Immobilization of thermostable exo-inulinase from mutant thermophilic *Aspergillus tamarii*-U4 using kaolin clay and its application in inulin hydrolysis

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**ABSTRACT**

In this study, attempts were made to immobilize purified exo-inulinase from mutant thermophilic *Aspergillus tamarii*-U4 onto Kaolinite clay by covalent bonding cross-linked with glutaraldehyde with an immobilization yield of 66% achieved. The free and immobilized inulinases were then characterized and characterization of the enzymes revealed that temperature and pH optima for the activity of the free and immobilized enzymes were both 65 °C and pH 4.5 respectively. The free inulinase completely lost its activity after incubation at 65 °C for 6 h while the immobilized inulinase retained 16.4% of its activity under the same condition of temperature and incubation time. The estimated kinetic parameters $K_m$ and $V_{max}$ for the free inulinase as estimated from Lineweaver-Burk plots were 0.39 mM and 4.21 μmol/min for the free inulinase and 0.37 mM and 4.01 μmol/min for the immobilized inulinase respectively. Inulin at 2.5% (w/v) and a flow rate of 0.1 mL was completely hydrolysed for 10 days at 60 °C in a continuous packed bed column and the operational stability of the system revealed that the half-life of the immobilized inulinase was 51 days. These properties make the immobilized exo-inulinase from *Aspergillus tamarii*-U4 a potential candidate for the production of fructose from inulin hydrolysis.

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1. Introduction

The use of homogenous hydrolytic enzymes in the improvement of several technological processes aimed at the manufacture of functional foods and pharmaceutical products is a common practice in related industries. Such functional foods include oligosaccharides with prebiotic properties and fructose with health benefits [1]. However, homogenous hydrolytic enzymes face limitations such as instability to various environmental factors during the production process [2]. Additionally, homogenous enzymes are not appropriate for multiple uses as a result of laborious separation from the reagent and reaction productions. To overcome these limitations, enzymes are usually immobilized onto carrier molecules. This transfers the enzymes from homogenous soluble catalysts into a heterogeneous catalyst system with improved resistance to denaturing environmental conditions and also with possibility of repeated use in biotechnological processes [3]. Furthermore, immobilization lowers down-stream requirements, enables repeated enzyme use and ultimately resulting in a lower operational cost.

One of such hydrolytic enzymes is the exo-inulinase (E.C. 3.2.1.80; β-), which cleaves β-2,1-links in inulin to fructose in a single step enzymatic reaction. The immobilization of this inulinase (using numerous carriers) for inulin hydrolysis has been widely studied by several authors [4–7]. However, one major disadvantage of these methods is that large amount of expensive materials are used as carrier molecules. Additionally, the coupling of enzymes by some covalent methods leads to the chemical modification of the enzyme due to the several steps involved. Hence, the use of readily available and simple, yet effective immobilization of inulinase onto cheap carrier molecules would be expected to play a significant role in the food industries in which fructose is widely used. In this study, we report the immobilization of exo-inulinase from mutant strain *Aspergillus tamarii*-U4 onto kaolin clay, a cheap and naturally-occurring support in the West African environment. The properties of the free and immobilized exo-inulinases are also described. Furthermore, the immobilized kaolin immobilized inulinase was also evaluated in inulin hydrolysis for fructose production in a fixed bed column reactor.

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2. Materials and methods

2.1. Sample collection

The Kaolin clay used in this study was mined at Ologun-Eru Ibadan, latitude 741.9°N and longitude 848°E, Oyo state Nigeria and identified as such with the help of Geologist at the Department of Geology University of Ibadan, Nigeria.

All other chemicals were of analytical grade purchased from local suppliers.

2.2. Microorganism and culture conditions

The Aspergillus tamarii-U4 used was from the Culture collection of the Department of Microbiology, University of Ibadan. It's a mutant strain generated from Aspergillus tamarii-INU4 (accession number KJ423064) by UV-treatment as previously reported [Garuba and Onilude unpublished]. The isolate was maintained on Potato Dextrose Agar slant and sub-cultured every four weeks.

