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Research article

ADSORPTION KINETICS STUDIES OF ENDOGLUCANASE FROM *RHIZOPUS ORYZAE* ON ACTIVATED CHARCOAL

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ABSTRACT: The adsorption kinetics and activities of endoglucanase enzyme from *Rhizopus oryzae* were evaluated using activated commercial charcoal as adsorbent. The effect of various experimental parameters on adsorption of endoglucanase such as initial enzyme concentration, amount of adsorbent, contact time and temperature were investigated. The pseudo-first-order and pseudo second-order kinetic models were used to describe the kinetic data which showed that the adsorption of the enzyme followed the pseudo-first order rate expression. The adsorbed enzyme was subjected to saccharification in presence of commercially available carboxy methyl cellulose and the reusability of the adsorbed enzyme was also tested.
Key words: adsorption, pseudo-first-order, pseudo second-order, saccharification, reusability.

INTRODUCTION

Cellulase, a multicomponent enzyme consisting of three different enzymes (endocellulases, cellobiohydrolase, and beta glucosidases) is responsible for the bioconversion of cellulose into soluble sugars and have immense applications in paper and food industries. For repeated utilization of the enzyme, cellulase has been immobilized on a number of insoluble and soluble carriers (Dourado *et al*, 2002; Wu *et al*, 2005; Sinegani *et al*, 2005; Li *et al*, 2004). Selection of an immobilization strategy greatly influences the properties of the resulting biocatalyst. Adsorption, being the physical immobilization of the enzyme has advantages of wide applicability and may provide relatively small perturbation of the enzymes native structure and function, which contributes to maintain sites of enzyme activity (Khan and Bokhar, 2008). Physical adsorption of an enzyme onto a solid relies on non-specific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid. It is the most widely accepted in environmental treatment applications throughout the world (Moreno-Pirajani and Giraldo, 2012). Activated charcoal is one of the finest adsorptive agents known and around 80% of the world production of activated charcoal is used in aqueous-phase adsorption of both organic and inorganic compounds (Cooney *et al*, 1995; Hayashi *et al*, 2005).

Although reports on adsorption of cellulase from *Aspergillus niger* (Daoud *et al*, 2010, Jabasingh, 2012) from *Penicillium notatum NCIM NO-923* (Das *et al*,,2010,) were available, detailed literature on studies on adsorption kinetics of fungal cellulases is very scanty.

The aim of the present work is to study the feasibility of using activated carbon in the batch model for adsorption of partially purified endoglucanase from *Rhizopus oryzae* and to elucidate the usefulness of pseudo-first- order and second-order models for the adsorption kinetics of enzyme.

MATERIALS AND METHODS

Microorganism: The endoglucanase producing fungal strain *Rhizopus oryzae* PR7 MTCC 9642 (Karmakar and Ray, 2013) was taken as working strain.

Extraction, Purification and Assay of Enzyme The grown culture was filtered through filter paper (Whatman No1) and filtrate was used centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. The enzyme was purified from the culture filtrate using ammonium sulphate, membrane filter of 30 kDa and 70 kDa.

To measure the activity of endoglucanase tubes containing the assay mixture (1ml) each containing an 0.5 ml of enzyme diluted with 0.1(M) phosphate buffer (pH-6) was incubated with 1 %(w/v) CM-cellulose for 10 minutes (Karmakar and Ray,2010) at 33°C . The reducing sugar released was measured by the dinitrosalicylic acid method (Bernfeld, 1955) taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of endoglucanase was defined as that amount of enzyme that liberated 1μ mole of glucose per ml per minute of reaction.

Reagents: All the reagents were of analytical grade and were obtained from Himedia (India) and Merck (Germany).

