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COMPARATIVE STUDIES ON THE KINETIC PARAMETERS OF IMMOBILIZED *CYPERUS ESCULENTUS L.* INVERTASE WITHIN POLYACRYLAMIDE AND CALCIUM ALGINATE GELS.

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ABSTRACT Invertase has significant application in the food processing industries. The partial purification and immobilization of the enzyme extracted from Tiger nut (*Cyperus esculentus* L.), within polyacrylamide and calcium alginate gels was studied. Some kinetic parameters/characterization of the immobilized enzyme systems was carried out using a batch process to identify the optimal conditions of the immobilized systems. The Evaluation Assays of varying temperature and pH were conducted on the immobilized systems. The polyacrylamide immobilized invertase had an optimum pH of 7 and optimum temperature of 40^0C with Michaelis constant, K_m of 1.50M and V_{max} of 33.11µmol/min. The calcium alginate immobilized invertase exhibited an optimum pH and temperature of pH 7 and 40^0C respectively with a K_m of 3.02M and a V_{max} of 20.88µmol/min. Turnover number calculated for both immobilized systems revealed a higher value of 22.07 obtained for polyacrylamide as compared with 6.91 for calcium alginate gel. Furthermore, the result on inhibition- effects on cations indicated that $CoCl_2$ and $FeCl_2$ activated immobilized calcium alginate gel while $MnSO_4$, $CaCl_2$ and $HgCl_2$ activated immobilized polyacrylamide gel. Thus, the immobilization of polyacrylamide gel gave better results considering the kinetic properties obtained.
Keywords: Invertase, Polyacrylamide gel, Calcium alginate gel, Immobilization, Entrapment.

INTRODUCTION

Invertases [β-D-fructofuranoside fructohydrolase (EC 3.2.1.26)] are disaccharidases which belong to the Gh32 family of glycoside hydrolases and they catalyse the hydrolysis of sucrose into an equimolar mixture of D-glucose and D- fructose (Fotopoulos, 2005; Mohandesi *et al*., 2016). The production of invert sugar (an equimolar mixture of glucose and fructose) is sweeter than sucrose and this is of high interest in various industrial applications (Mohandesi *et al*., 2016; Ward, 2012). Invertase has resourceful application in the bakery industry and a key ingredient in a number of sweets and confectionary products (Ward, 2012). The enzyme is used in the manufacturing of soft-centered candies and fondants, hydrolysis of inulin to fructose, production of lactic acid and glycerol, (Kotwal & Shankar, 2009). Other application of the enzyme is in the manufacturing of artificial honey, plasticizing agents used in cosmetics, drug and pharmaceutical industries, paper industries and as enzyme electrodes for the detection of sucrose (Kotwal & Shankar, 2009; Kulshrestha *et al*., 2013; Mohandesi *et al*., 2016). Hence, the market potential is in food industries (Du *et al*., 2013; Mohandesi *et al*., 2016)

The demand for industrial enzymes is on a continuous rise compelled by the growing demand for sustainable solutions (Adrio & Demain, 2014). Besides, the application of these enzymes for industrial purposes may be increased by their immobilization in an active state (Milovanovic *et al*., 2007). Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. (Datta *et al*., 2013). The different techniques used for immobilization of enzymes are, Adsorption, Entrapment/ microencapsulation, Crosslinking or covalently binding to a support and Affinity immobilization (Datta *et al*., 2013; Singh *et al*., 2013). The gel entrapment method has the advantage that it preservers a high level of enzyme activity since enzyme molecules are physically retained and shield

by the matrix and not chemically bound to it (Arumingtyas *et al*., 2015).

Matrix entrapment is done by mixing enzyme solution with polymer fluid in matrices such as agar, polyacrylamide, collagen ,Calcium-alginate (Sharma, 2012). Alginates (Alg) are one of the most used polymers due to their mild gelling properties and nontoxicity, improving enzyme stability and functional properties (Bonine *et al*., 2014). Moreover, alginate gel is an inert molecule, thus preventing contamination, and it also has no toxic effects (Aafia Aslam, 2006). Polyacrylamide, another commonly used matrix for the entrapment of enzymes, has the property of being non-ionic (Mahajan *et al*., 2010).