2.3. Inulinase production by Aspergillus tamarii-U4 in solid state fermentation and inulinase assay

Fifty grams (50 g) of dried wheat bran was inoculated with 4% inoculums [1] density of the spores of Aspergillus tamarii-INU4 at 65% moisture content. Incubation was done at 55 °C for 6 days. After the period of incubation, Sodium Acetate buffer (pH 4.8) 0.2 M, was added to the fermented matter at ten times (v/w) and incubated in a shaker incubator (New Brunswick scientific, Edison, New Jersey, U.S.A) at 50 °C with agitation at 150 rpm for 30 min. This whole content was then filtered using Whatman Filter paper No. 1 and the supernatant was used as the crude enzyme preparation. The inulinase activity was determined as described by Garuba and Onilude [8].

Briefly, 0.1 ml of the enzyme and 0.9 ml of a sodium acetate buffer (0.1 M, pH 5.5) containing 2% inulin was incubated at 50°C for 30 min. Thereafter, the enzyme was inactivated by keeping the reaction mixture at 90°C for 20 min. The reaction mixture was then assayed for reducing sugar liberated using the DNS method. The solution was placed in a boiling water bath for 10 min and allowed to cool at room temperature. A blank with distilled water instead of enzyme solution was run parallel. To 1.0 ml of each mixture, 1.0 ml of DNS reagent was added and placed in boiling water for 5 min. After cooling to ambient temperature, the volume was raised to 8 ml with distilled water. Absorbance of the reaction mixture was measured using a Jenway Spectrophotometer at 540 nm.

One unit of inulinase activity was defined as the amount of inulinase enzyme that produced 1 μmole of fructose per minute under defined assay conditions.

2.4. Purification of inulinase

The crude inulinase was purified by ammonium sulphate pre-precipitation followed by gel chromatography [5].

2.5. Preparation of Kaolin-clay and inulinase immobilization

Kaolin clay was first beneficiated as described below [9,10]. Clay lumps were crushed and screened with a 2 mm sieve. One hundred grams of resulting material was then dispersed in one litre of 30% Hydrogen peroxide (H₂O₂) solution so as to rid the sample of any coloured organic and inorganic matter present and the suspension agitated using a mechanical stirrer for 5 min and then allowed to stand for 2 h. The clear supernatant liquid was then decanted and the clay re-suspended in one litre of 0.5 M NaOH solution for a period of 2 h with moderate agitation with a mechanical stirrer. The resulting suspension was diluted with deionised water and later decanted. The residue was re-dispersed in the water, agitated and the process of sedimentation and siphoning was repeated until very little clay was left in the suspension. The resultant colloidal suspension was air-dried for three days and calcined at 700 °C in a furnace for 6 h. The calcined clay was then dealuminated by reacting it with 60% (w/v) sulphuric acid for 30 min followed by repeated fluxing with distilled water.

2.5.1. Acid activation of kaolin clay

Acid activation was carried out with reflux technique of 5.0 g of the calcined and dealuminated kaolin clay that was mixed with 100 ml of 6 M Hydrochloric acid (HCl) solutions at 90 °C and stirred for 3 h. The suspensions were decanted and the solids were washed with distilled water until no chloride anion was detected with AgNO₃. Acid activated kaolin was then dried at 100 °C for overnight [9].

2.5.2. Immobilization by adsorption

Immobilization by adsorption using clay was achieved using a modified method of Ajayi et al. [10]. Ten grams (10 g) of the beneficiated, calcined, dealuminated and acid activated clay sample was mixed with 20 ml of inulinase (containing 50–150 units) in 100 ml of 0.2 M sodium acetate (pH 6.0) in a beaker with constant stirring of the clay sample and the solution was then allowed to settle for 24 h. The top solution was decanted and the resulting thick clay immobilized-inulinase was collected, dried at room temperature for a period of 24 h using Whatman No 1 Filter paper. The immobilized inulinase was washed with 50 ml of n-hexane to remove any fatty material and other artifacts which could interfere with the enzyme activity and latter dried at room temperature using Whatman No 1 Filter paper.