Adsorption of endoglucanase : The activated carbon was used after washing well with double distilled water several times to remove soluble inorganic compounds followed by drying in oven at 50 °C for 72h to drive off the moisture. The adsorption of endoglucanase from aqueous solutions onto activated carbon samples was performed by using batch equilibrium technique. Typically, 30mg of activated carbon was introduced into glass tubes containing 12 ml of enzyme solution in phosphate buffer pH 6.0 with initial concentrations ranging from 3.32 to 6.54 mg/ml. These tubes were incubated in a temperature controlled water bath shaker for 1 h 20 min with constant shaking in order to reach the adsorption equilibrium. The adsorption temperature was kept at 30°, 40°, 50° and 60°C. Upon equilibration, the solid particles were removed by centrifuging and the equilibrium concentration of endoglucanase in the solution was determined by a spectrophotometer (Shimadzu, Japan) at wavelength (max) 280 nm.

Each experiment was duplicated under identical conditions and a negative control (without adsorbent) was simultaneously carried out to ensure that the adsorption was caused by the support and not by the container. The equilibrium adsorption capacity of endoglucanase on the activated carbon, Qe (mg g−1) was calculated according to the equation:

$$\mathbf{Qe} = \frac{(C_0 - C_e)\,V}{m} \quad \ldots\ldots\ldots (1)$$

where C_0 (g l^{-1}) is the initial concentration of cellulase solutions, Ce (g l^{-1}) is the equilibrium concentration of endoglucanase solution, V (ml) is the volume of endoglucanase solution, and m (g) is the mass of activated carbon. The percentage of immobilization of endoglucanase is calculated by the following equation:

$$\mathbf{\%} = \frac{(C_0 - C_e)}{C_0} 100 \quad \ldots\ldots\ldots\ldots (2)$$

Adsorption kinetics of endoglucanase : The immobilization experiments were performed in 100 ml conical flasks and the contents were incubated at preset temperature with shaking. The mass of activated carbon to volume of used endoglucanase was 0.25 g per 100 ml. The aqueous samples were withdrawn at preset time intervals and centrifuged, followed by measuring the endoglucanase concentration, Ct, using a spectrophotometer. The adsorption capacity of endoglucanase on the activated carbon at time t, Qt, was calculated according to the following equation:

$$\mathbf{Qt} = \frac{(C_0 - C_t)}{m}$$

Adsorption of cellulose in different concentrations of charcoal : Properly diluted endoglucanase (12 ml) was adsorbed in different concentrations (10, 20, 25,30,40,50 mg) of activated carbon samples in phosphate buffer pH 6.0 at 30°C for 1 hr.

Calculation of adsorption kinetics :

Pseudo first order model

The pseudo first order equation is generally expressed (Nassar, 1997) as:

$$dqt/dt = k1\,(qe - qt) \quad (1)$$

Where, qe and qt are the adsorption capacity at equilibrium and at time t, respectively (mg/g) and k1 is the rate constant of pseudo first order adsorption (1/min). After integration and applying boundary conditions, t = 0 to t = t and qt = 0 to qt = qt, Eq. 1 takes the form of Eq. 2.

$$\log (qe - qt) = \log (qe) - (k1t)/2.303 \quad (2)$$

A plot of log (qe - qt) versus t should give a straight line, if the sorption is controlled by this model. k1 and qe can be determined from the slope and intercept of the plot, respectively.

Pseudo-second order model

The pseudo second order adsorption kinetic rate equation as expressed by Ho *et al.,* (2000) is

$$dqt/dt = k2 (qe - qt)2 \quad (7)$$

Where, k2 is the rate constant of pseudo second order adsorption (mg/g/min). From the boundary conditions t = 0 to t = t and qt = 0 to qt = qt, the integrated form of equation (7) yields Eq. 8.

$$1/(qe - qt) = 1/qe + k2 \quad (8)$$

Reuse of adsorbed endoglucanase onto activated carbon: The endoglucanase assay experiment described above was repeatedly carried out to examine the efficacy of the immobilized endoglucanase onto activated carbon for the hydrolysis of carboxy methylcellulose. After the batch reaction of 30 min, the activated carbon was separated and washed exhaustively with the double distilled water for subsequent reuse. The remaining activity was measured with every reuse. The reusability was evaluated as the change in the percentage of the glucose productivity obtained with repeated use relative to the initial productivity.

Saccharification by adsorbed enzyme: The adsorbed endoglucanase (4.5 gm/l) were incubated with carboxy methylcellulose (10 mg/ml) pH 6.0 at 30°C for 30 minutes.

