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Immobilization of Cassava Linamarase on Kankara Kaolinite Clay

Olusegun. A. Ajayi[1*] Andrew, J. Nok[2] and Sam S. Adefila[3]
1. Department of Chemical Engineering, Ahmadu Bello University, Kaduna State. Nigeria
2. Department of Biochemistry, Ahmadu Bello University, Kaduna State. Nigeria
3. Department of Chemical Engineering, Covenant University, Ota, Ogun State. Nigeria
*E-mail of the corresponding author: segeaj@yahoo.com +2348033175209; +2348028845459

Abstract
The enzyme, linamarase was successfully extracted and immobilized on pretreated Kankara kaolinite clay, serving as substitute for other expensive catalysts supports. Acid treatment of the clay was observed to affect the activity of linamarase. The activity of the enzyme was higher in the cortex compared with both in the stem and the leaves. Enzyme activity was observed to increase with increase in the silica contents of the treated clay. Operational stability/activity of the immobilized enzyme was found to reduce by about 50% after 18days at room temperature, following first order denaturation reaction, with rate constant k obtained to be $0.03day^{-1}$ with R^2 0.957. The K_m and V_{max} were determined to $0.1986mM^{-1}$ and 10.01mM/min respectively, which is an indication of the enzyme's affinity to the support used. The specific surface area and pore size of kaolin were also found to decrease with rate of enzyme anchoring, pointing to occupation and/or blockage of the available pore. Kankara kaolinite clay is a promising cheaper support material for linamarase immobilization.
Keywords: linamarase, immobilization, kaolinite clay, cassava, metakaolin, cyanogen

1.0 Introduction

Cassava (*manihot esculanta crantz*) is a widely grown root crop in countries of the tropical regions of Africa, Latin America and Asia (Cock, 1985; Ndindu et al, 2003; Okafor, 1992) serving as the staple food for over 200 million Africans (Cock, 1985; CIAT,1991). About 10 million tonnes of cassava are processed for consumption as garri annually in Nigeria alone (Nok and Ikediobi,1990). However, the edible storage tuber contains varying amounts of cyanogenic glucoside, *linamarase* and lotaustralin (Yeoh and Troung, 1993; Yeoh et al,1998; Cooke et al, 1987). It has been established that roots from the same variety of cassava plant, may also differ in their cyanogen content, and that cassava roots show both axial and radial gradient in cyanogen content (Fokunang et al, 2001), whereas *linamarase* is present in all parts of the cassava plant, though in relatively small quantities (Miller and Conn, 1980).

Cyanogenic glucosides are groups of widely occurring natural substances that on hydrolysis yield ketone or aldehyde, sugar, as well as highly toxic cyanide ion (Fomunyam, 1984). Apart from cassava, bitter almonds, sorghum and lima beans are other major food sources of cyanogenic glycoside. Cyanogenic glucoside and the enzymes necessary for the release of hydrogen cyanide are all present separately in the plant (McMahon, et al,1985; Sundaresan, et al, 1987). Linamarin, is the predominant cyanogenic glucoside in the cassava, which accumulates to concentrations as high as 500mg/kg fresh weight in the root (White et al, 1994; Aletor, 1993).

Hydrolysis of linamarin carried out endogenously by *linamarase* results in the release of free cyanide. Accordingly, in processed cassava products such as flour and gari, variable amounts of residual cyanide, arising from linamarin and its breakdown product (acetone cyanohydin and free cyanide), have been reported (Akintonwa e al, 1994; Egan et al, 1998; Ikediobi and Onyike, 1982). These observations revealed the inefficiency of processing materials to eliminate all the cyanogens present.

Coincidentally, in recent years *linamarase* has attracted considerable industrial and academic interest in view of its potential use in the processing of edible cyanogenic plant tissues, particularly cassava, apricots and bitter almonds and the quantification of bound cyanide in the form of cyanogenic glucosides (CNG) in plant tissues and body fluids. For example, *linamarase* has been used successfully in a batch wise process to detoxify fermenting cassava during 'gari' production (CIAT,1991; Yeoh and Troung, 1993). In order to minimize wastes associated with batch wise use of the soluble enzyme, some attempts have been made to immobilize *linamarase* with a view to develop a system that enabled repeated use of the enzyme (Rao and Hahn, 1984; Nambisan and Sundaresan, 1985).