2.5.3. Immobilization by covalent method

To 2 g of kaolin clay, 100 ml of 0.2 M sodium acetate buffer (pH 6.0) and 8 ml of 2.5% glutaraldehyde was added. The content was stirred gently for 1 h at 25 °C, recovered by filtration using Whatman Filter paper No1, and washed with water. Each of the support matrixes was then mixed with 20 ml of inulinase (containing 50–150 units) and 100 ml of 0.2 M sodium acetate buffer (pH 6.0) and stirred gently for 24 h at 4 °C. The immobilized inulinase recovered by centrifugation (at 11,000g for 20 min) was washed thoroughly three times with distilled water and dried using Filter paper. The adsorbed inulinase was desorbed by adding 100 ml of 0.5 M NaCl in 0.2 M sodium acetate buffer (pH 6.0) with gentle stirring for 30 min using a magnetic stirrer. The preparation was recovered by centrifugation as described above and washed thoroughly three times with distilled water and dried using Whatman No 1 filter paper and stored at 4 °C.

2.5.4. Determination of the extent of immobilization

The filtrates obtained from centrifugation above and wastewater was collected for the determination of the unabsorbed protein content using the method of Lowry et al. [11]. The protein content of the immobilized enzyme was calculated as the difference between the protein content of the enzymatic solution and that found for the sum of the filtrates and waste waters. The extent of immobilization was calculated by the equation below:

\[
\% \text{ of immobilization enzyme} = \frac{\text{Amount of immobilized enzyme}}{\text{Amount of added enzyme}} \times 100
\]
2.6. Effect of temperature and pH on the activity of the free and immobilized inulinase

Effect of temperature on the activities of the free and immobilized inulinase was investigated at temperatures ranging between 40 and 70 °C in 0.2 M sodium acetate buffer (pH 4.8). Similarly, effect of pH on the activities of the free and immobilized inulinases was measured in a pH range of 3.5 to 7.0 using 0.2 M sodium acetate buffer (pH 3.0 to 5.0) and 0.2 M phosphate buffer (pH 6.0 to 7.0). Thermal stability of the free and immobilized inulinase preparations were also investigated by measuring residual activities of the enzymes at one hour interval during incubation at 65 °C for 10 h while pH stability of the free and immobilized enzymes were measured by determining the residual activities after incubating the inulinase preparations in a pH range of 3.5–7.0 using 0.2 M sodium acetate buffer (pH 3.0–5.0) and 0.2 M phosphate buffer (pH 6.0–7.0) for 6 h at 25 °C.

2.7. Kinetics of the free and immobilized inulinase

The kinetic constants $K_m$ and $V_{max}$, using inulin as the substrate, were evaluated by fitting the experimental data to the Michaelis-Menten model using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results and discussion

3.1. Exo-inulinase purification

In this study exo-inulinase produced by mutant thermophilic Aspergillus tamarii-U4 was purified to homogeneity and the degree of purification investigated by SDS-PAGE analysis of the enzyme. The result as shown in Plate 1 indicates that the enzyme is made up of a single isoform with an estimated band weight of 66 kDa.

Plate 1. Electrophoretogram depicting separation of protein in extracellular inulinase from from mutant thermophic Aspergillus tamarii-U4 (Lane 1) and protein Markers (kDa) (Lane 2) used in SDS-PAGE.

| Inulinase Type and Source | Type of Carrier Material | % Immobilization | Refs. |
|--------------------------|-------------------------|-----------------|-------|
| Exo-inulinase from A. fumigatus | Dowex | 63 | Gill et al. [5] |
| Exo-inulinase from Kluyveromyces sp Y-85 | Macroporous ionic polystyrene beads | 62 | Weling et al. [12] |
| Endo-inulinase from A. niger | Chitosan | 66 | Nguyen et al. [13] |
| Exo-inulinase from K. marxianus | Gelatin | 82.6 | de-Paula et al. [6] |
| Exo-inulinase from A. niger | Amino cellulofine | 96 | Nakamura et al. [14] |
| Frutozyme | Sepabeads | 100 | Ricca et al. [16] |
hyde onto chitosan. This 66% immobilization yield is, however, lower compared to 82.60% yield obtained when inulinase from *Kluyveromyces marxianus* was immobilized onto gelatin as reported by de-paula et al. [6]. A higher yield of 96% was also reported by Nakamura et al. [14] when inulinase from *Aspergillus niger* was immobilized onto Amino-Cellulofine [14]. In addition, Gill et al. [15] reported 100% yield using DEAE-Sephasel, QAE-Sephadex and Cona-linked amono-activated silica beads. A 100% immobilization yield was also reported by Ricca et al. [16] when commercial Fructozyme L from Novozymes was immobilized onto Sepabeads with amino groups (Sepabeads EC-HA).