Thin-layer chromatography: The end products of saccharification of carboxymethyl cellulaose by endoglucanase was analysed by chromatography on a pre coated TLC plate (Merck) using a solvent system of butanol: acetic acid: water: methanol (3:3:1 v/v), developing it with 0.1% methanolic orcinol in 10% H_2SO_4.

All experiments were done in triplicate and their values were averaged.

RESULTS AND DISCUSSION

Effect of contact time and initial enzyme concentration:

The effect of time on the adsorption of endoglucanase onto activated carbon was carried out in order to determine the equilibrium points. It was found that for all experiments (Fig. 1), the plots showed that kinetics of enzyme adsorption consisted of two phases; an initial rapid phase where adsorption was fast and a second slower phase where equilibrium was achieved. The curve was due to initial instantaneous external surface adsorption of endoglucanase followed by a gradual adsorption stage before reaching the point of equilibrium. The time to reach equilibrium was 50 min for the studied endoglucanase concentrations. This trend went in agreement with the reports of other investigators (Kadirvelu , 2009; Kannan , 2002; Garg, 2003).Based on these results, the contact time was fixed at 60 min for the rest of batch experiments to ensure that the equilibrium was reached.

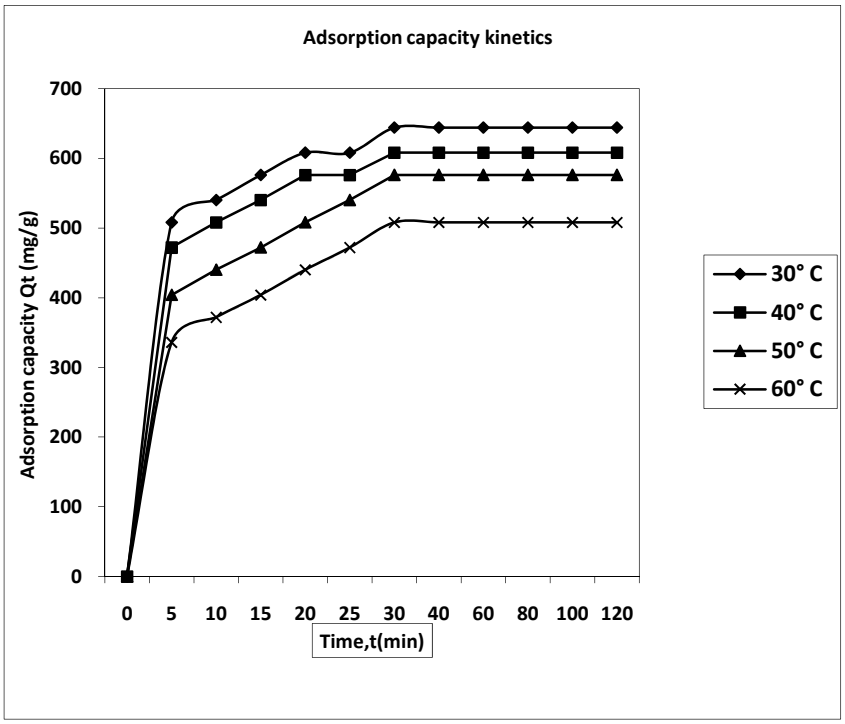

Fig 1. Adsorption capacity kinetic at different temperatures (initial cellulase concentration, C_0=4.5gm/Lt; amount of adsorbent=0.03gm; volume of sample, V=0.012Lt; initial pH=6)

Effect of concentration of charcoal: The effect of amount of charcoal on the adsorption capacity of endoglucanase (Fig. 2) showed that the percentage of adsorption increased with increase in charcoal amount up to 30 mg, further increase in the amount of charcoal showed no further adsorption of the enzyme. It might be due to the achievement of an equilibrium state at this concentration of charcoal.