There are no reports yet on the immobilization of invertase from *Cyperus esculentus* in the literature. These nutritious tubers are widely cultivated as human foods and livestock feeds across the Mediterranean areas (Klein *et al*., 2014), in most part of West Africa including Ghana and Nigeria, Spain and Arabian Peninsula. Studies have shown that *Cyperus esculentus* L. is rich in oil, minerals, Starch and vitamins E and C. The starch and oil are major macronutrients in the tiger nut tuber. As an high oil yield and more adaptable crop, tiger nut have more potential usage as food and industrial materials (Jing *et al*., 2016). In the present studies, invertase from *Cyperus esculentus* was partially purified and entrapped within polyacrylamide and calcium alginate gels. Subsequently, the kinetic properties of the immobilized invertase were investigated.

MATERIALS AND METHOD

Materials

All chemicals were of analytical grade and obtained from Sigma Co (St. Loius, MO, USA). Invertase was isolated from the seeds/ tubers of Tiger nut (*Cyperus esculentus L.*), which was purchased from a local market.

Methods

Extraction of Invertase

The fresh grains of Tiger nut, *Cyperus esculentus* were washed and homogenized in chilled 300ml of 50mM phosphate buffer at pH 7.5 containing 1mM 2-Mercaptoethanol, 5µm and 0.5M Nacl. The homogenate was filtered and the crude enzyme was centrifuged at 2700 x g for 15 minutes; the supernatant (invertase extract) was used for all assays.

Ammonium Sulphate Precipitation

Solid ammonium sulphate (56.1g) equivalent to 80% ammonium sulphate saturation was added to one hundred millimeters of the crude invertase enzyme and the mixture was kept for 35minutes then centrifuged at 27000xg for 15minutes. The precipitate was dissolved in 10ml of 10mM acetate buffer pH 4.5, which contains 50mM phosphate buffer at pH 7.5 overnight. Invertase activity and protein concentration were then assayed.

Protein Determination

Protein concentration was determined using Folin phenol reagent (Lowry *et al*., 1951) with Bovine Serum Albumin as a standard. The adsorption was read at 540nm.

Enzymatic Activity

The activity of invertase was assayed by addition of 0.2mls of standard sucrose solution (containing 1g sucrose dissolved in 100mls acetate buffer, pH 4.5) with 0.1ml of partially purified enzymes, the solutions were then incubated at 37^0C for 10minutes, and the enzymatic reaction was stopped by the addition of 1ml DNS solutions (3,5 dinitrosalicylate) and 2.7mls of acetate buffer (pH 4.5) was added and allowed to stand in boiling water for 5 minutes to generate colour, it was then cooled at room temperature. Absorbance was measured against a blank containing 1ml DNS and 3ml acetate buffer pH 4.5 at 540nm. A standard curve was prepared with sucrose. One-unit (IU) of activity was defined as the mass of enzyme required to hydrolyze 1-µmole of sucrose per minute under a precisely defined set of reaction conditions (Ward, 2012).

Entrapment Methods of Immobilization.

Enzyme Immobilization on polyacrylamide gel: The entrapment of Invertase on polyacrylamide gel was carried out on 12.5% (w/v) polyacrylamide gel and with the addition of 3.1ml invertase extract containing 0.408mg/ml protein with some modification (Trevan & Grover, 1979). Then, 4.2ml acrylamide/bisacrylamide solution was added followed by acetate buffer of pH 4.7. 10% Ammonium per sulphate was added followed by TEMED. The mixture was then allowed to polymerize at room temperature. The gel was then sliced into various equal sizes of 50mg beads.

Enzyme Immobilization on calcium alginate gel This was done by the method of (Kierstan & Bucke, 1977). Exactly, 3.063g of sodium alginate was dissolved in 8.75mls of phosphate buffer and autoclaved at 121^0C for 15minutes and cooled at room temperature; 17.5mls of the invertase extract was then

added and mixed before allowing it to stand for 10 minutes. The enzyme-alginate mixture was carefully pumped through a sterile syringe drop wise into a beaker containing 250mls of sterile 0.12M calcium chloride, in order for the mixture to form beads. The beads were then kept in solution for 1hour to ensure complete precipitation and hardening. The immobilized invertase activity was determined in the precipitated enzyme.