Accordingly, Ikediobi and others successfully immobilized *linamarase* on non-porous glass bead (1998). The use of kaolin as a support for immobilization of *lipase* was reported by Abdul Rahman and co-workers (2005); for radioactive waste immobilization (Osmanlioglu, 2002) and application of NaY zeolite in the immobilization of *lysozyme* (Chang et al, 2006). Suffice to note that, kaolin are negatively charged aluminosilicate crystalline structures consisting of a three-dimensional arrangement of SiO_2 and AlO_4^- tetrahedrally linked to each other by a shared oxygen atom. It presents an inert, stable and relatively good pore aperture for immobilization purposes, aside the mechanical strength and thermostability (Kilara, 1981). The

silica and alumina content of kaolin when upgraded, can serve as immobilization sites for various enzymes.

Gladly, Nigeria is blessed in abundance with kaolin clay (RMRDC, 2004; Ahmed and Onaji, 1987) which is a cheap source of both silica and alumina. This could serve as an alternative, after subjecting it to proper treatment. The elemental composition of Nigerian kaolin clay was reported (Atta et al, 2007) to contain the required components for the purpose of immobilization of *linamarase* via physical adsorption. Importantly, Onaji (1992) worked extensively on chemical and mineralogical analyses of some Nigerian clay, out of which, Kankara kaolin was not an exception. His finding on Nigerian kaolinite clay was later corroborated by the works of Chandrasekhar and Ramaswamy (2007) and Al-Shameri and Rong (2009) on other kaolin deposits. However, no report exists in the open literature on immobilization of *linamarase* on clay. In the present paper, *linamarase* was immobilized on calcined kaolinite clay and acid treated metakaolin. The activity and denaturation of the enzyme were also studied, as well as the kinetic behavior of the immobilized enzyme.

2.0 Materials and Methodology

2.1 Chemicals and Starting Materials

Fresh cassava plant from Giwa village near Zaria, Kaduna State, Nigeria, was harvested, washed and used appropriately. Similarly, clay of kaolin origin was mined from Kankara village of Katsina State, Nigeria, and used appropriately in this work. All reagents used in this work were of analytical standard.

2.2 Clay Beneficiation, Activation and Dealumination.

The mined clay was beneficiated using the wet method for 3 days, was dried and ground using jaw crushers (model PE 150*250 SBM) and ball mill. A part of the resulting powder with particle size of <75micron, was calcined at 700^0C in a furnace for 6hrs, prior to dealumination reaction. About 50g of the calcined clay (metakaolin) was made to react with three-fold 60wt% sulphuric acid (dealumination) for such reaction times as 5, 20, 40,60, and 80 minutes. The beneficiated, calcined clay and the dealuminated samples were sent for elemental, structural and BET surface area analyses. The elemental (XRF) analyses of the samples were done using a Model-PW2400, with X-ray tube of rhodium anode and scintillation detector with current at 40mA and voltage 40 mV, with accuracy of ±0.02. The structural (XRD) analysis was conducted using a Philips analytical X-ray instrument: X'Pert-MPD (PW 3020 vertical goniometer and PW 3710 MPD control unit) employing, Bragg–Brentano recording within the range of 10–70^0 at scanning rate of 2^0/min.

2.3 Linear Model for Methyl Umbelliferyl Galactoside (MU-Gal)

Known concentration of methyl umbelliferyl galactoside (Mu-Gal) was prepared with its pH adjusted to 9 using NaOH solution. The absorbance corresponding to these concentrations were measured at 390nm using spectrophotometer (Model JENWAY-6405 UV/VIS). A calibration curve was plotted from the data generated, with the aim of using the model obtained for subsequent determination of the enzyme concentration.

2.4 Extraction of *Linamarase* and Assaying with Mu-Gal

Linamarase was extracted from freshly harvested cassava tuber (cortex), leave and stem following the procedure fully described by Nok and Ikediobi (1990). The activity of *linamarase* was assayed using MU-Gal as an artificial substrate. The enzyme immobilized on stationary phase served as *linamarase*. The assaying was done as follows: 0.2ml of the solution containing 0.1M of the substrate was dispensed into packed columns (containing metakaolin with bed height of 2cm) with pH set to 5.5 using 1ml of acetate buffer and allowed to drain. The eluates were collected into separate tubes with pH adjusted to 9 using 0.1ml of 1.0M NaOH. The absorbance of the released methyl umbelliferyl was measured at 390nm.