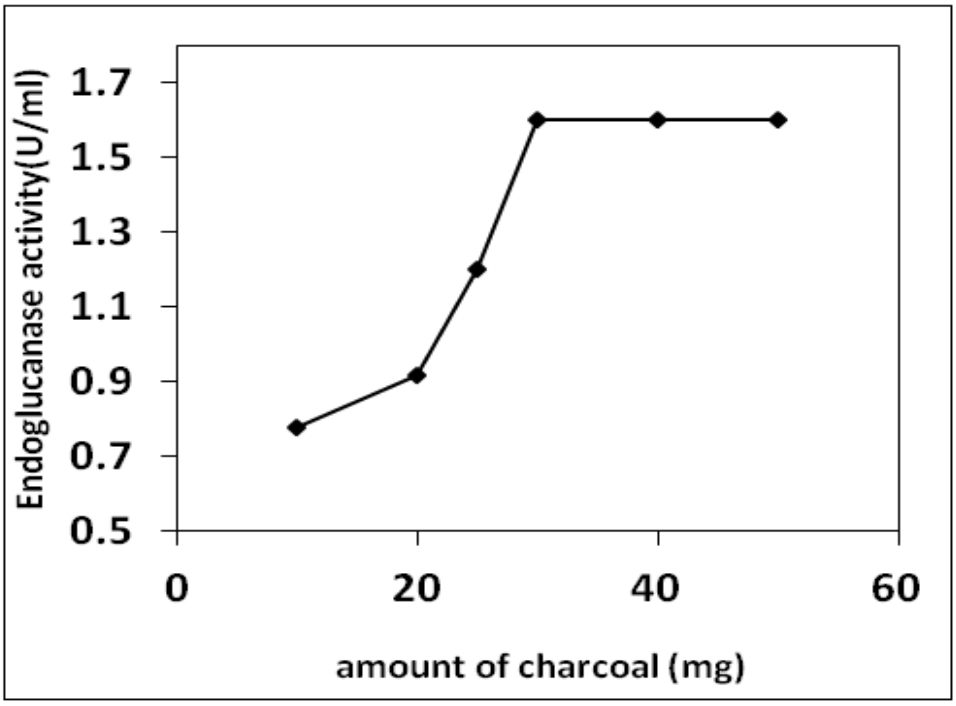

Fig 2. Enzyme adsorbed in different concentration of charcoal.

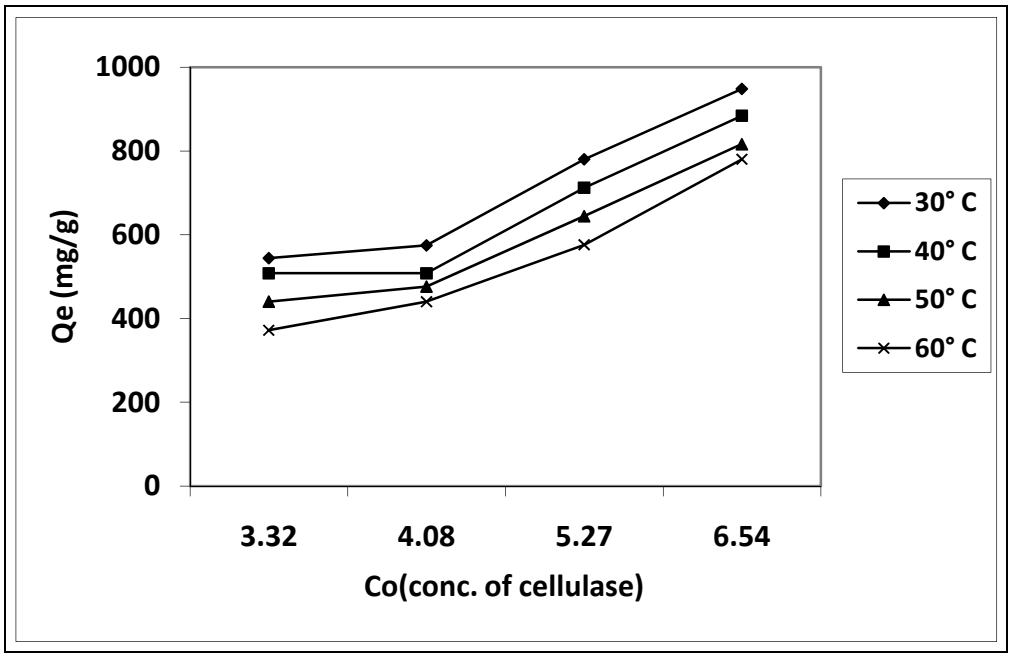

Fig 3. The amount of endoglucanase adsorbed on activated charcoal at equilibrium at different temperatures (amount of adsorbent=0.03gm; volume of sample, V=0.012Lt; initial pH=6)

