled due to activation by Co^{2+} and Mg^{2+}; rate of activation was dependent on the concentration of the metal ion. The ability of metals to bind with many ligands at a time helps to bring remote parts of the enzyme amino acid sequence together and also to establish an active conformation of the enzyme. According to

available literature, the favorable binding of the appropriate metal ion on the active site of an enzyme shift the enzyme conformation towards a more compact and organized structure by a process similar to induced fit mechanism. For example, large conformational changes were observed following the binding of the chloride ion to the active site of α-amylase from *Pseudoalteromonas haloplanktis* [12]. The incubation of xylanhydrolase produced by *Geobacillus stearothermophilus* KIBGE-IB29 in 1.0 mM concentration of $Ca^{2+}$and Mg^{2+} ions each, enhanced its catalytic activity up to 171% and 242%, respectively [36]

3.2 Stability of the Reactivated RSDA

The re-activated RSDA was stored in different metal ions at 10 ^{o}C and higher temperatures (70 ^{o}C and 80 ^{o}C) to determine the stability of the enzyme. Figure 3 shows that incubation in Mn^{2+} had a stabilizing effect on the RSDA irrespective of the temperature; the RSDA incubated at 10 ^{o}C in the presence of 10 mM Mn^{2+} retained 126.4% activity after 6 h incubation.

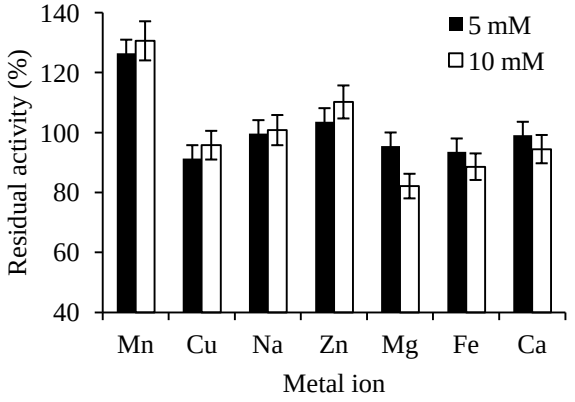

Figure 3. Effect of metal ion on the stability of the cobalt ion activated RSDA when kept at 10 ^{o}C for 6 h

In the presence of Zn^{2+} ion the residual activity was 110.2% while a slight loss of activity was observed due to exposure to other metal ions. At 70 ^{o}C, residual activity was 148.2% in the presence of Mn^{2+} ions, 128% in the presence of Fe^{2+} and approximately 100% in the presence of Cu^{2+}, Na^{+} and Zn^{2+} ions; a slight activity was lost in the presence of Ca^{2+} and Mg^{2+} ions (Figure 4).

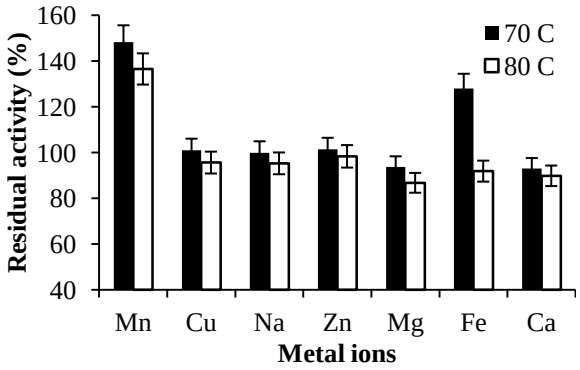

Figure 4. Effect of metal ions on high temperature (70 ^{o}C and 80 ^{o}C) stability of the Co^{2+} activated RSDA

At 80 ^{o}C, relative activity for RSDA incubated in the presence of Mn ion was 136.5%, in the presence of Zn^{2+} it was 98.1% and approximately 95.0% in the presence of Cu^{2+} and Na^{+} ions. Figure 5 indicates that the holoenzyme was slightly sensitive to calcium, and gradually lost activity with increasing concentration of Ca^{2+}. At higher concentrations of 7.5-10 mM Ca^{2+} sensitivity decreased and the apoenzyme maintained approximately 93% activity after incubation. Metal ions subtly control the reactivities of the enzyme, therefore the substitution may alter the enzyme activity, stability and even stereo-specificity [14,15]. From our RSDA stability studies, it is evident that Mn^{2+} ions remarkably stabilized the RSDA, both at low temperature (10 ^{o}C) and at high temperatures (70 ^{o}C and 80 ^{o}C).

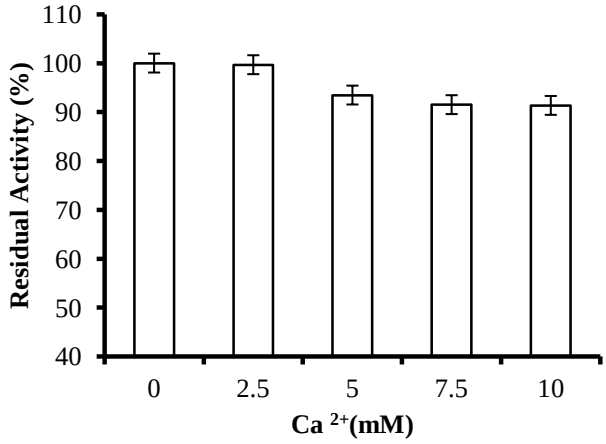

Figure 5. Effect of varying Ca^{2+} concentration on the stability of the Co^{2+} reactivated RSDA