One unit of *linamarase* activity is defined as that amount of enzyme, which produced one micro-mole of MU-Gal per min under the described assay conditions. The protein concentration was determined following the Lowry method (Okafor, 1992).

2.5 Immobilization of *Linamarase* on Kaolin

10g of beneficiated clay sample was weighed and poured into a beaker. The extracted enzyme solution with known concentration and fixed volume (30ml) was added into the beaker successively and accompanied by rigorous stirring at a fixed rate for the clay sample. The resultant mixture was allowed to settle for 24 hours. A thick immobilized enzyme was collected by filtration, washed with n-hexane and dried at room temperature for another 24hrs. The enzyme was washed with n-hexane to remove fatty materials and other artifacts, which could inhibit the activity of the enzyme (Nok and Ikediobi, 1990). The aforementioned procedure of immobilization was done for metakaolin and samples dealuminated at various reaction times. The extracted *linamarase* was thereby, physically absorbed on the samples and their activities were determined as described in Section 2.4.

2.6 Protein Concentration and Volume of Enzyme

The procedure described in Section 2.5 was repeated only for metakaolin but the volume of enzyme employed was varied from 5ml to 30 ml, in the interval of 5ml. The protein concentration was determined with increase in volume of enzyme employed for constant mass of clay. BET analysis was carried out on metakaolin and metakaolin-immobilized material.

2.7 Operational Stability and Kinetics Studies

The native and immobilized *linamarase* were separately, stored at 25^0C for a period of 18 days and the residual *linamarase* activity was assayed at 3 day intervals to monitor the stability of the enzymes. To establish this, a first order process was assumed, and a plot of log of protein denaturation against time in days, was obtained, to ascertain the assumption. The stability of the immobilized enzyme was also estimated from it. The K_m and V_{max} were also determined, using the Lineweaver-Bulk double reciprocal plots.

3.0 Results and Discussion

3.1 Characterization of Kaolinite Clay Support

The elemental analysis conducted on the samples and protein concentration is represented in Table 1.It could be inferred from Table 1 that the Kankara clay used was actually of kaolinite type, as is also supported by the XRD analysis shown in Figure 1, despite the presence of some impurities like muscovite, clinochlore, etc. The SEM image for the beneficiated kaolin shown in Figure 2 depicts the plate-like structure of kaolin and other inherent phases. The phases identified by XRD analysis were confirmed by the morphology seen in Figure 2. The thermal treatment carried out on the clay makes its pore relatively more available for occupation by the enzyme.

The effect of acid treatment was clearly seen in samples A to E, with relative increase in silica to alumina ratio resulting from the removal of alumina by sulphuric acid from the clay framework. The dealuminated samples were acidic in nature with low pH, even after fluxing repeatedly with water. Addition of base reagent to the already dealuminated samples, needed to increase the pH, might attack the residual silica content which is part of the component required for immobilization purposes.

Therefore calcined clay was chosen since the structure is more reactive and less acidic (conducive environment for enzyme activities). The BET surface area of metakaolin and immobilized calcined clay was found to be 16.1205m²/g and 11.5724m²/g, respectively, which is an indication of pore occupation/blockage or eclipsed by the introduced enzyme – *linamarase* -. The turtose pores of kaolin could accommodate the *linamarase*, a relatively large molecule, which has the possibility of denying access to the adsorbate in reaching some part of the surface. This phenomenon could reduce the measurable or accessible surface area, resulting from diffusional resistance and inhibition caused by the anchored *linamarase* (Lenarda et al, 2007 and Upadhyay, 2006). This observation was noticed in the SEM image for the *linamarase* immobilized metakaolin shown in Figure 3. The blurred sticky material noticed in Figure 3 was attributed to the anchored *linamarase*.

3.2 Standardization Curve for MU-Gal

Figure 2 shows a linearized standard curve for the relationship between known concentrations and absorbance for MU-Gal. The empirical model developed from Figure 4, was used subsequently for the purposes of assaying.

3.3 Protein in *linamarase*: Volume of Enzyme Effect

Figure 5 reflects the effect of varied enzyme volume on protein concentrations. It shows that protein concentration increased with volume of enzyme for fixed quantity of support. The slight drop in the specific surface area of the anchored material points to the availability of more pores/space for enzyme immobilization. This observation further buttresses the suitability and inert environment for *linamarase* immobilization by metakaolin. Expectedly, the enzyme release protein proportionately to its volume.