Effect of Optimum pH on the Activity of Immobilized Enzyme

The effect of pH was assayed as proposed by Fischer and Kohles (1951) in which 5 test tubes each containing six beads and 0.4mls of sucrose solution (1g sucrose dissolved in 100mls of acetate buffer pH 1-6; phosphate buffer pH 7 and Tris-HCl of pH 8) were incubated at 37^0C for 10minutes. The reaction was stopped by the addition of 2mls DNS solutions and 5.4mls of the various buffers. The mixture was allowed to stand in boiling water for 5 minutes to generate colour and cooled at room temperature. Absorbance was read against a blank containing 2mls DNS and 6mls of the various buffers at 540nm.

Effect of Optimum Temperature on the Activity of Immobilized Enzyme

A temperature study of the immobilized invertase was carried out over the range of 30^0C to 80^0C with interval of 10^0C, using Fischer and Kohles (1951). The beads and sucrose solution were incubated at various temperatures for 10 minutes. The reaction was finally stopped by the addition of DNS phosphate buffer. The reaction conditions were as described above.

Effect of Cations on the Activity of Immobilized Enzyme

Beads and sucrose solution (1g of sucrose dissolved in 100mls of phosphate buffer containing 5mM of cations $CoCl_2$, $Fecl_2$, $Fecl_3$, $MgCl_2$, $MnSO_4$, $CaCl_2$ and $HgCl_2$) were incubated at 37^0C for 10minutes. The reaction conditions were as described above.

Kinetic Characterization of Immobilized Enzyme: In determining the kinetic parameters, K_m and V_{max}, of the immobilized invertase, the lineweaver-Burks plot was used. Both K_m and V_{max} were determined from the intercepts at X- and Y- axis respectively. The enzyme turnover number (K_{cat}) was also estimated.

Data Analysis: Data presented were the average of at least two measurement, numerical and graphical representation were generated using Microsoft excel 2016.

RESULTS AND DISCUSSION

Influence of pH on Immobilized Enzyme: The effect of pH on the immobilized enzymes is presented in figure 1. Both polyacrylamide and calcium alginate immobilized enzyme systems gave an optimum pH of 7, while at pH 8, no reaction was observed. The pH of 7 which is a neutral pH was found to be common to both immobilize systems. This appears consistent with the pH of most physiological systems. Since both immobilized systems are by entrapment, as such much different in optimum pH of activity of both immobilized enzymes is not expected to be different. However, Kulshrestha *et al.,* 2013 recorded a pH of 4.5 and a pH of 4.8 was obtained by (Ward, 2012).

Influence of Temperature on Immobilized Enzymes: Temperature studies of immobilized polyacrylamide and calcium alginate gels at different temperatures shows a maximum activity of 40^0C for both systems (fig 2), although the activity of calcium alginate immobilized invertase was generally higher. This could be as a result of the substrate having much accessibility to the enzyme in the entrapped medium at this temperature and much of the enzyme have not been destroyed by the heat neither the immobilized matrices. The extent of optimum temperature displacement for immobilized enzymes depends on the type of matrix as well as on interactions between the enzyme and the matrix (Dwevedi, 2016). A similar observation of 40^0C was recorded by (Ward, 2012).

Kinetic Studies of the Immobilized Enzymes: The lineweaver Burk's plot for polyacrylamide immobilized invertase and calcium alginate gels are presented in table1. K_m and V_{max} as well as the turnover numbers were determined. K_m and V_{max} for the immobilized polyacrylamide gel calculated from lineweaver burk's plot were 1.50M and 33.11µmol/min and for Calcium alginate beads 3.02M and 20.88µmol/min respectively. However, the Michaelis constant (Km) for the free and immobilized invertase were 93.19mM and 139.19mM in alginate gel (Chi & City, 2008). The ratio of V_{max}/K_m which is a measure of catalytic efficiency of the immobilized invertase was found to be $22.07min^{-1}$ for polyacrylamide and $6.91min^{-1}$ for calcium alginate beads respectively. The kinetic parameters were significantly different for the two immobilized systems. Since turnover number represents an index of

physiological efficiency, it is glaring that polyacrylamide immobilized invertase possesses a comparatively lower turnover number. This can be explained on the basis of accessibility of the substrate to the enzyme which, in practice, is not a strange phenomenon in immobilized system. In such systems, the feasibility of the reaction is a function of diffusional effect.