A common disadvantage of most of these immobilization techniques is the use of synthetic and expensive carrier molecules that are not readily available. Furthermore, it’s necessary sometimes to use large amount of the carrier molecules [5]. The immobilization of exoinulinase onto kaolin clay in this study could be advantageous since kaolin clay is naturally occurring, readily available and cheap material. This will in turn have a low cost effect on the immobilization technique as opposed to the use of expensive synthetic materials that are not readily available. Furthermore, Kaolin is reported to have reduced susceptibility to microbial attack compared to wide range of carriers reported in [10].

The expressed activity of the kaolin-immobilized inulinase was higher compared to the free inulinase (data not shown). This observation could be as a result of the change in the orientation and conformations of the enzyme facilitating the accessibility of inulin substrate into the active sites of the enzyme as well as the prompt release of the product into the bulk medium as suggested by Basso et al. [17]. The increased activity as observed in this study could be advantageous since most enzymes immobilization techniques are reported to cause a reduction in enzyme activity resulting from unfavourable conformational changes in the active sites of the enzymes [16].

### 3.3. Effect of temperature on the activity and stability of free and immobilized inulinase

Based on the results of the immobilization yields, covalently-bonded kaolin clay was selected for further studies. As presented in Fig. 1 the optimum temperature of activity for both the free and immobilized inulinase preparations was observed to be 65 °C. However, an enhanced activity was observed with the immobilized inulinase compared with the free inulinase. A further increase in temperature beyond the optimum led to a decrease in activity. Similar observation has been reported by Yewale et al. [7]. This observation is, however, a deviation from the report of de Paula et al. [6] who observed a shift from 55 °C to 60 °C in the optimum temperature of activity of inulinase from *Kluyveromyces marxianus* var. *bulgaricus* immobilized onto gelatin cross-linked with glutaraldehyde. A decrease in activity beyond the optimum temperature could be as a result of thermal inactivation of the enzyme as suggested by Sanjay and Sugunan [18]. The result of the thermal stability of the free and immobilized inulinase as presented in Fig. 2 showed that the immobilized inulinase retained 4.4% of its activity compared to the free inulinase.

![Fig. 1](image1.png)

*Fig. 1. Effect of different temperatures on the activity of free and immobilized exoinulinase produced by mutant *Aspergillus tamarii*-U4. Data are means of three replicates with standard deviation.*

![Fig. 2](image2.png)

*Fig. 2. Thermal stability of free and immobilized inulinase produced by Mutant strain *Aspergillus tamarii*–U4. Data are means of three replicates with standard deviation.*

![Fig. 3](image3.png)

*Fig. 3. Effect of pH on the activity of free and immobilized inulinase (A) and the stability of the free and immobilized inulinase produced by Mutant strain of *Aspergillus tamarii*-U4. Data are means of three replicates with standard deviation.*
activity after incubation at 65 °C for 7 h while the free inulinase completely lost its activity when incubated for 6 h at the same temperature. Immobilization by covalent bonding has been reported to increase the resistance of the inulinase to thermal unfolding [18]. This increased resistance could result from the cross-linking of inulinase enzymes with carrier at many points thereby making the spatial configuration of immobilized enzyme more rigid and difficult to unfold when heated thus it could retain its stable configuration and stability is therefore enhanced [7]. This enhanced stability at elevated temperatures has been suggested to be advantageous for the scale up process as it allows greater solubility of inulin at elevated temperature giving effective higher substrate concentration. Additionally, there will be lower risk of microbial contamination at higher temperature [7,18].