3.4 Operational Residual Activity of Enzyme

Figure 6 shows the rate of decrease of *linamarase* activity as a function of prolonged period of application *viz-a-viz* storage time, in days, for both the native and immobilized *linamarase*. The activity of the immobilized *linamarase* was retained, dropping to about 50% its initial value only after 18 days of experimental consideration. This implies that calcined kaolin clay was able to encapsulate and retain enzyme activity for a relatively long (storage) period at room temperature,compared to native raw *linamarase*. This observation was found to agree with the works of AbdulRahman et al (2005) and Zhai et al (2010).

3.5 Determination of Michealis-Menten constants

The K_m and V_{max} were determined using Lineaweaver-burk plot (represented in Figure 7) which gives the respective values to be 0.1986mM-1 and 10.01mM/min. These values indicate high affinity between the substrate and the support irrespective of the storage period. The denaturation rate constant for the metakaolin-immobilized linamarase was determined to be 0.035 with R^2 0.952, as shown in Figure 8.

4.0 Conclusion

The suitability of Nigerian kaolinite clay to serve as support for linamarase enzyme extracted from cassava was investigated. The results obtained indicated the prospective use of this support, since the clay contains in various proportions, the required compound (i.e.silica and alumina) for enzyme support purpose. Though not considered in this work, it is however, assumed that when fully developed, the usage of kaolin as source of silica and/or alumina will prove to be more cost effective and competitive in comparison with the conventional expensive alginate compounds.

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Table 1: Elemental analysis of the clay samples used.

Element (wt,%)	BF	CC	A	B	C	D	E
SiO_2	42.03	43.36	46.8	57.82	65.51	75.55	90.43
Al_2O_3	31.82	32.87	27.43	5.06	4.45	3.22	3.23
Fe_2O_3	3.42	3.5	2.48	0.52	0.44	0.28	0.26
CaO	0.88	0.4	0.44	0.36	0.54	0.44	1.08
MgO	1.41	1.05	1.22	0.52	0.5	0.48	0.5
K_2O	1.47	1.46	1.22	1.07	1.16	1.33	1.37
Na_2O	0.37	0.36	0.35	0.34	0.35	0.35	0.38
Si/Al	2.245	2.242	2.900	19.425	25.026	39.887	47.595
Protein Conc. (mg/ml)	0.4299	0.487	0.5098	0.527	0.5298	0.5612	0.7925

Legend

BF	Beneficiated kaolin clay
CC	Calcined kaolin clay @700^{0}C for 6hrs
A, B, C, D and E	Dealuminated metakaolin for 5,20,40,60 and 80 minutes

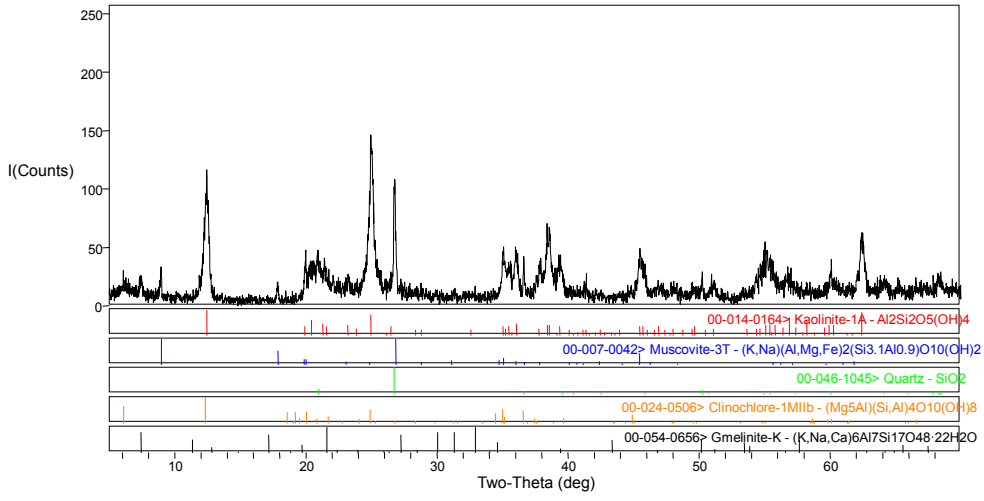

Figure 1: X-ray Diffratogram for beneficiated kaolinite clay.