The extra stability of *B. amyloliquefaciens* and *B. licheniformis* heat stable α-amylases was attributed to the occurrence of a Ca^{2+}-Na^{+}-Ca^{2+} metal triad in the main Ca^{2+} binding site, bridging domains A and B of these enzymes [8,37]. The Ca^{2+} ion present in an amylase molecule is co-ordinated by ligands belonging to the two domains of the catalytic site; an $(\alpha/\beta)_8$ barrel and a large loop. These ligands are essential for the enzymes catalytic activity and thermostability [38]. In structural metal sites, the metal ion mainly stabilizes the tertiary structure of the enzyme in a manner analogous to disulphide bonds [10] which may be achieved by replacing the lack of disulphide bonds in the protein-ligand complex [39]. Apart from co-operation binding between metal ions and protein ligands, metal ions can also activate or stabilize proteins indirectly through electrostatic stabilization [40,41]. Checking the effect of varying concentration of Ca^{2+} ions on stability, it was observed that calcium inactivation was concentration dependent and at minimal quantities (1-2.5 mM) no activity was lost. This shows that the RSDA was stable at low calcium concentration. Metal profile of α-amylase activity was enhanced in the presence of Ca^{2+}, Cs^{1+}, Mn^{2+} and Co^{2+}, and inhibited in the presence of Na^{1+}, Mg^{2+}, Ag^{1+} and Cu^{2+} [42]. Irfan et al. [43] reported the production of a Ca^{2+} stable amylase from *Bacillus subtilis* grown on wheat bran.

3.3 Kinetic constants of native and EDTA-treated RSDA

To determine the kinetic constants for starch hydrolysis using the RSDA, the enzyme was incubated in varying starch concentrations and the K_m /V_{max} calculated from the Lineweaver-Burk plot. Evaluation of the kinetic constant of the native RSDA and cobalt activated RSDA showed an alteration in the Michaelis Menten constant (K_m) as seen in Figure 6. The K_m of the re-activated RSDA was 0.18 mg/ml while the K_m of native RSDA was 0.3 mg/ml. The maximum reaction rate in form of the V_{max} for the cobalt reactivated RSDA and native RSDA were 27.3 and 30.0 U/mg, respectively.

The lower K_m observed for the cobalt activated RSDA compared to the native RSDA is an indication of improved affinity of the metal activated and stabilized RSDA for raw potato starch. This suggests a favorable modification of the quaternary structure of the enzyme, or the introduction of complementary charge properties between metal ions and the enzyme residues. Metals also facilitate faster ligand exchange which ensures a more efficient product turnover due to rapid product dissociation.

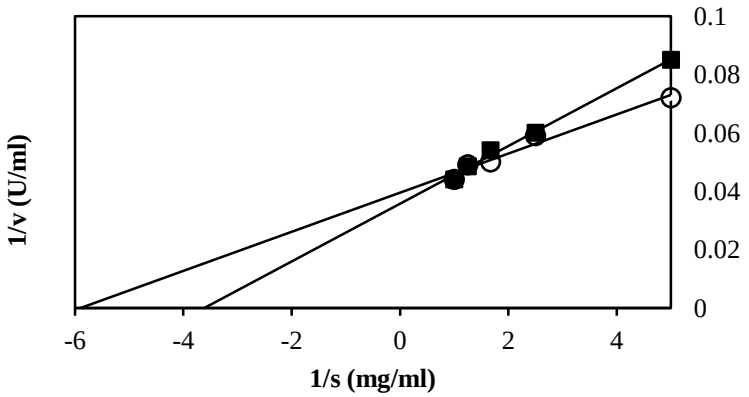

Figure 6. Lineweaver-Burk diagram for the native RSDA and the RSDA reactivated with Co^{2+} ions. *Raw potato starch concentration varied from 0.2 to 1 mg/ml in 0.2 M citrate-phosphate buffer pH 6 (**circle**-* RSDA reactivated with Co^{2+} ions; **square** – native RSDA*)*

4. CONCLUSION

The above results show that EDTA- inactivated RSDA from *Aspergillus carbonarius* could be re-activated using metal ions. Moreover, metal ion substituted RSDA was more active and stable than the native type. Also of interest is the observation that the metal modified RSDA had a lower K_m indicating improved affinity for its raw starch substrate. Ewert et al. [42] reported that the inactive apo form of aminopeptidase (Pep) A secreted by a strain of Lactobacillus was reactivated by metal ions and these ions modified the enzyme character. According to the authors, the optimal pH, temperature and substrate specificity of Pep A can be tailored using different divalent transition metal ions.

Metal ion substitution is a simple, cheap, and cost effective tool for improving amylase activity, stability and catalytic efficiency. Though many works evaluate the effect of metal ions on enzyme activity and stability, few attempt to tailor the enzyme activity, stability or even kinetics

using this approach. This method does not need knowledge of the residues in the enzyme protein or their functions nor does it require specialized equipment and therefore remains appealing. Moreover, this can be combined with either immobilization or further enzyme modification using physical, chemical or biological agents for more improved results. Considering that poor activity and stability are key factors which have limited the large scale use of RSDAs, such a procedure will enable their use in numerous industrial bioprocesses where starch hydrolases are needed.

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