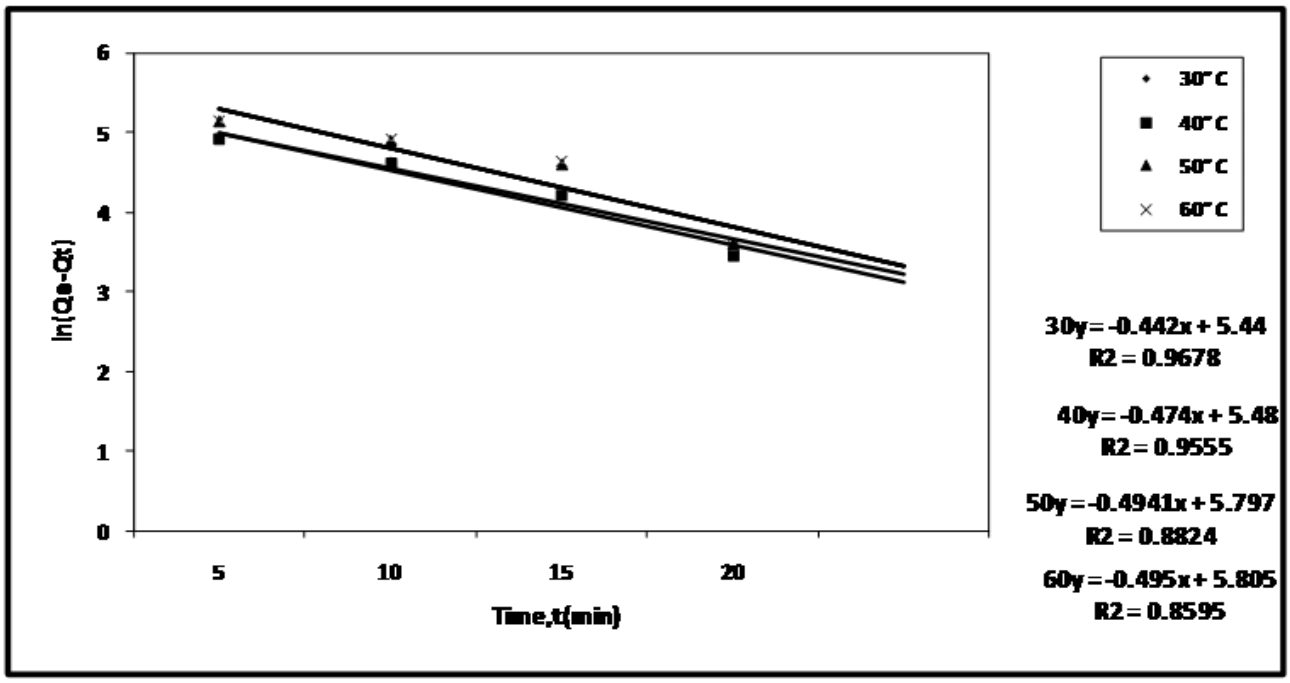

Fig 4. Pseudo 1st order plots for adsorption of endoglucanase on activated charcoal

Effect of temperature on adsorption:

The different concentrations of endoglucanase adsorbed on the activated charcoal at different temperatures (30°, 40°, 50°, 60° C) was investigated which showed that the maximum adsorption had taken place at a temperature of 30°C after which it declined (Fig. 3).Since the adsorption decreased with increase of temperature, this system was considered to be exothermic. The increase in temperature might not favour the adsorption of endoglucanase enzyme molecules further on the active sites of the support due to the high vibrational energy generated at higher temperatures.