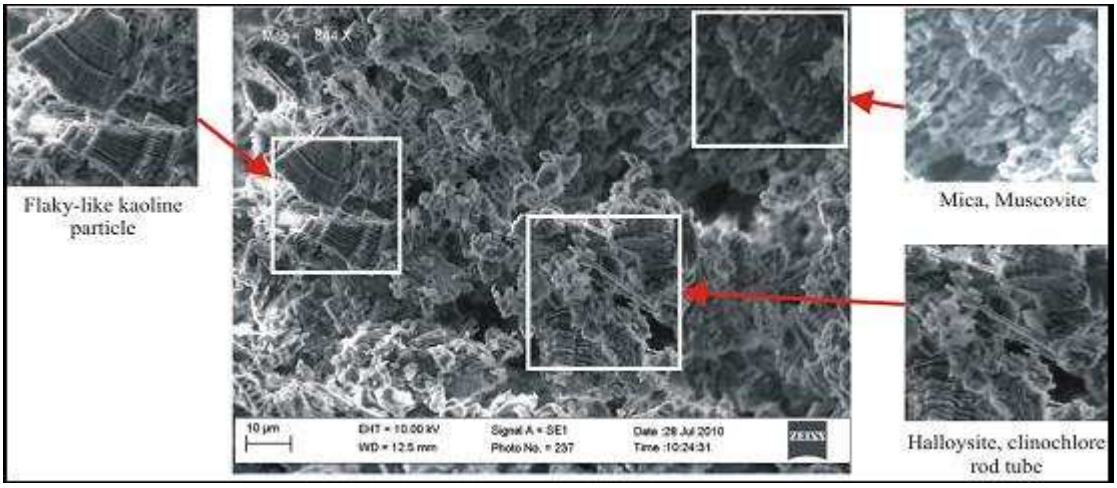

Figure 2: SEM image for the beneficiated kaolin

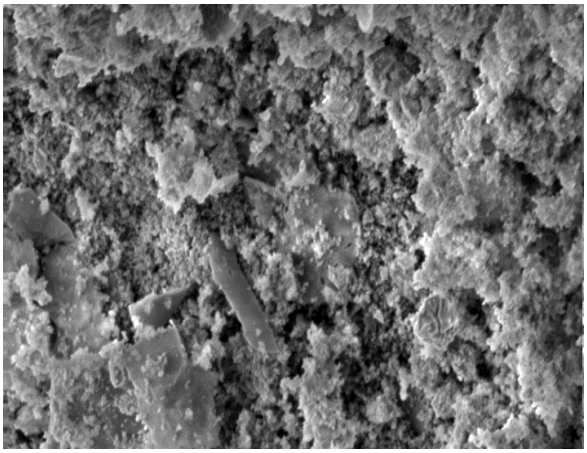

Figure 3: SEM image for metakaolin-linamarase immobilized

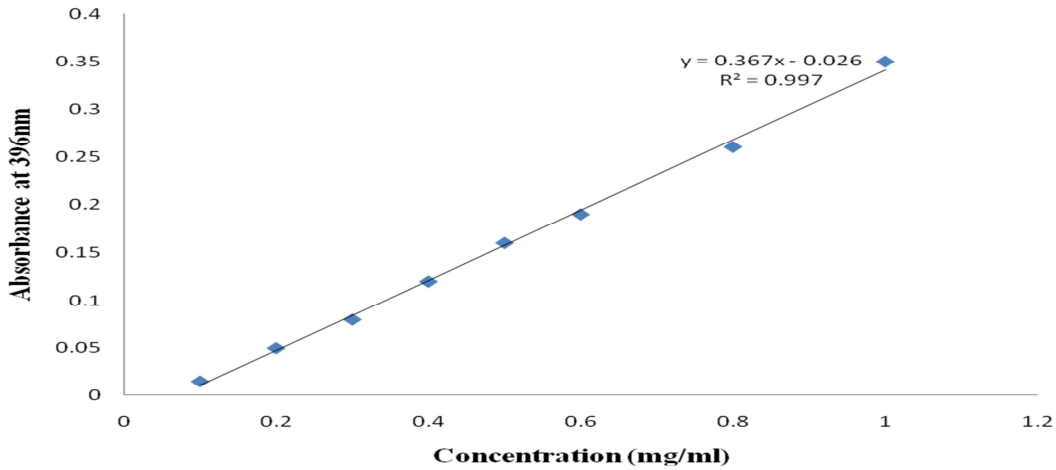

Figure 4: Calibration curve for determination of enzyme concentration measured at 396nm