Effect of Cations on Immobilized Invertase: The activity of cations on immobilized invertase is shown in fig 5. $CoCl_2$ and $FeCl_3$ acted as an activator while, $FeCl_2$, $MgCl_2$, $MnSO_4$, $CaCl_2$, $HgCl_2$ as an inhibitor in calcium alginate gel. Furthermore, $CoCl_2$, $MnSO_4$, $CaCl_2$, $HgCl_2$ all acted as an activator and $FeCl_3$, $FeCl_2$, $MgCl_2$ as inhibitors in polyacrylamide gels. The activity of immobilized enzymes is known to be a function of microenvironment. The experiment on the effect of cations, exemplified this phenomenon. Both $CoCl_2$ and $FeCl_3$ did activate immobilized calcium alginate gel and $CoCl_2$, $MnSO_4$, $CaCl_2$, $HgCl_2$ all activates immobilized polyacrylamide gel. This observation indicates denaturation of proteins by forming ligands complexes. However, $FeCl_2$ inhibited both immobilized systems. The presence of metal ion Mn^{2+} increasing the enzymatic activity has been reported for several invertases, while in comparison, ions including Ni^{2+} and Cu^{2+} showed inhibitory effect, this also suggests the presence of thiol groups or His residues that are important for enzyme activity (Zhou *et al*., 2020). The difference in ion effects shows the ability of metal ions to modify the affinity of enzyme for a substrate under conditions similar to those in which the enzyme pre-existed in vivo.

CONCLUSION: The entrapment method become increasingly popular in terms of enzyme immobilization (Labus *et al*., 2020). In this study the entrapment of *Cyperus esculentus L*. Invertase within polyacrylamide and calcium alginate gel have been achieved and from the results obtained it is also clear that immobilization on polyacrylamide gel gave better results considering the kinetic properties obtained of lower Km and higher turnover number.

RECOMMENDATION: Future investigation should endeavor at adopting logistic and sensible entrapment techniques along with innovatively modified supports to improve the state of the enzyme immobilization and provide new perspectives to the industrial sector (Datta *et al*., 2013).

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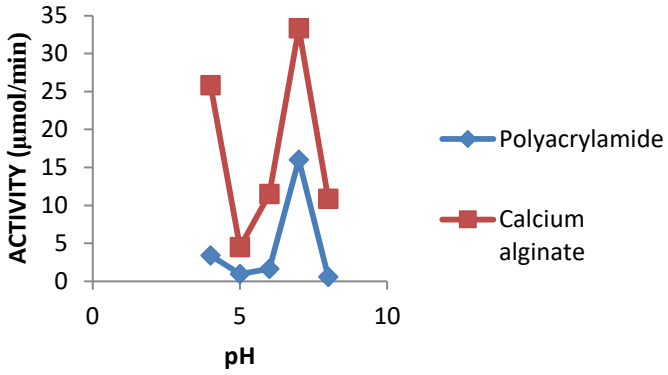

Figure 1. Effect of pH on the activity of immobilized invertase.

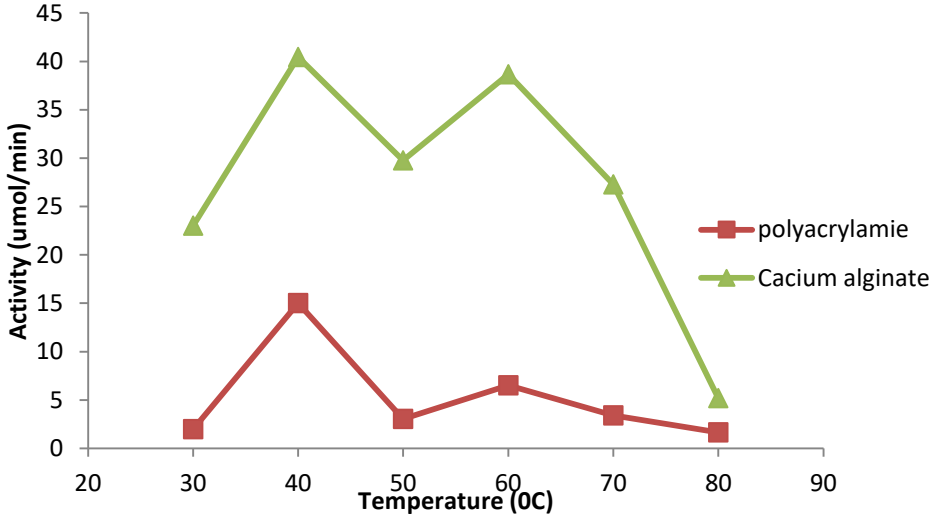

Figure 2. Effect of Temperature on the activity of immobilized invertase.