Kinetic studies

Both pseudo-first-order and pseudo-second-order kinetic models were applied to describe the adsorption kinetics of the present enzyme. Fig 4 depicted the pseudo-first order plots for adsorption of endoglucanase on activated charcoal at different initial temperatures. Similar result has been presented in literature (Abechi *et al,* 2013; Hamid, 2014, Demirbas, 2004). Though the pseudo second order adsorption kinetic rate equation as expressed by Ho *et al.,* (2000) was also studied (data not shown), it was found the adsorption kinetics of the present enzyme predominantly followed the pseudo-first order plot.

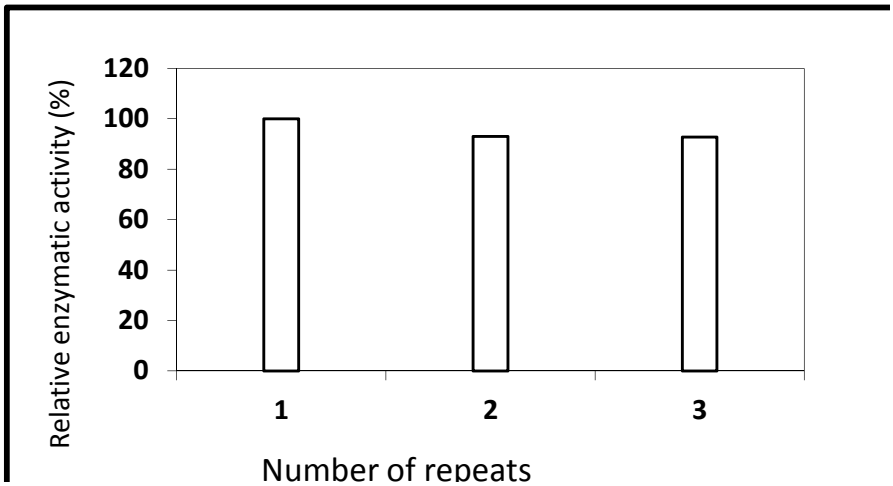

Fig 5. Recycling of adsorbed enzyme onto activated carbon in hydrolysis of carboxymethylcellulose (initial concentration of carboxy methylcellulose was 1 mg/ml; the amount of enzyme was 4.5 gm/l; pH and temperature were 6.0 and 30°C, respectively; each reaction time was 60 min).

Recycling of adsorbed enzyme

The reusability of adsorbed endoglucanase in the hydrolysis of carboxy methylcellulose was examined by studying the change in the enzyme activity with the repeated utilization in the enzymatic hydrolysis of carboxy methylcellulose. The activity after each round of substrate hydrolysis was determined and expressed as the percentage relative to that in the first use. The results obtained with the immobilized cellulase are shown in Fig. 5. The glucose yields decreased at the initial stage and became a constant value (about 80%) of the initial productivity after using it three times. This result suggests that enzymes leaked at the initial stage of the repeated batch enzyme reactions, but the system then stabilized for repeated batch enzyme reactions. This result suggested that the activated carbon was effective in maintaining the cellulose activity during the hydrolysis of carboxy methylcellulose and might contribute to reducing the inhibition effect caused by soluble sugars produced.

Catalytic property of the adsorbed endoglucanase

The effect of hydrolysis time in the catalytic property of carbon adsorbed endoglucanase for carboxy methylcellulose hydrolysis was carried out. The obtained results (Fig. 6) showed the accumulation of glucose with time for each amount of enzyme adsorbed. The rate of glucose accumulation increased with amount of adsorbed enzyme. It illustrated glucose profile as a function of amount of enzyme adsorbed onto activated carbon. The glucose concentration was maximum at 30^{o} C, increased almost linearly with time and gave highest yield at 90 min. For the other enzyme concentrations, the increase in glucose concentration slashed down after an initial rise.