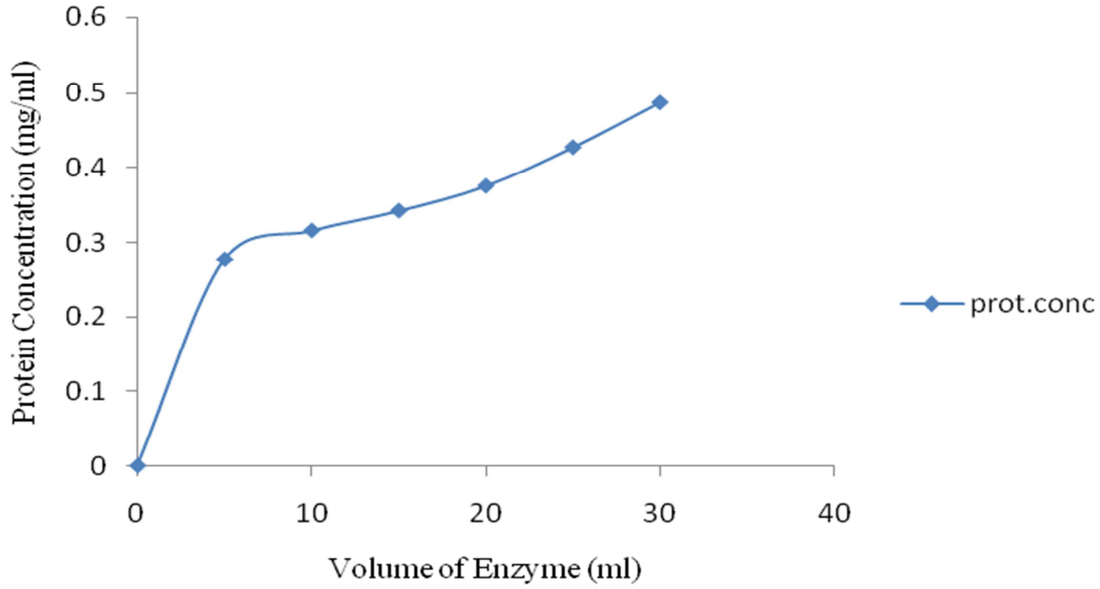

Figure 5: *Linamarase* activity as a function of volume of enzyme used.

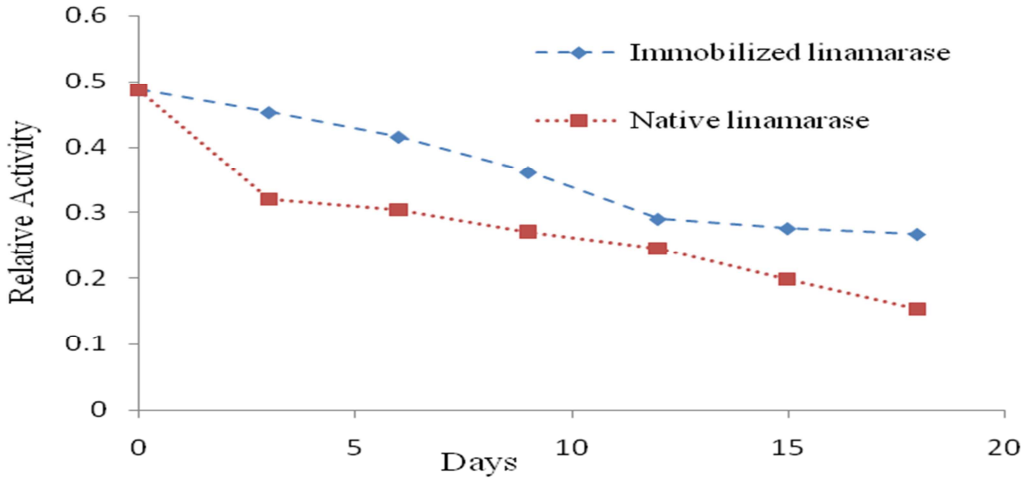

Figure 6: Relative activity of native and immobilized linamarase from 0 to 18 days