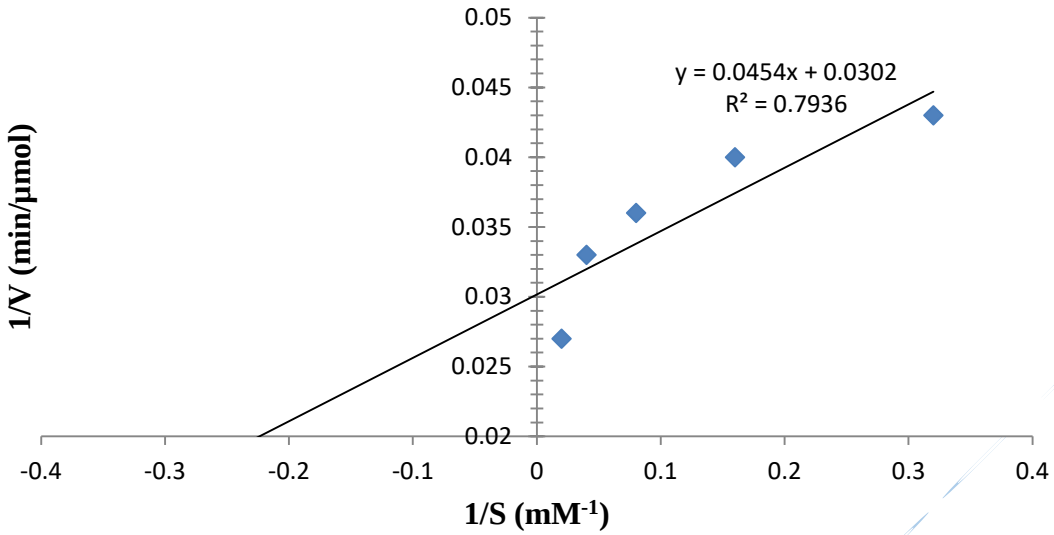

Figure 3. Estimation of Kinetic parameters of polyacrylamide immobilized invertase.

S: Substrate concentration; V: Invertase specific activity.

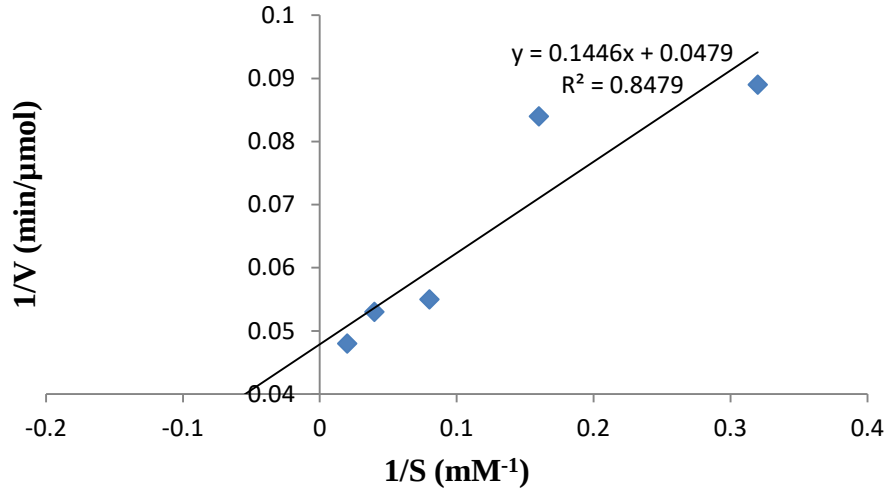

Figure 4. Estimation of Kinetic parameters of calcium alginate immobilized invertase.

S: Substrate concentration; V: Invertase specific activity.

Figure 5: Effects of cations on the activity of immobilized invertase.

Table 1. Kinetic parameters of immobilized *Cyperus esculentus* invertase.

Immobilization supports	Km(M)	Vmax(µmol/min)	Vmax/Km(min^{-1})
Polyacrylamide gel	1.50	33.11	22.07
Calcium alginate gels	3.02	20.88	6.91