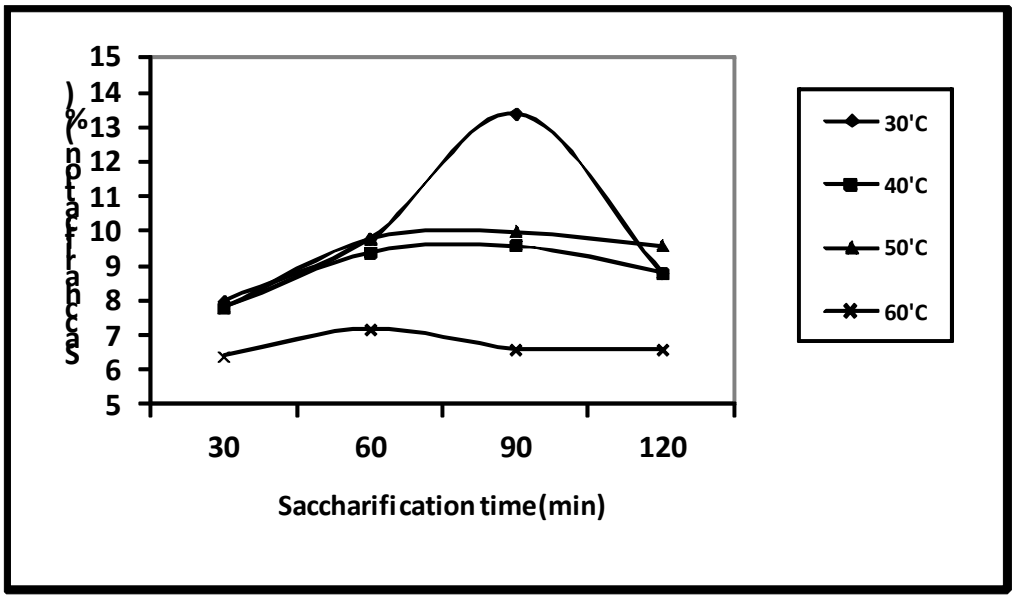

Fig 6. Saccharification by adsorbed endoglucanase (Concentration of carboxymethylcellulose 10 mg/ml; adsorbed enzyme 4.5 gm/l; pH 6.0; temperature 30°C).

Thin layer chromatography

Glucose was found to be the final product of bioconversion of both adsorbed and free enzyme tested (Fig 7), a result similar to that reported from hydrolysis by cellulases of *Thermomonospora* sp (Ferchak *et al*, 1980) and *Trichoderma viridae* ITCC 1433 (Herr, 1980). Cellobiose was also detected as an intermediate product but not end product after hydrolysis of cellulose.

CONCLUSION

From the experiments, it can be concluded that partially purified endoglunase from *R. oryzae* could be adsorbed on activated charcoal following a pseudo first order adsorption kinetics. The charcoal adsorbed enzyme could also effectively saccharify its substrate to produce glucose like that of the free native enzyme. This revealed that charcoal was not affecting the active site of the enzyme, although it could increase the reusability of the enzyme.

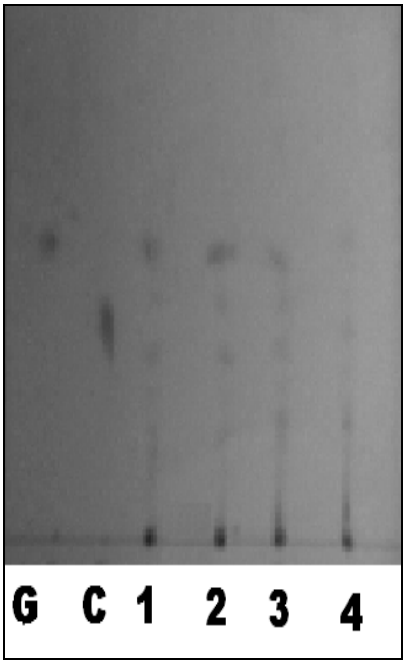

Fig 7. Thin layer chromatographic analysis of the end products of saccharification of the adsorbed and free endoglucanase enzyme of *R.oryzae.*
G:Glucose , C: Cellobiose , 1: Adsorbed enzyme, 2: Adsorbed enzyme, 3: Free enzyme, 4: Free enzyme, Substrate: 10mg/ml, temperature :33°C,time: 60 min, pH: 6

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