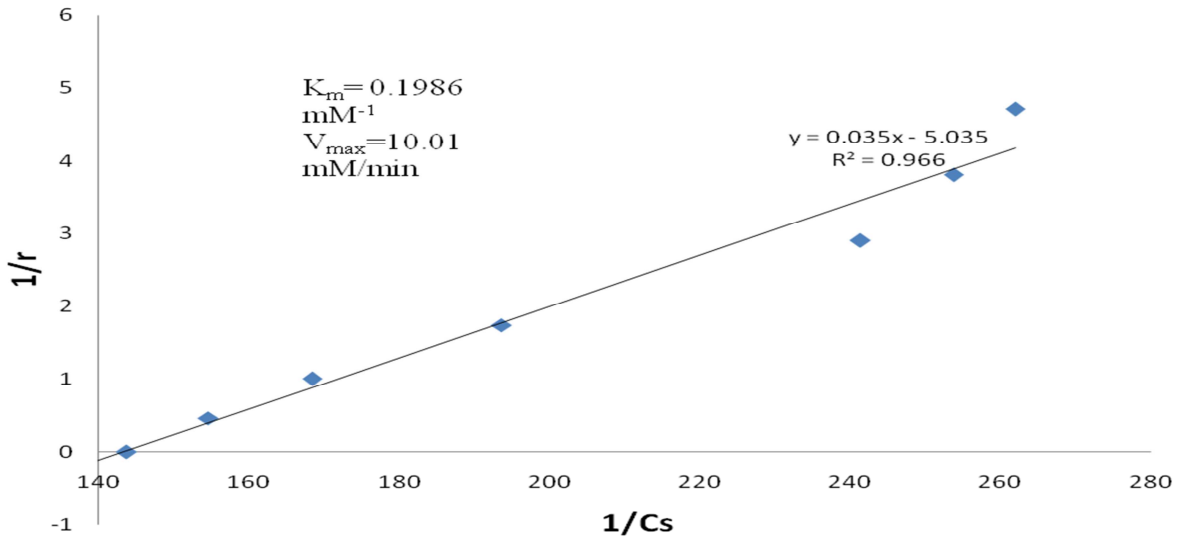

Figure 7: Lineaweaverburk plot for the immobilized *linamarase*

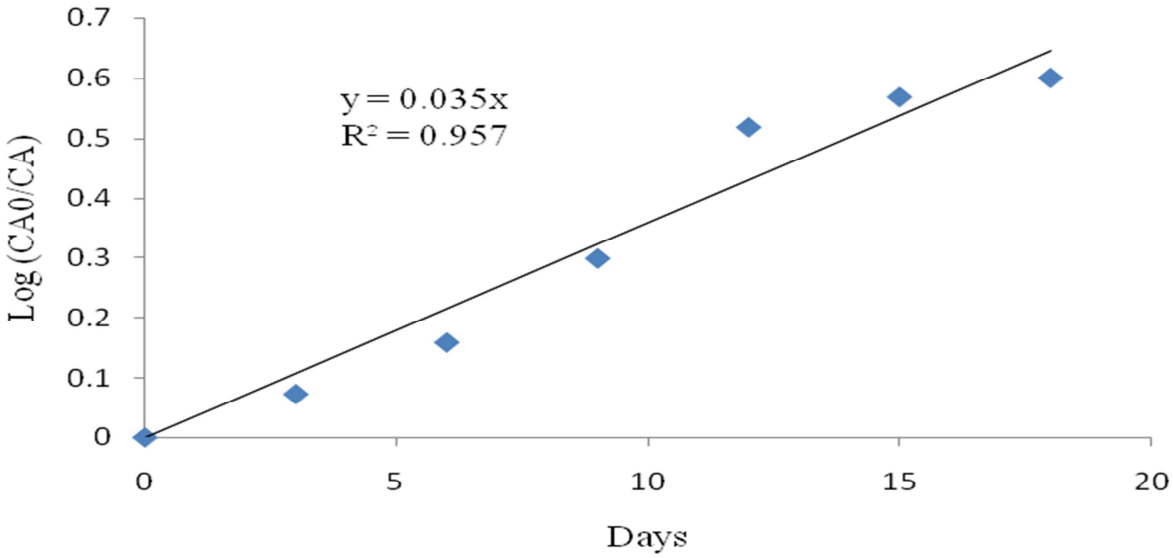

Figure 8: Test for first-order mechansim for linamarase denaturation reaction for 18days