3.4. Effect of pH on the activity and stability of free and immobilized inulinase

The results of the effect of pH on the activity and stability of the free and immobilized inulinase from Aspergillus tamarii-U4 is presented in Fig. 3(a) and (b). As shown, pH 4.5 was optimum for the activities of both free and immobilized inulinase preparations. Catana et al. [19] on the other hand observed an increase towards the alkaline medium in the optimum pH of inulinase after immobilization by entrapment in gel. This difference in results could be ascribed to the variation in the microenvironment provided by the two enzyme support systems. A broader-shaped curve observed for immobilized inulinase as compared to the free enzyme in this study is similar to the reports of Catana et al. [14] and de-Paula et al. [6]. This broader-shaped curve might be due to the strong interaction between supports and covalently bound enzyme which affect the intra-molecular forces responsible for maintaining the conformation of the enzyme ultimately leading to enhanced enzyme stability [18].

3.5. Estimated kinetic parameters of the free and immobilized inulinase

The estimated kinetic parameters $k_m$ and $V_{max}$ for the free inulinase as estimated from Lineweaver-Burk plots were 0.39 mM and 4.21 μmol/min for the free inulinase and 0.37 mM and 4.01 μmol/min for the immobilized inulinase respectively. The results suggest a reduction in kinetic parameters. Similar reduction in kinetic parameters of inulinase after immobilization has been reported by Gill et al. [20] for inulinase from Aspergillus fumigatus and Aspergillus niger NCIM 945 immobilized [7]. This reduction has been attributed to changes in effective localized charges near

| Flow rate (mL/min) | Temperature/°C conversion |
|-------------------|--------------------------|
|                   | 30  | 60 | 100 | 40 | 93 | 21 | 80 | 2 | 61 | 0 | 39 |

![Fig. 4. Influence of storage at 25 °C on the activities of free and immobilized inulinases from mutant strain Aspergillus tamarii-U4 Data are means of three replicates Data are means of three replicates with standard deviation.](image)

![Fig. 5. Operational stability of the immobilized inulinases in continuous production of fructose for 50 days the hydrolysis of inulin. Data are means of three replicates with standard deviation.](image)
the active site of the enzyme owing to the proximity between the enzyme and support [5]. Additionally, enzymes have been reported to generally experience some conformational changes which can result in substrate affinity or allosteric properties and subunit dissociation [21].

3.6. Influence of storage on the activity of free and immobilized inulinase

As shown in Fig. 4 an enhanced stability was observed for immobilized inulinases compared with the free when the both inulinase enzymes were stored at 25 °C for 30 days. This suggests that the immobilized inulinases were less sensitive to the aging process.

3.7. Inulin hydrolysis by free and immobilized inulinases

The effect of flow rate and temperature on inulin hydrolysis was investigated 2.5% (w/v) inulin solution. As revealed in Table 2, inulin hydrolysis decreased with an increase in flow rate. Similar observation was earlier reported by Yun et al. [4] and Yewale et al. [7]. This decrease could be a result of the low residence time associated with increased flow rate resulting in a reduced contact time between the enzyme and the substrate.

The operational stability of inulin hydrolysis at 60 °C and a flow rate 0.1 mL/min was investigated and the % inulin hydrolysis monitored every 24 h for 50 days. The results as presented in Fig. 5 showed a consistent 100% hydrolysis till the 10th day beyond which, a gradual decrease in % hydrolysis was observed. The estimated half-life of 51 days for the immobilized inulinase in this study is high compared with that estimated for inulinase from Aspergillus fumigatus with half-lives of 35 and 22 days, when immobilized on chitin and QAE-Sephadex respectively [20]. This high half-life could also be advantageous in the continuous production of fructose by inulin hydrolysis as suggested by Yewale et al. [7].

4. Conclusion

Conclusively, this study presents the immobilization of purified exo-inulinase from mutant thermophilic Aspergillus tamarii-U4 using kaolin-clay by cross-linking with glutaraldehyde with a 66% immobilization yield. To the best of our knowledge, this is the first report presenting the immobilization of inulinase using clay. The optimum temperature of 65 °C and pH of 4.5 for the activity of the immobilized exo-inulinase coupled with its half life of 51 days during continuous hydrolysis makes this immobilized form a potential candidate for large scale production of fructose from inulin. Efforts are however being made to scale up this process.

Conflict of interest

The authors declare that they have no conflicting interests.

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